RIJKSUNIVERSITEIT ANTWERPEN

FACULTEIT VAN DE GENEESKUNDE

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EXPERIMENTAL INDUCTION OF DIARRHOEA IN NEWLY-WEANED PIGLETS AND EFFECT OF ANTISECRETORY DRUGS

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Proefschrift tot het verkrijgen van de graad van Doctor in de Diergeneeskundige Wetenschappen

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Gent 1990

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Abbreviations

AC : adenylate cyclase
ADP : adenosine diphosphate
AP : activator protein
ATP : adenosine triphosphate
C : control
Ca^{++} : calcium
cAMP : cyclic adenosine monophosphate
cGMP : cyclic guanosine monophosphate
CH : chloramphenicol
Cl^{-} : chloride
CT : cholera toxin
ETEC : enterotoxigenic *Escherichia coli*
GC : guanylate cyclase
GDP : guanidine diphosphate
GTP : guanidine triphosphate
IP : inhibitor protein
K^{+} : potassium
LT : heat-labile enterotoxin
Na^{+} : sodium
NSAID : non-steroidal anti-inflammatory drugs
PG : prostaglandin
PWD : post-weaning diarrhoea
ST : heat-stable enterotoxin
TGE : transmissible gastroenteritis
TGEV : transmissible gastroenteritis virus

Chapter I: PATHOPHYSIOLOGICAL MECHANISMS OF DIARRHOEA AND ANTISECRETORY DRUG THERAPY : A REVIEW

The word diarrhoea originates from the Greek terms 'dia' (= through) and 'rhein' (= to flow). Diarrhoea can best be defined as an increase in the faecal waterexcretion. It is the most important cause of dehydration an hypovolaemia in young animals and children.

This review is focused on the physiology of the intestinal secretion and absorption, on pathophysiological mechanisms of diarrhoea, especially post-weaning diarrhoea in piglets, and on antisecretory drugs for treatment of diarrhoea.

I.A. The mechanisms of water and electrolyte transport in the normal intestinal tract

I.A.1 The movement of water in and out the intestinal tract.

Water is the major component of the intestinal content. It moves across the intestinal mucosa secondary to the flow of electrolytes and soluble nutrients. Absorption or secretion of electrolytes and nutrients occurs actively or passively (Argenzio and Whipp, 1980). The net-movement of water across the intestinal mucosa is thus the result of absorptive and secretory processes (Binder, 1979).

Water from salivary, gastric, biliary, pancreatic and small intestinal secretions together with fluid contained in the diet is presented to the small intestine. In men, normally between 7 and 9 liters water is daily delivered to the small intestine. The normal small intestine absorbs approximatively 80 percent of this load. About 1.5 liters per day enters the large intestine (Binder, 1979).

Compared to the small intestine, the colon absorbs a much smaller quantity of fluid but does this much more efficiently. Of the 1.5 liter that daily passes the ileocaecal valve, the colon absorbs all but 0.1 to 0.2 liter (or a 90 % efficiency). The colon can increase this absorption until a maximal capacity of approximatively 5 liters in men. This is an important regulatory mechanism (Binder, 1979). Thus there is a net absorption of fluid across the intestine.

Aldosterone which becomes released by the adrenal glands as a result of dehydration can increase the colonic absorption of sodium (Na^+) and water (Levitan and Ingelfinger, 1965). An increased small intestinal fluid load to the large intestine will only result in diarrhoea when this fluid load exceeds the maximal fluid absorptive capacity of the colon. In neonatal animals, this compensatory mechanism is not well developed. This can explain why small intestinal fluid loss results in a more severe disease in newly born animals than in adults (Argenzio, 1985).

The intestinal motility, the permeability of the mucosa, the digestion, absorption and secretion are important in the maintenance of this state of a net-water absorption.

I.A.2. Intestinal motility

Movement of contents through the small and large intestine are controlled by the intrinsic activity of the smooth muscles together with the modulating action of the autonomic nervous system and the gastrointestinal hormones (Rozé, 1980). A normal intestinal motility is not only important for a normal movement of contents but, in the small intestine, also for the maintenance of a state in which few micro-organisms are colonizing the small intestine.

The intestinal smooth muscle contractions are regulated by cyclic depolarizations in the muscle membrane (slow waves). The slow waves appear to be generated in cells of the longitudinal muscle layer or in cells that are closely associated with the longitudinal layer and are electronically spreading to the circular muscle layer. Slow waves are more frequent in the duodenum than in the ileum so that intestinal content is pushed forward (Alvarez and Mahoney, 1922; Bortoff, 1965).

The nervous system regulates the intestinal motility through intrinsic and extrinsic reflex arcs (Fig. 1). A variety of sensory receptors (mechano-, chemo-, osmo-, nociceptive and very few temperature and pressure receptors) in the intestinal wall give information about the intestinal content and the mechanical condition of the gut wall to the enteric nervous system.

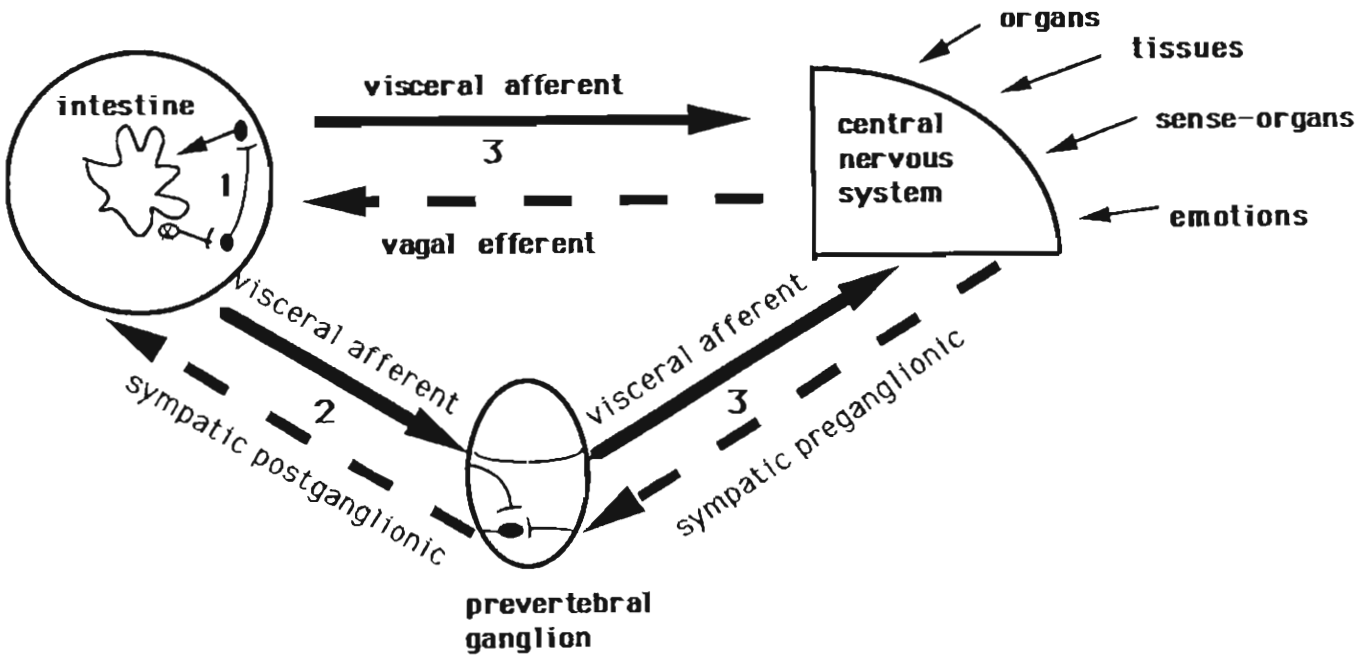


Fig.1. Schematic representation of the intrinsic and extrensic reflex arcs: 1 = intramural reflex arc; 2 = extrinsic reflex arc with synaptic contact in the prevertebral ganglion; 3 = extrensic reflex arcs: visceral afferent nerve fibres make reflex connections with preganglionic autonomic neurons in spinal cord and brainstem; x = receptor in the intestinal wall. After Ooms (1978-1979).

The enteric nervous system is composed out of several nervous plexuses (the subserous plexus, the myenteric plexus, a deep muscular plexus, the submucous plexus and mucosal plexus) and can act autonomically (Goyal, 1983). Frequently, however, its action is regulated by the central nervous system and the sympathetic and parasympathetic nerves. The information of the intestinal receptors usually comes via visceral afferent nerves in the extrinsic reflex arcs. These reflex arcs pass via the prevertebral ganglion and/or the central nervous system. In the central nervous system, this information becomes integrated with multiple inputs from other tissues (muscles, skin), organs and sense-organs (eyes, taste, smell...) and from emotional stimuli (Ooms, 1978-1979). All this information results in a response via efferent nerves which influences the intrinsic reflex arcs. The parasympathetic nerves increase the contraction, whereas the sympathetic nerves inhibit contractions (Ruckebusch, 1981). Furthermore in men, serotinergic nerves have been identified in the myenteric plexus. Serotonin stimulates small intestinal contractions (Daniel, 1968; Wood, 1979).

The intestinal motility is also regulated by the gastrointestinal endocrine system. In men, this system is formed by at least 16 different endocrine-paracrine cells located in the gastrointestinal mucosa between the other epithelial cells (Solcia *et al.*, 1981). In pigs, at least 12 different endocrine cells have been identified in the gastrointestinal tract (Ito *et al.*, 1986). These endocrine-paracrine cells produce peptides which become released by chemical and mechanical stimuli from the intestinal content onto the mucosa, by stimuli of nerves and by the action of drugs and hormones which reach the endocrine-paracrine cells via the circulation (Ooms, 1978-1979; Uvnas-Wallenstein, 1980). The peptides can be released into the gut lumen (exocrine) (Uvnas-Wallenstein, 1980), into the circulation (endocrine) (Solcia *et al.*, 1981) or into its environment (paracrine). In the latter case, a neighbouring cell is the target cell (Solcia *et al.*, 1981; Dockray *et al.*, 1983). Some of these released peptides can influence the intestinal motility by directly acting on intestinal smooth muscle cells or by an effect on the nerves reflex arcs (Rozé, 1980).

The interactions between the nervous and the endocrine system and the fact that the peptides released by the enteroendocrine cells are also found in neurons and nerves of the enteric but also of the extramural nervous system where they function as neurotransmitters, make it extremely difficult to evaluate the role of the enteroendocrine cells in intestinal motility, secretion, absorption and disease (Rozé, 1980; Dockray *et al.*, 1983).

I.A.3. Intestinal permeability, absorption and secretion

The mucosal barrier

In the small intestine, the functions of digestion and absorption are facilitated by a large surface area created by folds, villi and by microvilli (Trier and Madara, 1981). The villi are finger-like projections which are lined by columnar epithelial cells (enterocytes), goblet cells and few enteroendocrine, tuft (caveolated cells), cup cells and within the epithelium overlaying the lymphatic nodules also membranous epithelial cells (M cells) (Madara and Trier, 1987). The villi increase the absorptive surface 7- to 14-fold.

The enterocytes on villi have at their luminal site projections which are called microvilli and which form the brush border. The microvilli amplify approximatively 30-fold the surface of the cells in contact with the intraluminal content. In a neonatal piglet the small intestinal absorptive surface is approximatively 114 m^2 (Whipp, 1978). A continuous filamentous-appearing glycoprotein coat, the glycocalyx, is applied directly to the outer surface of the microvillous membrane. In this surface coat, enzymes are present which play a role in the last steps of digestion (Trier and Madara, 1981; Egberts *et al.*, 1984). There is evidence showing that specific receptors are located on the microvillous membrane-surface coat complex. These receptors selectively bind substances prior to their absorption (Egberts *et al.*, 1984). They can probably also play an important role in bacterial colonization (Dean and Isaacson, 1985; Laux *et al.*, 1986).

The goblet cells produce mucus which formes a layer over the glycocalyx (Mouwen *et al.*, 1983). The enteroendocrine cells release hormones probably at the basal site of the cell in the propria. The function of tuft and cup cells is still unknown.The M cells transport intraluminal antigen to the gut-associated lymphoid tissue (Madara and Trier, 1987).

The crypt epithelium is composed of at least five distinct cell types which are active secretory cells: the Paneth, goblet, enteroendocrine, undifferentiated cells and few tuft cells (Trier and Madara, 1981).

The significance of the Paneth cells, present in crypts in most mammalian species but not in dogs, cats and pigs small intestine, is not yet known (Trier, 1966; Wheeler and Wheeler, 1964). They seem to contain lysozyme, IgA and IgG in men and have phagocytic capacity in rats.

A major function of the undifferentiated crypt cells is the constant renewal of the intestinal epithelium. There are indications that the undifferentiated cells serve as the precursor for all other differentiated intestinal epithelial cells. Following proliferation of these cell, the newly formed immature cuboïdal cells migrate onto the villi, mature meanwhile as they approach the villous tips, where they become extruded and lost into the lumen (Moon, 1978). In the neonate, complete epithelial cell renewal takes 7 to 10 days, whereas this occurs in 2 to 4 days in the adult. In the pig the adult type of gut develops by 3 to 4 weeks of age. The undifferentiated crypt cells secrete water and electrolytes in the crypt lumen (Troughton and Trier, 1969; Cheng and Leblond, 1974; Madara, 1982; Bye *et al.*, 1984).

The mucosa of the colon differs from that of the small intestine in that villi are absent. Crypts, however, extent from the muscularis mucosae to the flat absorptive surface. The epithelium of the lower half of the crypts is composed of proliferating undifferentiated cells, mucus-secreting goblet cells and endocrine epithelial cells. In the upper half of the crypts, undifferentiated cells differentiate to absorptive epithelial cells. The morphology of this latter cell type differs from that of absorptive cells in the small intestine: the microvilli are less abundant and numerous membrane bounded glycoprotein-containing vesicles are present in the apical cytoplasm. The function of these vesicles is not known, their presence suggests that these cells may secrete glycoproteins as well as absorb water and electrolytes.

The cells of the epithelial layer are connected to each other by intercellular attachment zones or junctional complexes which consist out of 3 parts: zonula occludens (tight junctions), zonula adherens (intermediate junctions) and macula adherens (spot desmosomes). The tight junction is

located at the upper part of the lateral plasma membrane and completely encircles the cells. It has important implications with regard to transport phenomena. It allows passive transport of water, cations and probably also small water-soluble non-electrolytes. Its permeability decreases from the duodenum to the rectum. Most dietary nutrients and selected ions can not pass the tight junction and can only pass the epithelial layer via the transcellular route as further described (Turnberg, 1978; Whipp, 1978; Powell, 1981; Binder, 1983).

The intestinal epithelial cell layer forms thus a selective permeability barrier. Transport across this barrier can occur by 2 routes: passive through tight junction and the intercellular space (intercellular route) and active or passive across apical and basolateral cell membranes and the cytoplasm of an enterocyte (transcellular route) (Turnberg, 1978; Powell, 1981)(Fig. 2).

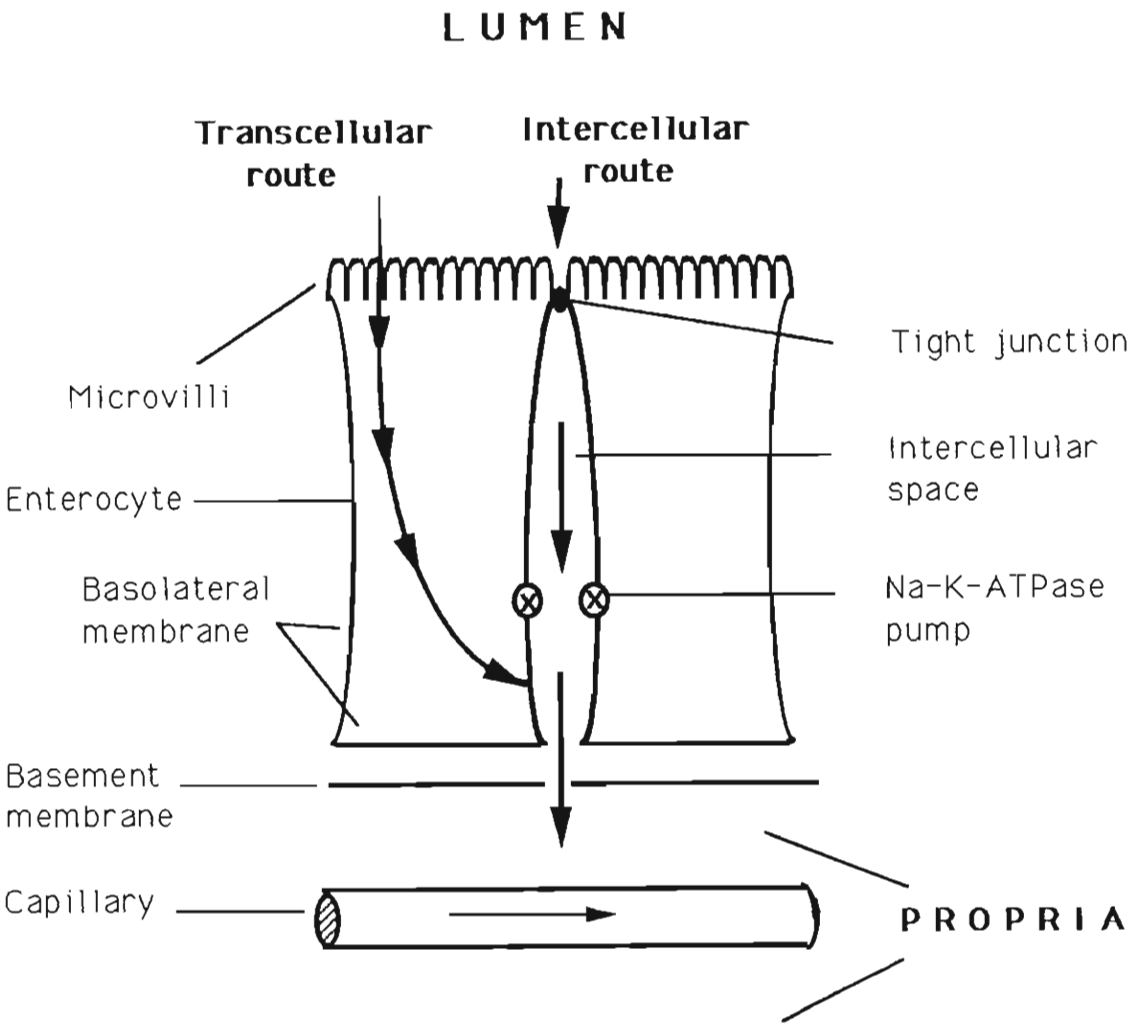


Fig. 2. Schematic representation of two permeation routes across the intestinal epithelium: the transcellular route across apical and basolateral membranes and the cytoplasm of an enterocyte, the intercellular route through tight junction and the intercellular space. After Powell (1981) and Binder (1983).

Transport processes

The absorption process involves the movement of nutrients and electrolytes accompanied by the transport of water. The secretion process involves the secretion of electrolytes or the loss of electrolytes and nutrients followed by the loss of water from the plasma to the lumen. In the small intestine, absorption occurs primarily by villous enterocytes and secretion by crypt cells (Moon, 1978; Aitken, 1984). Under normal circumstances villous absorption exceeds crypt secretion and results in a net absorption in the small intestine and also across the entire intestine.

The movement of water is secondary to both osmotic and hydrostatic pressure gradients established via active solute (ion) transfer. The movement occurs through transcellular and intercellular routes (Whipp, 1978; Argenzio and Whipp, 1980; Powell, 1981) (Fig. 2). This latter pathway is quantitatively most important and depends principally upon the active transport of Na^+ from the enterocytes into the intercellular space. The resulting osmotic gradient across the tight junctions induces water movement into the intercellular space. This space becomes distended, the hydrostatic pressure increases and the isotonic fluid moves through the basement membrane into the interstitium and then into the capillaries and lymphatics (Mailman, 1982; Binder, 1983). Changes in the interstitial hydrostatic or oncotic pressure and in the intraluminal hydrostatic and osmotic pressure will influence transport processes.

The permeability of the intercellular pathway determines to a large degree the net absorption of fluid since ions absorbed via the transcellular route can diffuse back into the intestinal lumen through the tight junction. As described above, the tight junction becomes less permeable from duodenum till rectum. Therefore, water absorption in the large intestine is very efficient.

Three different mechanisms are responsible for electrolyte movement across intestinal mucosa: passive diffusion, convection and carrier-mediated active transport.

Passive diffusion

Electrolyte movement across the tight junction and the intercellular space is a passive process. As mentioned above, the permeability of the tight junctions decreases from duodenum to rectum. In the aqueous channels of the tight junction, fixed negative charges are present. This results in a higher rate of cation movement through the tight junction (Powell, 1981).

The electrolyte transport through epithelial cells (transcellular transport) occurs either passive or active.

The forces responsible for passive ion movement are chemical concentration gradients or electrical potential concentration gradients (Turnburg, 1978; Whipp, 1978; Freel, 1979; Argenzio and Whipp, 1980)

Convection

Convection or solvent drag is solute movement secondary to water flow. This electrolyte transport occurs in the jejunum and is a result of the high permeability of the jejunal mucosa in comparison with the mucosa of the more caudal intestine (Fordtran *et al.* , 1965; Fordtran *et al.* , 1968).

Active absorption in the small intestine and colon

Active transport is an energy-dependent process that results in the net movement against or in the absence of an electrochemical concentration gradient. It can be competitively inhibited. There is ample evidence that active absorption processes are located in villous epithelial cells and active secretion processes are present in crypt epithelium.

* Transcellular electrogenic Na^+ absorption is a two-step process that occurs along the entire intestinal tract (Fig. 3). Na^+ movement into the cell across the luminal cell membrane occurs as a result of a low intracellular Na^+ concentration and electronegativity of the cell interior (electrochemical concentration gradient). Both are induced by the extrusion of Na^+ from the cell across the basolateral membrane in the intercellular space by the ATP dependent Na^+-K^+-ATPase pump (Fig. 3). The serosal side becomes electrically positive compared to the mucosal solution. This leads to a driving force for the diffusional flow of chloride from mucosa to serosa across the epithelium (Schultz and Zalusky, 1964). In the large intestine there are two main differences with the small intestine. First, the tight junctions are less permeable resulting in higher potential differences. This provides a greater driving force for Cl^- absorption. Second, the permeability of the luminal membrane increases as the luminal Na^+ concentration decreases. This enables Na^+ to be absorbed in spite of very low luminal concentrations. Aldosterone probably plays a role in this mechanism (Frizell and Schultz, 1978).

* Neutral Na^+- Cl^- coupled transport (Fig. 3) has been identified in ileum and colon (Frizell *et al.*, 1979). It is not associated with the development of an electrochemical concentration gradient. Na^+ and Cl^- are coupled on a carrier mechanism that mediates the neutral entry of Na^+ and Cl^- into the cell. Na^+ is actively pumped out off the cell by the Na^+-K^+-ATPase pump. The energy for the Cl^- transport across the mucosal membrane is provided by the Na^+ gradient.

* A Na^+-H^+ exchange process (Fig. 3) (Na^+ absorption and H^+ secretion) has been described in the small intestine. This process is stimulated by luminal bicarbonate. (Rector, 1983; Powell, 1987).

* Cl^- absorption and bicarbonate secretion are closely linked (Fig. 3) in the ileum of most animal species and exist in the colon (Hubel, 1968; Tai and Decker, 1980). This mechanism is not affected by the absence of Na^+. It is an active process.

* The absorption of several non-electrolytes across the apical cell membrane (Fig. 3) (e.g. D-hexosen, L-amino acids, oligopeptides, diglycerides, triglycerides and some vitamins and bile salts only in the ileum) is coupled to the absorption of Na^+ and is also carrier mediated. Na^+ is actively extruded across the basolateral membrane by the above described Na^+-K^+-ATPase pump. This process is present in the small intestine and not in the colon (Goldner *et al.*, 1969 ; Schultz and Curran, 1970).

* Short-chain fatty acids (pyruvate, acetate, proprionate and also butyrate) are formed by the bacterial flora in the caecum and colon by fermentation of non-absorbed carbohydrate. They are primarily absorbed by a non-active transport process (non-ionic diffusion) and further, by ionic diffusion of their Na^+ or K^+ salt into the cells. The presence of these short-chain fatty acids can stimulate the colonic absorption of Na^+ and water 2- to 6-fold (Hildman *et al.*, 1980; Ruppin *et al.*, 1980).

Active secretion in the small intestine and colon

* An electrogenic Cl^- secretion (Fig. 3) exists in the jejunum which is initiated either by an increased mucosal cyclic AMP concentration or by increases in the intracellular calcium (Ca^{++}) concentration. A model has been proposed for the Cl^- secretion which says that there is a coupled entrance of Cl^- and Na^+ at the basolateral side of the cell, similar to the coupled entry at the mucosal side. The driving force for the coupled entrance is the Na^+-K^+-ATPase pump. Na^+ is pumped outside and K^+ inside the cell, whereafter K^+ is recycling back across a K^+-permeabel basolateral membrane. The intracellular Na^+ concentration decreases and Na^+ is reentring the cell together with Cl^-. The coupling of Na^+ and Cl^- results in a high intracellular concentration of Cl^-. Chloride will not pass the mucosal membrane since this membrane is relatively impermeabel to Cl^-. This model hypothesizes that changes in cAMP or Ca^{++} increase the Cl^- conductance across the mucosal membrane and thus the active Cl^- secretion (Berridge, 1984; Frizell *et al.*, 1979; Petzinger, 1984).

* Bicarbonate secretion into the lumen occurs in the ileum and colon (Fig. 3). This secretion is mainly due to a Cl^- - bicarbonate exchange (Cl^- absorption and bicarbonate secretion). In the ileum, however, the bicarbonate secretion is probably partly Na^+-dependent (Smith *et al.*, 1985).

* In the colon, a net K^+ secretion occurs which is the resultant of a passive K secretion (potential-dependent) and an active K^+ transport (active absorption and secretion) (Binder and Sandle, 1987).

I.A.4. Mediators of intestinal secretion and absorption

A summary of the mechanisms which are proposed to be involved in the regulation of intestinal secretion and absorption of electrolytes by enterocytes are schematized in Figure 3. Three important intracellular substances are involved in the regulation of secretion and absorption of intestinal epithelial cells nl. Ca^{++}, cAMP and cGMP. Their concentration can be influenced by hormones, neurotransmitters, drugs and bacterial toxins.

Intracellular mediators

Calcium

A decrease in the intracellular Ca^{++} concentration results in a stimulation of the active Na^+ and Cl^- absorption (Donowitz and Asarkof, 1982) , whereas the Ca^{++} ionophore A 23187 which facilitates the transport of calcium across the cell membrane increases the active secretion of Cl^- (Naftalin, 1981). The effects of Ca^{++} in the cell are largely dependent on the intervention of calmodulin, a specific receptor protein. Ca^{++}-calmodulin probably influence the intestinal ion transport by phosphorylation of intestinal proteins (Cheung, 1982; Donowitz *et al.*, 1984a). There are also indications that a part of the effects of Ca^{++} is mediated by the initiation of the arachidonic metabolism and increasing the prostaglandin production (Smith and Field, 1980; Smith and McCabe, 1984). The intracellular Ca^{++} concentration is regulated by extracellular substances which either influence the influx of Ca^{++} into the cell such as serotonin or regulate the intracellular Ca^{++} concentration indirectly by affecting the concentration of cAMP (Naftalin, 1981).

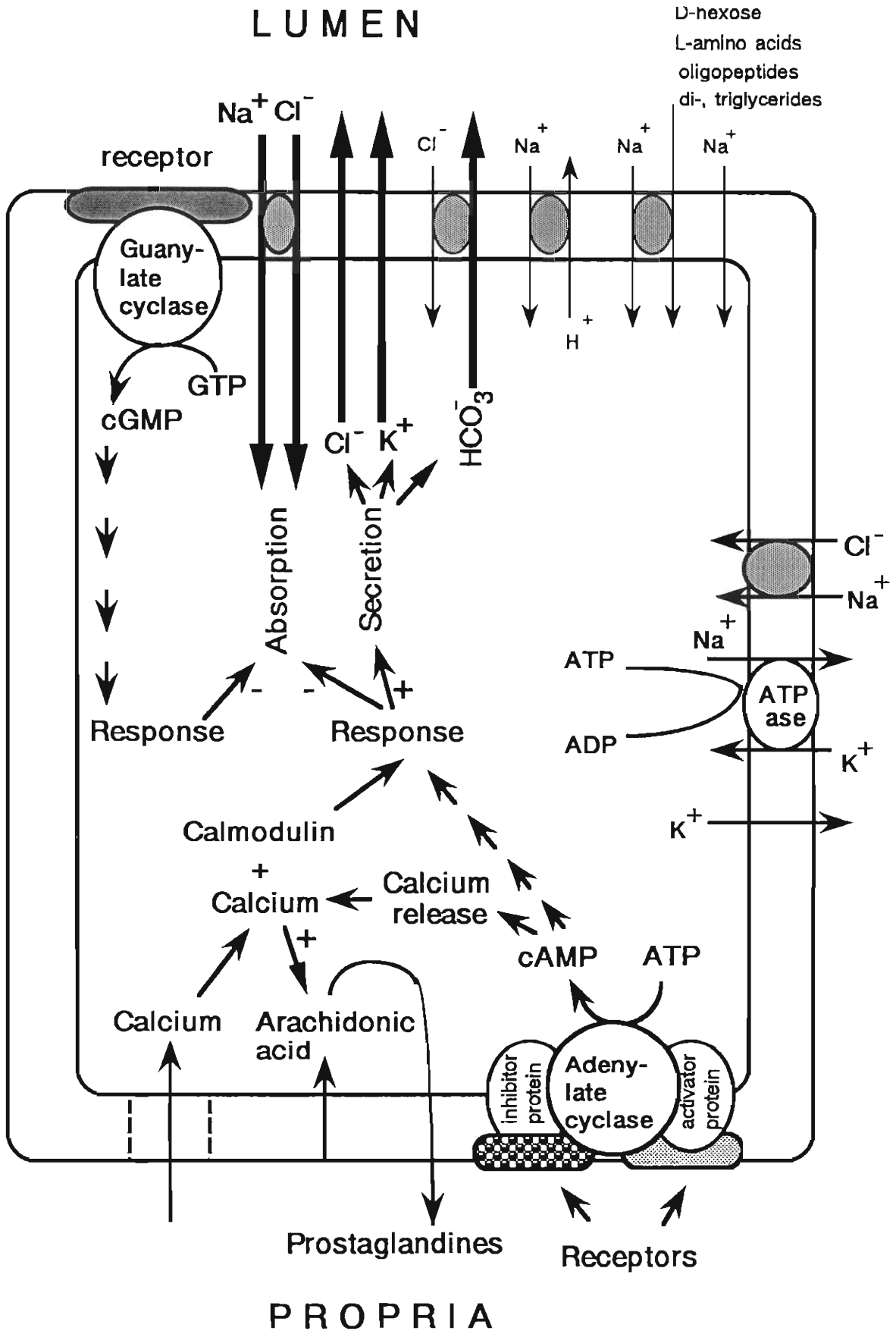


Fig. 3. Schematic representation of the site of action of 3 intracellular substances nl. calcium, cAMP and cGMP on secretion and absorption of electrolytes by enterocytes. The transport processes are drawn if they are present in the same cell.

Cyclic adenosine monophosphate (cAMP)

An adenylate cyclase enzyme complex is localized in the membrane of enterocytes, which is regulated by hormones, neurotransmitters, drugs and bacterial toxins. This complex is composed out of stimulating and inhibiting receptors which are linked to the enzyme through membrane-associated coupling proteins (in Fig. 3, 4 and 6 presented by inhibitor protein = IP and activator protein = AP). Binding to the receptors will cause an interaction of the receptor with the membrane associated coupling proteins which will influence the activity of adenylate cyclase when GTP is present. Activation of this complex results in the production of cAMP (cyclic adenosine monophosphate) out of ATP (adenosine triphosphate). In crypt cells, cAMP stimulates active anion secretion. In villous cells, cAMP stimulates the active anion secretion and also inhibits the coupled Na^+-Cl^- absorption across the luminal membrane of the enterocytes (Nellans *et al.*, 1973; Frizzel *et al.*, 1976). The exact mechanisms of these effects are not known. Possibly, they are caused by phosphorylation of Na^+-Cl^- carriers or channels and Cl^- channels in the membrane by a cAMP-dependent protein kinase and/or by inducing an increase of intracellular Ca^{++} (Hardcastle *et al.*, 1984; Lazo *et al.*, 1984). Ca^{++} would subsequently act on the membrane carrier or channels.

cAMP has been observed to increase the Cl^- permeability in the paracellular pathway (Powell, 1981).

Cyclic guanosine monophosphate (cGMP)

A third intracellular mediator is cGMP. Its concentration is regulated by a guanylate cyclase enzyme complex which is mainly located in the brush border membrane of enterocytes but is also present in basolateral membranes and is also controlled by cell surface receptors (Guerrant *et al.*, 1980; Walling *et al.*, 1978). In rabbits, increase in cGMP results in an inhibition of the Na^+-Cl^- absorption of villous enterocytes and has almost no effect on Cl^- secretion by crypt cells (Guandalini *et al.*, 1982: Petzinger, 1984). Administration of the thermo-stable enterotoxin type a, which stimulates cGMP production, to small intestinal enterocytes of piglets, however, did not induce antiabsorptive effects, but increased Cl^- secretion in crypt and in villous enterocytes (Ahrens and Panichkriangkrai, 1985; Panichkriangkrai and Arhens, 1988).

Extracellular mediators

Serotonin (5-hydroxytryptamine; 5-HT)

5-HT is present in enteroendocrine cells and within neurons where it is stored together with a specific binding protein (Jurio and Gabella, 1974; Ito *et al.*, 1987). Its release from enterochromaffin cells into the circulation can be induced by luminal instillation of hypertonic glucose solutions, by acidification of the duodenum, by hydrostatic pressure on the mucosa and also by cholera toxin and the thermo-labile enterotoxin of enterotoxigenic *Escherichia coli* (Kellum and Jaffe, 1976; Berridge, 1984). Furthermore, several circulating substances and drugs have been shown to influence the release of 5-HT from enterochromaffin cells such as catecholamines, morphine, nicotine and reserpine and to a lesser extent acetylcholine. The effect of the catecholamines can be antagonized by d,l-propanolol. In cats and guinea-pigs, 5-HT can also be

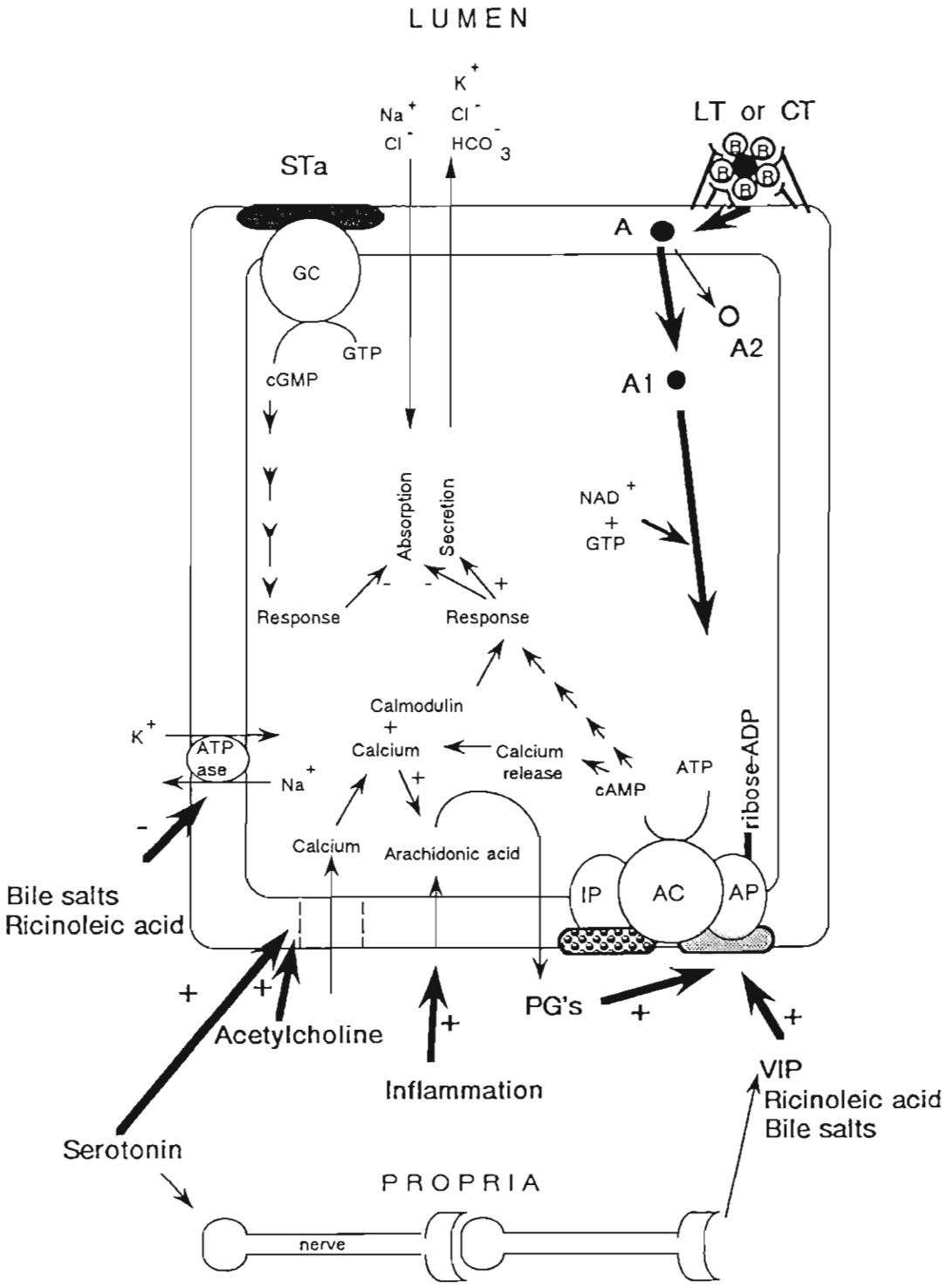


Fig. 4. Schematic representation of the sites of action of some secretagogues on mechanisms involved in secretion and absorption of electrolytes by enterocytes. The secretagogues mostly act by increasing the concentration of one or more of the three intracellular mediators: calcium, cAMP and cGMP. Thermo-labile (LT) and cholera toxin (CT) bind with their B subunits on ganglioside receptors on the cell. The A subunit penetrates into the membrane where it becomescleaved in A1 and A2 fragments. The A1 fragment acts on the activator protein (AP) resulting in an activation of adenylate cyclase. The thermo-stable enterotoxin type a (STa) activates guanylate cyclase (GC) (see also Fig. 3)

released from enterochromaffin cells by stimulation of the vagus (Ahlman *et al.*, 1978).

5-HT can exert a direct effect on enterocytes (paracrine effect) or can stimulate nervous reflex arcs. One of the transmitters released at the efferent end of the reflex arc is vasoactive intestinal peptide (VIP). The effect of VIP on enterocytes is further described. The direct effect of 5-HT is exerted by increasing the entry of Ca^{++} into the cells (Fig. 4). Serotonin has also been observed to induce the release of prostaglandins (Cassuto *et al.*, 1979; Donowitz *et al.*, 1980; Hardcastle *et al.*, 1981; Berridge, 1984; Cooke and Carrey, 1985)

5-Hydroxytryptophan (5-HTP)

5-HTP is a precursor of 5-HT whereto it becomes converted in the gut (Gershon and Ross, 1966).

Vasoactive intestinal polypeptide (VIP)

VIP is a circulating peptide hormone and a neurotransmitter in the gut (Cooke *et al.*, 1984; Cooke *et al.*, 1985; Schwartz *et al.*, 1974). VIP release can be induced by different substances acting from the luminal site onto the intestinal mucosa such as lipids, hypertonic NaCl solution, diluted HCl solution and isotonic phenylalanine, by systemic agents such as Ca^{++}, neostigmine, pentagastrin, oxytocin in the dog and by stimulation of the thoracal vagus in calves. VIP release is also induced by the action of cholera toxin on the small intestine in cats (Cassuto *et al.*, 1979).

Effects of VIP are vasodilation, relaxation of non-vascular smooth muscles, stimulation of bile flow and stimulation of intestinal secretion (Mailman, 1978;Cooke *et al.*, 1984; Cooke *et al.*, 1985; Schwartz *et al.*, 1974)

VIP stimulates adenylate cyclase activity, resulting in an increase in cAMP and subsequently in a decrease in the net Na^+ absorption and a conversion of the net Cl^- absorption to net Cl^- secretion (Fig. 4)(Simon *et al.*, 1981). This effect is not due to increased formation of tissue prostaglandins (Binder *et al.*, 1980). The effect on bicarbonate absorption is not clear. Either a decrease in the net absorption or no effect have been observed upon intravenous administration of VIP (Krejs *et al.*, 1980b ; Davis *et al.*, 1981).

Acetylcholine and cholinergic agonists

Cholinergic agonists inhibit the Na^+ and Cl^- absorption or stimulate Na^+ and Cl^- secretion (Isaacs *et al.*; 1976; Tapper *et al.*, 1978). These effects have been observed in the small intestine and colon in rat and men. They are mediated by receptors on enterocytes from both villi and crypts. Acetylcholine probably also acts on ion transport indirectly via regulation of the intrinsic neural activity (Charney and Donowitz, 1976; Browning *et al.*, 1978). The observed effects on ion transport are indicative for a function of the parasympatic nervous system in the intestinal electrolyte and fluid transport since acetylcholine is a neurotransmitter in the intestine. The effect of acetylcholine on ion transport is Ca^{++} dependent (Fig. 4) (Hubel *et al.*, 1980).

Catecholamines

Adrenalin can stimulate neutral Na-Cl absorption in both the small and large intestine and

decreases the bicarbonate secretion. Especially α_2-adrenergic agonists are very potent stimulators of absorption (Field and McColl, 1973; Tapper *et al.*, 1981; Parsons *et al.*, 1984). The absorption is probably due to a direct effect on epithelial cells since α_2-adrenoreceptors have been demonstrated on intestinal epithelial cells (Field and McColl, 1973; Chang *et al.*, 1983). Isoproterenol can increase the absorption of Na^+, Cl^- and water in the jejunum and ileum and of K^+ in the ileum (Morris and Turnberg, 1981).

The mechanism by which α_2-agonists stimulate absorption is still uncertain. Clonidine, an α_2-adrenoceptor agonist, can decrease Ca^{++} entry into the cell suggesting a close linkage between the receptor and the Ca^{++} entry mechanism (Donowitz *et al.*, 1986). Furthermore, clonidine decreases the basal cAMP content in rabbit ileum, but not in rat ileum (Laburthe *et al.*, 1982; Nakaki *et al.*, 1983; Nakaki *et al.*, 1982b). α_2-Adrenoceptor agonists (clonidine, adrenalin and methoxamine) inhibit the cholera toxin-induced intestinal secretion (Nakaki *et al.*, 1982a) and they can reduce the intracellular cAMP concentration induced by choleratoxin, enterotoxin, theophylline, prostaglandins and VIP (Nakaki *et al.*, 1982b).

Aldosterone

The mineralocorticoid aldosterone can increase Na^+ and Cl^- absorption and K^+ secretion in the colon similar to its effects in the kidneys. Aldosterone causes these effects by opening a Na^+ channel in the apical cell membrane and/or by increasing the sensitivity of these channels, probably by inducing a K^+ channel and by increasing the amount of Na-K-ATPase and the activity of this enzyme. (Field, 1978; Perrone *et al.*, 1984; Binder and Sandle, 1987). Aldosterone exerts no effect on intestinal electrolyte and fluid transport in jejunum and has minimal effect in the ileum. Na^+ depletion is the major stimulant for aldosterone secretion. Therefore, the effects of aldosterone represent an important compensatory mechanism to dehydration.(Binder, 1983; Bywater, 1983; Perrone *et al.*, 1984; Rafestin-Oblin *et al.*, 1984).

Glucocorticoids

Dexamethasone and prednisolone administration in experimental animals has been observed to increase Na^+, Cl^- and water absorption and K^+ secretion in both small intestine and colon. The effects of glucocorticoids in the small intestine are not inhibited by aldosterone blockers (Field, 1978; Charney *et al.*, 1981; Perrone *et al.*, 1984). Probably, glucocorticoids exert these effect on Na-K transport through different receptors than mineralocorticoids. Different cytosolic receptors have been observed in the intestine of rats (Lentze *et al.*, 1985).

The antisecretory effects of glucocorticoids together with their antiinflammatory property, could explain the beneficial effect of glucocorticoids in several diarrhoeal disorders.

Prostaglandins

Prostaglandins (PG) induce secretion by activating adenylate cyclase so decreasing NaCl absorption and increasing Cl^- secretion (Fig. 4) (Kimberg *et al.*, 1971). PGE_2 stimulates the enzyme in the small and large intestine whereas PGD_2 and PGI_2 only activate the enzyme in large intestine. Activation occurs in a dose-related way, indicating a specific drug-receptor interaction.

$PGF_{2\alpha}$ has a less consistent effect (Pierce *et al.*, 1971; Bukhave and Rask-Madsen, 1980). Prostaglandins also stimulated duodenal bicarbonate secretion (Flemstrom and Mylander, 1981; Isenberg *et al.*, 1985). The mechanism of activation of adenylate cyclase is not the same as for VIP since meclofenamic acid antagonizes the action of PG on adenylate cyclase but not of VIP (Simon *et al.*, 1981).

PG's in the intestine are mainly formed by lamina propria cells, whereas the break down mainly occurs in the epithelial cell layer. The formation is caused by inflammation and tissue injury (Fig. 4). Prostaglandins may be produced as a result of activating Ca^{++}-mobilizing receptors on epithelium or on subepithelial tissue (Berridge, 1982; Berridge, 1984; Donowitz, 1985). It has been suggested that the endogenous production of prostaglandins may mediate several diarrhoeal disorders such as salmonellosis and ulcerative colitis in men (Gianella *et al.*, 1975).

Histamine

Histamine has been observed to inhibit the net Cl^- absorption in the ileum of the rabbit. It probably also inhibits bicarbonate secretion. The effect is mediated via H1 receptors (Fromm and Halpern, Linaker *et al.*, 1981).

Opiates and opioid peptides

Synthetic enkephalins, codeine and morphine stimulate active Na^+ and Cl^- absorption in the ileum but have less potent effect on the ion transport in the colon (Fogel and Kaplan, 1984). Opiates can inhibit several secretory processes. It has been suggested that the action of codeine is primary on transit time and not on electrolyte transport.

Glucagon

Infusion of glucagon in the superior mesenteric artery induced intestinal secretion. This secretion was the result of an increased capillary pressure, increased permeability and net Cl^- secretion (Granger *et al.*, 1980; Kaufman *et al.*, 1980).

Somatostatin

Somatostatin is found in both the central nervous system and the intestine. It can induce an increase in the intestinal transit time, stimulate the active Na-Cl absorption in the ileum and colon, inhibit the bicarbonate secretion of pancreas and the bicarbonate secretion in the ileum. Furthermore, it has an inhibitory effect on fluid secretion induced by cholinergic agonists and VIP. It does not act by changing the cAMP or cGMP concentration but seems to inhibit the action of both intracellular mediators of secretion. Further, somatostatin can inhibit the effect of serotonin and other Ca^{++}-dependent intestinal secretory processes (Carter *et al.*, 1978; Guandalini *et al.*, 1980; Dharmsathaphorn *et al.*, 1980; Dobbins *et al.*, 1981). Therefore, the action of somatostatin may be to prevent a change in intracellular Ca^{++} concentration, to block activation of calmodulin or to inhibit the effect of calmodulin (Furness and Costa, 1979).

Bile salts and hydroxy-fatty acids

Bile salts decrease absorption in the jejunum and colon more than in the ileum. They can stimulate net secretion of K^+ in the small intestine (Parkinson and Olson, 1963; Kelly *et al.*, 1980). Bile salts have been observed to activate adenylate cyclase and to increase cAMP levels in the intestinal mucosa of rat and rabbit (Conley *et al.*, 1976; Binder *et al.*, 1978; Binder, 1983), but not in small or large intestine of men (Simon *et al.*, 1981). Furthermore, a decrease in Na-K-ATPase activity and an increase in the mucosal permeability have been observed (Fig. 4) (Hepner and Hofmann, 1973; Binder, 1979). It has been postulated that bile salts stimulate the intestinal fluid secretion via an activation of enteric nerves (Karlström *et al.*, 1986).

Ricinoleic acid

Ricinoleic acid, the active ingredient of castor oil, can induce net intestinal secretion via multiple mechanisms. This hydroxy-fatty acid has been observed to decrease K^+ absorption, active Na^+ absorption and Na-K-ATPase activity and to increase K^+ secretion. An increase in the mucosal cAMP concentration in the small intestine and colon has not consistently been observed (Fig. 4) (Binder *et al.*, 1978; Simon *et al.*, 1981; Binder, 1983). Ricinoleic acid has also been observed to increase the passive Ca^{++} permeability in the jejunal brush border and to increase the permeability of the intestinal barrier (Gaginella and Phillips, 1976; Gaginella *et al.*, 1977; Maenz and Forsyth, 1982). As for bile salts, Karlström *et al.* (1986) hypothesized that nervous reflexes are involved in the intestinal fluid secretion due to Na ricinoleate.

I.B. Pathophysiological mechanisms of diarrhoea

I.B.1. Changes in intestinal motility

Diarrhoea is mostly accompanied with disorders of the intestinal motility. These disorders, however, only have been extensively studied for a limited number of diseases. It was observed that a *Strongyloides* infestation in sheep was accompanied with a decreased motility in the abomasum, an increased jejunal motility and a decreased motility in the spiral colon (Bueno *et al.*, 1980). The alterations in motility are thus not always the same in different regions of the intestinal tract during an infection. Furthermore, the relationship between motility and intestinal transit time has not yet been completely clarified. Read *et al.* (1984) found a weak positive correlation between the frequency of contractions in the cranial jejunum and the transit time of 50 % of the food into the caecum. Hypermotility, however, not always results in an increased transit time. Hypermotility in the colon may result in constipation (Snape, 1983). It is therefore extremely difficult to interpretate the results of intestinal motility studies. Hypermotility and hypomotility will be discussed separately.

Hypermotility

Hypermotility can be primary or secondary. Primary hypermotility can occur as a result of stress, transport, fear or the direct action of products released by tumors or micro-organisms and can lead to diarrhoea. Often, however, hypermotility during diarrhoea is induced by fluid accumulation in the intestine. This fluid accumulation causes a distension of the intestinal wall and, via receptors, a reflectory (intestino-intestinal excitatoric reflex) increase in the motility (secondary hypermotility) (Ooms, 1978-1979; Argenzio, 1980; Argenzio and Whipp, 1980; Ruckebusch, 1981). Besides, due to fluid accumulation, mucosal damage seen during some infections may induce hypermotility. This was observed following infection with an invasive strain of *E. coli* (Burns *et al.*, 1978) and during the intestinal phase of infection with Trichinella spiralis in rats (Palmer *et al.*, 1984). Several invasive bacteria and bacterial enterotoxins have been mentioned to induce propulsive contractions in the ileum of rabbits so for instance *Salmonella typhimurium*, *Vibrio cholerae*, *Escherichia coli* and the enterotoxins of both latter micro-organisms (Mathias *et al.*, 1982; Borriello, 1984).

The fluid loss due to hypermotility is the result of the decreased transit time and a subsequent maldigestion and malabsorption (see I.B.3.).

Hypomotility

Hypomotility can be caused by irritation of the peritoneum (peritonitis), anaesthesia, laparotomy, administration of drugs (e.a. opiates, ...) (Bueno *et al.*, 1978; Ruckebusch, 1981; Malagelada, 1984), by the thermo-stable enterotoxin of *Escherichia coli* (Pesti, 1979; Mathias *et al.*, 1982) and by stimulation of painreceptors in the gut wall. Severe distension of an intestinal segment can reflectoric (intestino-inhibitory reflex) induce a reduced intestinal motility (Ooms, 1978-1979; Argenzio, 1980; Ruckebusch, 1981). *Strongylides* infestation in ponies has been observed to reduce ileal, caecal and colonic motility, probably due to a mechanical effect of the parasites (Bueno *et al.*, 1979). Furthermore, endotoxins have been observed to decrease the gastrointestinal motility in neonatal calves. Endotoxins can be present in the intestinal tract during some bacterial enteritis (Bueno *et al.*, 1980).

A decrease in the intestinal motility results in retention of digesta and subsequently in bacterial overgrowth (Borriello, 1984; Scott and Cahall, 1982). Bacterial fermentation of the intestinal content results in the formation of more and smaller molecules causing an increase in the intraluminal osmolarity. This causes flow of water from the interstitium into the intestinal lumen (Caspary *et al.*, 1981). Due to the bacterial fermentation metabolites such as deconjugated bile acids, hydroxylated fatty acids and organic acids will be formed which can stimulate the intestinal secretion in small and large intestine. They can also damage the enterocytes so increasing the intestinal permeability and enhancing the malabsorption. The deconjugation of the bile salts can results in fat maldigestion and malabsorption and in steatorrhea (Donaldson and Toskes, 1983; Borriello, 1984). Due to the fluid accumulation in the lumen a distension of the gut wall occurs and a reflectoric increase of the intestinal motility can be the result. Furthermore, some of the

metabolites can increase intestinal motility (Mathias and Clench, 1985). Hypermotility can thus follow on a period of hypomotility.

I.B.2. Changes in intestinal permeability

An increase in the intercellular or transcellular permeability can result in fluid accumulation in the intestinal tract and subsequently in diarrhoea.

Intercellular permeability

The intercellular permeability can increase due to for the intestine "stressing" situations such as an increased interstitial (in thepropria) hydrostatic pressure (Cooper *et al.*, 1978; Moon,

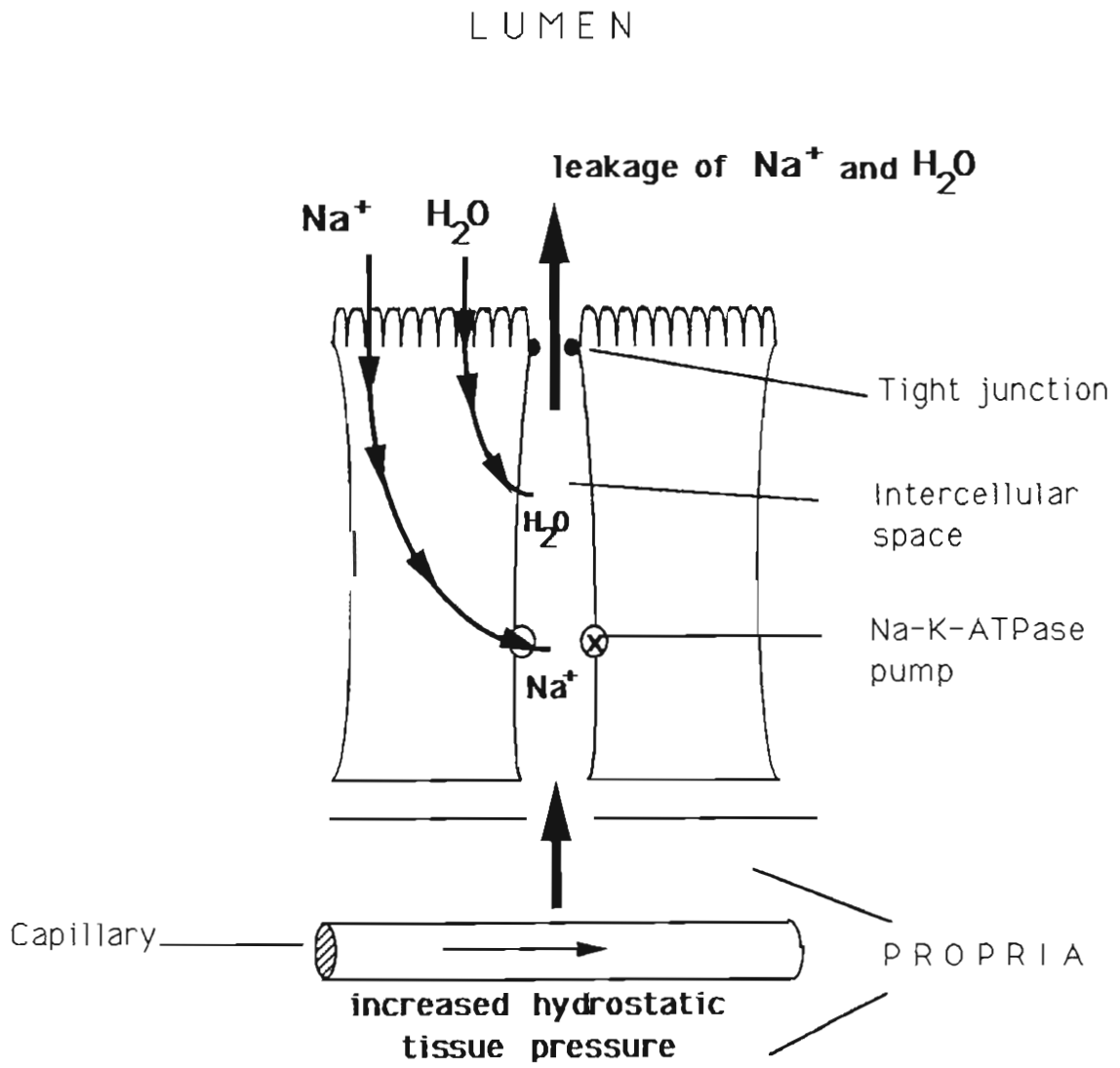


Fig. 5. Proposed model to explain the effect of increased hydrostatic tissue pressure on the intercellular permeability and so in the flow of water and electrolytes across the intestinal mucosa. Water and electrolytes "leak" through the open tight junction down the pressure gradient from blood to the intestinal cavity . After Argenzio and Whipp (1980).

1978; Turnberg, 1978; Argenzio and Whipp, 1980; Powell, 1981). The consequence of this elevated interstitial pressure will depend on the degree of widening of the tight junctions. A slight widening results in "leakage" of Na^+ and water from the intercellular space into the lumen (Fig.5). A larger widening will results in the passage of proteins from the interstitium into the lumen. The colloid osmotic pressure in the interstitium and subsequently in plasma will decrease and more water will pass through the epithelial barrier into the lumen (protein-losing enteropathy). An increased interstitial hydrostatic pressure can be caused by a rise in the venous pressure (Argenzio and Whipp, 1980; Mailman, 1982) , by lymphatic obstruction (Moon, 1978), by a decreased plasmic colloid osmotic pressure (Argenzio and Whipp, 1980) and by an increased capillary permeability as occurs during inflammation (Moon, 1978; Argenzio and Whipp, 1980; Morris, 1984).

Metabolites of bacterial fermentation such as dihydroxy-bile salts and hydroxy fatty acids have also been mentioned to increase the intercellular permeability. The hydroxy fatty acid ricinoleic acid (the active principle of castor oil) has probably a similar effect (Powell, 1981).

Malnutrition and an increased intraluminal osmotic pressure can probably also induce an increase in the intercellular permeability (Cooper *et al.* , 1978).

Transcellular permeability

Destruction of enterocytes results in an increase in the transcellular permeability (Moon, 1978). The interruption in the epithelial barrier will result in "leakage" of proteins from the propria and submucosa into the intestinal lumen (protein-losing enteropathy). The decreased colloid osmotic pressure will result in fluid loss into the intestinal cavity, this together with a decrease in the absorptive surface will result in diarrhoea. If endothelial cells of blood-vessels in the propria and/or submucosa become damaged, than a hemorrhagic exsudate will flow into the intestinal lumen.

Damage of intestinal epithelial cells can occur following viral or bacterial infections and parasitic infestations, due to the action of toxins on the intestinal mucosa or as a result of villous hypoxy (Moon, 1978; Harris, 1980; Mailman, 1982; Jodal *et al*, 1984; Keljo *et al.*, 1985).

I.B.3. Maldigestion-malabsorption

Maldigestion

Maldigestion refers to the situation in which the intestinal absorptive capacity is intact but in which there is an incomplete digestive breakdown of large molecules. The undigested molecules are not absorbed and accumulate within the lumen of the intestine, increasing the intraluminal osmotic pressure and the flow of fluid from blood into the intestinal lumen. In the large intestine the undigested food will enhance bacterial growth and fermentation. This further increases the number of osmotic active particles and the influx of water and electrolytes. If carbohydrates are involved in the incomplete digestion, they will be fermented to lactate. The luminal bicarbonate

buffer will become overwhelmed, the pH will decrease and will probably further interfere with the absorptive processes in the colon (Powell, 1981). The maldigestion thus leads to a malabsorption (Moon, 1978; Argenzio and Whipp, 1980; Caspary *et al.*, 1981).

The absence or deficit in a digestive enzyme can be absolute and congenital or due to a disease (hepatic, pancreatic disease, ...). It, however, can also be relative as occurs following a sudden change in diet. The enzymes of the intestine have to adapt themselves to the quantity (Wittenbrink *et al.*, 1984) and type (Etheridge *et al.*, 1984) of the diet in the lumen. Temporary diarrhoea can occur.

Malabsorption

Maldigestion is characterized by structural abnormalities in the intestinal mucosa which result in impairment of the intestinal absorptive capacity. Mostly the structural abnormality is accompanied with a decrease in the epithelial surface area. The degree of the damage can vary from an effect on microvilli with an impaired celfunction (Boch *et al.*, 1982; Cox, 1983) to a destruction of enterocytes, as occurs following viral infections (coronaviruses, rotaviruses and parvovirus) (Aitken, 1984; Moon, 1978; Ducatelle *et al.*, 1981), infections with invasive bacteria, with coccidia and parasitic infestations such as *Trichostrongylus colubriformis*.

Destruction of villous enterocytes can lead to a mild or severe villous atrophy depending on the degree of the damage. The result is an impaired absorption of among others fat, glucose, Na^+, iron and water (Moon, 1978). Villous regeneration occurs from the undifferentiated crypt epithelial cells. These cells proliferate and the newly formed cells migrate onto the villi. The immature cells are not capable of absorbing glucose. Since the absorption of glucose is linked with this of Na^+, less Na^+ and subsequently less water will become absorbed.

Hypersensivity to dietary antigen has also been mentioned to induce a malabsorption. The hypersensitivity response, especially in type IV reaction, can induce a proliferation of crypt cells resulting in the elongation of crypts, an increase in villous height and an increased shedding of enterocytes at the villous top. So, the villi become covered with more immature than mature enterocytes and the absorptive capacity of the intestine decreases (Fergusan and McDonald, 1977; Mowat and Fergusan, 1981).

The thermo-stable enterotoxin type b (STb) produced by some enterotoxigenic *Escherichia coli* strains has been observed to induce a distinct villous atrophy, partly responsible for the small intestinal fluid loss induced by this enterotoxin. The mechanism by which the villous atrophy is induced, is not known (Whipp *et al.*, 1987).

Destruction of crypt epithelial cells is the characteristic feature of an infection with the canine parvovirus type 2 and feline parvovirus (Pedersen, 1987; Appel and Parrish, 1987). Complete villous atrophy without regeneration can occur.

Some antibiotics have been observed to induce malabsorption such as peroral neomycine therapy in men (Jacobson *et al.*, 1960) and chloramphenicol in calves (Mero *et al.*, 1985).

I.B.4. Hypersecretion

Some bacteria produce exotoxines which increase the small intestinal secretion (Fig. 4). These toxins act by stimulating the production of one or more of the intercellular mediators of the intestinal secretion. Hypersecretory diarrhoea is caused by enterotoxins of *Vibrio cholerae* , *Escherichia coli* , *Salmonella* Typhimurium and some other bacteria (Table 1) (Peterson *et al.*, 1983; Ruiz-Palacios, *et al.*, 1983; Malagelada, 1984; Harris, 1988; Charney *et al.*, 1976).

Table 1. Some enterotoxin producing bacteria stimulating the intestinal secretion and the site and mode of action of their enterotoxins.

Bacteria	Enterotoxin	
	Site of action	Mode of action
Vibrio cholerae (cholera toxin)	small intestine	activation of cAMP
Enterotoxigenic *E. coli* (LT enterotoxin)	small intestine	activation of cAMP
Enterotoxigenic *E. coli* (STa enterotoxin)	small intestine	activation of cGMP
Enterotoxigenic *E. coli* (STb enterotoxin)	small intestine	?
Salmonella serovars (enterotoxin)	not determined	activation of cAMP
Aeromonas species (enterotoxin)	small intestine	activation of cAMP
Pseudomonas aeruginosa (enterotoxin)	not determined	activation of cAMP
Shigella dysenteriae (enterotoxin)	not determined	activation of cAMP
Campylobacter jejuni (enterotoxin)	not determined	activation of cAMP
Yersinia enterocolitica (enterotoxin)	small intestine	activation of cGMP
Klebsiella pneumoniae (enterotoxin)	not determined	activation of cGMP

Some of these toxins can stimulate adenylate cyclase to such an extent that a secretory diarrhoea occurs. This diarrhoea is accompanied with loss of fluid that is isotonic, alkaline (HCO_3^-) and rich in electrolytes (Na^+, K^+, Cl^-). The structure of the mucosa remains intact. The absorption of glucose, amino acids and bile salts is unchanged. The osmotic gradient remains the same (Moon, 1978). The mechanism of this secretory diarrhoea is best studied for cholera toxin (CT) and the thermo-labile (LT) enterotoxin of *Escherichia coli* (Field, 1979; Holmgren, 1981; Berridge, 1984; Petzinger, 1984)(Fig. 4). Both enterotoxins are so called A-B toxins.The term **A-B** toxin refers to a multi-unit toxin in which the **B** component is involved in the **binding** of the toxin to the target cell and the **A** component **activates** adenylate cyclase. CT and LT are composed out of 5 B subunits and 1 A subunit. The 5 B subunits can adhere to ganglioside receptors on enterocytes. For CT these receptors are GM1 gangliosides, for LT GM1, GD1b gangliosides and an unidentified monoganglioside (Holmgren *et al.*, 1985) . Following binding, the A subunit passes into the membrane where it becomes reduced to form A1 and A2 fragments. The A1 fragment is an ADP-ribosyltransferase which catalyses the transfer of ADP-ribose of nicotinamide

adenine dinucleotide (NAD) to the activator protein which subsequently can activate adenylate cyclase. The adenylate cyclase is located at the basolateral membrane of the cell. Activation of adenylate cyclase results in an increased production of cAMP which will act as a second messenger (Moss *et al.*, 1984).

Besides their effect on adenylate cyclase, LT and CT have been observed to stimulate the release of serotonin by enterochromaffin (=enteroendocrine) cells (Cassuto *et al.*, 1981; Berridge, 1984). CT stimulates the secretion of intestinal mucin in the rat and rabbit small intestine (Yardley *et al.*, 1972; Forstner *et al.*, 1981). Lundgren and coworkers have suggested that the enteric nervous system is involved in cholera toxin induced secretion in cat and rat (Cassuto *et al.*, 1983; Eklund *et al.*, 1987). There still is controverse to whether PGE_2 plays a role in CT induced diarrhoea. In rat ileal loops no release of PGE_2 could be demonstrated due to the action of cholera toxin (Smith *et al.*, 1985), whereas in cholera patients in Bangladesh elevated jejunal PGE_2 concentrations were observed (Speelman *et al.*, 1985).

Some ETEC strains produce heat-stable enterotoxins (ST). Two different types were identified namely the ST a and the ST b enterotoxins. The ST a enterotoxin is methanol soluble and induces intestinal secretion in infant mice and newborn pigs but not in 8-week-old pigs (Burgess *et al.*, 1978). In 8-week-old pigs the net absorption is reduced (Whipp *et al.*, 1981). STa binds to receptors on enterocytes and subsequently activates an enzyme complex located in the membrane of the cell, the guanylate cyclase complex, resulting in an increased production of cGMP (Frantz *et al.*, 1984). The STb enterotoxin is relatively methanol insoluble and induces intestinal secretion in newborn pigs and 8-week-old pigs but not in suckling mice (Burgess *et al.*, 1978). The exact mechanism of action of the STb enterotoxin has not yet been completely elucidated. It has been observed that this enterotoxin induces a slight villous atrophy through an unknown mechanism. Furthermore, the toxin can probably induce an impaired active alanine absorption (Whipp *et al.*, 1987). Weikel *et al.* (1986) observed that ST b stimulates the active secretion of an anion which is not Cl^-. This anion was thought to be bicarbonate. Eklund *et al.* (1985) proposed that a nervous mechanism is mediating part of the secretory response to a mixture of ST a and ST b enterotoxins. The STb enterotoxin is susceptible to trypsine degradation (Whipp, 1987).

In the pig colon, ST a and cholera toxin abolish the net Na^+ absorption, reduce the net Cl^- absorption and elicit the net bicarbonate secretion, probably due to an uncoupling of the Na^+-H and Cl-HCO_3 exchange processes. ST a selectively activates cGMP, whereas cholera toxin selectively activates cAMP (Argenzio and Whipp, 1981).

Some strains of *Salmonella* Typhimurium induce an inflammation in the intestinal submucosa which results in the production of prostaglandins. The effect of prostaglandins on the intestinal electrolyte and water transport has been described above. Prostaglandins induce hypersecretion in the small, as well as in the large intestine (Gianella *et al.*, 1975). There is evidence that *Salmonella* produces a cholera-toxin-like toxin. The role of this toxin in the diarrhoea still has to be determined (Peterson *et al.*, 1983).

Enterotoxins with effect on the intestinal fluid transport are also produced by *Yersinia enterocolitica*. An LT and ST enterotoxin have been described. The effect of the LT is not known. The ST enterotoxin increases the intracellular cGMP concentration. Furthermore, this enterotoxin

causes morphological changes in the villous enterocytes and propria of the intestinal mucosa (Harris, 1988).

Other enteropathogenic bacteria which have been observed to produce enterotoxins are *Campylobacter jejuni* and *Pseudomonas aeruginosa.*

I.C. Etiology and pathophysiology of post-weaning diarrhoea (PWD) in piglets.

In young piglets, diarrhoea is an important cause of poor feed conversion, growth retardation and death as a result of severe dehydration. Three diarrhoeal disease entities can be recognized in piglets: neonatal diarrhoea, diarrhoea between 3 days and 3 to 4 weeks of age and post-weaning diarrhoea (PWD).

The neonatal piglet has a very efficient small intestinal absorption of water and electrolytes, whereas its large intestinal absorption is not very efficient. Furthermore, the regeneration capacity of its small intestinal villi is slow. It takes approximatively 7 days to completely renew its villous-enterocytes (Moon *et al.*, 1978). Infections increasing small intestinal fluid loss can therefore easily induce severe diarrhoea (Argenzio, 1985).

When piglets become older, the efficiency of their small intestinal absorption decreases and the rapidity with which villous epithelial cells can be replaced increases. The replacement of cells is 3 times higher in the 3-week-old than in the neonatal piglet (Moon *et al.*, 1975; Moon *et al.*, 1978). The large intestine becomes anatomically and functionally more developed and is capable to increase the normal fluid absorption capacity six times in the 3-week-old piglet, whereas no such increased fluid compensation occurs in the neonatal piglet (Argenzio *et al.*, 1984). The older piglet is therefore much more resistant to the diarrhoea inducing effect of small intestinal enteropathogens. Furthermore, the older piglet is less susceptible to intestinal secretion due to LT-enterotoxin (McEwan *et al.*, 1990).

Enterogenic *Escherichia coli* (ETEC) has been implicated as an important causal agent of post-weaning diarrhoea in piglets. However, experimental infection with ETEC of 3- to 4-week-old piglets does not consistently induce diarrhoea (Smith and Halls, 1968 ; Cox *et al.*, 1986). This could be due to the age-related resistance of piglets to small intestinal diarrhoea. An infection with ETEC alone can be insufficient to induce enough small intestinal fluid loss for diarrhoea to occur. In the presence of complicating factors the compensatory capacity of the intestine can become overwhelmed resulting in diarrhoea.

One of these complicating factors is induced by the weaning process itself. In the normal small intestine there is a net absorption of fluid. Post-weaning, however, only a small amount of net water absorption or even a net secretion has been shown to occur in the piglets' small intestine (Hamilton, 1974; Hamilton and Roe, 1977). An explanation for this labile fluid balance is given by morphological studies of the effect of weaning on the small intestine. These studies revealed that villous atrophy, sometimes together with crypt hypertrophy, occur post-weaning (Kenworthy, 1976; Deprez, 1988; Miller *et al.*, 1984b), resulting in a decrease in the small intestinal absorptive capacity (Miller *et al.*, 1984a). The small intestine of the weanling piglet is thus in a delicate

situation with regard to the net fluid balance. When newly-weaned piglets are infected with ETEC, the produced enterotoxins will induce the secretion of more fluid in the small intestine, so that the absorptive capacity of the colon can become overcome and post-weaning diarrhoea can occur.

Other factors which either cause small intestinal malabsorption and/or factors which enhance the colonization of the small intestine with ETEC can play a role in the multifactorial diarrhoea syndrome occurring in newly-weaned piglets. Some of these factors are given below.

I.C.1. Malabsorption

Change from milk diet to a lesser digestible dry diet

The villous atrophy which can be observed post-weaning has been attributed to the change from milk diet to a lesser digestible dry diet (Kenworthy, 1976; Deprez, 1988). The mechanism by which the dry diet causes this reduction in villous height has not yet been clarified. A mechanical effect of the dry food on the villous mucosa, an inflammatory response to bacterial toxins and/or to digestion products of the host or of his residual gut microflora (Kenworthy, 1976) and a hypersensitive response to new dietary antigens (Miller *et al.*, 1984b) have been mentioned as plausable explanations.

Enteric viral infection

Villous atrophy can also be induced by infections with enteropathogenic viruses such as rotavirus and transmissible gastroenteritis virus (TGEV) (Moon *et al.*, 1973; Shaw *et al.*, 1989). Concurrent infections of ETEC and rotavirus or TGEV have been reported in piglets with post-weaning diarrhoea (Tzipori *et al.*, 1980b; Wilson and Francis, 1986; Pritchard, 1987). A combined experimental infection of newly-weaned piglets with rotavirus and ETEC caused a more severe diarrhoea than an infection with either pathogen alone (Tzipori *et al.*, 1980a; Kyriakis, 1982; Lecce *et al.*, 1982). Underdahl *et al.* (1972) obtained similar results using a combined infection with a cell-cultured attenuated strain of TGEV in combination with a mildly virulent *E. coli* strain. Pritchard (1987) observed a more severe diarrhoea and mortality in endemically with TGEV infected herds when a concurrent infection with ETEC occurred.

Other enteropathogenic microorganisms

An infections with coccidia (Coussement *et al.*, 1981) or *Cryptosporidium* (Tzipori *et al.*, 1981) can occur in weaned piglets and is capable to induce villous atrophy. The role of both parasites in weanling diarrhoea has not yet been determined.

Thermo-stable enterotoxin type b (STb)

It has been shown that the STb enterotoxin can induce partial villous atrophy in jejunal loops of 2- to 6-week-old piglets exposed during 2 hours to the enterotoxin (Rose *et al.*, 1987; Whipp *et al.*, 1987).

Antibiotics

In men, it is well known that oral administration of neomycine can induce a reversible villous atrophy resulting in malabsorption (Jacobson *et al.*, 1960; Roger *et al.*, 1966). In calves, oral chloramphenicol administration in a dose of 50 mg/kg 2 times a day has been observed to induce a reduction in villous height and crypt depth (Mero *et al.*, 1985). The mechanism for the chloramphenicol-induced villous atrophy is unknown.

I.C.2 Colonization of the small intestine with ETEC

Several factors have been proposed to enhance the colonization of the small intestine with ETEC.

Withdrawal of lactogenic antibodies

The suckling piglet is protected from enteric infection by immunoglobulins (anti-adhesive, anti-toxic, plasmid curing) and probably also by other anti-bacterial agents such as lactoferrin and transferrin present in colostrum and milk (Nagy *et al.*, 1979; Sellwood, 1983; Porter *et al.*, 1978). Immunoglobulins in sow colostrum and milk can inhibit the adhesion of the ETEC strains to the small intestinal mucosa (Nagy *et al.*, 1976). This anti-adhesive capacity of sows' milk remains present in vaccinated sows until 4 to 5 weeks post partum (Nagy *et al.*, 1979). Besides its anti-adhesive effect, sows' milk can contain antibodies which can cure K88 plasmids out of *E. coli* strains, and which can block the plasmid-transfer of drug resistance (Porter *et al.*, 1985). Furthermore, milk of immune sows exerts an opsonic activity suggesting an important role for opsonophagocytosis as a defence mechanism against ETEC colonization (Sellwood, 1983). Recently, Deprez *et al.* (1986) demonstrated that sows' milk inhibits the rise of fecal hemolytic *E. coli* shedding. So at weaning, the milk diet is replaced by a dry diet without immunoglobulins or other immune factors, allowing ETEC to proliferate.

Malabsorption-Maldigestion

The malabsorption as a result of small intestinal villous atrophy and the maldigestion occurring as a result of the change at weaning from a good digestible milk diet to a lesser digestible dry diet results in an increased amount of nutrients in the small intestinal lumen. It has been suggested that this increased amount of nutrients can induce a proliferation of ETEC, so enhancing

the colonization of villi with ETEC (Lecce *et al.*, 1983; Tzipori *et al.*, 1984). Deprez (1988), however, could inhibit the villous atrophy occurring post-weaning by adding water (2 volumes) to the dry diet (1 volume). Although villi remained normal, there still was a proliferation of hemolytic *E. coli* strains. This indicates that the malabsorption occurring post-weaning is not the most important factor inducing proliferation of *E. coli*.

Rotavirus

Lecce *et al.* (1982) demonstrated that pigs which became inoculated with hemolytic *E. coli* during the acute but not the postrecovery phase of a rotavirus infection developed a shift of their normal bacterial flora from nonhemolytic to hemolytic *E. coli*. They suggested that a rotavirus infection interferes with the normal clearing mechanisms of the gut either by nonspecifically negating a gross protector of gut colonization or by a specific effect such as altering the binding sites on villous enterocytes so that the adhesion of ETEC strains to villi becomes facilitated. Benfield *et al.* (1988) investigated the effect of a rotavirus infection on a subsequent inoculation with K99 ETEC in 3-day-old gnotobiotic piglets. They could not demonstrate a stimulating effect of the rotavirus infection on the ability of the K99 ETEC to colonize the small intestine. The combined inoculation resulted in diarrhoea which was an additive effect of both agents and not a synergistic effect such as has been reported in calves.

K99 *E. coli* can colonize the caudal small intestine of neonatal piglets. Three-week-old piglets, however, are resistant to adhesion of $K99^+$ *E. coli* to their villi. It still has to be determined if a rotavirus infection in newly-weaned piglets could make them susceptible again to an infection with $K99^+$ *E. coli*. This could perhaps explain the presence of these fimbriae producing ETEC in piglets with post-weaning diarrhoea (Francis *et al.*, 1982; Wilson and Francis, 1986; Söderlind *et al.*, 1988).

Antibiotics

It has been observed that oral administration of antibiotics immediately post-weaning can select for antibiotic resistant ETEC strains which then rapidly can become the dominant strain in the faecal flora (Hinton *et al.*, 1985). Antibiotics such as ampicillin, chloramphenicol, furazolidone and cloxacillin Na^+, can increase the colonization of the intestinal tract by potentially pathogenic microorganisms by suppressing the normal anaerobic flora. These antibiotics can induce an increase in the number of fecal coliforms within 4 days after administration (Kaufman, 1984).

Fimbriae and receptors or receptor-sites

K88 (F4), K99 (F5), P987 (F6) and F41 fimbriae producing ETEC strains can induce colibacillary diarrhoea in neonatal piglets (Morris and Sojka, 1985; Parry S.H. an Rooke D.M., 1985; Renault and le Bourhis, 1985; Nakazawa *et al.*, 1987; Söderlind *et al.*, 1988). It has been shown that the epithelium in the small intestine develops resistance to adhesion of K99 and $P987^+$

E. coli with increasing age (Runnels *et al.*, 1980; Dean *et al.*, 1989). Probably such an age-related resistance also exists for the F41 fimbriae. This can explain why in cases of weanling diarrhoea most often K88 fimbriae producing ETEC strains are isolated. Nevertheless, K99 or P987 positive ETEC strains can be identified in faeces from piglets with PWD (Wilson and Francis, 1986; Söderlind *et al.*, 1988). In one study, however, (Nakazawa *et al.* 1987) more K99 than K88 positive ETEC strains were isolated from piglets with PWD. A possible explanation for this finding could be that the K99 strains they found produced not only K99 fimbriae but also other unknown fimbriae. Arguments in favor of this hypothesis are the fact that several authors described the isolation of K88, K99, F41 and P987 fimbriae negative ETEC strains from piglets with PWD (Wilson and Francis, 1986; Nakazawa *et al.*, 1987; Söderlind *et al.*, 1988; Nagy *et al.*, 1990) and that recently a new fimbria has been identified on ETEC strains isolated from piglets with PWD nl. F107 (Stamm *et al.*, 1990). The ETEC strains produced these fimbria *in vivo* but not in culture. Another explanation for the presence of K99 positive ETEC in some cases of PWD could be that not all piglets become resistant to K99 adhesion at the same age.

The age of the piglets does not influence the adhesion of K88 positive *E. coli* to villi. Of the K88 fimbriae, 3 serological variants have been identified nl K88ab, K88ac and K88ad (Guinée and Jansen, 1979).Not all piglets posses receptors or receptor sites for one or more of these K88 variants; in some piglets one or more of the K88 variants show a strong, in some a weak and in others no adhesion to their villi (Bijlsma *et al.*, 1982; Sellwood, 1983; Bijlsma and Bouw, 1985). Decreased adhesion, as well as resistance to adhesion are most probably genetically determined and result in a congenital resistance of piglets to K88-mediated colibacillosis (Bijlsma and Bouw, 1985).

PH in the gastrointestinal tract

The gastric acidity has been mentioned to play an important role in protection against infection with ETEC. It has been suggested that the pH of the gastric content increases after weaning to non-bactericidal values which allows infection with and proliferation of ETEC in the stomach. Subsequently ETEC can infect and colonize the small intestine (Schulman, 1973). Prohaszka and Baron (1980) could experimentally show that weaning piglets to a diet with a high protein supply resulted in a rise in gastric pH above 5 and that feeding this diet caused an increase in the faecal flora of hemolytic *E. coli* and ETEC strains accompanied with severe diarrhoea. Hampson *et al.* (1985) found a significant correlation between the gastric pH and the number of coliform organisms in the gastric content. They, however, could not demonstrate a rise in gastric pH after weaning. A higher pH in gut contents is observed in iron deficient piglets and could be caused by an atrophic gastritis and hypochlorhydria seen in these piglets (Larkin and Hannan, 1985).

Francis *et al.* (1989) demonstrated a correlation between the K99 fimbriae expression and pH of the intestinal fluid. Low pH values, such as normally occur in the proximal intestinal fluid, suppressed the K99 production. A higher gastric pH will result in an increased pH in the cranial small intestine. It is not known of pH also influences the production of other fimbriae.

Environmental factors

Several conditions have been observed to influence the onset of diarrhoea and oedema disease after weaning. Madec and Josse (1983) described 10 predisposing factors that were connected and had a cumulative effect nl. (1) overcrowding, (2) a high temperature fluctuation, (3) bacterial pollution of drinking water, (4) a low feeding level of the sow, (5) a recent episode of transmissible gastroenteritis virus or porcine epidemic diarrhoea virus infection, (6) an average weight of the piglets at weaning of less than 9 kg, (7) a high heterogenicity in the weaning weights, (8) a feed intake of the piglet during the suckling period of less than 150 g/kg body weight at weaning, (9) the presence of diarrhoea during the suckling period and (10) a housing system without straw bedding. Van Veen *et al.* (1985) also found more PWD and bowel oedema when piglets were reared in a fully slatted concrete floor system than in a solid concrete floor with straw bedding.

I.D. Antisecretory drugs and treatment of diarrhoea

Drug therapy is potentially of use in both acute and chronic diarrhoeal diseases. The goal of drug therapy for diarrhoeal disease is to decrease stool water. This goal can be achieved in two ways. First drugs can increase absorption of water and electrolytes by increasing active electrolyte absorption, decreasing basal anion secretion or decreasing intestinal motility. Second, drugs can decrease hypersecretion. Some drugs exert both effects.

Figure 6 is a schematic representation of the sites of action of several antisecretory drugs.

I.D.1.Opiates

Opiates were the first class of effective antidiarrhoeal agents. Mostly used for the treatment of diarrhoea are codeine, loperamide and diphenoxylate. These drugs act by altering water and electrolyte transport and motility.

Throughout the body multiple opiate receptors have been identified. Codeine acts predominantly on opiate receptors of the μ type. Its action results in a decrease in jejunal motor activity (Schiller *et al.*, 1982). This effect seems to be the result of an increased segmentation of the circular muscle of the intestine causing a disruption of the propulsive activity. A high concentration of the drug also induces an increase in active electrolyte absorption and probably also inhibits the hypersecretion (McKay *et al.*, 1981). The problem with using codeine for treatment of diarrhoea is that the diarrhoea is not always controlled with the normally used dosages. As the dose is increased, adverse reactions and sedation are induced.

Like codeine, loperamide also binds to opiate receptors of the μ type and has an effect on the intestinal motor function (Schiller *et al.*, 1984). Furthermore, loperamide is observed to inhibit the activity of the Ca^{++}-calmodulin-stimulated phosphodiesterase. The calmodulin inhibitory effect of loperamide is not an opioid agonist effect and is as potent as the effect of diphenoxylate and

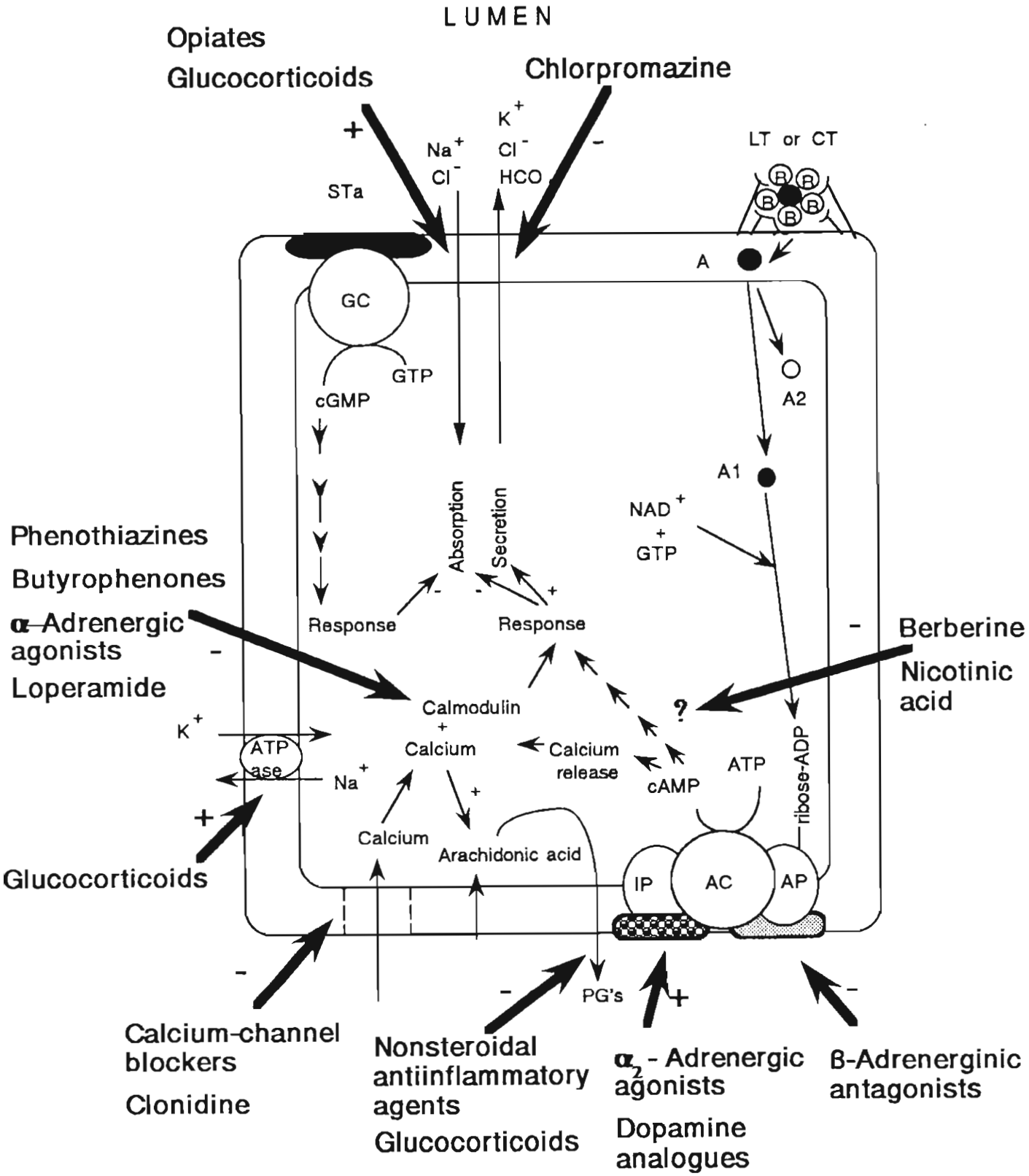


Fig. 6. Sites of action of some antisecretory drugs on secretory and absorptive transport of electrolytes by enterocytes (see also legend of Fig. 3 and 4).

even more potent than the effect of chlorpromazine and promethazine (Zavecz *et al.*, 1982; Clouet *et al.*, 1983). Loperamide reverses cholera toxin-induced secretion in rat jejunum, and prostaglandin E2-induced secretion of water, Na^+, K^+ and Cl^- in rabbit ileum. Furthermore, it reverses LT *E. coli* enterotoxin-induced secretion without affecting cAMP levels in rabbit ileum and, in a variable manner, ST *E. coli* enterotoxin- and cGMP-induced secretion. (Sandhu *et al.*, 1981; Hughes *et al.*, 1982; Turnberg, 1983). Loperamide is not indicated for treatment of inflammatory and invasive diarrhoea, due to its effects on the motor activity of the intestine.

Enkephalines act on δ opiate receptors. Although no such receptors are found on enterocytes, enkephalines can stimulate the basal absorption. This occurs perhaps via the release of neurohumoral substances (Dobbins *et al.*, 1981).

I.D.2. Glucocorticoids

Glucocorticoids can stimulate active Na^+ absorption in the jejunum, ileum and colon in the rat, rabbit and monkey (Charney *et al.*, 1975; Charney and Donowitz, 1976; Binder, 1978; Sellin and Field, 1981). This increase involves a primary increase in Na^+ entry across the apical membrane but also a stimulation of the Na^+-K^+-ATPase in the cell membrane. Glucocorticoids have antiinflammatory effects and can inhibit the arachidonic metabolism by both the cyclooxygenase and the lipoxygenase pathways. Glucocorticoids increase bicarbonate secretion in rabbit ileum and Cl^- secretion in rat small intestine (Binder, 1978; Sellin and Field, 1981). The overall effect, nevertheless, is an increased absorption.

Glucocorticoids have to many side effects when used for a longer period and can therefore not be used in the treatment of chronic diarrhoeal diseases.

I.D.3. Adrenergic receptor agonists

Adrenergic receptor agonists are a promising group of potentially antidiarrhoeal drugs. They act by increasing the basal absorption. The most useful of these drugs are the α_2-adrenergic agonists which work at the lowest concentration such as clonidine. They act on α_2-receptors. It has been hypothesized that they act by inhibiting adenylate cyclase activity. They can inhibit PGE_2, cholera toxin- and the heat-stable enterotoxin(STa)-mediated activation of the adenylate cyclase-cAMP system (Field and McColl, 1973; Chang *et al.*, 1982, Nakaki *et al.*, 1982). Furthermore clonidine has been observed to inhibit the intestinal motility. This effect can enhance his antidiarrhoeal activity (Schiller *et al.*, 1985)

The α_2-adrenergic agonists can induce other effects such as hypotension and chronic depression which probably could limit the usefulness of the currently available agents of this group (Schoener and Pitts, 1985). There are subtle differences between intestinal and other α_2-receptors. It is not impossible that α_2-agonist can be developed with a specificity for the gastrointestinal tract (Darmsathaphorn *et al.*, 1983).

I.D.4. ß-Adrenergic antagonists

The ß-adrenergic antagonist propanolol inhibits the basal absorption in human small intestine (Morris and Turnberg, 1981). In the intestine of rats and rabbits, however, propanolol has no effect on basal absorption, but is antisecretory for cholera toxin and dibutyryl cAMP (Taub *et al.*, 1977; Donowitz and Charney, 1979). It has been hypothesized that ß-adrenergic agonists stimulate the adenylate cyclase enzyme complex, increasing the intracellular concentration of cAMP. The antagonists block this stimulation. Propanolol also inhibits calmodulin, the Ca^{++} receptor protein.

Propranolol is antihypertensive (Traub and Rosenveld, 1985).

I.D.5. Dopamine analogues

Dopamine analogues such as bromocriptine can stimulate absorption in rat ileum and colon and can overcome cholera toxin-induced secretion. They act predominantly via α_2-receptors and partly by stimulating dopamine receptors (Donowitz *et al.*, 1983b).

Side effects of bromocryptine are nausea and vomiting. These effects, however, only occur during the first days of the treatment. Therefore, this drug will only be useful for the management of chronic diarrheal disease.

I.D.6. Somatostatin

Somatostatin is a 14-amino acid cyclic peptide that is found in the intestinal mucosa in endocrine cells. It has no effect on basal water and electrolyte transport in canine, rat and human jejunum and/or colon . It stimulates active Na^+ and Cl^- absorption *in vitro* and can inhibit secretion stimulated by the intracellular mediators Ca^{++}, cAMP and cGMP (Carter *et al.*, 1978; Dharmsathaphorn *et al.*,1980; Guandalini *et al.*,1980; Krejs *et al.*, 1980a).

Somatostatin will not be directly useful in the treatment of diarrheal disease since it has to be administered intravenously and has a short half life time. Analogues will have to be developed which can be given intramuscularly or subcutaneously and have a longer half-life.

I.D.7. Calcium-channel blockers

Verapamil has been best studied. It can increase basal ileal absorption of water, Na^+ and Cl^- when administered to the serosal ileal surface. In the colon, the same activity is observed when this agent is added to the luminal surface. No effect on cAMP -induced secretion could be demonstrated. A side effect of verapamil administration is constipation. *In vitro* verapamil inhibits the intestinal secretion caused by increased Ca^{++} entry across the ileal basolateral membrane. (Donowitz and Asdarkof, 1982; Donowitz, 1983; Donowitz *et al.*, 1983a; Donowitz *et al.*, 1984b; Donowitz *et al.*, 1985).

Nifedipine can inhibit the secretory action of cholera toxin and of the heat-stable enterotoxin

of *E. coli*. Diltiazem has been observed to have this effect on the ST enterotoxin induced secretion. This effect occurs at a step before the increase in cGMP content (Larsen J.-J., 1982).

I.D.8. Calcium-calmodulin antagonists

Calmodulin is one of the major intracellular receptors for Ca^{++}. An inhibitory effect on Ca^{++}-calmodulin has been attributed to a number of drugs such as phenothiazines (chlorpromazine), butyrophenones (haloperidol), diphenylbutylpiperidines, antimalarial drugs, anticholinergic agents, antihistaminica and α-adrenergic agonists (Prozialeck and Weiss, 1982; Weiss *et al.* , 1982).

Weiss *et al.* (1982) gave an explanation for the fact that so many drugs have a Ca^{++}-calmodulin-inhibitory activity. They suggested that Ca^{++}-calmodulin antagonists need to have a hydrophobicity, a positive charge and separation of hydrophobic areas from the positive charge by at least three carbon atoms (Weiss *et al.* , 1982). When Ca^{++} binds to calmodulin, then a hydrophobic domain becomes exposed. This domain is essential for interactions with receptor proteins.The phenothiazines appear to bind to this region of calmodulin (Cheung, 1982).

The best studied Ca^{++}-calmodulin antagonists in animal models and in humans are the phenothiazines, such as chlorpromazine and trifluoperazine. Chlorpromazine has been observed to inhibit cAMP-induced hypersecretion due to cholera toxin, *E. coli* enterotoxin, VIP and prostaglandins (Simon *et al.* , 1981) and cGMP-mediated secretion due to heat-stable *E. coli* enterotoxin (Lönnroth and Munck, 1980; Lönnroth and Jennische, 1982). Chlorpromazine has also been mentioned to exert these effects due to a membrane stabilizing activity (Lönnroth and Jennische, 1982; Weiss *et al.* , 1982).

The phenothiazines induce, besides their effect on the intestinal fluid transport, other pharmacological activities such as sedation, anti-emetic effects, sometimes hypothermia and an a-lytic effect (Booth, 1977; Baldessarini, 1980). These side effects could compromise their usefulness for treatment of diarrhoea.

I.D.9. Non-steroidal antiinflammatory agents

The mechanism of nonsteroidal antiinflammatory agents in the treatment of diarrhoea is difficult to assess since they have several effects. They can act by inhibiting the arachidonic metabolism via the cyclooxygenase pathway, by stimulating the intestinal absorption, by inhibiting the activation of the adenylate cyclase-cAMP system by cholera toxin and by inhibiting the cAMP protein kinase activity (Vane, 1971; Wald *et al.* , 1977; Kantor and Hampton, 1978) .

Indomethacin has been observed to inhibit the secretion caused by *Salmonella*, *Vibrio cholerae* , cholera toxin, *Shigella*, and heat-stable *E. coli* enterotoxin (Gots *et al.* 1974; Madsen and Knoop, 1978; Smith *et al.* , 1981). Aspirin can inhibit the intestinal secretion caused by cholera toxin, heat-labile and heat-stable *E. coli* enterotoxin (Finck and Katz, 1972; Wise *et al.* , 1983). Bismuth subsalicylate orally administered becomes hydrolyzed into bismuth compounds and salicylate. The latter acts as a prostaglandin synthesis inhibitor and so decreases the intestinal

secretion, whereas bismuth perhaps reduces the multiplication and the number of ETEC (Feldman *et al.*, 1981; Graham *et al.*, 1983).

I.D.10. Nicotinic acid

Nicotinic acid can inhibit cholera toxin-induced rabbit jejunal secretion due to a decrease in the activation of the adenylate cyclase-cAMP system. No effect was observed on the basal secretory rate (Turjman *et al.*, 1977). In pigs, nicotinic acid could prevent net fluid secretion induced by cholera toxin and heat-stable enterotoxin of *E. coli* without changing the cyclic nucleotide concentration in intestinal mucosa. Here it was thought to act at a step beyond the activation of the cyclic nucleotides (Forsyth *et al.*, 1981).

I.D.11. Berberine

Berberine is an alkaloid from the root and bark of the plant Berberis aristata and has been used in India as an antidiarrhoeal medication for thousands of years.

In vivo, berberine inhibits secretion induced by cholera toxin and LT enterotoxin of *E. coli* in rabbit ileum (Askri and Scheftel, 1988), by cholera , STa enterotoxin of *E. coli* and methylprednisolone in rat jejunum and ileum (Tai *et al.*, 1981; Swabb *et al.*, 1981; Sack and Froehlich, 1982) and by ST a enterotoxin in pig jejunum (Zhu and Ahrens, 1982). Berberine does not seem to inhibit the adenylate cyclase system, but is thought to act at a step after adenylate cyclase activation (Tai *et al.*, 1981). Berberine has also broad spectrum activity against bacteria, protozoa and fungi.

Chapter II: AIMS OF THE EXPERIMENTAL STUDIES

The aim of the present studies was to evaluate the therapeutic usefulness of some antisecretory drugs for treatment of diarrhoea with subacute dehydration in piglets as can occur the first weeks post weaning. Therefore, a reproducible diarrhoea model had to be developed.

Post-weaning diarrhoea (PWD) is an important cause of poor feed conversion and sometimes of death as a result of severe dehydration. This diarrhoea can have a high morbidity and causes a subacute dehydration with sometimes a mortality of near 20 %. The etiology of post-weaning diarrhoea is multifactorial. Besides enterotoxigenic *Escherichia coli* (ETEC), other factors have been mentioned to play a role in this diarrhoea syndrome. Therefore treatment performed by oral rehydration (water, electrolytes and glucose) and the administration of antibiotics is not always succesful. Furthermore, antibiotic therapy can result in the development of antibiotic-resistant pathogenic bacteria. The antibiotic therapy can inhibit the development of an intestinal mucosal immunity against ETEC, since antibiotics will decrease or inhibit the bacterial proliferation which is necessary for the development of a good intestinal mucosal immunity (Deprez, 1988). There is thus a need for other therapeutics in the treatment of post-weaning diarrhoea.

As mentioned above, enterotoxigenic *E. coli*, involved in the postweaning diarrhoea syndrome, posses two important virulence factors namely fimbriae and enterotoxins.

Fimbriae are thread-shaped, nonflagellar, immunogenic protein polymers located on the surface of the bacteria (Klemm, 1985; Parry and Rooke, 1985). The fimbriae allow the ETEC bacteria to adhere to receptor or receptor sites on the brush border of villous enterocytes and so to resist the flushing stream of nutrients and mucus in the digestive tract, enabling the ETEC bacteria to colonize the small intestinal villous mucosa.

Besides producing fimbriae, ETEC strains elaborate heat -labile and/or heat-stable enterotoxins. These enterotoxins induce diarrhoea by stimulating the secretion and/or inhibiting the absorption of water and electrolytes in the small intestine (Field, 1979; Berridge, 1984, Petzinger, 1984).

Although ETEC play an important role in the etiology of post-weaning diarrhoea, inoculating newly-weaned piglets with ETEC does not consistently produce disease (Smith and Halls, 1968). Other factors have been mentioned to play a role in post-weaning diarrhoea such as the change from a milk diet to a lesser digestible dry diet, a direct mechanical effect of the new diet on the intestinal mucosa the withdrawal of lactogenic antibodies and a concurrent infection with enteropathogenic viruses such as rotavirus (Tzipori *et al.*,1980a; Lecce *et al.*, 1982; Miller *et al.*, 1984b; Sellwood, 1984; Wilson and Francis, 1986). Several of these factors can induce a less or more pronounced villous atrophy. This atrophy results in a decrease in the absorptive surface in the small intestine and thus in intestinal malabsorption. It has been postulated that this malabsorption can enhance the proliferation of *E. coli* in the small gut and so can facilitate the colonization of the villous mucosa with ETEC (Lecce *et al.*, 1983; Tzipori *et al.*, 1984). Lecce *et al.* (1982) also hypothezised that an infection with rotavirus could induce changes in the plasmamembrane of villous enterocytes which might result in an increased binding of ETEC.

From a pathophysiological point of view post-weaning diarrhoea is thus a combination of

small intestinal malabsorption and hypersecretion. Treatment could therefore be performed by administering drugs that enhance absorption and/or decrease secretion of fluid and electrolytes in the small intestine. Several drugs have been shown to exert these effects *in vitro*, *in situ* and sometimes also *in vivo*. They are called antisecretory drugs and include neuroleptics (chlorpromazine), calcium-channel blockers (verapamil), non-steroidal antiinflammatory agents (indomethacin, aspirin), α_2-adrenergic agonists (clonidine) and ß-adrenergic antagonist (propranolol) (Lönnroth *et al.*, 1979; Donowitz *et al.*, 1986). Of these drugs, chlorpromazine has been shown to significantly reduce fluid losses in neonatal colibacillosis. None of these drugs, however, have been evaluated in piglets in a post-weaning diarrhoea model. Treatment of diarrhoea with antisecretory and/or absorption stimulating drugs can have some interesting advantages on antibacterial therapy. These drugs, when used in animals with diarrhoea and dehydration, can have a direct beneficial effect since they immediately inhibit fluid loss in the small intestine. Furthermore, treatment with these drugs will allow piglets to develop an active immunity against the ETEC strain involved in the weaning diarrhoea since bacterial growth is not inhibited.Piglets will therefore become protected against reinfections with the same fimbriae and enterotoxin producing E. coli strains.

To evaluate the effect of antisecretory drugs on diarrhoea in newly-weaned piglets, a reproducible diarrhoea model was developed. This model allowed us to study clinical, pathological, hematological, biochemical and hemodynamic changes following diarrhoea so that the effect of antisecretory drugs on some of these parameters could be evaluated. In this model piglets were pretreated with chloramphenicol (colonization-resistance suppressing antibiotic) and subsequently inoculated with the enteropathogenic transmissible gastroenteritis virus (TGEV) and two K88ac fimbriae possessing ETEC strains. This model combined most likely malabsorption (TGEV) and hypersecretion (ETEC).

K88ab, K88ac, K88ad, K99 (F5), P987 (F6) and F41 fimbriae can be identified on ETEC strains isolated from piglets with neonatal colibacillary diarrhoea. K88ac fimbriae positive ETEC were chosen to inoculate 3- 4-week-old newly weaned piglets since K88ac strains are most often isolated from piglets with post-weaning diarrhoea and since it was demonstrated in the present thesis using an *in vitro* adhesion assay that K88ac fimbriae producing *E. coli* adhere significantly more to villi of most newly-weaned piglets than K88ad, K99(F5), P987(F6) and F41 fimbriae$^+$ *E. coli*.

K88$^+$ *E. coli* adhered weak or did not adhere at all to villi of some piglets. It has been shown that this resistance to adhesion of K88$^+$ ETEC strains (no receptors) or decreased adhesion is genetically determined and results in a congenital resistance of piglets to K88-mediated colibacillosis (Sellwood, 1983, 1984a; Bijlsma and Bouw, 1985). Thus when inoculating piglets with K88$^+$ ETEC to induce diarrhoea, the genetic susceptibility of the piglets for adhesion of the K88$^+$ ETEC to villous enterocytes has to be known. In alive piglets, this can only be determined using by surgery collected small intestinal mucosa of which villi, villous enterocytes or brush borders are separated and subsequently are used in an *in vitro* adhesion assay. It was examined whether this procedure could be replaced by a non-invasive test which could easily be performed in alive piglets.

Since the presence of K88 receptors is genetically determined, it is important to have information on the genetic susceptibility of Belgian piglets for K88ac$^+$ ETEC adhesion. When

only few piglets possess K88 receptors, than it could be interesting to interfere in the breeding using K88 negative boars.Therefore, the prevalence of piglets with receptors for $K88^+$ *E. coli* was determined in a Belgian piggery.

Besides ETEC, villous atrophy is important in the etiology and pathogenesis of post-weaning diarrhoea. During these studies, the hypothesis was investigated that the infection with an enteropathogenic virus can cause changes in membrane nature which might alter the binding sites on villous enterocytes so that the adhesion of ETEC to villi becomes facilitated (Lecce *et al.*, 1982).

Chapter III: EXPERIMENTAL STUDIES

III.A. *In vitro* adhesion of fimbriae positive *Escherichia coli.*

III.B. A diarrhoea model in newly-weaned piglets

III.C. Effect of antisecretory drugs in an experimental diarrhoea model in newly-weaned piglets

III.A. *IN VITRO* ADHESION OF FIMBRIAE POSITIVE *ESCHERICHIA COLI*

III.A.1. INTRODUCTION

The ability to adhere to specific host tissues is of primary importance in diseases such as bacterial diarrhoea. It determines the site of infection by facilitating specific surface to surface interaction between the bacteria and the host epithelium and allows the bacteria to resist and circumvent the flushing and cleaning mechanisms that protect the intestinal tract. The attachment can be mediated by cell-surface structures termed "adhesins" which adhere to specific receptors present on the enterocytes. For some bacteria such as for enterotoxigenic *Escherichia coli* (ETEC), the adhesins have been identified, for other bacteria the adhesins still have to be determined.

Following infection with ETEC, these *E. coli* strains have to adhere to the small intestinal villous mucosa, whereafter colonization of the small gut can occur. Adhesion is thus an essential step in the pathogenesis of colibacillary diarrhoea. The adhesins of ETEC are thread-shaped, nonflagellar, immunogenic protein polymers built out of a protein subunite which is called piline. The adhesins are located on the surface of the ETEC and are called fimbriae. They enable the ETEC to adhere to specific receptors on the brush border of villous enterocytes in the small intestine (Gaastra and Graaf, 1982; Klemm, 1985). On the basis of morphological, functional and immunological characteristics and using haemagglutination and in vitro adhesion assays, several fimbriae have been identified on ETEC strains at present : Type 1 (F1), CFA/I (F2), CFA/II (F3) (cs1-, cs2-, cs3-variants), E8775 (cs4-, cs5-, cs6-variants), PCFO159, K88 (F4)(K88ab-, K88ac-, K88ad-variants), K99 (F5), P987 (F6), F41, CS31A, ATT25 (F17), ATT 111 (F111) (Nagy *et al.*, 1977; Guinée and Jansen, 1979; Moon, 1979; Pohl *et al.*, 1982; Levine, 1987; Girardeau *et al.*, 1988; Lintermans *et al.*, 1988; Bertels *et al.*, 1989; Stamm *et al.*, 1990).

The K88, K99, P987, F41 and F111 fimbriae are identified on ETEC strains isolated from piglets (Wilson and Francis, 1986; Pohl *et al.*, 1988; Pohl *et al.*, 1989; Activity report 1988-1989, NIDO, Ukkel, Belgium). In neonatal colibacillary diarrhoea, K88, K99, F41 and P987 fimbriae$^+$ strains are frequently identified. In piglets with post-weaning diarrhoea mostly K88 and rarely K99 or P987 fimbriae$^+$ strains are isolated (Wilson and Francis, 1986; Broes *et al.*, 1988; Söderlind *et al.*, 1988) and no reports were found describing the isolation of F41$^+$ *E. coli* strains from piglets with weaning diarrhoea. Of the 3 K88 variant (nl. K88ab, K88ac and K88ad), the K88ac variant is most often identified (Guinée and Jansen, 1979). It was tried to find an explanation for these observations by comparing the quantitative adhesion of different fimbriae producing *E. coli* strains to villi of 4- to 5-week-old newly-weaned piglets using an *in vitro* adhesion assay (Girardeau, 1980). The results from this comparative study showed that significantly more K88ab or K88ac$^+$ *E. coli* adhered to the villi of most newly-weaned piglets than K88ad, K99, P987, F41 or K99/F41$^+$ bacteria. K88ac or K88ab fimbriae producing strains are, therefore, most indicated to be used in an experimental post-weaning diarrhoea model in piglets. It has, however, been demonstrated that in some pigs one or more of the K88 variants only adhere weakly or do not adhere to the brush border of the villous enterocytes. Weak adhesion and the absence of adhesion

are genetically determined and result in a congenital resistance of piglets to K88 mediated adhesion (Sellwood, 1983, 1984). When using $K88^+$ ETEC to experimentally inoculate piglets, the genetic susceptibility of piglets for adhesion of $K88^+$ ETEC to their villi has to be determined. In vivo, this has to be performed using surgically obtained villi, villous enterocytes or brush borders in combination with an *in vitro* adhesion assay (Snodgrass et al., 1981; Bijlsma et al., 1985b). Disadvantages of routinely using abdominal surgery are the increased susceptibility to infections as a result of the abdominal surgery and the occurrence of intestinal adhesions (Bueno et al.,1978; Snodgrass et al., 1981; Altura, 1983; Papa et al., 1983). It has been described that ETEC can adhere to buccal epithelial cells (Thorne et al, 1979). Buccal epithelial cells are easier to obtain from alive piglets. It was therefore examined whether the adhesion of $K88^+$ *E. coli* to buccal epithelial cells reflects the adhesion to villi.

The presence of a high percentage of K88 resistant piglets in the swine population could be interesting since it would make it possible to further select for K88 resistant swine by mating sows with K88 resistant boars. Therefore, the prevalence of piglets with receptors for adhesion of $K88^+$ *E. coli* was determined in a Belgian piggery.

III.A.2. COMPARISON OF THE *IN VITRO* ADHESION OF K88, K99, F41 AND P987 POSITIVE *ESCHERICHIA COLI* TO INTESTINAL VILLI OF 4- TO 5-WEEK-OLD PIGLETS.

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ABSTRACT

In vitro adhesion of K88ab, K88ac, K88ad, P987, K99, F41 and K99/F41 positive *Escherichia coli* strains to duodenal, jejunal and ileal villi was determined in 4- to 5-week-old piglets. The $K88^+$ strains adhered strongly to villi in most tested piglets, the $K99^+$ and $F41^+$ strains adhered weakly or not, whereas the $P987^+$ and $K99/F41^+$ strains only adhered weakly to villi in all piglets. The number of bacteria adhering to villi was highest for the $K88ab^+$ and $K88ac^+$ strains (55 ± 5 to 81 ± 4 *E. coli*/250 µm villous length) and decreased in the following order K88ad > P987 > K99/F41 > K99 > F41.

There was no difference in the adhesion to villi of the different small intestinal segments for $P987^+$ and $F41^+$ strains. The $K99^+$ strain adhered significantly more to villi of the caudal half of the small intestine, the $K99/F41^+$ strain to jejunal and ileal and the $K88^+$ strains to jejunal villi in comparison to duodenal ones.

INTRODUCTION

Enterotoxigenic *Escherichia coli* (ETEC) are an important causal agent of acute diarrhoeal disease in young animals and children (Morris and Soyka, 1985). Adhesion is the first step in the pathogenesis of ETEC induced diarrhoea. It allows the bacteria to resist the flushing mechanism in the small intestine and to colonize small intestinal villi. The adhesion occurs to specific membrane receptors or receptor sites on villous enterocytes of the small intestine and is mediated by long threadlike protein polymers present on the surface of these ETEC which are called fimbriae or pili (Klemm, 1985). Fimbriae which are found on ETEC strains isolated from neonatal piglets with diarrhoea are K88 (F4), K99 (F5), 987P (F6) and F41 fimbriae (Morris and Sojka, 1985; Parry S.H. and Rooke D.M., 1985; Renault and Le Bourhis, 1985; Pohl *et al.*, 1988; Pohl *et al.*, 1989). In piglets with weanling diarrhoea often K88 fimbriae possessing ETEC strains (Wilson and Francis, 1986), and infrequently 987P and K99 fimbriae producing strains are identified (Francis *et al.*, 1982; Wilson and Francis, 1986; Broes *et al.*, 1988; Söderlind *et al.*, 1988). No reports were found which describe the isolation of $F41^+$ *E. coli* strains from piglets with postweaning diarrhoea.

The purpose of the present study was to compare the *in vitro* adhesion of ETEC strains

producing K88ab, K88ac, K88ad, K99, F41 and P987 fimbriae to villi of duodenum, jejunum and ileum in 4- to 5-week-old just-weaned Belgian piglets. The results were discussed in relation with literature data regarding the appearance of these fimbriae in ETEC induced postweaning diarrhoea.

MATERIAL AND METHODS

Bacterial strains

The *E. coli* strains used and their characteristics are listed in Table 1. The $K88^+$ strains and the E57 strain (control strain without fimbriae) were inoculated on tryptone soy agar (Oxoid) whereas the $K99^+$, $F41^+$, $K99/F41^+$ and $P987^+$ strains were inoculated on Minca (bioMerieux) supplemented with 1 % (vol/vol) PolyViteX (bioMerieux)(Guinée *et al.*, 1977). All strains were incubated overnight at 37 °C or during 2 days at 18 °C, whereafter they were examined for production of fimbriae with a slide agglutination test using specific antisera against K88ab, K88ac, K88ad, K99, F41 and P987 fimbriae (RIVM, Bilthoven, The Netherlands). The strains were subsequently suspended and diluted in sterile physiological saline until an A_{620} of 1.2 (approximately 4.5×10^9 bacteria ml^{-1} suspension, as determined by counting colony forming units).

Animals

The animals used in this study were 33, 4- to 5-week-old, female piglets (Belgian Landrace (BL) x BL or Piétrain x BL) from the same commercial farm. Sows were vaccinated 6 and 2 weeks prior to delivery with a polyvalent *E. coli* vaccine containing K88ac and K88ab fimbriae possessing *E. coli* strains (Gletvax®, Wellcome or Stellamune coli 5®, Smith-Kline RIT). Piglets were weaned when 3- to 4-week-old. The piglets were euthanatized with an overdose of methomidate (Hypnodil®, Janssen) between day 4 and day 10 after weaning.

Small intestinal villi

Immediately after euthanasia, the abdomen of the piglets was opened and 15 cm long intestinal segments were excised at 4 sites nl. 2 cm caudal to the pylorus (duodenum), at one- and two-thirds of the distance between the pylorus and the ileocaecal valve (cranial and caudal jejunum) and also 2 cm cranial to this valve (ileum). The segments were opened and washed in a Krebs buffer containing 1 percent formaldehyde after which villi were gently scraped from the mucosa with a glass slide, washed several times in the same buffer and stored in this buffer. The formaldehyde was added to the buffer to stop the enzymatic reaction which would result in lysis of epithelial cells. Within 3 weeks the *in vitro* villous adhesion assay was performed.

Table 1: Characteristics of *E. coli* strains used

Strain	Serotype[a]	Fimbriae	Antigen number[b]	Source[c]
GIS 25	O149:K91:K88ac	K88ac	F4	RIT
156KP83	O149:K91:K88ac	K88ac	F4	NIDO
G7	O8:K87:K88ab	K88ab	F4	NIDO
E68 type 1	O141:K85ab:K88ab	K88ab	F4	NIDO
H56	O8:K87:K88ad	K88ad	F4	Bijlsma (1982)
19KP74	O141:K85:K88ad	K88ad	F4	NIDO
B41	O101:K99:F41	K99/F41	F5/F41	NIDO
B41MC	O101:F41	F41	F41	NIDO
72KP85	O?:K99	K99	F5	NIDO
126A	O8:K85:K99	K99	F5	NIDO
P81-6O3A	O?:987P	P987	F6	NIDO
E57	O138:K81	none		NIDO

[a]O? = serogroup not determined
[b]Ørskov and Ørskov (1984)
[c]NIDO = P. Pohl and P. Lintermans, National Institute of Veterinary Research, Brussels Belgium; RIT = Smith-Kline RIT, Genval, Belgium.

Villous adhesion assay

The *in vitro* adhesion assay was based on the technique described by Girardeau (1980). The stored villi were placed in a Krebs buffer without formaldehyde and after 1h the buffer was renewed. This step was repeated 4 times. About 50 villi were transferred with a pipette into 1 ml 0.1 M phosphate buffer (pH 6.7) containing 1 percent (W/V) D-mannose (Difco), after which 0.1 ml of a bacterial suspension in sterile physiological saline, containing one of the strains mentioned in Table 1, was added. The presence of D-mannose in the buffer results in an inhibition of the cellular adhesion of bacteria by Type 1 pili. These pili are produced by most pathogenic and nonpathogenic *E. coli* from a wide variety of host species.

After gently mixing villi and bacterial suspension for 1h at room temperature, some villi were removed with a pipette, placed under a cover slip and examined by light microscopy at magnification x 600. The adhesion of bacteria was evaluated quantitatively by counting the number of visible bacteria adhering along 50 µm villous brush border at 5 different places villus^{-1} (250 µm villus $^{-1}$) and on 5-6 villi per small intestinal segment. Villi of each segment were tested twice.

The mean bacterial adhesion per 250 µm villous length and per small intestinal segment was calculated for each of the fimbriae, using only the data from piglets which showed adhesion of the fimbriae positive strain to villi of at least one of the four intestinal segments. For the K88 variant^{+} strains the mean bacterial adhesion was separately calculated for piglets which showed a weak adhesion (mean adhesion to villi of all 4 segments < 30 bacteria/250 µm villous length) or a strong adhesion (mean adhesion to villi of all 4 segments > 40 bacteria/250 µm villous length).

Villi of all 33 piglets were tested for *in vitro* adhesion of the K88^{+} strains and strain E57 to their villi, whereas only 30 piglets were assayed for the *in vitro* adhesion of the other fimbriae

positive strains to their villi.

Statistical procedures

Data were presented as means ± standard error of the mean (SEM) and were analysed by a two-level nested analysis of variance with unequal sample sizes. Significance was accepted at the $P < 0.05$ level.

RESULTS

When strains were grown at 18 °C, no fimbriae could be identified with the slide agglutination test using specific antisera and no *in vitro* adhesion to villi was observed. When grown at 37 °C, only the control strain (E57) was negative in the slide agglutination test. This strain did not adhere *in vitro* to villi.

The number of piglets which showed strong, weak or no adhesion of a fimbriae$^+$ *E. coli* strain to their villi is presented for each of the tested fimbriae in Table 2. The K88$^+$ strains rarely did not adhere, sometimes adhered weakly, but mostly adhered strongly to the villi. The P987$^+$ and K99/F41$^+$ strains always adhered weakly. The K99$^+$ and F41$^+$ strains each adhered weak to villi of 23 and did not seem to adhere to villi of 7 piglets (one piglet showed no *in vitro* adhesion of the K99$^+$ and the F41$^+$ strains, 6 piglets no adhesion of the K99$^+$ strain and 6 other piglets no adhesion of the F41$^+$ strain).

Table 2: Number of piglets with strong, weak or no *in vitro* adhesion of fimbriae positive *Escherichia coli* strains to their villi

Adhesion	Number of piglets with adhesion of						
	Fimbriae	K88ab*	K88ac*	K88ad*	P987**	K99**	F41**	K99/F41**
Strong[A]		30	29	24	0	0	0	0
Weak[B]		3	2	8	30	23	23	30
Absent		0	2	1	0	7	7	0

* Total number of tested piglets = 33; ** Total number of tested piglets = 30
[A] strong: mean adhesion to villi of all 4 intestinal segments > 40 bacteria/250 µm villous length
[B] weak: mean adhesion to villi of all 4 intestinal segments < 30 bacteria/250 µm villous length

The mean number (± SEM) of bacteria adhering to 250 µm villous length of duodenal, jejunal and ileal villi are presented in Fig. 1 for piglets showing strong adhesion of K88ab$^+$ (n=30), K88ac$^+$ (n=29) or K88ad$^+$ strains (n=24) to their villi and for piglets with a weak adhesion of the P987$^+$ (n=30), K99$^+$ (n=23), F41$^+$ (n=23) and K99/F41$^+$ strians (n=30). The number of bacteria adhering to villi was highest for K88ab$^+$ and K88ac$^+$ strains and decreased in

the following order for the K88ad$^+$ > P987$^+$ > K99/F41$^+$ > K99$^+$ > F41$^+$ strains. In the highly K88 susceptible piglets between 42 ± 5 and 81 ± 4 bacteria/250 µm villous length were counted. This was significantly higher than the number of K88 variant$^+$*E. coli* adhering to villi in the weakly K88 susceptible piglets (between 5 ± 2 and 24 ± 7 bacteria/250 µm villous length, $p < 0.05$; data not shown in Figure 1) and than the number of the other fimbriae positive strains adhering to these villi (Fig. 1). The number of P987$^+$ *E. coli* adhering to villi was significantly higher than the number of F41$^+$, K99$^+$ and K99/F41$^+$ *E. coli* and the number of K99/F41$^+$ *E. coli* adhering to caudal jejunal and ileal villi was significantly higher than the number of F41$^+$ *E. coli*.

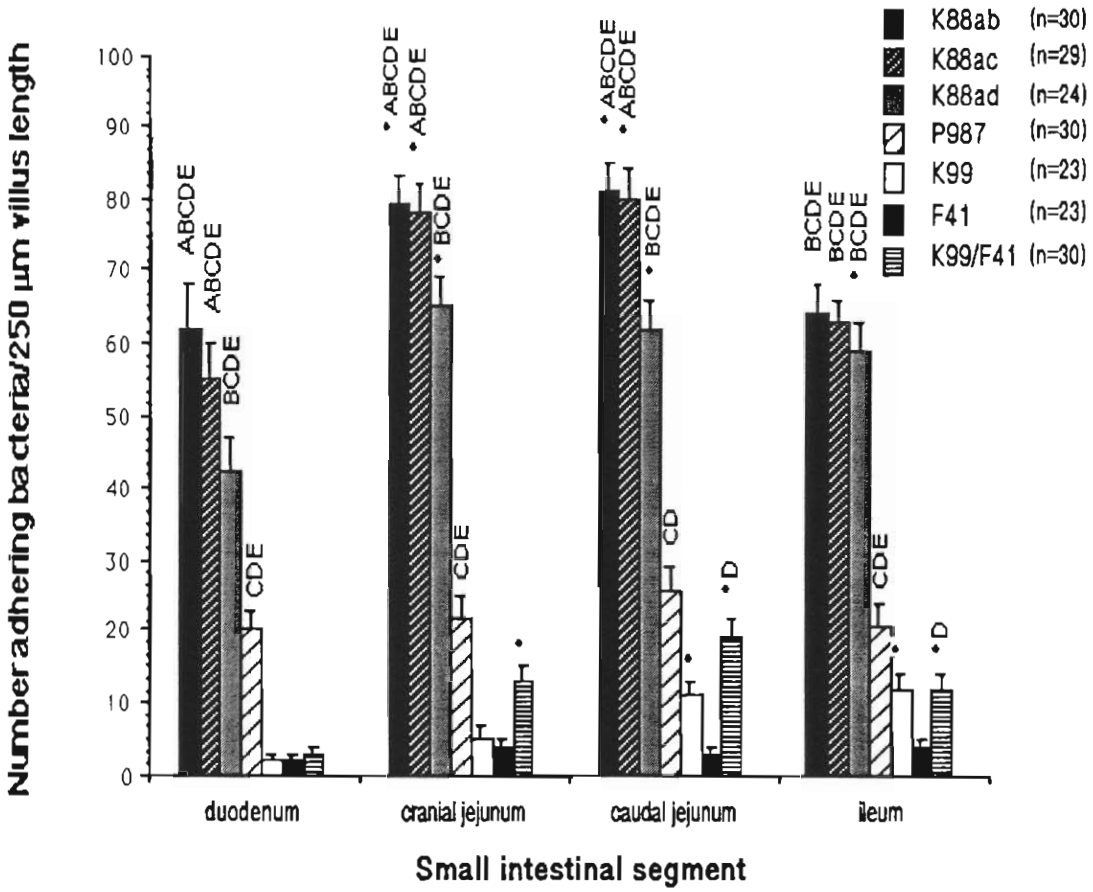


Fig. 1: Adhesion of K88 variant (strong adhesion) and of P987, K99, F41 and K99/F41 (weak adhesion) positive *E. coli* strains to duodenal, jejunal and ileal villi of 4- to 5-week-old piglets. Significantly different ($p < 0.05$) in comparison with ^{A} K88ad-, ^{B} P987-, ^{C} K99-, ^{D} F41-, ^{E} K99/F41-mediated adhesion to villi of the same intestinal segment; * significantly different ($p < 0.05$) with adhesion of the same fimbriae to duodenal villi.

There was no difference in number of bacteria adhering to villi between the 4 small intestinal segments for the K88 variant$^+$ strains in the weakly K88 susceptible piglets (data not shown in Fig. 1), for the P987$^+$ and for the F41$^+$ strains (Fig. 1). This was not the case for the K88 variant$^+$ strains in the highly K88 susceptible piglets, for the K99/F41$^+$ and the K99$^+$ strains. All 3 K88 variant$^+$ strains adhered significantly more to jejunal than to duodenal villi and the K88ad$^+$ strain also to ileal than to duodenal villi of piglets highly susceptible to K88 adhesion. The K99$^+$ strains adhered significantly more to villi of the caudal half than to villi of the cranial half of the small intestine and the K99/F41$^+$ strain to jejunal and ileal villi than to duodenal villi.

DISCUSSION

Of the K88 variant$^+$ *E. coli* strains, the K88ac are most often isolated from piglets with diarrhoea (activities reports 1985 to 1988, National Institute for Veterinary Research, Brussels; Westerman *et al.*, 1988). It has been suggested that differences in susceptibility of the pigs to adhesion of the K88 variant$^+$ ETEC could cause selection to one of the variants (Gaastra and De Graaf, 1982). However, no difference in adhesion of the K88ab$^+$ and K88ac$^+$ strains to villi was observed. Both K88 variants did adhere significantly more to duodenal and jejunal villi than the K88ad$^+$ strains. The adhesion of these latter strains, however, was still too pronounced to explain its low appearance.

In the present study, in just-weaned piglets the mean number of K88$^+$ bacteria adhering to villi in the highly K88 susceptible animals was significantly higher than the mean number of P987$^+$, K99$^+$, F41$^+$ and K99/F41$^+$ adhering bacteria. Runnels *et al.* (1980) obtained similar differences for the adhesion of K88$^+$ and K99$^+$ *E. coli* to enterocytes of six-weeks-old piglets, whereas they observed a less pronounced difference in one day old piglets. Their results suggest that the epithelium in the small intestine may develop resistance to K99-mediated adhesion with increasing host age. Recently an age-related resistance to colonization of porcine intestinal epithelium has also been described for the P987$^+$ *E. coli* (Dean *et al.*, 1989). This resistance was, however, not due to a decrease in the number of P987 receptors in older pigs since there was no difference in the *in vitro* adhesion to isolated brush borders of neonatal and older pigs. The resistance became apparent upon experimental infection of pigs and was probably caused by the release of P987 receptors into the intestinal lumen in the older pigs. In the present study, the F41$^+$ strain adhered extremely weak to villi of 23 of the 30 tested 4- to 5-week-old piglets. This is in accordance with the fact that no reports were found which describe the isolation of F41$^+$ strains from outbreaks of postweaning diarrhoea. Several studies, however, describe the isolation of F41$^+$ strains from the faeces of piglets with neonatal diarrhoea (Francis *et al.*,1982; Wilson and Francis,1986; Söderlind *et al.*, 1988). These data could indicate that there is an age-related resistance to adhesion of the F41 fimbriae to villous enterocytes in pigs.

The presence of receptors for K88 fimbriae is genetically determined. Piglets can posses a high or a weak number of receptors, or receptors can be absent (Sellwood, 1984; Bijlsma *et al.*,1982). In the present study, most piglets possessed a high number of receptors and only few piglets a weak number or no receptors for one or more of the 3 K88 variants.

It is not known if the presence of receptors for K99, F41 or P987 fimbriae is genetically determined. Runnels and Moon (1984) found obvious differences in adhesion of K99$^+$ strains to isolated intestinal epithelial cells of neonatal piglets of different litters. They suggested that this difference could be congenitally or genetically determined. Grimes *et al.* (1986) observed no adhesion of K99$^+$ strains to small intestinal villous brush borders in 5 % of 242 tested slaughter age pigs. They had indications that the absence of adhesion was due to the release of K99 receptors from the brush borders during storage. In our study, the K99$^+$ strains and the F41$^+$ strain did not adhere to the villi of 7 of the30 tested piglets. Since only few F41$^+$ bacteria adhered to villi of 23 of the 30 tested piglets (between 2 and 4 ± 1 bacteria/250 µm villous length and per small intestinal segment), the absence of adhesion to villi of the7 of the 30 tested piglets could not be interpreted as being a congenital or genetic resistance to adhesion. The K99$^+$ strain, however, revealed a slightly more pronounced adhesion to villi (between 2 ± 1 and 12 ± 2 bacteria/250 µm

villous lenght and per small intestinal segment). In one piglet the number of K99$^+$ *E. coli* adhering to villi of the caudal small intestine resembled (85 K99$^+$ *E. coli* /250 µm villous length) the adhesion of the K88$^+$ strains to villi of highly susceptible piglets. Differences between piglets in adhesion of the K99$^+$ strain to their villi could reflect congenital or genetic differences. It could, however, also indicate that age-related resistance does not appear for all pigs at the same age.

All piglets possessed receptors for P987 fimbriae and the P987$^+$ strain adhered equally to villi of all tested piglets. This indicates that there is probably no genetically determined difference in number of receptors as is the case for the K88 fimbriae.

In contrast with the K99$^+$ and the F41$^+$ strains, the K99/F41$^+$ strain adhered weakly to villi in all piglets and adhesion of the latter strain to caudal jejunal villi and ileal villi was significantly higher than the adhesion of the F41$^+$ strain. This could reflect more avid forces of the K99/F41$^+$ strain, or could indicate that a polyfimbriated K99/F41$^+$ *E. coli* strains has the possibility to adhere in a higher number to villi than strains which produce only F41 or K99 fimbriae. The association of several fimbriae at the surface of a single bacterium could lead to a more effective colonization of the intestine if the different fimbriae recognize different receptor structures. This seems to be the case for the K99 and F41 fimbriae (Mouricout and Julien, 1987).

In the present study, most K88 receptors were found in jejunum and most K99 receptors in the caudal small intestine. A similar result was obtained for the *in vitro* adhesion of K99$^+$ *E. coli* to brush borders of the small intestine of calves (Sellwood and Lees, 1981). Results are in agreement with the observation that K88$^+$ strains *in vivo* colonize the entire small intestine (Arbuckle, 1970; Hohmann and Wilson, 1975) and K99$^+$ strains the caudal small intestine (Moon *et al.*, 1977; Moon *et al.*, 1979; Runnels *et al.*, 1987; Benfield *et al.*, 1988). P987$^+$ and probably also F41$^+$ strains colonize the caudal small intestine. However, no differences in the number of P987 and F41 receptors on villous enterocytes of duodenum, jejunum and ileum could be demonstrated. Besides the number of receptors, other mechanisms can determine the small intestinal colonization of an ETEC strain. It has recently been shown that intestinal fluid of the most proximal part of the small intestine is capable of suppressing the expression of K99 fimbriae, but not of K88$^+$ fimbriae (Francis *et al.*, 1989). It is not impossible that the same mechanism also can influence the expression of other fimbriae.

In conclusion, the *in vitro* adhesion assay revealed that of the tested fimbriae positive *E. coli* strains, K88ab$^+$ and K88ac$^+$ strains adhered most numerous to villi. The K88 mediated adhesion was most pronounced to jejunal villi. Results suggest that K88ab$^+$ or K88ac$^+$ ETEC are probably the most indicated strains to be used in an experimental postweaning diarrhoea model in the piglet.

Veterinary Microbiology, 15 (1987) 201-207
Elsevier Science Publishers B.V., Amsterdam—Printed in The Netherlands

III.A.3. *IN VITRO* ADHESION OF K88AB -, K88AC- AND K88AD -POSITIVE *ESCHERICHIA COLI* TO INTESTINAL VILLI, TO BUCCAL CELLS AND TO ERYTHROCYTES OF WEANED PIGLETS

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ABSTRACT

The phenotype of 21 weaned piglets, concerning adhesion of *Escherichia coli* possessing K88ab, K88ac or K88ad fimbriae to pig cells, was determined in an *in vitro* assay. Comparison was made with adhesion of these three K88 variant strains to buccal mucosal epithelial cells and to erythrocytes (haemagglutination) in the same piglets. Whereas adhesion of the three K88 variant strains to intestinal villi was piglet specific, buccal cell adhesion (BCA) and haemagglutination (HA) were not. The K88ab strain was weakly adhesive or non-adhesive in the BCA and negative in the HA test. K88ac strains consistently gave negative and K88ad consistently gave positive results in both assays. After washing the bacteria with phosphate-buffered saline, the K88ab strain revealed a positive HA test. Neither the BCA, nor HA test can be used to determine the pig intestinal adhesive phenotype.

INTRODUCTION

An important virulence factor of enterotoxigenic *Escherichia coli* (ETEC) strains is their ability to adhere with fimbrial adhesins to receptors or receptor sites on villous enterocytes (Sellwood, 1984; Klemm, 1985; Bijlsma and Bouw, 1985) . By this property, these strains can escape the natural flushing mechanism in the small intestine and colonize the small gut. A wide variety of fimbrial adhesins (fimbriae) have been reported (Klemm, 1985), of which K88 (F4) fimbriae are most commonly found on ETEC isolated from cases of diarrhoea in piglets. At present, three serological variants of this adhesion antigen— K88ab, K88ac and K88ad—have been identified (Guinée and Jansen, 1979). Using these three variants, Bijlsma *et al.* (1982) demonstrated the existence of five different porcine intestinal adhesive phenotypes and 15 intermediate phenotypes in which adhesion with one or more of the K88 variant strains was incomplete (Bijlsma and Bouw, 1985). *In vivo* identification of these phenotypes could be

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performed using either a jejunum resection (Bijlsma *et al.*, 1985b) or a biopsy technique (Snodgrass *et al.*, 1981), in combination with an *in vitro* adhesion assay. Disadvantages of routinely using such techniques are the increased susceptibility to infections as a result of the abdominal surgery (Bueno *et al.*, 1978; Altura, 1983; Papa *et al.*, 1983) and the occurrence of intestinal adhesions, as observed by Snodgrass *et al.* (1981), at necropsy of piglets several months after obtaining small intestinal biopsies. Therefore, determination of the adhesive phenotypes by a simple and non-invasive technique would be desirable. The aim of this study was to determine whether the buccal cell adhesion assay could be used for this purpose. Thorne *et al.* (1979) demonstrated the adherence of several ETEC strains to human buccal mucosal cells. We investigated the possibility of attachment of three K88 variant strains to porcine buccal cells and whether this attachment reflected adhesion to intestinal villi.

MATERIALS AND METHODS

Animals and samples

Twenty-one, 3-4-week-old, female, just-weaned piglets from primiparous sows were purchased from the same commercial farm and were individually housed. Piglets were of mixed breeding (Pietrain x Belgian Landrace) . On Day 1 of the experiment, a Silastic catheter was implanted in the left common carotid artery as previously described (Schrauwen and Houvenaghel, 1985). Heparinized blood samples were taken. These samples were tested for mannose-resistant haemagglutination and the piglets were then divided into three groups. In order to induce diarrhoea and dehydration, piglets of Group 1 were infected with ETEC (Cox *et al.*, 1986a), piglets of Group 2 with transmissible gastroenteritis virus combined with ETEC (Cox *et al.*, 1986b), whereas piglets of Group 3 served as controls. Infected piglets which developed shock either died spontaneously or were killed with an overdose of methomidate (Hypnodil ®, Janssen). The control piglets and the piglets which recovered from the experimental infection were killed with an overdose of methomidate 10 days after catheterization. Immediately, the abdomen was opened and 15-cm long segments were excised at four different places along the small intestine (duodenum, cranial jejunum, caudal jejunum and ileum) . The segments were opened and washed in Kreb's buffer pH 7.2 at 4 °C (NaCl 0.12 M, KCl 0.014 M, $NaHC0_3$ 0.025 M and KH_2P0_4 0.001 M) supplemented with 1% formaldehyde. The villi were then gently scraped from the mucosa with a glass slide, washed three times with the Kreb's buffer and stored in this buffer until assayed within 3 weeks. Simultaneously, buccal mucosal epithelial cells were scraped from the oral mucosa of the piglets, washed and maintained in the same buffer for not longer than 3 weeks.

Table I: Strains and serotypes of ETEC used in the study

Strain	Serotype		
GIS 25	O149	K91	K88ac
156KP83	O149	K91	K88ac
G 7	O8	K87	K88ab
H 56	O8	K87	K88ad
19KP74	0141	K85	K88ad

Bacterial strains

The standard ETEC strains (GIS 25, G 7, H 56) and field isolates (156KP83, 19KP74) used are listed in Table I. For long-term storage, the strains were maintained at - 20 ° C in tryptone soy broth (TSB) (Oxoid: CM129) supplemented with 20 % (v/v) glycerol. The strains were inoculated overnight on blood agar base (Oxoid:CM55) plus 5 % (v/v) whole defibrinated sheep blood at 37 ° C, examined for production of K88ab, K88ac or K88ad fimbriae with a slide agglutination test, using specific antisera (RIVM, Bilthoven, The Netherlands) and re-inoculated on TSB and tryptone soy agar (TSA) (Oxoid: CM131), for 24 h at 37 ° C. The bacterial mass in broth cultures or suspensions was estimated by measuring the absorbance (optical density) with a spectrophotometer at 620 nm (Spectronic 21 MV; Bausch and Lomb), whereafter the number of bacteria was determined by counting colony forming units (CFU) per ml culture or suspension. After 24 h incubation, the different TSB cultures showed a mean absorbance of 0.5 ($\pm 1.9 \times 10^9$ CFU ml^{-1}) . These cultures were tested for mannose-resistant haemagglutination MRHA. Strains on TSA were suspended and diluted in phosphate-buffered saline (PBS) until an absorbance of 1.1 ($\pm$ 3.9 X 10^9 CFU ml^{-1} suspension) . These suspensions were used in the villous adhesion and the buccal cell adhesion assays.

Villous adhesion assay

The villous adhesion assay was based on the technique described by Girardeau (1980). The stored villi were placed in Kreb's buffer without formaldehyde for ± 1 h, after which the supernatant was decanted. This step was repeated three times. About 30-40 villi were transferred with a pipette into 1 ml 0.1 M.phosphate buffer (pH 6.7) containing 1% (w/v) D-mannose (Difco) and 0.1 ml of a bacterial suspension in PBS was added. D-Mannose inhibits the cellular adhesion of bacteria by Type 1 pili. After gently mixing for 1 h at room temperature, some villi were removed with a pipette, placed on a glass slide under a cover slip and examined by light microscopy at magnification x 600. Adhesion was registered as positive when many bacteria covered the brush borders of the villous enterocytes, when only a few bacteria were observed adhesion was registered as weak positive.

Buccal cell adhesion (BCA) assay

The same technique was used as for the villous adhesion assay.

Mannose-resistant haemagglutination (MRHA)

This test was performed with the heparinized blood samples taken at the beginning of the experiment. Before assaying, the porcine erythrocytes (RBC) were washed in PBS and centrifuged at 1.4 X g for 5 min. Washing and centrifugation were repeated three times. The RBC were then suspended to 3% (v/v) in PBS containing 2.5% (w/v) a-methyl-D-mannopyranoside (a mannose analogue) (Janssen Chimica). As D-mannose, a-methyl-D-mannopyranoside inhibits haemagglutination of erythrocytes by Type 1 pili. One millilitre of the erythrocyte suspension was supplemented with 1 ml of a 24-h TSB culture (in glass tubes) . The reaction was read after an incubation period of 3 h at 4 ° C. When MRHA occurred, erythrocytes formed a sheet on the bottom of the glass tube. In the case of a negative MRHA, the erythrocytes formed a red spot.

Effect of cultivation and washing of K88 variant strains on MRHA of pig erythrocytes

Bacteria were inoculated in TSB or on TSA. After 18 h at 37°C, the mean optical density of the TSB cultures was 0.4. Bacteria grown on TSA were suspended as described above to an optical density of 0.7. Each culture or suspension was divided in three equal volumes. The first part of the TSB culture or the TSA suspension, TSBC and TSAS, respectively, was tested for MRHA. A second part was centrifuged at 1.4 x g for 5 min, the supernatant discarded and the bacteria were washed in PBS. Centrifugation and washing were repeated three times, after which the MRHA was assayed. The washed bacteria which had been grown in TSB (WB) had an absorbance of 0.2, the washed bacterial suspension (WS) an absorbance of 0.4. Since washing lowered the bacterial concentration, we tested the effect of this decrease by diluting the third part of broth cultures and bacterial suspensions to the same concentration as WB and WS, respectively (diluted broth culture = DB and diluted suspension = DS) . These suspensions were then tested for MRHA.

RESULTS AND DISCUSSION

The result of the *in vitro* adhesions of ETEC strains possessing K88ab, K88ac or K88ad fimbriae to intestinal villi, to buccal epithelial cells and the results of the MRHA are summarized in Table II.

No obvious differences were observed between adhesion of bacteria to villi of duodenum, cranial jejunum, caudal jejunum and ileum in the same piglet. The piglets studied belonged to Phenotypes A, B, D and E, but not to Phenotype C (susceptible to adhesion of both the ab and ad variants). Of the 21 piglets tested, 67% revealed Phenotype A (susceptible to adhesion of all three

Table II: Adhesion of K88-positive ETEC to intestinal villi, buccal cells and erythrocytes of piglets

n*	Villous adhesion**				Buccal cell adhesion			MRHA***		
	ab	ac	ad	Phenotype†	ab (n)††	ac	ad	ab	ac	ad
14	+	+	+	A	± (12),-(2)	-	+	-	-	+
1	+	+	-	B	±	-	+	-	-	+
0	+	-	+	C						
2	-	-	+	D	±	-	+	-	-	+
4	-	-	-	E	±(3), - (1)	-	+	-	-	+

*n = number of piglets; ** ab, ac, ad = the three K88 ETEC variants; + = adhesion; ± = weak adhesion ; - = no adhesion; *** MRHA= mannose-resistant hemagglutination; †Intestinal adhesive phenotypes according to Bijlsma *et al.* (1982) . †† the K88ab strain showed no buccal cell adhesion in 2/14 piglets with Phenotype A and 1/4 with Phenotype E.

K88 variants), 5% Phenotype B (adhesion of ab and ac variants), 9% Phenotype D (adhesion of ad variant), whereas 19% were totally resistant to adhesion (Phenotype E) . The adhesion to villi was piglet specific (i.e., differences were between piglets) and K88 variant specific (i.e., differences were between adhesion of the three K88 variant strains to villi of the same piglet), which is in agreement with the results of Bijlsma *et al.* (1982).

Adhesion to the buccal mucosal cells was not piglet specific, but only K88 variant specific: both K88ac strains showed no *in vitro* adhesion (sometimes a few bacteria could be seen adhering), both K88ad strains adhered intensely to the cells and the K88ab strain showed only a weak adhesion (some pigs were negative), indicating a lack of correlation with intestinal adhesion.

The MRHA was also K88 variant specific. The K88ab and K88ac strains were consistently negative and the K88ad strains were consistently positive for haemagglutination. This haemagglutination pattern was different from the MRHA observed by Bijlsma *et al.* (1985b). Under their experimental conditions, the G7 K88ab strain always caused haemagglutination. As shown in Table III, this difference was probably due to the fact that we did not wash the bacteria before use in the MRHA. Comparison of MRHA and villous adhesion revealed no correlation.

It was concluded that *in vitro* adhesion to buccal cells cannot replace the villous adhesion assay for phenotyping piglets for receptors for the K88 antigen on their villi.

Table III: The effect of washing K88-possessing ETEC on MRHA of porcine erythrocytes

	K88ab	K88ac	K88ad
TSBC and TSAS	-	-	+
WB and WS	+	-	+
DB and DS	-	-	+

TSBC = tryptone soy broth culture of ETEC; TSAS = suspension of tryptone soy agar-cultivated bacteria; WB and WS=bacteria cultivated in TSB or on TSA, respectively, and washed three times; DB and DS = diluted TSB culture or TSA suspension, respectively; + = haemagglutination; - = no haemagglutination.

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III.A.4. THE PREVALENCE OF *IN VITRO* ADHESION OF K88-POSITIVE *ESCHERICHIA COLI* TO VILLI OF JUST-WEANED PIGLETS IN A BELGIAN PIGGERY*.

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ABSTRACT

In the present study, 4- to 5-week-old female piglets (Belgian Landrace (BL) x BL, Piétrain x BL) were assayed for *in vitro* adhesion of K88ab, K88ac and K88ad *Escherichia coli* to their small intestinal villi. The piglets, which were the progeny of 43 primiparous sows, were from a commercial piggery. Adhesion of the 3 K88 variants was observed in 80.7 % of the piglets, whereas only 3.6 % were totally resistant to K88 adhesion. During the study, the percentage of piglets genetically susceptible to adhesion of the 3 K88 variant *E. coli* strains increased significantly from 69.2 % during the first 7 months to 94.3 % during the last 7 months ($p < 0.01$) of the study. Simultaneously with this increase, post-weaning diarrhoea occurred.

INTRODUCTION

Besides the production of enterotoxins, enterotoxigenic strains of *Escherichia coli* (ETEC) possess another important virulence factor, namely fimbriae. These are thread-shaped, nonflagellar, immunogenic protein polymers located on the surface of the bacteria and enabling them to adhere to receptors on villous enterocytes in the small intestine (Klemm, 1985; Parry and Rooke, 1985). Specific adherence allows the ETEC bacteria to resist the flushing mechanism of nutrients and mucus in the intestine, so facilitating colonization of the small intestinal villous mucosa.

On the basis of morphological, functional and immunological characteristics and using haemagglutination and in vitro adhesion assays, 16 different fimbriae have been identified on ETEC strains at present (Pohl *et al.*, 1982; Klemm, 1985; Parry and Rooke, 1985; Levine, 1987).

*translated from: Cox E., Houvenaghel A., 1988. *In vitro* adhesie van K88 positieve *Escherichia coli* aan het darmepitheel van pasgespeende biggen op een Belgisch varkensbedrijf. VL. Diergen. Tijdschr., 57, 90-96.

Of these, the K88 (F4) fimbriae possessing ETEC have been isolated only in pigs (Klemm, 1985; Parry and Rooke, 1985) where they are an important cause of diarrhoea in neonatal and newly-weaned piglets (Wilson and Francis, 1986). Up till now, three serological variants of the K88 adhesion antigen have been identified namely K88ab, K88ac and K88ad (Guinée and Jansen, 1979). It was demonstrated using an *in vitro* adhesion assay that in some pigs one or more of the K88 variants only adhered weakly or did not adhere to the brush border of the villous enterocytes, indicating that in these pigs there was a decreased number of receptors or that the receptors for K88 ETEC were absent (Bijlsma *et al.*, 1982; Sellwood, 1983; Bijlsma and Bouw, 1985). Decreased adhesion, as well as resistance to adhesion (no receptors) are most probably genetically determined and result in a congenital resistance of piglets to K88 mediated colibacillosis (Bijlsma and Bouw, 1985). Indeed, whenever such piglets are naturally or experimentally infected with K88 ETEC, they develop no or only a weak diarrhoea (Sellwood, 1983, 1984). It was suggested that K88 resistance is more widespread in certain pig breeds (Edfors-Lilja *et al.*, 1986). In a study about the prevalence of intestinal receptors for K88 ETEC in 10 Chinese pigs, no receptors were encountered (Chappuis *et al.*, 1985), while in Australia, 87.7 % of the pigs in a commercial herd had receptors for K88ac ETEC (Snodgrass *et al.*, 1981).

The aim of the present study was to determine in a Belgian piggery the prevalence of piglets with receptors for one or more of the K88 variant *E. coli* strains using an *in vitro* villous adhesion assay (Girardeau, 1980). The piglets were divided into five groups according to the five intestinal adhesive phenotypes described by Bijlsma et al. (1982). Pigs with *in vitro* adhesion of the 3 variants belong to phenotype A, piglets with K88ab and K88ac adhesion belong to phenotype B, piglets with K88ab and K88ad adhesion belong to phenotype C, piglets with only adhesion of K88ad belong to phenotype D and K88 resistant piglets belong to phenotype E.

MATERIALS AND METHODS

Animals

A total of 140 female piglets (4-to 5-weeks-old; 5 to 8.3 kg; Belgian Landrace (BL) x BL or Piétrain x BL) were assayed for the in vitro adhesion of K88ab, K88ac and K88ad ETEC to their villi. The piglets were form one commercial piggery and were the progeny of 43 primiparous sows (gilts) and at least 15 boars (in 11 litters the exact boar was not known since mating with 2 different boars had taken place). The sows were vaccinated subcutaneously 6 and 2 weeks prior to delivery with a polyvalent *E. coli* vaccine containing among others O149:K91:K88ac antigens (Gletvax ®, Wellcome or Stellamune coli®, Smith-Kline RIT). The number of piglets tested in each litter varied: six piglets were tested in one litter, 5 piglets in another litter and between 2 and 4 piglets in all other litters.

The piglets were immediately after weaning individually housed and were allowed to drink UHT sterilized whole cow's milk ad libitum. Then piglets were either not infected, or infected with ETEC, or with transmissible gastroenteritis virus (TGEV) and ETEC (Cox *et al.*, 1986b). Some piglets of the latter group were treated with an antisecretory drug (Cox *et al.*, 1987). The piglets died as a result of diarrhoea and hypovolaemic shock or they were euthanatized with an

overdose of methomidate (Hypnodil ®, Janssen) within 10 days after the start of the experiment.

Samples

Immediately after death or euthanasia, the abdomen was opened and 15-cm long intestinal segments were excised from duodenum (2 cm caudal to the pylorus), cranial jejunum (1/3 of the total length of the small intestine), caudal jejunum (2/3 of the total length of the small intestine) and ileum (2 cm cranial to the ileocaecal valve). These segment were opened, and washed in a Krebs buffer containing 1 % formaldehyde, whereafter villi were gently scraped from the mucosa with a glass slide. Villi were washed several times in the Krebs buffer with formaldehyde and subsequently stored in this buffer. The *in vitro* villous adhesion assay was performed no longer then 3 weeks following sampling of the villi.

Bacterial strains and in vitro villous adhesion assay

The preparation of the bacterial suspensions and the *in vitro* villous adhesion assay were performed as recently described (Cox and Houvenaghel, 1987). Briefly, forty to fifty villi were incubated for 1hour at room temperature with a bacterial suspension of one of the following K88 ETEC strains: GIS 25 (O149:K91:K88ac), 156KP83 (O149:K91:K88ac), G7 (O8:K87:K88ab), E68 type 1 (O141:K85:K88ab), H56 (O8:K87:K88ad) and 19KP74 (O141:K85:K88ad). The bacterial suspension contained approximately 4.5 x 10^9 bacteria ml^{-1} suspension. Subsequently, an examination by light microscopy at a magnification of 600 x was carried out to determine whether bacterial adhesion to the villous-brush border had occurred. No distinction was made between weak and normal adhesion. A possible interference of the experimental *in vivo* infections with ETEC or with TGEV and ETEC on the interpretation of the *in vitro* adhesion assay was avoided as much as possible carrying out the test on villi of duodenum, jejunum and ileum (duodenum is not or almost not infected with TGEV) and by examining the villi by light microscopy before the adhesion assay was performed. When bacterial adhesion to the villi was observed, the number of bacteria per 250 µm villus length was counted and compared with the number counted after the *in vitro* adhesion.

After the *in vitro* villous adhesion assay was performed, the piglets were divided into one of the five above-mentioned phenotypes. During the first 7 months of this study, 65 piglets were tested (group I), during the subsequent 7 months, 40 piglets (group II) and during the last 7 months, 35 piglets (group III). The 3 groups were statistically compared by means of the chiquadrate test.

RESULTS

In all piglets adhesion of a K88 variant strains was observed either to villi of all 4 intestinal segments or no adhesion occurred. The prevalence of the porcine phenotypes concerning *in vitro* adhesion of K88 positive *E. coli* to villi is presented in Table 1. During the complete period of 21 months, a total of 80.7 % of the tested piglets belonged to phenotype A. Twenty-nine of these

piglets were obtained from 8 different litters which were the progeny of one boar and 8 primiparous sows. Only 19.3 % (27) of all tested piglets had an intestinal adhesive phenotype different from A. These 27 piglets belonged to 18 different litters (at least 10 boars and 18 gilts). Five of these 27 piglets were resistant to adhesion of the 3 K88 variant strains (phenotype E).

The percentage of piglets susceptible to adhesion of the 3 K88 variant strains to their villi (phenotype A) increased significantly during the period of 21 months during which the adhesion assays were performed, while the phenotypes B, C, D and E decreased. The percentage of piglets with the A phenotype rose from 69.2 % during the first 7 months (period I), to 87.5% the subsequent period (period II) and to 94.3 % during the last 7 months (period III). Comparison of the percentage of piglets with receptors for K88ab (the sum of phenotypes A, B and C), for K88ac (phenotypes A and B) or for K88ad (phenotypes A, C and D) between the 3 successive 7 month periods revealed a significant increase ($p < 0.05$). This increase was most pronounced for piglets with the K88ac receptors and amounted 15.9 % (from 78.4 % during the first period to 94.3 % during the third period).

Table 1: The prevalence of porcine phenotypes concerning *in vitro* adhesion of K88ab, K88ac and K88ad-positive *E. coli* to villi of weaned piglets from a Belgian piggery.

adhesion of K88 E. coli[a]			phenotype[b]	period[c] % piglets			Total % piglets
K88ab	K88ac	K88ad		I (n=65)	II (n=40)	III (n=35)	(n=140)
+	+	+	A	69,2	87,5	94,3	80,7
+	+	-	B	9,2	5,0	0,0	5,70
+	-	+	C	7,7	5,0	5,7	6.4
-	-	+	D	6,2	2,5	0,0	3,6
-	-	-	E	7,7	0,0	0,0	3,6

[a] + = adhesion, - = no adhesion; [b] adhesive phenotypes according to Bijlsma et al. (1982); [c] Period October 1985 - April 1986: I = 1st, II = 2nd, III = 3rd successive period of 7 months; n = number of piglets.

During the course of the study, a gradually worsening weanling diarrhoea was observed in the piggery. Diarrhoea occurred in 2 to 3 % of the weaned litters during the first period. The occurrence of diarrhoea increased to about 30 % of the newly-weaned litters during the second period and to 100 % halfway through the third period. About 340 litters were weaned per period

of 7 months. The morbidity within the affected litters varied greatly during the first period but subsequently increased to become mostly 100 % during the third period. Nevertheless, the percentage of mortality in the piglets did not increase. In faecal samples of piglets with diarrhoea during the second period, but especially during the third period, rota- and pararotavirus (diagnoses by ELISA, Dr. P. Callebaut, Laboratory of Veterinary Virology, State University of Ghent) and O149:K91:K88ac *E. coli* could be identified (serotyping, Dr P. Pohl and Dr. P. Lintermans, National Institute for Veterinary Research, Brussels).

DISCUSSION

In the present study, the *in vitro* adhesion of K88 fimbriae producing *E. coli* to the brush border of villous enterocytes was examined in 4- to 5-weeks-old female piglets. Since neither age (Runnels *et al.,* 1980), nor sex difference (Bijlsma *et al.,* 1985a) influences K88 adhesion in pigs, the obtained results present a good picture on the prevalence of pigs with K88 receptors on the Belgian piggery.

A very high percentage of piglets had receptors for the 3 K88 variant strains and was, therefore, susceptible to K88 mediated colibacillary diarrhoea. Only about 4 % of the piglets was totally resistant to K88 adhesion. The piglets studied were purebred Belgian Landrace or crossbred Belgian Landrace x Piétrain, so it can be assumed that K88ab, K88ac and K88ad receptors appear frequently in both Belgian breeds.

In Belgium, mostly K88ac fimbriae producing *E. coli* strains are isolated from the faeces of piglets with colibacillary diarrhoea (activities report 1985-86, National Institute for Veterinary Research, Brussels). Pigs with receptors for K88ac ETEC are, therefore, probably more susceptible to colibacillosis. About 87 % of the piglets of the piggery under study had K88ac receptors. The same high percentage of K88ac susceptible pigs was observed by Snodgrass *et al.,* (1981) in an Australian farm. However, their and our data are from one farm only and are, therefore not representative for the situations in the two countries. In a Dutch study (Bijlsma *et al.,* 1985a), in which pigs from different slaughterhouses were examined, considerably better results were obtained since almost 50 % of the tested animals were completely resistent to K88 adhesion (phenotype E).

It was seen in the present study that the percentage of piglets with K88 receptors increased gradually, though significantly, during the 21 months in which piglets were tested for *in vitro* adhesion of the K88 variant strains to their villi. At the same time, weanling diarrhoea gradually developed in the piggery. K88ac *E. coli* were isolated from piglets with diarrhoea. These findings suggest that the increase of piglets with K88 receptors may have been a causal factor in the occurrence of weanling diarrhoea in the piggery. However, when only the increase in the percentage of K88ac susceptible piglets (from 78.4 % in the first period to 94.3 % in the third period) is considered, the change is too small to draw such a far-reaching conclusion. The results of the present study, however, indicate that in the evaluation of farms with weanling diarrhoea the genetic susceptibility of the piglets to adhesion of K88 *E. coli* to their villi has to be taken into account. This could allow to interfere in the breeding using K88 negative boars (phenotype E).

Such an intervention would not only result in a direct decrease of the diarrhoea induced by K88 ETEC, but will finally make vaccination against K88 positive *E. coli* unnecessary.

Walters and Sellwood (1984) observed no differences in growth rate between K88 receptor positive and negative piglets. The results of a recently performed Swedish study (Edfors-Lilja *et al.*, 1986), on the other hand, indicate that K88 receptor positive piglets have a poorer daily weight gain during the first weeks of live, but grow better during the fattening period. In their study, however, weak piglets, very small piglets and piglets with defects were not included. This selection probably influenced the results of their study since precisely these piglets are more susceptible to diarrhoea and the negative effects of diarrhoea. Nevertheless, their study shows that it is necessary to have information about the physiological function of the K88 receptor before pigs without K88 receptors should be bred on a large scale. At present, intervention in the breeding, however, is still very difficult. Indeed, the presence of K88 receptors in pigs can only be demonstrated on the villi of the small intestine. In the present study, this was assayed postmortem. It is possible to identify the adhesive phenotypes in live pigs when a small intestinal resection (Bijlsma *et al.*,1984) or biopsy (Snodgrass *et al.*, 1981) is carried out and the susceptibility to K88 adhesion to villi is examined using an *in vitro* adhesion assay (Girardeau, 1980; Sellwood and Lees, 1980; Thorns and Morris, 1985) or an enzyme immunoassay (Chandler *et al.*, 1986). It is, however, difficult to investigate the genetic susceptibility to K88 adhesion in live pigs on a large scale using these techniques.

III.B. A DIARRHOEA MODEL IN NEWLY-WEANED PIGLETS

III.B.1. INTRODUCTION

In order to evaluate the effect of antisecretory drugs on diarrhoea with subacute dehydration in newly-weaned piglets, it was necessary to develop a consistently reproducible diarrhoea model.

As has been described in chapter I.C. enterotoxigenic *Escherichia coli* (ETEC) have been implicated as an important causal agent in the etiology of post-weaning diarrhoea (PWD). Following infection, there strains adhere with fimbriae to the small intestinal villous mucosa and subsequently colonize the small gut. Furthermore they elaborate heat-labile (LT) and/or heat-stable (ST, type a and/or type b) enterotoxins which induce a net secretion of water and electrolytes in the small intestine. LT increases the intracellular concentration of cyclic adenosine monophosphate (cAMP) resulting in an increased secretion and a decreased absorption of water and electrolytes (Field, 1979; Berridge, 1984). STa increases the intracellular level of cyclic guanosine monophosphate (cGMP) mainly resulting in a decreased absorption (Dreyfus *et al.*, 1984). STb has been observed to induce a slight villous atrophy so decreasing the absorptive surface in the small gut and to stimulate the active secretion of an unidentified anion (Whipp *et al.*, 1985; Weikel *et al.*,1986).

It was tried to induce diarrhoea and subacute dehydration in newly-weaned piglets by administering drugs or toxins which stimulate small intestinal secretion (secretagogues) and so mimiced the effect of ETEC or by inoculating piglets with K88ac$^+$ ETEC. As secretagogues piglets were given LTp-enterotoxin, cholera toxin, castor oil, DL 5-hydroxytryptophan or a combination of castor oil and DL-5-hydroxytryptophan.

Inoculation of piglets with ETEC was performed using two ETEC strains which both produced K88ac$^+$ fimbriae since K88ac$^+$ *E. coli* were seen to adhere numerously to villi of newly-weaned piglets (see chapter III.A.2). Both strains produced STa, STb and LT enterotoxins. Besides ETEC, factors which either enhance the colonization of the intestinal tract with ETEC and/or induce small intestinal malabsorption can play a role in the etiology of the PWD syndrome. Therefore, three different inoculation experiments were performed. In the first experiment, piglets were infected with both ETEC strains alone. In the second experiment, they were orally pretreated with chloramphenicol and subsequently inoculated with both ETEC strains. In the third experiment, the chloramphenicol pretreatment was followed by an inoculation with transmissible gastroenteritis virus (TGEV) and one day later with ETEC.

Chloramphenicol was given to the piglets, since chloramphenicol has been mentioned to decrease the colonization resistance in the small intestine by suppressing the normal anaerobic flora. This colonization resistance suppressing effect results in an increase in the number of fecal coliforms within 4 days administration (Kaufman, 1984), so that it could be expected that chloramphenicol treatment makes the intestine more susceptible to colonization with ETEC.

Villous atrophy is a consistent finding in piglets with PWD. It can be induced by an infection with an enteropathogenic virus such as rotavirus or transmissible gastroenteritis virus

(TGEV) (Moon *et al.*, 1973; Shaw *et al.*, 1989). Concurrent infection of ETEC and rotavirus or TGEV have been reported in piglets with post-weaning diarrhoea (Tzipori *et al.*, 1980b; Wilson and Francis, 1986; Pritchard, 1987). A combined experimental infection of newly-weaned piglets with rotavirus and ETEC caused a more severe diarrhoea than an infection with either pathogen alone (Tzipori *et al.*, 1980a; Kyriakis, 1982; Lecce *et al.*, 1982). Underdahl *et al.* (1972) obtained similar results using a combined infection with a cell-cultured attenuated strain of TGEV in combination with a mildly virulent *E. coli* strain. Pritchard (1987) observed a more severe diarrhoea and mortality in endemically with TGEV infected herds when a concurrent infection with ETEC occurred. Since, rotavirus is enzootic in Belgian piggeries and most piglets are infected with rotavirus before the age of 6 tot 7 weeks (Debouck, 1984), piglets were inoculated with TGEV in stead of rotavirus.

Only chloramphenicol pretreatment followed by an inoculation with transmissible gastroenteritis virus (TGEV) and one day later with ETEC consistently induced diarrhoea. In this model clinical, hematological, biochemical, hemodynamic, histopathological and ultrastructural changes were studied.

III.B.2. EXPERIMENTAL INDUCTION OF DIARRHOEA IN NEWLY WEANED PIGLETS

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ABSTRACT

To induce diarrhoea and hypovolaemia, newly-weaned conventionally bred piglets (3- to 4-weeks-old), were either given secretagogues or were inoculated with enterotoxigenic *Escherichia coli* (ETEC).

Choleratoxin (n=2), *E. coli* heat-labile enterotoxin (n=2) or castor oil (n=2) were given intragastrically or piglets were intraperitoneally injected with DL 5-hydroxytryptophan (n=3). These substances induced a transient diarrhoea without clinical symptoms of dehydration. Therefore, a combination of castor oil and DL 5-hydroxytryptophan was given two times a day during 3 consecutive days to 3 piglets. Although diarrhoea lasted for 5 days, still no hypovolaemia occurred.

Probably the secretagogues have to be given continuously to mimic the continuous release of enterotoxins during secretory colibacillary diarrhoea. It was, therefore, tried to reproduce colibacillosis in the just-weaned piglets. Animals were inoculated with K88ac fimbriae producing ETEC strains (O149:K91:K88ac; LT, Sta and STb positive)(n=7), or pretreated with chloramphenicol followed by the ETEC inoculation (n=8), or pretreated with the antibiotic, inoculated with an enteropathogenic coronavirus, transmissible gastroenteritis virus (TGEV) and subsequently inoculated with ETEC (n=18). Only the last procedure induced a reproducable diarrhoea (93 %) and dehydration resulting in a mortality of 80 %.

It was concluded that the latter experimental procedure could be used to study diarrhoea and hypovolaemia in newly-weaned piglets and to evaluate the effect of potentially antisecretory drugs on postweaning diarrhoea in piglets.

INTRODUCTION

Enterotoxigenic *Escherichia coli* (ETEC) are implicated as an important causal agent in diarrhoea in men and young animals. These ETEC strains possess important virulence factors namely fimbriae and enterotoxins. The fimbriae are long protein polymers on the surface of the bacteria which allow them to adhere to receptor or receptor sites on villous enterocytes and subsequently to colonize the small intestinal mucosa (Klemm, 1985). Furthermore, they elaborate

heat-labile (LT) and/or heat-stable (ST, type a and/or type b) enterotoxins which induce a loss of fluid and electrolytes in the small intestine resulting in a watery diarrhoea. The LT-enterotoxin exerts this effect by increasing the intracellular level of cyclic adenosine monophosphate (cAMP) in small intestinal enterocytes. cAMP increases in crypt enterocytes the secretion and it decreases in villous enterocytes the absorption of water and electrolytes (Field, 1979; Berridge, 1984). STa acts by increasing the intracellular level of cyclic guanosine monophosphate (cGMP). cGMP mainly decreases the absorption and has almost no effect on secretion (Dreyfus *et al.*, 1984). The mechanism of the STb-enterotoxins is not yet known. It has been observed to induce a slight reduction in villous length so decreasing the absorptive surface in the small gut (Whipp *et al.*, 1985).

ETEC is implicated as an important causal agent of postweaning diarrhoea (PWD) in piglets. Inoculation of weaned piglets with ETEC, however, does not always result in disease (Smith and Halls, 1968). Besides the presence of enterotoxigenic *E. coli* (ETEC) in the intestine other factors have been mentioned to play a role in PWD such as the genetic susceptibility of the host to adhesion of K88 fimbriae producing ETEC strains to small intestinal villi (Bijlsma *et al.*, 1982; Sellwood, 1984), nutritional, environmental and immunological factors (Miller *et al.*, 1984; Sellwood, 1984) and infection with enteropathogenic viruses (Lecce *et al.*, 1982; Pritchard, 1987; Wilson and Francis, 1986).

The aim of the present study was to find a model for studying diarrhoea and subacute dehydration in just-weaned piglets. This was tried either by administering drugs which stimulate the small intestinal secretion or by inoculating the piglets with ETEC.

MATERIALS AND METHODS

Pigs

The experiments were performed on 45, just-weaned, female piglets of mixed breeding (3 to 4 weeks old and weighing 5 to 7.2 kg), from a commercial farm. Immediately after weaning, the piglets were individually housed at room temperature (26 ± 2 °C). During the experiments they were allowed to drink milk ad libitum (UHT sterilised whole cow's milk).

Experimental procedure

In a first experiment, 1 or a combination of 2 secretion inducing substances were administered to piglets. They were given LT-enterotoxin (Smith-Kline RIT)(n=2, intragastrically in a dose 14 times the dose provoking severe diarrhoea in neonatal piglets; Dr. L. Dobrescu, personal communication), cholera toxin (Difco)(n=2, intragastrically 1 mg/piglet), castor oil (n=2, intragastrically 60 ml and 7 h later 30 ml/piglet), DL 5-hydroxytryptophan (5-HTP) (Aldrich Chemical Co.) (n=3, intraperitoneally 50 mg/kg twice with an interval of 1 hour). Three piglets were given a combination of castor oil (60 ml/piglet) and 5-HTP (50 mg/kg) simultaneously twice a day during 3 consecutive days. A silastic catheter (Dow Corning) was implanted in the left carotid artery in 1 of these 3 piglets as described by Schrauwen and Houvenaghel (1985) for haematocrit determination. All piglets were observed for duration and severity of diarrhoea and for

symptoms of dehydration.

In a second experiment, 7 piglets were catheterized on day 1. These piglets were inoculated on day 4 with 2 ETEC strains (group ETEC). Before the inoculation, piglets were starved during 3 h, were subsequently given 62 ml of a 1.4 % NaHCO3 solution to neutralize gastric acidity (prevention of ETEC destruction), whereafter they were inoculated intragastrically with 10 ml of a bacterial suspension of field-isolate 156KP83 (O149:K91:K88ac, LT, STa, STb) and 10 ml of suspension containing the laboratory strain GIS 25 (O149:K91:K88ac, LT, STa, STb). The inoculum was prepared from 24h cultures on Brain Heart Infusion agar (Oxoid, England) of both strains which were suspended and diluted in sterile salt solution to a final concentration between 0.7 and 1.0 x 10^9 viable bacteria/ml.

In a 3rd experimental group (group CH-ETEC), 8 catheterized piglets were pretreated on day 1, 2 and 3 with chloramphenicol (1875 mg/l milk; Chloromycetin®, Parke-Davis) before they were inoculated with the same 2 ETEC strains on day four.

A 4th experimental group consisted out of 8 catheterized (group CH-TGE-ETEC) and 10 non-catheterized piglets (group CH-TGE-ETEC-NC). In this experiment, the 3 days oral antibiotic treatment was followed by an inoculation with transmissible gastroenteritis virus (TGEV) on day 4. TGEV was given orally after 3 hours of starvation. Pigs were inoculated with 1.66 x 10^6 pig infective dose. On day 5, piglets were inoculated with the 2 ETEC strains as described.

Control piglets were treated with chloramphenicol on days 1, 2 and 3 and were given 62 ml NaHCO3 solution and 15 min later 20 ml physiological salt solution on day 5 (group CH; n = 2), were pretreated with chloramphenicol and infected with TGEV on day 4 (group CH-TGE; n = 4), or received no treatment (group C; n = 3).

During the experiments the following parameters were daily registered in all experimentally inoculated piglets: survival, appearance and severity of diarrhoea, weight-change and body temperature. In catheterized piglets, the Hct was also daily determined. Piglets of both control groups were observed daily for the presence of diarrhoea. Faecal swabs for detection of hemolytic *E. coli* were taken on day 3 in all piglets. These swabs were inoculated onto blood agar plates at 37 °C for 24 hours.

Pigs were euthanatized with an overdose methomidate (Hypnodil®, Janssen). This occurred for 2 piglets of the ETEC group, 2 of the CH-ETEC group, 1 of the CH group, 2 of the C group and 2 of the TGE group on day 8, for piglets with severe diarrhoea when shock occurred and for all other piglets at the end of the tenth day.

Small intestinal segments were excised from the caudal jejunum (2/3 of the total length of the small intestine) and from the ileum (2 cm cranial to the ileocaecal valve). Segments of 15 cm in length were opened. Villi were scraped from the mucosa and the ability of a K88ac strain to attach to the intestinal villi of the piglets was assayed in vitro by the technique of Girardeau (1980).

Specimens of the caudal end of the excised small intestinal segments of catheterized piglets were fixed in 10 % phosphate-buffered formalin for histological examination. This was not done for piglets of the CH-TGE-ETEC-NC group or the CH-TGE group.

Statistical procedures

Values were given as means ± SEM and analysed for statistical significance using a one-way analysis of variance. Simple contrasts were used to assess statistical differences between values of the third day and the values after ETEC infection. The Mann-Whitney U test was used to demonstrate statistical differences between time-matched values of the different variables and between the duration of diarrhoea in both groups.

RESULTS.

Administration of secretion inducing drugs induced a transient diarrhoea without clinical symptoms of dehydration. The induction time was 1 h for 5-HTP and castor oil, 2h for LT-enterotoxin and 12 h for choleratoxin. The faeces were pasty to semiliquid and diarrhoea lasted 2 h for cholera toxin, 12 h for 5-HTP, 24 h for LT-enterotoxin, 30 h for castor oil and 5 1/2 day when a combination of castor oil and 5-HTP was given during 3 consecutive days. No increase in Hct was observed in the catheterized piglet given this combination. The Hct remained approximatively 34. 5-HTP induced a temporary erythema.

Five piglets, the 2 piglets of the ETEC group which were euthanatized on day 8 of the experiment and 3 of the CH-TGE-ETEC-NC group euthanatized on day 10 showed no in vitro adhesion of the K88ac strain to their villi. Data of these piglets, which developed diarrhoea but all survived the observation period, were not included in the results. In all other piglets a significant in vitro villous adhesion was observed showing that these piglets were genetic susceptible to K88ac adhesion to their villi.

In none of the piglets, ETEC could be isolated from rectal swabs taken before the inoculation on day 3. Nevertheless, diarrhoea was observed on day 3 in 12.5 % of the piglets not pretreated with chloramphenicol (40 % piglets of the ETEC group and none of the C group) and in 69 % of the piglets pretreated with the antibiotic namely in 75 % of piglets of the CH-ETEC and CH-TGE-ETEC groups, in 71 % piglets of the CH-TGE-ETEC-NC group (Fig. 1) and in 50 % of piglets of the CH and CH-TGE group.

In the ETEC-group, inoculation of the 5 piglets which were genetic susceptible to K88ac adhesion resulted in diarrhoea or an aggravation of diarrhoea in 60 % of the piglets (Fig. 1), in a transient but not significant drop in weight gain (Table 1), and in a significant decrease in haematocrit (Fig. 2). No significant changes in body temperature were registered (Fig. 3). All piglets survived the infection (Fig. 4). The mean duration of the diarrhoea was 2.2 ± 1.0 days. At necropsy no macroscopic lesions were observed. The histological examination revealed the presence of coliform bacteria on villi of 1 of the piglets euthanatized on day 7 after ETEC inoculation.

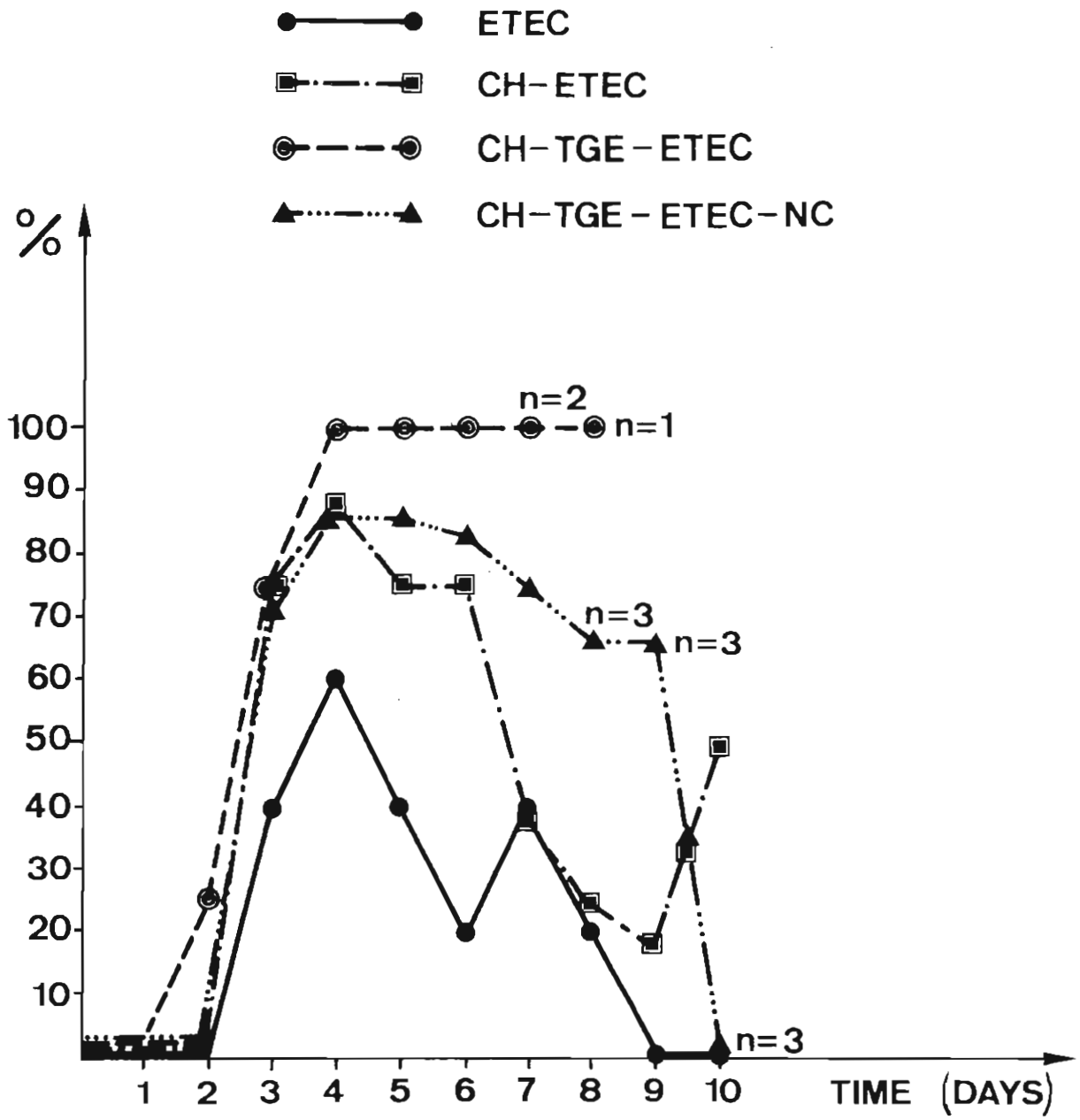


Fig. 1. Percent piglets with diarrhoea following different inoculations (ETEC: inoculation with ETEC of 5 piglets with a catheter in the left carotid artery; CH-ETEC: pretreatment with chloramphenicol during 3 days followed by an inoculation with ETEC of 8 catheterized piglets; CH-TGE-ETEC: the chloramphenicol pretreatment of 8 catheterized piglets is immediately followed by an inoculation with transmissible gastroenteritis virus and one day later by an inoculation with ETEC; CH-TGE-ETEC-NC: the same procedure as for the CH-TGE-ETEC group in 7 piglets without an arterial catheter).

Table 1. Daily weight change in g ± SEM following different inoculations of newly-weaned piglets (see Fig. 1 for the legend).

Time in days	ETEC	CH-ETEC	CH-TGE-ETEC	CH-TGE-ETEC-NC
1	-385 ± 90	-236 ± 30	-142 ± 70$^{(a)}$	- 43 ± 50
2	-385 ± 90	+ 92 ± 80	+ 48 ± 30	+153 ± 60
3	+156 ± 80	+291 ± 70	+176 ± 60	+173 ± 60
4	+ 87 ± 50	- 22 ± 40**	- 151 ± 140*	- 9 ± 60*
5	- 28 ± 90	+126 ± 70	- 447 ± 100$^{***(a)}$	- 236 ± 140**
6	+139 ± 20	+107 ± 70	- 360 ± 50$^{**(aa)}$	- 265 ± 150**
7	+130 ± 20	+125 ± 60	- 455, -325	- 83 ± 130
8	+170 ± 120	+252 ± 120		+129 ± 30
9	+153 ± 50	+241 ± 100		+ 43 ± 100
10	+230 ± 120	+346 ± 120		+191 ± 20

Significant differences from the values prior to infection (day 3) are indicated by $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. Significant differences between the ETEC group and the CH-TGE-ETEC group are indicated by $^{a}p < 0.05$; $^{aa}p < 0.01$.

On day 4, inoculation induced in the CH-ETEC group diarrhoea or an aggravation of diarrhoea in 88 % of the piglets (Fig. 1), a significant weight loss (Fig. 2) and a slight, but not significant, increase in haematocrit (Fig. 3). The following days the mean weight gain increased and the haematocrit gradually decreased. On days 5 and 6 several piglets started vomiting and one piglet of this group died 5 days after inoculation (Fig. 5). There was no significant change in body temperature (Fig. 4). The mean duration of the diarrhoea for this group was 4.3 ± 0.5 days. At necropsy no lesions were observed. Coliform bacteria were seen by light microscopy on villi of both piglets euthanatized 5 days after ETEC inoculation and 2 piglets euthanatized 7 days after inoculation.

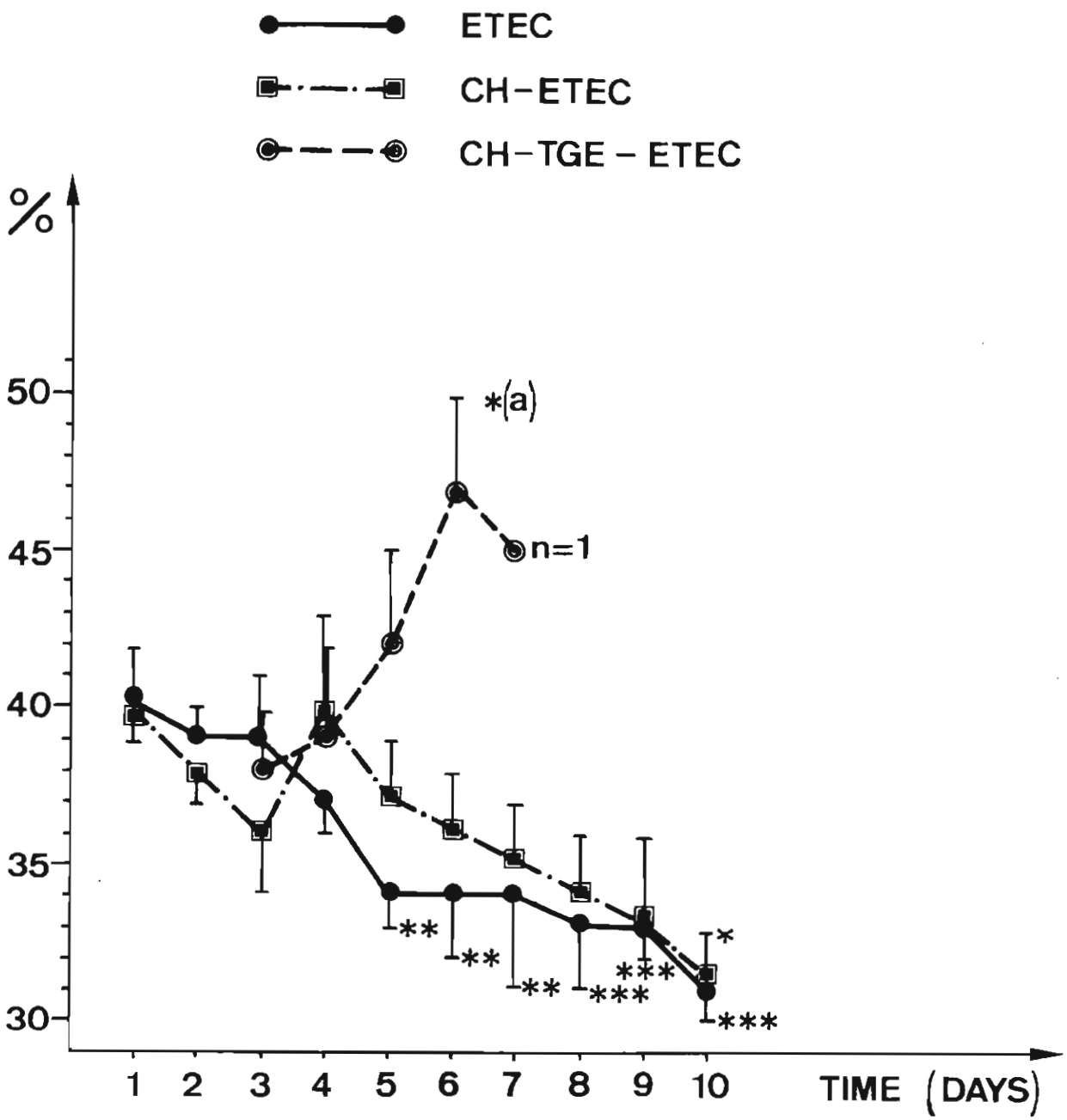


Fig. 2. Changes in haematocrit following different inoculations of newly-weaned piglets (see Fig. 1 for the legend). The values represent means ± SEM. Significant differences from the values prior to infection (day 3) are indicated by * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Significant difference between the ETEC group and the CH-TGE-ETEC group is indicated by [a] $p < 0.05$.

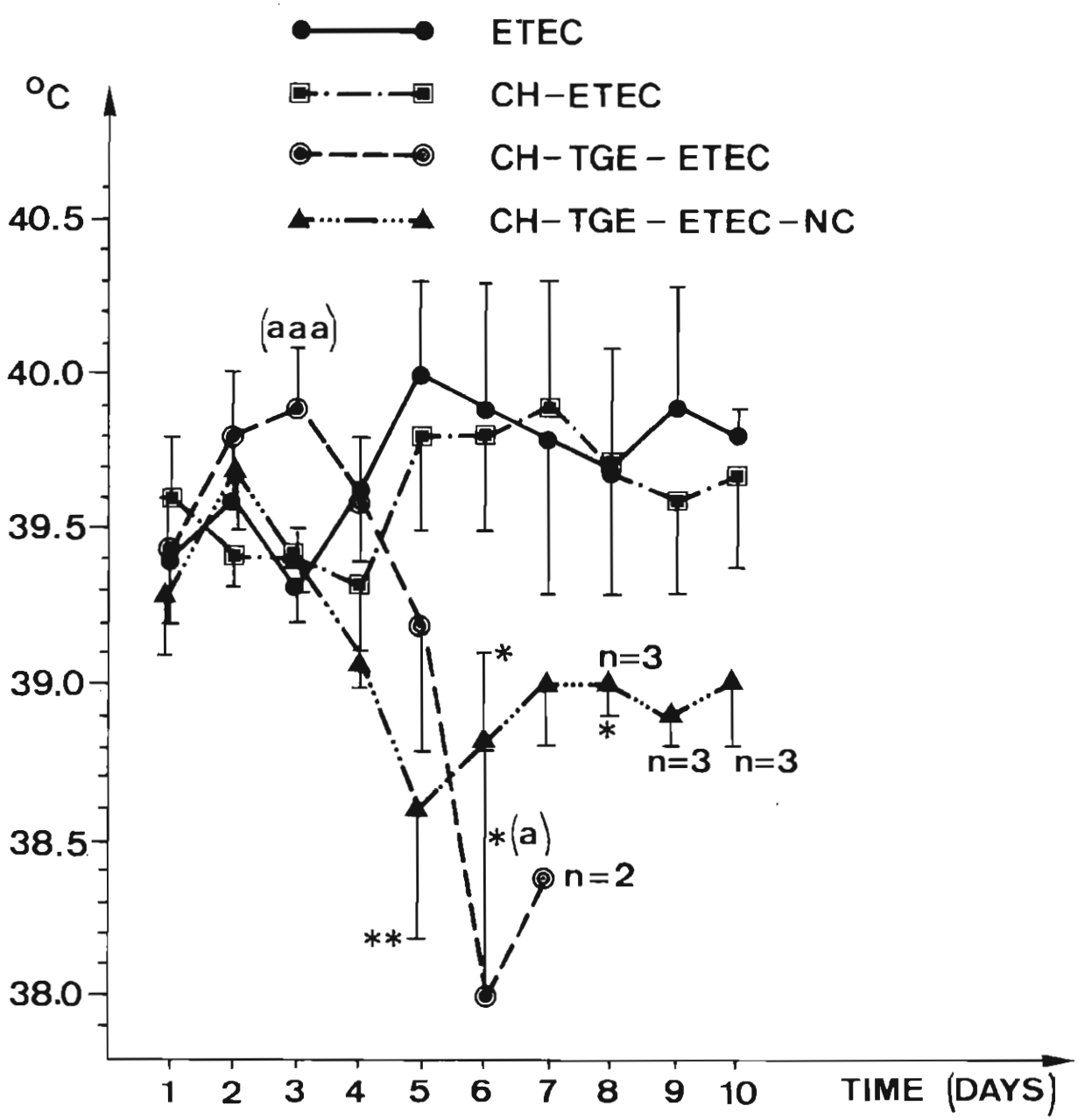


Fig. 3. Changes in body temperature following different inoculations of newly-weaned piglets (see Fig. 1 for the legend). The values represent means ± SEM. Significant differences from the values prior to infection (day 3) are indicated by * $p < 0.05$; ** $p < 0.01$. Significant differences between the ETEC group and the CH-TGE-ETEC group are indicated by ^{a} $p < 0.05$; ^{aaa} $p < 0.001$.

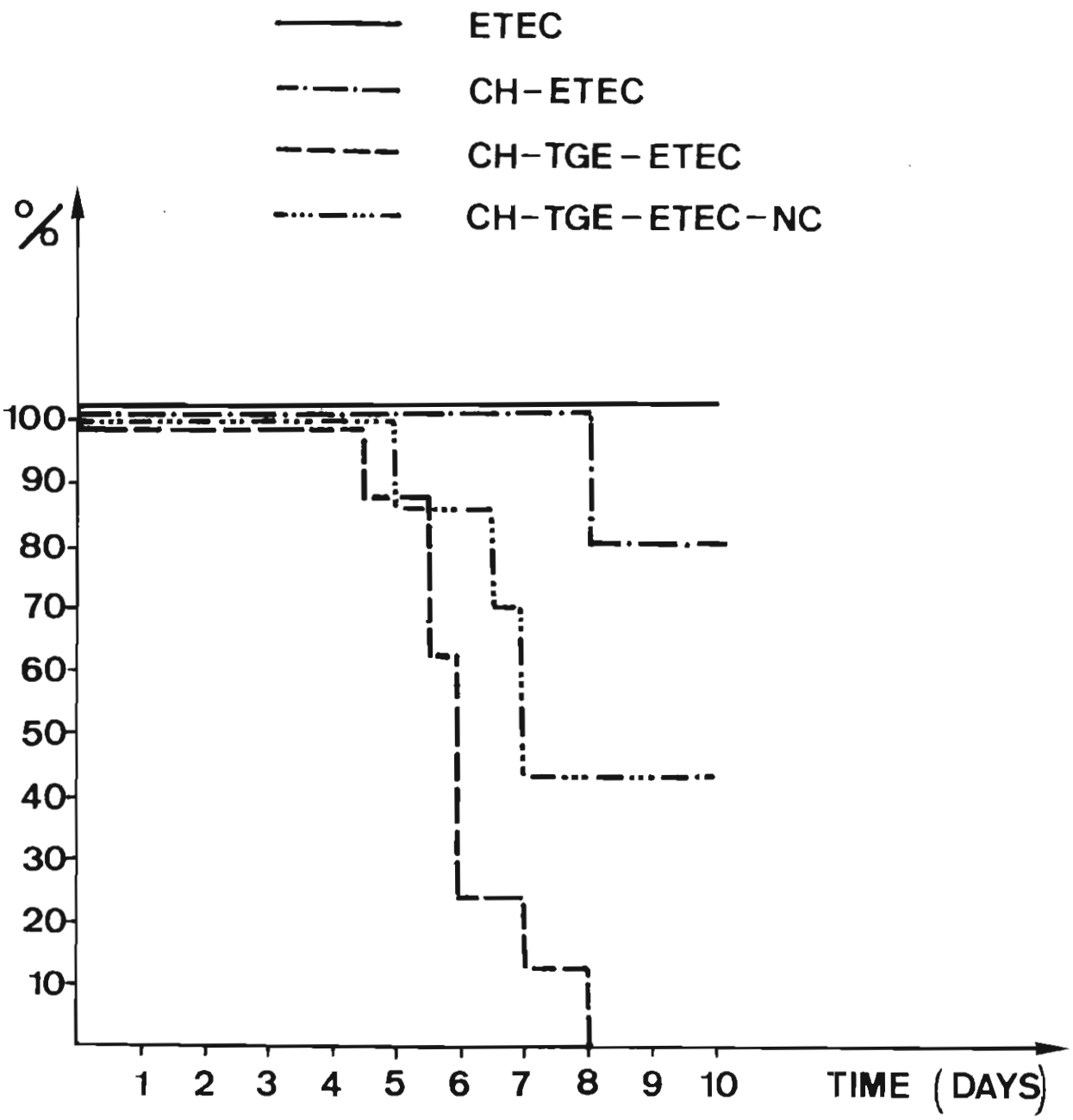


Fig. 4. Survival rate following different inoculations (see Fig. 1 for the legend)

In the CH-TGE-ETEC group, a severe persisting diarrhoea occurred in all piglets (Fig. 1) and vomiting in most, resulting in a significant weight loss (Table 2), haemoconcentration (Fig 2.), decrease in body temperature (Fig. 3) and a 100 % mortality within 5 days following the TGEV inoculation (Fig. 4). The mean survival time of the piglets was 3.3 ± 0.5 days. The gross changes at necropsy of shocked piglets were: cyanosis of the extremities, severe congestion of the fundus mucosa of the stomach, watery content in and congestion of the thin-walled small intestine and in few piglets congestion of the colon wall. Light microscopic examination of caudal jejunum and ileum of the piglets showed severe villous atrophy and adhesion of coliform bacteria to the villi.

In the CH-TGE-ETEC-NC group, 1 of the 7 K88ac susceptible piglets did not develop diarrhoea (Fig. 1). Inoculation induced a significant weight loss (Table 2) and decrease in body temperature (Fig. 3). Three of the piglets (2 with diarrhoea) survived the experiment (Fig. 4). The mean survival time of the piglets which developed shock was 3.4 ± 0.5 days.

In group CH, diarrhoea occurred in one of both piglets at the third day of the chloramphenicol treatment and resolved within 1 day. In the C group, 2 piglets showed diarrhoea 6 days after weaning during 1 and 2 days respectively and one of both animals started vomiting. At necropsy no lesions were seen. Histological examination of the caudal small intestine showed long villi. In the CH-TGE group, 1 of 4 piglets did not develop diarrhoea, whereas 2 piglets showed diarrhoea following the chloramphenicol pretreatment and the 4th piglet following the TGEV inoculation. In the 3 latter piglets, diarrhoea remained present until they were euthanatized on day 8, 10 and 10 respectively.

Comparison of ETEC and CH-ETEC groups showed a significantly longer duration of the diarrhoea in the CH-ETEC group ($p < 0.05$). However, Hct, weight change, body temperature and survival did not differ significantly.

Comparison of CH-ETEC and CH-TGE-ETEC groups revealed a significant higher weight loss on days 5 and 6, a significant increase in haematocrit on day 5, a significant decrease in body temperature on day 6 and a significant higher mortality in the CH-TGE-ETEC group.

Comparison of the registered parameters between the catheterized and non-catheterized piglets pretreated with chloramphenicol and inoculated with TGE and ETEC, revealed a similar but less severe clinical response in the non-catheterized piglets. In the CH-TGE-ETEC-NC group, one piglet did not develop diarrhoea following inoculation, the weight loss on day 5 was significantly less pronounced and mortality was lower.

DISCUSSION

In the present study, several substances were administered to piglets to induce diarrhoea and dehydration. Substances used stimulate the intestinal loss of water and electrolytes by effects on the active small intestinal secretion and absorption. LT-enterotoxin and cholera toxin stimulate adenylate cyclase and so the production of cAMP and induce the release of serotonin (5-HT) by enterochromaffin cells (Berridge, 1984). Serotonin induces diarrhoea by stimulating intestinal motility and secretion (Doherty, 1981). Hypersecretion is provoked by stimulating the influx of Ca^{2+} into enterocytes and by inducing the release of vasoactive intestinal peptide (VIP) from

afferent nerves (Cassuto *et al.*, 1983). VIP subsequently activates adenylate cyclase. 5-HTP is converted to 5-HT (Gershon and Ross, 1966). Castor oil is an hydroxy fatty acid and is mentioned to induce hypersecretion by stimulating adenylate cyclase (Binder, 1983). All tested products induced diarrhoea. However, no dehydration occurred even though diarrhoea could last for several days. The induced small intestinal fluid loss was probably not severe enough so that a compensation could occur by an increased fluid absorption in the colon (Argenzio,1985). Furthermore, an increased motility of the small intestine could have resulted in a quick clearance of the oral administered secretagogues from the intestinal tract. This increased motility is either caused by a direct effect of the secretagogues (e.g. LT-enterotoxin) (Borriello, 1978) or by a reflex due to a distension of the gut by local accumulation of fluid. To induce hypovolaemia, hypersecretion inducing drugs or toxins probably have to be given continuously as occurs during diarrhoea caused by ETEC. Therefore, it was tried to induce hypovolaemia by inoculating newly-weaned piglets with K88 positive ETEC.

Inoculation of piglets with ETEC did not consistently induce diarrhoea. This is in accordance with previous reports (Smith and Halls, 1968; Arbuckle, 1970; Sarmiento *et al.*, 1988). Besides the presence of ETEC other mechanisms have been mentioned to play a role in PWD such as nutritional, environmental and immunological factors (Miller *et al.*, 1984), the genetic susceptible to adhesion of K88 ETEC to their villi (Sellwood, 1984) and infection with enteropathogenic viruses (Lecce *et al.*, 1982; Wilson and Francis, 1986; Pritchard, 1987). The absence of diarrhoea in 2 piglets of the ETEC group was not due to an inherited resistance to adhesion of the K88 ETEC to their villous enterocytes. Indeed both animals possessed K88 receptors on their villi. The absence of diarrhoea in both piglets, however, argues for the fact that no or only a weak colonization of the small intestine occurred with the enterotoxigenic *E. coli*.

The digestive tract has a natural resistance to colonization with potentially pathogenic microorganisms due to a cooperative and synergistic activity of the anaerobic microflora and the host. This colonization resistance could perhaps explain why 2 piglets of the ETEC group did not develop diarrhoea following ETEC inoculation. It was, therefore, tried to enhance diarrhoea by making the gut more susceptible to colonization. This can be performed by oral administration of antibiotics which selectively suppress the anaerobic gut flora such as chloramphenicol (Kaufmann, 1984). In the present study, the pretreatment of piglets with chloramphenicol enhanced the occurrence and the duration of diarrhoea following ETEC inoculation. Besides its effect on the colonization resistance, oral chloramphenicol treatment has been observed to induce diarrhoea in calves which has been attributed to a reduction in villous height and in crypt depth induced by the antibiotic (Mero *et al.*, 1985). In the present study, diarrhoea occurred in 69 % of the piglets pretreated with chloramphenicol. No ETEC could be isolated from rectal swabs of these animals. It is plausible that the diarrhoea was partly the result of a direct effect of chloramphenicol on the small intestinal mucosa.

Although chloramphenicol pretreatment followed by ETEC inoculation consistently induced diarrhoea which was more severe than diarrhoea as a result of ETEC inoculation alone, there was still no pronounced dehydration. Several studies suggest the concurrent involvement of rotavirus in

the complex etiology of PWD (Tzipori *et al.*, 1980; Lecce *et al.*, 1983). Since rotavirus is enzootic in Belgian piggeries and most pigs become infected before the age of 6 to 7 weeks (Debouck *et al*, 1984), an inoculation with another enteropathogenic virus i.e. transmissible gastroenteritis virus (TGEV) was used prior to the ETEC-inoculation. TGEV infects and destroys enterocytes on the villous epithelium resulting in a pronounced villous atrophy and crypt hyperplasia and in a malabsorption diarrhoea. In 3-week-old animals transmissible gastroenteritis may lead to a debilitating diarrhoea with a mortality rate of less than 3 % (Moon *et al.*, 1973). In the present study, the combination of chloramphenicol pretreatment, TGE inoculation and ETEC inoculation induced diarrhoea and dehydration which were invariable fatal in catheterized piglets. Histological examination of jejunal and ileal small intestinal segments revealed a pronounced villous atrophy and a moderate to high number of coliform bacteria on the surface of the villi. These results indicate that malabsorption contributed to the observed diarrhoea and that the atrophic villi possessed receptors for K88ac$^+$ ETEC. The effect of virus-induced villous atrophy on adhesion of K88ac$^+$ ETEC was also examined in vitro (chapter III.B.3.). In the latter study, it was shown that the villous atrophy was attended with a temporarily diminished susceptibility of villous enterocytes to adhesion of K88ac$^+$ *E. coli*. This could indicate that ETEC are not important in the severe diarrhoea upon dual inoculation of piglets with TGEV and ETEC. An electron microscopic study of the small intestinal mucosa of the dually inoculated piglets revealed, however, a colonization of villi with ETEC in most piglets with severe diarrhoea, suggesting an increased small intestinal fluid loss due to the ETEC strain (chapter III.B.4.).

There was a remarkable difference in clinical response following chloramphenicol pretreatment, TGEV and ETEC inoculation between catheterized and non-catheterized piglets. Symptoms were significantly more severe in the operated piglets. It is known that surgery leads to a reticulo-endothelial cell depression, so making the host more susceptible to infection (Altura, 1983).

In conclusion, it was only possible to induce a reproducible diarrhoea and dehydration in newly-weaned piglets when ETEC inoculation was preceeded by a pretreatment with chloramphenicol and an inoculation with TGEV. This diarrhoea model could be useful to study diarrhoea and hypovolaemia in newly-weaned piglets and to evaluate the effect of potentially antisecretory drugs on post-weaning diarrhoea in piglets.

Veterinary Microbiology, 17 (1988)159-169
Elsevier Science Publishers B.V., Amsterdam—Printed in The Netherlands

III.B.3.EFFECT OF EXPERIMENTALLY-INDUCED VILLOUS ATROPHY ON ADHESION OF K88AC-POSITIVE *ESCHERICHIA COLI* IN JUST-WEANED PIGLETS

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ABSTRACT

Three- to four-week-old, just-weaned piglets were infected with transmissible gastroenteritis (TGE) virus and the next day with K88ac + enterotoxigenic *Escherichia coli* (ETEC) . Histological examination of caudal jejunum and ileum of piglets killed 2-3 days after virus challenge (1-2 days after ETEC infection) revealed severe villous atrophy especially in the jejunum compared with controls ($P < 0.05$) . Four-5 days after TGE virus infection villous length increased and after 7 days it was near normal. Villi scraped from jejunal and ileal mucosa of the piglets were incubated *in vitro* with K88ac+ *E. coli* and the number of bacteria adhering to 250 µm villous brush border was counted. Attachment of bacteria to villi of piglets killed 2-3 days after TGE virus infection was significantly decreased in comparison with adhesion to villi of non-infected piglets or of piglets killed 7 days after the virus infection. Correlation between in vitro adhesion and villous height was 0.6649 ($P < 0.001$). The results suggest that the experimentally-induced villous atrophy was attended with a temporarily diminished susceptibility of villous enterocytes to adhesion of K88ac + *E. coli.*

INTRODUCTION

Enterotoxigenic *Escherichia coli* (ETEC) are one of the most important causal agents of acute diarrhoeal disease in man and young animals (Morris and Sojka, 1985; Black, 1986; Taylor and Echeverria, 1986; Wilson and Francis, 1986) . ETEC strains possess long threadlike protein polymers (fimbriae) on their surface which mediate adhesion to specific membrane receptors or

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receptor sites on villous enterocytes of the small intestine. Specific adherence allows the ETEC strains to resist the flushing mechanism in the small intestine and to colonize the tissues (Klemm, 1985). Children in developing countries and domestic livestock throughout the world often show a combination of ETEC and enteric viral infection (mostly rotaviruses, sometimes coronaviruses) (House, 1978; Newsome and Coney, 1985; Wilson and Francis, 1986) . This dual infection induces a more severe diarrhoea than monoinfections. In calves even synergistic effects have been reported (Hess *et al.,* 1984). The enteropathogenic viruses damage the small intestinal villi, resulting in villus atrophy (Moon, 1978). Such damage can change membrane nature (Egberts *et al.,* 1984) and might subsequently influence attachment of ETEC to enterocytes. Lecce et al. (1982) postulated that the injury caused by a rotavirus infection could alter the binding sites on villous enterocytes so that the adhesion of ETEC strains to villi is facilitated. In this communication we describe the effect on intestinal villous height during experimentally-combined infections of piglets with a coronavirus (transmissible gastroenteritis virus) and K88ac fimbriae-possessing ETEC (Cox *et al.,* 1986b) and the effect of villous shortening on adhesion of K88ac *E. coli* to villi of piglets.

MATERIALS AND METHODS

Bacterial strains

The characteristics of ETEC strains used are listed in Table 1. The enterotoxin production was determined by L. Okerman (Faculty of Veterinary Medicine, Ghent, Belgium). The thermo-labile (LT) enterotoxin was identified with a reversed passive latex agglutination kit (VET-RPLA kit, Oxoid), whereas for the production of the thermo-stable enterotoxin type a (STa) the suckling mouse bioassay was used (Scotland *et al.,* 1985). The thermo-stable type b (STb) enterotoxin was determined by DNA colony hybridization with a gene probe by J.G. Mainil as reported (Mainil *et al.,* 1986). The strains were stored at -20°C in tryptone soy broth (Oxoid) supplemented with 20% (v/v) glycerol.

Animals

Twenty-six, 3- to 4-week-old female piglets from primiparous sows were purchased from the same commercial farm. Sows were vaccinated 6 and 2 weeks prior to delivery with a polyvalent *E. coli* vaccine containing among others, 0149:K91:K88ac antigens (Gletvax®; Wellcome). The vaccination of gilts results in passive immunity of suckling piglets against K88 ETEC-mediated neonatal enteric colibacillosis via colostrum and milk (Kohler, 1978). Two days post-weaning, however, 3-week-old piglets are susceptible again to *E. coli* enteritis (Nagy *et al.,* 1979) . Immediately after weaning, piglets were individually housed and during the experiment they were allowed to drink ultra high temperature sterilized whole cow's milk ad libitum.

Table 1: Characteristics of E. *coli* strains used

Strain	Serotype	Fimbriae	Enterotoxin[1]	Source[2]
GIS 25	0149:K91:K88ac	K88ac	LT, STa, STb	RIT
156KP83	0149:K91:K88ac	K88ac	LT, STa, STb	NIDO
E57	0138:K81	None	STa, STb	NIDO

[1]LT, heat-labile enterotoxin; STa, heat-stable enterotoxin type a; STb, heat-stable enterotoxin type b. [2]RIT, Smith-Kline RIT, Genval, Belgium; NIDO, P. Pohl and P. Lintermans, National Institute for Veterinary Research, Brussels, Belgium.

Experimental procedure

During 3 days prior to infection 18 piglets were treated with chloramphenicol (Chloromycetin®, Parke-Davis) suspended in their milk (1875 mg l^{-1} milk). This antibiotic suppresses the anaerobic microflora and may result in colonization of the intestine with potentially pathogenic microorganisms (Kaufman, 1984) . After a starvation period of 3 h, piglets were orally infected with TGE virus (1.66 X 10^6 pig infective dose) on Day 4. The TGE virus was supplied by Prof. Dr. M. Pensaert (Faculty of Veterinary Medicine, Ghent, Belgium). On the fifth day, again after a starvation period of 3 h, these 18 piglets were given 62 ml of a 1.4% $NaHC0_3$ solution to neutralize gastric acidity (prevention of ETEC destruction); 15 min later each piglet was intragastrically inoculated with 10 ml bacterial suspension of strain GIS 25 and with 10 ml suspension of strain 156KP83. The bacterial suspensions were prepared by inoculating the strains overnight on brain heart infusion agar (Oxoid) at 37°C. Afterwards they were examined for production of K88ac fimbriae with a slide agglutination test. They were then suspended and diluted in sterile physiological saline until an A620 of 0.4 (approximately 1.2 X 10^9 bacteria ml^{-1}) . Six piglets were killed 2-3 days (Group 1), 3 piglets 4-5 days (Group 2) and the 9 remaining piglets (Group 3) 7 days after the TGE virus challenge. Eight piglets were kept as uninfected controls (Group C) and were killed between 5 and 10 days after weaning. Piglets were killed with an intraperitoneal injection of methomidate (Hypnodil®, Janssen).

Samples

Immediately after euthanasia, the abdomens of the piglets were opened and 15-cm long intestinal segments were excised at two sites: at two-thirds of the distance between the pylorus and the ileocaecal valve (caudal jejunum) and also 2 cm cranial to the ileocaecal valve (ileum). The segments were opened and washed in a Krebs buffer containing 1% formaldehyde after which villi were gently scraped from the mucosa with a glass slide, washed several times with the same buffer and stored in this buffer. The formaldehyde was added to the buffer to stop all enzymatic reaction which would lyse epithelial cells. Within 3 weeks the *in vitro* villous adhesion assay was performed.

Except for 1 piglet in Group 1 and 1 in Group 3, specimens of the caudal end of the

excised small intestinal segments were fixed in 10%o phosphate-buffered formalin for histological examination.

Villous adhesion assay

This assay was based on the *in vitro* technique described by Girardeau (1980) . The stored villi were placed in a Krebs buffer without formaldehyde and after 1 h the buffer was renewed. This step was repeated 4 times. About 50 villi were transferred with a pipette into 1 ml 0.1 M phosphate buffer (pH 6.7) containing 1 % (w/v) D-mannose (Difco), after which 0.1 ml of a bacterial suspension in sterile physiological saline, containing strains GIS 25, 156KP83 or E57 (Table 1), was added. The presence of D-mannose in the buffer results in an inhibition of the cellular adhesion of bacteria by Type 1 pili. Bacterial suspensions were prepared by inoculating the 3 strains overnight on 5 % sheep blood agar (Oxoid) at 37°C, examining them for production of K88ac with a slide agglutination test, using a specific antiserum (RIVM, Bilthoven, The Netherlands), reinoculating them on tryptone soy agar (TSA) (Oxoid) during 24 h at 37°C and retesting them for production of fimbriae. The strains on TSA were suspended and diluted in sterile physiological saline until an A620 of 1.2 (approximately 4.5 x 10^9 bacteria ml^{-1} suspension). After gently mixing villi and bacterial suspension for 1 h at room temperature, some villi were removed with a pipette, placed under a cover slip and examined by light microscopy at magnification x 600. When no adhesion was observed piglets were considered as genetically resistant (Bijlsma *et al.,* 1982; Sellwood, 1983). When adhesion occurred, however, it was quantified by counting the number of visible bacteria adhering along 50 µm villous brush border at 5 different places villus $^{-1}$ (250 µm $villus^{-1}$) and on 5-6 villi small intestinal $segment^{-1}$. Villi of each segment were tested twice. The mean bacterial adhesion 250 µm villous $length^{-1}$ and experimental $group^{-1}$ was calculated for piglets with a K88ac adhesive phenotype.

Histological examination

The small intestinal specimens fixed in formalin were dehydrated, embedded in paraplast, cut at 5 µm and stained with hematoxylin and eosin. Only for K88ac genetically-susceptible piglets were villous height (from the tip of the villus to the villus/crypt junction) and crypt depth (junction of villus/crypt to base of the crypt) measured, using an ocular micrometer, for 10 well-oriented villi $segment^{-1}$. Subsequently the mean villous height and crypt depth intestinal $segment^{-1}$ and $experimental\ group^{-1}$ were calculated.

Statistical procedures

Data were presented as means ± standard error of the mean (SEM) and were analysed by a two-level nested analysis of variance (ANOVA) with unequal sample sizes. Significance was accepted at the $P < 0.05$ level. Pearson product-moment correlation (R) was calculated between mean villous height in an intestinal segment and the mean number of bacteria adhering *in vitro* to villi of the same segment.

RESULTS

The *in vitro* adhesion assay revealed that the villi of all piglets in Group 1 and Group 2, of 6 of the 9 piglets in Group 3 and of 5 of the 8 piglets in the control group (Group C) were susceptible to adhesion of K88ac ETEC (Table 2) . Only data of susceptible piglets were included in the results. The infected piglets developed severe diarrhoea, whereas some started vomiting. All piglets slaughtered 2-5 days after the TGE virus infection (Groups 1 and 2) were severely dehydrated and markedly depressed. In the other infected piglets, faecal fluid loss and dehydration seemed to be less pronounced and recovery occurred 5-6 days after the virus challenge. These piglets were killed 7 days after TGE virus inoculation (6 days after the ETEC inoculation; Group 3). At that time only one piglet showed diarrhoea. One of the control piglets spontaneously developed diarrhoea during the observation period, which was still present at necropsy. The other control piglets remained healthy.

Mean villous height and crypt depth in jejunum and ileum of K88ac-susceptible piglets

TABLE 2 : Pig phenotypes relating to adhesion of K88ac-positive *E. coli* to their villi

Group	N [1]	Phenotype	
		Adhesive	Non-adhesive
C	8	5	3
1	6	6	0
2	3	3	0
3	9	6	3

[1]Number of pigs tested in each group.

in the different groups are illustrated in Fig. 1. In Group 1, a significant villous atrophy occurred in the caudal jejunum, whereas in the ileum a similar but not significant villous atrophy was observed. Villi or villous tips were covered with cuboidal enterocytes. Patchy to continuous attachment of coliform bacteria to villi was observed in the jejunum of 4 piglets and the ileum of 3 piglets of Group 1. In one piglet no adhesion of bacteria to jejunal and ileal villi was observed. In intestinal segments where adhesion was absent, villi were extremely short, less than 120 µm. In the other small intestinal segments, villous length was above 180 µm. Four to five days after the TGE virus infection (34 days after the ETEC infection; Group 2) villi were still slightly shortened (Fig.1). Villous enterocytes were low to high columnar. A patchy bacterial adherence with irregularly arranged groups of bacteria of various sizes occurred in the jejunum of one piglet and the ileum of all 3 piglets. In piglets killed 7 days after the TGE virus infection (6 days after the ETEC infection; Group 3), villi were almost as high as in control piglets (Fig.1). Villous

enterocytes were high columnar. No bacterial adhesion was observed. In the control group, on the contrary, multifocal adhesion of coliform bacteria was observed on villi in 2 piglets. One had diarrhoea when euthanatized, the other was healthy. A slight villous atrophy was observed in both piglets. The other control piglets had long slender villi covered with high columnar enterocytes. Crypt depth did not differ significantly between the 4 groups (Fig. 1).

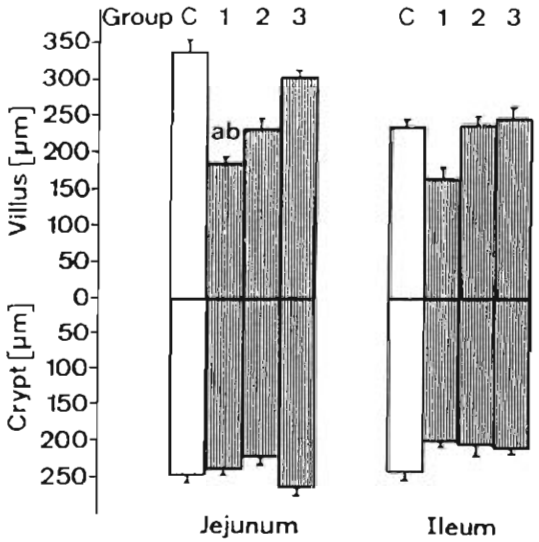


Fig. 1. Mean (± SEM) villous height and crypt depth in caudal jejunum and ileum of non-infected piglets (empty bars: Group C: n = 5) and of piglets infected with TGE virus and enterotoxigenic *E. coli* (striped bars). Piglets of Group 1 (n=6), Group 2 (n=3) and Group 3 (n=6) were euthanatized 2-3, 4-5 and 7 days after TGE virus challenge, respectively. Ten villi and crypts were measured small intestinal segment $^{-1}$. a, in comparison with Group C; b, in comparison with Group 3; $P < 0.05$.

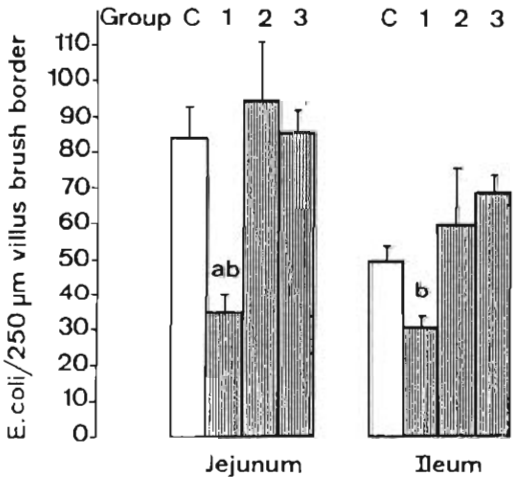


Fig. 2. Number of K88ac-positive E.coli adhering along 250 µm villous brush border of villi isolated from jejunal and ileal segments of piglets. Villi were obtained from non-infected piglets (empty bars : Group C: *n* = 5) and from piglets infected with TGE virus and enterotoxigenic *E. coli* (striped bars) and euthanatized 2-3 (Group 1: n = 5), 4-5 (Group 2: n = 3) and 7 days (Group 3: n = 5) after TGE virus inoculation. Bars represent mean number of bacteria 250 μm^{-1} villi and experimental group^{-1} ± SEM. a, in comparison with group C; b, in comparison with Group 3; $P < 0.005$.

The bacteria adhering after incubating villi for 1 h with strains 156KP83 or GIS 25 was a combination of *in vivo* adhesion (oral infection with both strains) and of *in vitro* adhesion (*in vitro* incubation of villi with one of both strains). The *in vitro* adhesion of the E57 strain was evaluated by comparing the counts of non-incubated villi with values obtained after incubating the villi. Whereas both K88ac-strains adhered equally to villi, no adhesion of the E57 strain was observed. As can be seen in Fig. 2, adhesion of K88ac-positive strains of jejunal villi was significantly decreased for piglets of Group 1 in comparison with non-infected piglets (Group C) and with piglets recovering from the experimental infection (Group 3). Adhesion to ileal villi of Group 1 was significantly decreased in comparison with adhesion in Group 3. In Group 2 the adhesion to jejunal and ileal villi was similar to the adhesion in Groups 3 and C.

The correlation between mean villus height intestinal segment^{-1} and the mean number of bacteria adhering along 250 µm villus brush border of the same segment was R = 0.6649 ($P < 0.001$), indicating that a significant correlation existed between both parameters (Fig. 3).

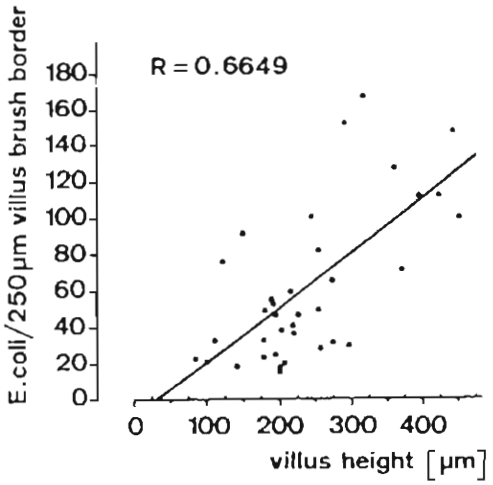


Fig. 3. Relationship between adhesion of K88ac-positive *E.coli* to villi and villous height. Each mark (n = 36) represents the mean values from jejunum or ileum of 1 piglet (n = 18). The fitted line represents the least square linear regression (equation: *E.coli* 250 µm^{-1} brush border = -10.84 + 0.302 x villous height), whereas R is the correlation coefficient.

DISCUSSION

The villus height measured in piglets of Groups 1 (48-72 h after TGE), 2 (96-120 h after TGE) and 3 (168 after TGE) in our study is comparable with the atrophy measured by Sheperd *et al.* (1979) at 40, 72 and 144 h, respectively, after experimental infection of 3-week-old piglets with TGE virus. This suggests that villous atrophy measured in this study was primarily a result of the TGE virus infection, However, Hess *et al.* (1984) observed a more pronounced villous atrophy in calves simultaneously infected with ETEC and rotavirus than in calves infected with ETEC alone or with rotavirus alone, and Rose *et al.* (1987) proved that the STb enterotoxin is capable of causing

partial villous atrophy in young pigs. So, the ETEC infection could have aggravated the virus-induced villous shortening in our experiment.

In group 1, *in vivo* adhesion of coliform bacteria to villi was noted in histological specimens of most piglets. No adhesion, however, was observed in specimens with severe atrophic villi (smaller than 120 µm). Since K88-positive ETEC specifically adhere to brush border membranes from genetically-suspectible pigs, this absence of *in vivo* adhesion on these severe shortened villi could have indicated that piglets had a non-adhesive phenotype (Bijlsma *et al.*, 1982; Sellwood, 1983). The *in vitro* adhesion assay, however, revealed attachment of K88ac ETEC to the atrophic villi. Thus, the piglets belonged to the adhesive phenotype. Nevertheless, after the *in vitro* incubation of villi with K88ac *E. coli*, the number of bacteria adhering along 250 µm villous brush border was still significantly less in this group than in Groups 3 and C. The atrophic villi in Group 1 were covered with cuboidal to low-columnar cryptlike cells. An electron microscopic study of the ultrastructure of these cells showed a short and sometimes incomplete microvillar border (Cox *et al.*, 1988). As reported by Pensaert et al. (1970b) and Shepherd et al. (1979), the cuboidal shape and the short and irregular brush border are indications of the immature nature of these enterocytes. In Group 2 (4-5 days after TGE virus infection) bacterial adhesion was again more pronounced and reached values comparable with results in Groups 3 and C. At this time villous height increased and villous enterocytes regained their normal structure. Transmission electron microscopic examination of the enterocytes of piglets in this group revealed microvilli which were longer than in Group 1 (Cox *et al.*, 1988a). In Group 3 (piglets killed 7 days after the viral infection) villous length, villous structure and adhesion to the villi were similar to the control group. Total recovery had probably occurred. This is in accordance with the results of Shepherd et al. (1979) who observed recovery of villous structure 3-6 days after TGE virus infection. Recently we evaluated the *in vitro* adhesion of K88ac *E. coli* to villi of 5 piglets killed 2 days after an oral inoculation with the 2 ETEC strains used in this study. The number of bacteria adhering to jejunal villi (81 ± 6 *E. coli* 250 μm^{-1} villous brush border) and to ileal villi (51 ± 5 *E. coli* 250 μm^{-1} villous brush border) was the same as in Group C in this experiment (E. Cox *et al.*, unpublished observation, 1987), which indicates that the decreased *in vitro* adhesion in Group 1 was not due to a direct effect of the ETEC strains. The decreased *in vitro* adhesion on villi 2-3 days after TGE virus challenge was probably the reflection of a temporary change in brush border membrane receptors occurring as a result of the viral infection. The enterocytes covering the villi during the phase of cell replacement following the virus infection (Pensaert *et al.*, 1970a, 1970b; Shepherd *et al.*, 1979) are not fully differentiated and probably possess a decreased number of receptors or receptor sites for K88ac-positive ETEC. This is in agreement with earlier studies showing that K88-positive strains adhere to the brush border of villous enterocytes but not to the undifferentiated crypt cells (Arbuckle, 1970; Bertschinger *et al.*, 1972; Hohmann and Wilson, 1975) .

Apart from virus-induced villous shortening, the variation in adhesion could have been due to the presence of piglets with a weak-adhesive phenotype (Sellwood, 1983; Bijlsma and Bouw, 1985). This phenotype is also an inherited characteristic.

Our findings indicate that the experimentally-induced villous atrophy probably results in a

temporary diminished susceptibility of villous enterocytes to K88ac ETEC adhesion. When villi regenerate, enterocytes seem to regain their normal adhesion capacity. Further experiments are requested to determine the exact mechanism for the observed phenomenon.

J. Vet. Med.B. 35, 317-330 (1980)
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ISSN 0931 - 1793

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III.B.4. ELECTRON MICROSCOPIC STUDY OF AN EXPERIMENTAL COMBINED INFECTION OF JUST-WEANED PIGLETS WITH TRANSMISSIBLE GASTROENTERITIS VIRUS AND K88ac POSITIVE ENTEROTOXIGENIC *ESCHERICHIA COLI.*

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ABSTRACT

Pretreatment of just-weaned piglets (n = 15; 3 to 4 weeks) with chloramphenicol, followed by an experimental combined infection with transmissible gastroenteritis virus (TGEV) and 24 h later with K88ac positive *E. coli* (LT, STa and STb enterotoxin positive) resulted in severe diarrhoea (100 %) and clinical dehydration (73 %). Seriously ill piglets intensely shed the inoculated *E. coli* and excreted TGEV. Scanning electron microscopy of the small intestinal surface of piglets killed before expiry (30 h to 5 d post TGEV infection) showed severe villous atrophy, villous fusion, colonization of villous-sites and -base, seldom of villous tips, with coliform bacteria, and at some sites denudation of villous tips. Transmission electron microscopy of the mucosa revealed mainly 3 cell types: cuboidal to low columnar enterocytes, highly vacuolated and often lipid droplets-containing cells, and especially in the duodenum of some piglets severely degenerated cells. Bacteria could be observed in close association with the microvillar border of all 3 cell types.

INTRODUCTION

Although post-weaning diarrhoea in piglets is often associated with a colonization of the small intestine with enterotoxigenic *Escherichia coli* (ETEC), challenging animals with known

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pathogenic *E. coli* strains does not consistently produce disease (Arbuckle, 1970; Smith and Halls, 1968). It has therefore been suggested that the occurrence of *E. coli* enteritis in weaned piglets is influenced by host predisposing factors (Smith and Halls, 1968). One of these factors is the genetic susceptibility of piglets to adhesion of K88 fimbriae possessing ETEC strains to receptor sites on villous enterocytes. Absence of receptors is inherited and results in resistance of piglets to infection by K88 positive *E. coli* (Shepherd *et al.*, 1979). Villous atrophy probably is also a predisposing factor. It has been postulated that villous atrophy and the subsequent malabsorption, which consistently are observed in newly weaned pigs (Hamilton and Roe, 1977; Kenworthy, 1976), can favor colonization of the gut by ETEC (Lecce *et al.*, 1983). The etiology of this villous atrophy in just-weaned piglets is unclear. An immunologic reaction to dietary antigens (Miller *et al.*, 1983), an effect of digestion products of host and its resident gut microflora on the intestinal mucosa (Kenworthy, 1976), and/or enteric viral infections (Lecce *et al.*, 1983) are mentioned as plausable causes. A role of enteropathogenic viruses in the post-weaning diarrhoea syndrome has been supported by recently performed experiments (Lecce *et al.*, 1983; Tzipori *et al.*, 1980) in which post-weaning diarrhoea could be consistently reproduced by infecting piglets with rotavirus and 24 h later with enterotoxigenic *E. coli*. Besides rotavirus, coronavirus can be identified in the faeces of piglets with colibacillary diarrhoea (Pritchard, 1987; Wilson and Francis, 1986). We recently reported the effect of an experimental combined infection of just-weaned piglets with transmissible gastroenteritis virus (TGEV) (a coronavirus) and K88ac+ ETEC on diarrhoea and survival (Cox *et al.*, 1986b). Whereas TGEV infection of 3-week-old piglets results in a mortality rate of less than 7 % (Moon *et al.* 1973), the experimental mixed infection induced a mortality rate of 100 %. The purpose of the present study was to describe morphological and ultrastructural changes of the small intestinal mucosa in newly weaned piglets dually inoculated with TGEV and ETEC and to relate these changes to possible mechanisms of diarrhoea.

MATERIALS AND METHODS

Animals

Experimental animals were 23 piglets (female, just-weaned, 3 to 4-week-old) of mixed breeding (Pietrain x Belgian Landrace), from primiparous sows and from the same commercial farm. Sows were vaccinated 6 and 2 weeks prior to delivery with a polyvalent *E. coli* vaccine containing among others, O149: K91: K88ac antigens (Gletvax ®, Wellcome or Stellamune coli 5®, Smith-Kline RIT). Immediately after weaning, piglets were individually housed (room-temperature of 27 ± 2 °C) and were allowed to drink UHT sterilized whole cow's milk ad libitum.

Bacterial strains

Piglets were inoculated with 2 ETEC strains (GIS 25 and 156KP83). Both were hemolytic O149:K91:K88ac strains, thermo-labile (LT), thermo-stable type a (STa) and thermo-stable type b (STb) enterotoxin positive. LT and STa were determined with a reversed passive latex agglutination kit (Oxoid) and the suckling mouse bioassay (Scotland *et al.*, 1985) respectively, and LT, STa and STb genes were determined by DNA colony hybridization with gene probes (Mainil

et al., 1986).

Experimental procedure

During 3 d and prior to infection 15 piglets (group A) were treated with chloramphenicol (Chloromycetin ®, Parke-Davis) suspended in their milk (1875 mg/litre). This antibiotic probably suppresses the anaerobic intestinal microflora increasing the susceptibility of the gut to colonization with potentially pathogenic microorganisms (Kaufman, 1984). On day 4, piglets were starved during 3 h after which they were orally infected with TGEV (1.66×10^6 pig infective dose/piglet). On day 5, they were starved again during 3 h, then 62 ml of a 1.4 % NaHCO3 solution was given to neutralize gastric acidity (prevention of ETEC destruction) and 15 min later they were intragastrically inoculated with 10 ml bacterial suspension of the GIS 25, and 10 ml of the 156KP83 strain. Bacterial suspensions were prepared by inoculating strains overnight on brain heart infusion agar (Oxoid) at 37 °C and subsequently suspending and diluting them in sterile physiological saline until an A620 of 0.4 (approximately 1.2×10^9 bacteria/ml, as determined by viable count). Control piglets either were treated with chloramphenicol on days 1, 2 and 3 and were given 62 ml NaHCO3 solution and 15 min later 20 ml physiological salt solution on day 5 (group CH; n = 4) or received no treatment (group C; n = 4). During the experiment clinical symptoms and severity of diarrhoea were daily registered. The severity of diarrhoea was evaluated by the same person who arbitrarily scored the consistency of the faeces (- = normal, + = pasty, ++ = semiliquid, +++ = watery).

Detection of hemolytic E. coli and enteropathogenic viruses

Faecal swabs were taken daily, plated out on blood agar base (Oxoid: (M55)) plus 5 % (v/v) whole defibrinated sheep blood and incubated aerobically overnight at 37 °C. The approximate percentage of hemolytic coliform colonies among the aerobic flora grown on the plate were scored (+ = 0 to 25 %, ++ = 25 to 50 %, +++ = 50 to 75 %, ++++ = 75 to 100 %). From each plate a few hemolytic *E. coli* strains were isolated and serologically identified at the National Institute for Veterinary Research, Brussels, Belgium, with a slide agglutination test.Faecal samples for virological examination were taken from piglets of group A prior to and one day after inoculation and a third time when watery diarrhoea occurred and in control groups at corresponding times. The faecal samples were frozen at -20 °C and tested for the presence of TGEV, rota- and rotalikevirus by an enzyme-linked immunosorbent assay at the Laboratory of Virology, Faculty of Veterinary Medicine, Ghent, Belgium.

Electron microscopy

Infected piglets were euthanatized when symptoms were severe enough that piglets were not expected to survive, or otherwise 7 d after TGEV inoculation (10 d after weaning). Control piglets were sacrificed at regular intervals during the experiment (1 and/or 2, 4 and 7 d after TGEV infection). Piglets were anaesthetized with methomidate (Hypnodil ®, Janssen). Intestinal specimens were removed from duodenum (2 cm caudal to the pylorus), caudal jejunum (2/3 of the

total length of the small intestine) and ileum (2 cm cranial to the ileocaecal valve) and immediately fixed in cold (4 °C) 2 % glutaraldehyde 0.1 M phosphate buffer, after which piglets were euthanatized with an overdose of methomidate. Tissues were post-fixed in 1 % osmium tetroxide. For scanning electron microscopy (SEM) the specimens were then dehydrated, critical-point dried, sputtered with a gold layer and examined by a Philips 501 scanning electron microscope. For transmission electron microscopy (TEM) fixed tissues were block-stained with 2 % (weight/volume) uranyl acetate, dehydrated, embedded in Taab 812 resin (Taab Laboratories, Reading, England), after which ultra-thin sections were contrasted with lead citrate and examined in a Philips 201 transmission electron microscope.

Susceptibility of piglets to K88ac+ ETEC adhesion to their villi.

Four intestinal segments (15 cm), three immediately adjacent to the specimens sampled for electron microscopy and a fourth from the cranial jejunum were excised, opened and washed in a Krebs buffer containing 1 % formaldehyde (volume/volume). Formaldehyde was added to the buffer to stop all enzymatic reaction which would lyse enterocytes. The villi were then gently scraped from the mucosa with a glass slide, washed 4 times in the buffer and stored in this buffer. Within 3 weeks the ability of both K88ac+ ETEC strains to adhere to the isolated intestinal villi was assayed *in vitro* by the technique described by Girardeau (1980). The *in vitro* villous adhesion assay was performed to identify if piglets were genetically susceptible to adhesion of K88ac+ ETEC strains (Bijlsma *et al.*, 1982).

RESULTS

As determined in the *in vitro* adhesion assay, only 1 of the chloramphenicol treated control piglets (piglet CH4) showed no attachment of K88ac+ ETEC to its villi. All other piglets were susceptible.

Immediately after weaning all piglets were healthy. On the 3rd day of the chloramphenicol pretreatment, however, in 73 % of the infected piglets (group A) and in 50 % of the piglets of group CH faeces became pasty to semiliquid. In group CH diarrhoea resolved within 1 d. In 2 piglets of group C diarrhoea occurred 6 d after weaning during 1, respectively 2 d and one of both piglets started vomiting. After the experimental TGEV-ETEC infection all inoculated piglets experienced a semiliquid to watery diarrhoea and vomiting occurred in 60 % of the piglets. In 11 of the 15 infected piglets the severe fluid loss resulted in serious dehydration and depression. These piglets were sacrificed before expiry (Table 1). In 3 of the 4 remaining piglets diarrhoea was less pronounced and resolved within 3 d following ETEC exposure. These 4 piglets were killed on day 10.

TABLE 1:Diarrhoea, ETEC shedding and colonization of the intestine by coliform bacteria in piglets pretreated with chloramphenicol and inoculated with TGEV and O149:K91:K88ac ETEC (group A), in non-infected, chloramphenicol-treated piglets (group CH), and in untreated controls (group C).

Piglets	Killed			Diarrhoea		Adhesion to villi[4]		
	days p.i.[1]	faecal score[2]	ETEC score[3]	onset days p.i.	duration days	duodenum	jejunum	ileum
Group A								
A1	1 (+6 h)	++	+++	-2	3	++	-	+
A2	1 (+6 h)	+++	+++	-2	3	-	+	++
A3	2	+++	++++	1/2	1 1/2	+++	+++	+++
A4	2 1/2	+	++++	1	1 1/2	+	+	+
A5	2 1/2	+++	++++	1	1 1/2	-	++	++
A6	2 1/2	+++	++++	-1	3 1/2	++	+	+
A7	3	+++	+++	1	2	-	+	-
A8	3	+++	++	0	3	-	-	-
A9	4	+++	++++	1	3	-	+	+
A10	5	++	+++	0	5	-	++	+
A11	5	++	+++	0	5	-	++	++
A12	7	-	-	-1	6	-	-	-
A13	7	-	-	0	6	-	-	-
A14	7	-	+	0	3	-	-	-
A15	7	-	-	0	5	-	+	-
Group CH								
CH1	1 (+6 h)	-	-	0	1	-	-	-
CH2	2	-	-	-	-	-	-	-
CH3	4	-	-	-	-	-	-	-
CH4	7	-	-	0	1	-	-	-
Group C								
C1	2	-	-	-	-	-	-	-
C2	4	+	++	2	2	-	-	+
C3	7	-	-	2	1	-	-	-
C4	7	-	-	-	0	-	-	-

[1]p.i.: post TGEV inoculation. [2]Faecal score at killing: - = normal, + = pasty, ++ = semiliquid, +++ = watery. [3]ETEC score at killing: + = 0 to 25 %, ++ = 25 to 50 %, +++ = 50 to 75 %, ++++ = 75 to 100 % hemolytic *E. coli* of total aerobic bacteria in faecal swabs. [4]Determined by SEM: - = no distinct adhesion, + = patches of bacteria on villi, ++ = severe adhesion at the basal region of the villi, +++ = continuous layers of bacteria.

No or only a few non-typable hemolytic *E. coli* were isolated from faecal swabs taken before infection. Within 1 d after inoculation, however, in 14 of the 15 infected piglets at least 50 and mostly 100 % of the total aerobic growth on blood agar plates were hemolytic *E. coli* of the serogroup used as inoculum. In the 11 piglets with severe diarrhoea and clinical signs faecal

ETEC shedding remained high until they were killed (Table 1). In the other 4 piglets ETEC excretion was lower than 25 % (n=1) or declined to less than 25 % within 1 to 3 d. One control piglet (C2) shed 0149:K91:K88ac *E. coli* on day 6 (Table 1).

Faecal rotavirus excretion occurred in 3 infected piglets (A1, A2 and A4) and in all chloramphenicol-treated controls, whereas following TGEV inoculation TGEV could be detected in faecal samples of all piglets of group A, except in both piglets killed 30 h after inoculation. Rotalikevirus was not identified in the 3 groups and TGEV not in both control groups.

In both control groups SEM of the intestinal mucosa of piglets killed early after weaning revealed shortened villi (Fig. 1), whereas later on jejunal villi, and in group C also duodenal villi, were longer with mostly transverse grooves (Fig. 2). In group CH, however, duodenal villi remained short (Fig. 3). Villi had a variable shape (finger-, leaf- or tongue-like) and in some intestinal segments villous fusion and/or an increased cell loosening at villous tips occurred. At higher magnification the bulging outlines of the surface of individual epithelial cells (more prominent in group CH (Fig. 3) and the individual microvillous tips were apparent on villi at many sites. Only occasionally mucus clumps were noted at the orifices of goblet cells. In the ileum of one piglet of group C adhesion of coliform bacteria to villi was observed (Table 1).



Fig.1. Caudal jejunum of a chloramphenicol-treated control piglet, 2 d after treatment. Villi are shortened and flattened, crypts are elongated (bar = 100 µm).



Fig.2. Caudal jejunum of a chloramphenicol-treated control piglet, 7 d after treatment. Most villi are long, with transverse folds (bar = 100 µm).



Fig.3. Duodenum of a chloramphenicol-treated control piglets, 2 d after treatment. Villi are short and fused. Openings of crypts are visible (1). On villi and especially at villous tips epithelial cells are rounded and bulging in the lumen (bar = 100 µm).

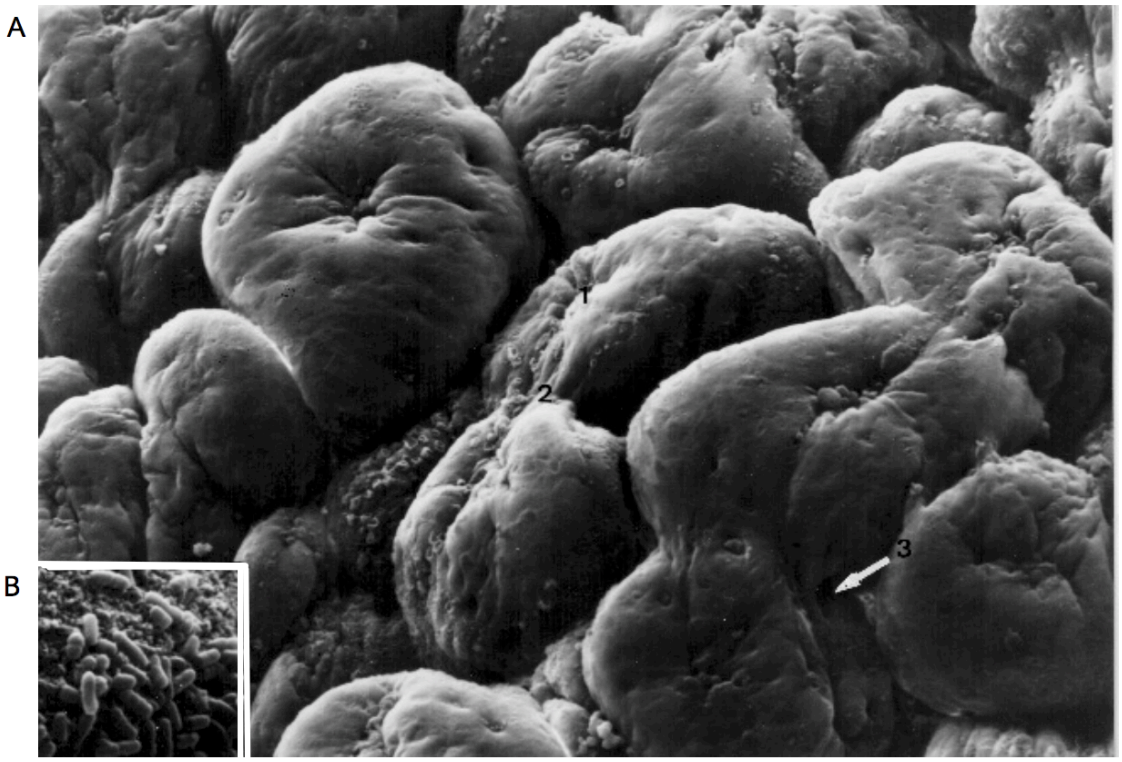


Fig. 4. A. Short and blunt villi in the caudal jejunum of a piglet, killed 2 1/2 d post TGEV infection: depression on villous tips (1); villous fusion (2); opening of crypt (3) (bar = 10 µm). B. Detail of villous-base; coliform bacteria lie scattered on the mucosa (bar = 1 µm).

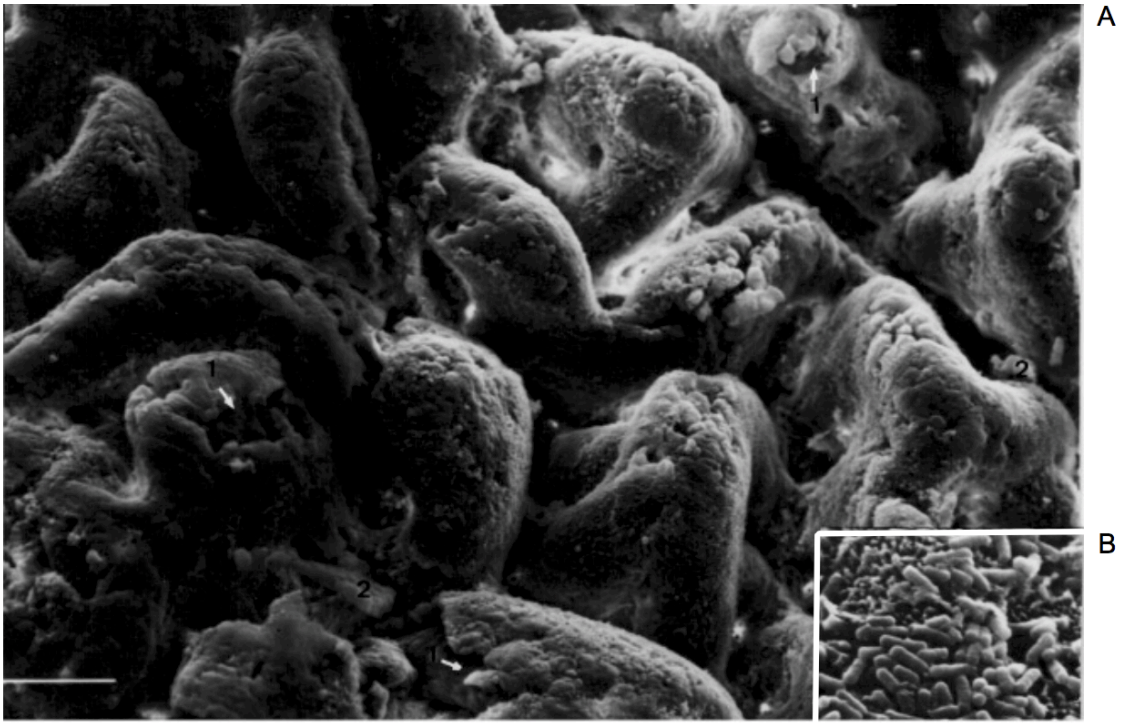


Fig. 5. A. Caudal jejunum of a piglet, killed 2 d post TGEV infection. Villi are short. Small to large epithelial defects on villous tips (1); cellular debris (2) (bar = 100 µm). B. Heavy colonization of the epithelial surface with coliform bacteria (bar = 1 µm).

In infected piglets, euthanatized 30 h to 5 d after TGEV inoculation, villi were severely shortened and openings of Lieberkuhn's crypts were visible at the base of the villi. Villi had a variable appearance. At some sites they were leaf- or tongue-like, at other sites they were blunt (Fig. 4.A) or ridge-shaped. In both piglets sacrificed 30 h after TGEV inoculation villous fusion already occurred and an increased desquamation of epithelial cells could be seen at villous tips in the caudal small intestine leaving occasional denuded areas on the villous surface. One day later, lesions were more severe (Fig. 5.A). In piglets killed later on, sometimes mucosal damage was noted at duodenal villous tips, whereas jejunal and ileal villi mostly appeared intact. In some areas large amounts of mucus covered the villous surface. Seven days after TGEV infection jejunal and ileal villi werE elongated. At higher magnification coliform bacteria could be observed on villi of 10 of the 11 piglets, killed 30 h to 5 d after TGEV inoculation (Table 1; Fig. 4.B). In one piglet with extremely short villi and in 3 surviving piglets, however, almost no coliform bacteria were seen. Bacterial colonization mostly occurred in caudal jejunum and ileum at villous-sites and -base (Fig. 4.B), seldom at villous tips (Fig. 5.B). Less adhesion seemed to occur to cells with severely shortened microvilli (Fig. 6). In piglets with increased mucus production, bacteria were often trapped in the mucus layer.

In group C TEM of villi showed high columnar enterocytes. The microvillar border of these cells consisted of long and regular microvilli. A broad terminal web and a well developed

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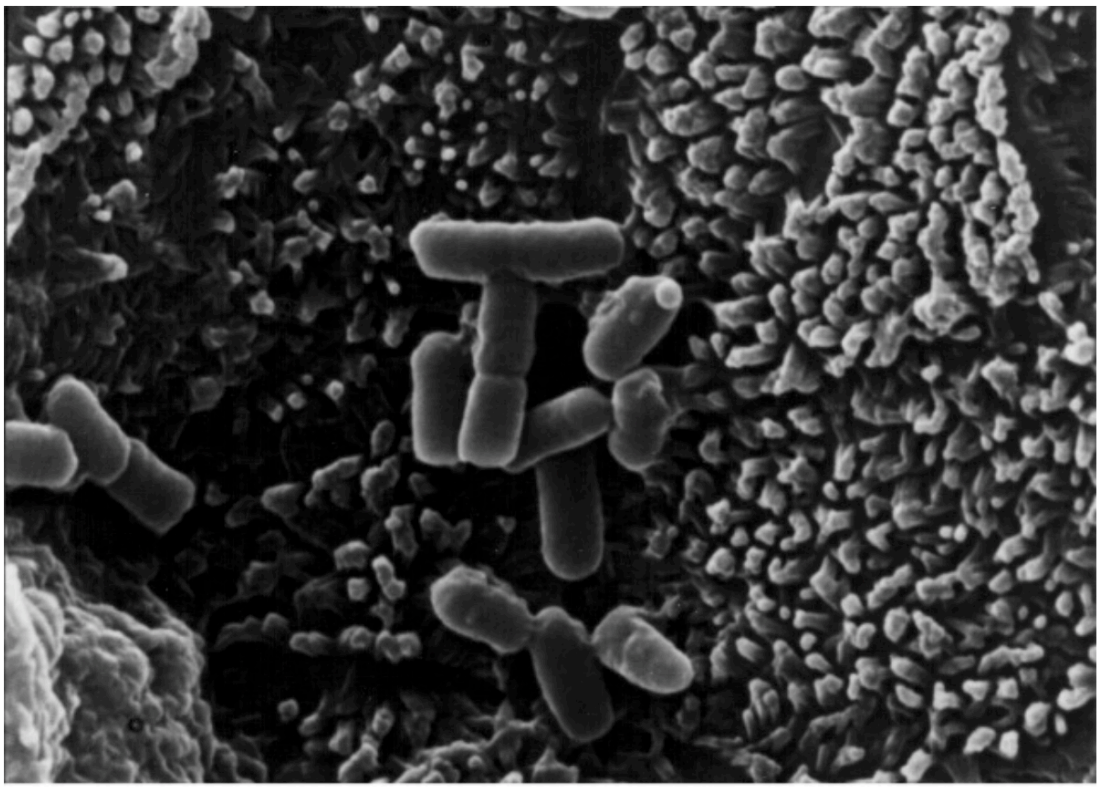


Fig. 6. Bacteria in close contact with shortened microvilli in ileum of an infected piglet, 2 1/2 d post TGEV infection. The coliform bacteria seem to attract the underlying microvilli (bar = 1 µm).

endoplasmic reticulum were observed. Mitochondria could be slightly swollen. Sometimes absorptive cells in both caudal intestinal segments contained vacuoles. In group CH low columnar cells were more apparent than in group C. These cells regularly were bulging in the lumen and often contained lipid droplets and/or swollen mitochondria. Sometimes microvilli were shortened. No viral particles were observed in both control groups.

In infected piglets killed 30 h to 3 d following TGEV challenge, enterocytes on villi in jejunum and ileum mostly were cuboidal to low columnar with a shortened (Fig. 7) and sometimes irregular or even absent microvillar border. In cuboidal cells the terminal web area and the rough endoplasmic reticulum (RER) were slight developed. In low columnar cells a well developed terminal web could be observed and the cisternae of the endoplasmic reticulum were often distended (Fig. 8), whereas regularly large lipid droplets were noted. In both cuboidal and low columnar cells mitochondria could be slightly to severely swollen with distorted cristae (Fig. 7). At 4 to 5 d after TGEV exposure more columnar cells with longer and more microvilli were seen. In duodenal segments of 5 piglets sacrificed between 2 to 4 d after virus infection some

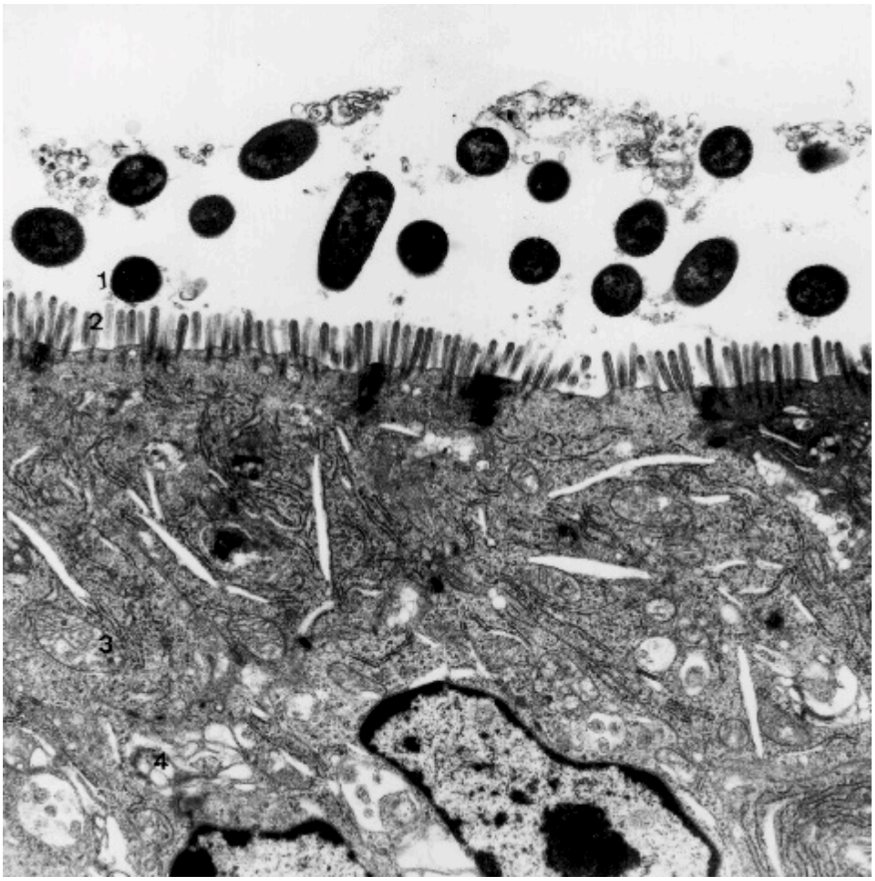


Fig. 7. Epithelial cells in the caudal jejunum, 2 1/2 d post TGEV infection. Bacteria (1) near the short microvillar border (2); cytoplasm with slightly swollen mitochondria (3) and vacuoles (4) (bar = 1 μm).

villi were covered with degenerated epithelial cells, which often formed an intact layer, separated from the lamina propria by a subepithelial space (Fig. 9). Only in one piglet similar lesions were observed in the posterior small intestine.

By TEM virus particles were detected in an occasional cell in both caudal intestinal segments of piglets A2, A3 and A5 and in the jejunum of piglet A6. Bacteria with the morphology of *E. coli* were located along the brush border, but not in direct contact with microvilli (Fig. 7). Colonized cells sometimes contained vacuoles and/or lipid droplets whereas their microvilli appeared normal in length or were shortened (Fig. 7). Only very rarely bacteria were observed near an extremely short and irregular microvillar border.

In duodenal segments bacteria could be observed in close association with degenerated cells. On day 7 after TGEV infection the ultrastructure of enterocytes resembled this of untreated piglets.

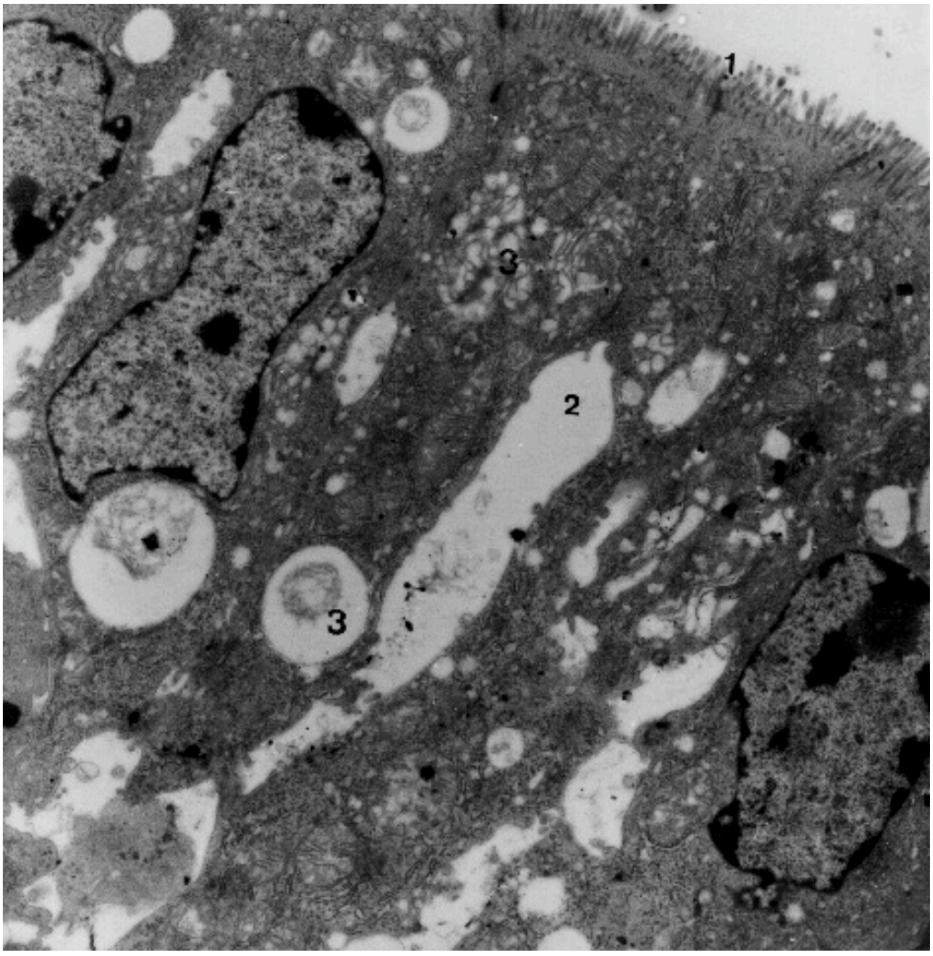


Fig. 8. Villous enterocytes in the caudal jejunum, 2 1/2 d post TGEV infection: shortened and irregular brush border (1); distended intercellular space (2); highly vacuolated cytoplasm, containing small irregular and larger globular vacuoles (3) (bar = 1 µm).

DISCUSSION

In this study chloramphenicol pretreatment followed by a combined TGEV-ETEC infection produced a profuse fatal diarrheal disease in most inoculated piglets, whereas in 50 % of the non-infected piglets a mild diarrhoea of 1 or 2 d duration could be observed. The severe diarrhoea was accompanied with an intense faecal shedding of the ETEC strains and with an excretion of TGEV.

In an experiment previously performed in our laboratory an enhanced occurrence and duration of diarrhoea and an increased mortality (from 0 to 12.5 %) were observed when an experimental ETEC infection was preceded by an oral chloramphenicol treatment (Cox *et al.*, 1986a). In the present study 63 % of all chloramphenicol-treated animals (n = 19) developed diarrhoea during the treatment, whereas in 2 untreated controls (n = 4) diarrhoea occurred at least 3 d later. Chloramphenicol has been mentioned to exert a "colonization-resistance-suppressing" effect in the gut so probably provoking a proliferation of coliform bacteria (Kaufman, 1984). In

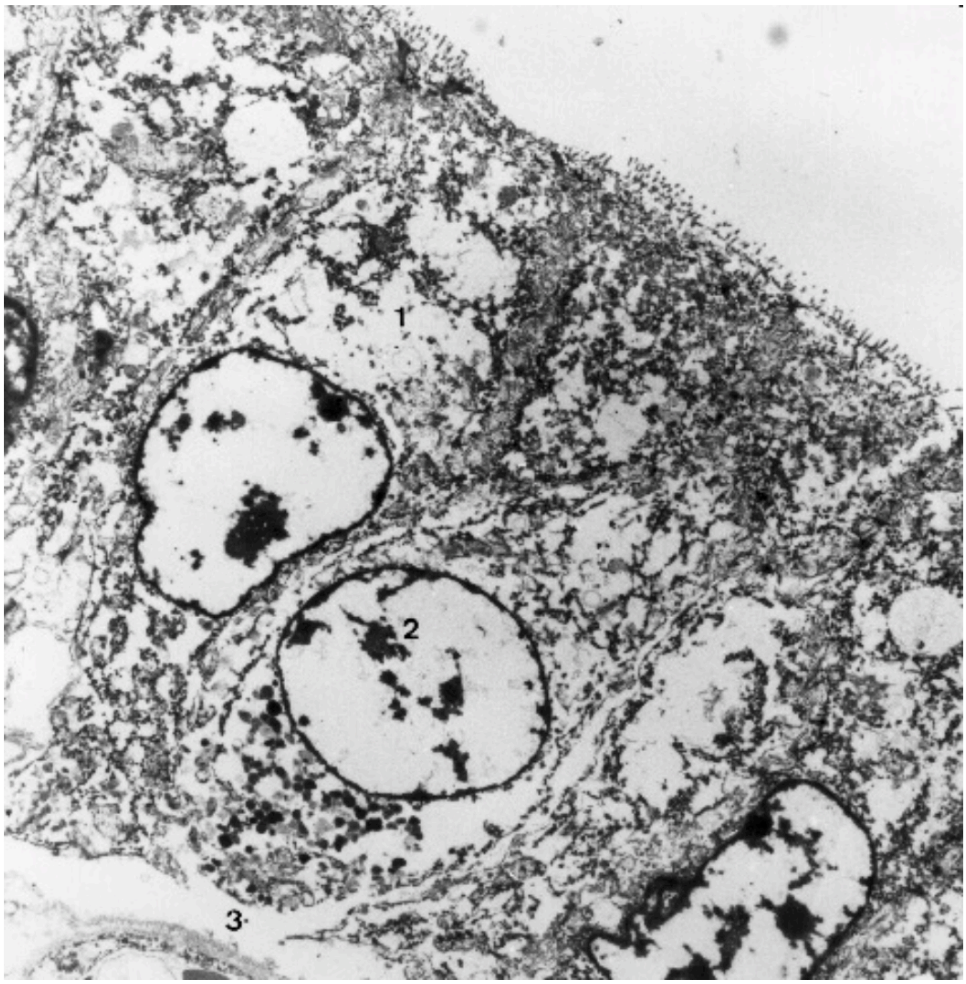


Fig. 9. Severely degenerated epithelial cells in the duodenum of a piglet 2 1/2 d post TGEV inoculation: cytoplasm contains degenerated organelles (1); pale, round nucleus (2); subepithelial space (3)(bar = 1 μm).

the present study no pathogenic hemolytic *E. coli* could be isolated from piglets during or immediately after chloramphenicol treatment. Rotavirus, however, was treated piglets (3 of group A and all piglets of group CH). In neonatal calves a 5 d oral chloramphenicol treatment also caused diarrhoea in 67 % of the treated animals (Mero *et al.*, 1985). No signs of enteric bacterial or viral overgrowth and/or imbalance were observed and diarrhoea was entirely attributed to a reduction in villous height and crypt depth. In our study duodenal villi in chloramphenicol-treated controls were shorter than in untreated animals. This, however, could have been the result of the rotavirus infection. Villous atrophy is a consistent finding in piglets with post-weaning diarrhoea and has in part been attributed to rotavirus infections (Lecce *et al.*, 1983). Since rotavirus is enzootic on Belgian piggeries (Debouck, 1984), we exposed piglets to TGEV, which also can be isolated from piglets with weanling diarrhoea (Pritchard, 1987). Whereas rotavirus only infects the upper part of the villi, TGEV multiplies in and destroys the major part of the villous epithelium, inducing a more pronounced villous atrophy, especially in the jejunum (Moon, 1978). As stated in the results, SEM revealed severe villous shortening in all infected piglets killed 30 h to 5 d after virus infection, comparable with the morphological changes described by Waxler (Waxler, 1972). Only 7 d after TGEV inoculation villous height was increased again. TEM examination of the intestinal mucosa at 30 h post TGEV infection showed cuboidal to low columnar enterocytes resembling the immature cryptlike cells covering the villi during the stage of regeneration following TGEV infection (Pensaert *et al.*, 1970b; Shepherd *et al.*, 1979). Later on highly vacuolated and often lipid droplets-containing cells were seen. These cells are also observed in porcine epidemic diarrhoea virus infections in piglets (Ducatelle *et al.*, 1982). Severe villous atrophy, villous fusion and presence of undifferentiated and functionally deficient cells can result in a severe "malabsorption syndrom".

Besides villous atrophy, SEM examination of the mucosa of diarrhoeic piglets showed colonization of villi with *E. coli*. Only in one of the severely ill piglets no adhesion was observed. Attachment of bacteria was already apparent 6 h after ETEC exposure. Similar results were obtained in monoinfections with ETEC (Arbuckle, 1970). In our study bacterial adhesion mostly occurred to the basal regions of the villi. Only in one piglet the entire villous mucosa was colonized. By TEM adhesion of *E. coli* was mostly seen to cells with an intact microvillar border and rarely to cuboidal cells with an extremely short and irregular arrangement of microvilli. We recently described that *in vitro* adhesion of K88ac+ *E. coli* to jejunal villi of piglets euthanatized during the early phase of the dual infection is significantly decreased in comparison with adhesion to villi of control piglets or of piglets killed later after infection (Cox *et al.*, 1988b). Both findings seem to indicate that the immature enterocytes covering the villi during the regeneration stage following TGEV exposure are less susceptible to adhesion of K88+ ETEC, which in turn is supported by in vivo studies showing that K88+ strains adhere to the brush border of villous enterocytes but not to the undifferentiated crypt cells (Arbuckle, 1970; Bertschinger *et al.*, 1972). Nevertheless the severe clinical symptoms in piglets with bacterial colonization of villi suggest the significance of ETEC adhesion in our model.

In an important number of severely ill piglets, killed before expiry, degenerative epithelial

lesions were observed in the cranial small intestine. These lesions were not correlated with presence or absence of bacterial adhesion. They, however, seemed to resemble intestinal mucosal damage noted in different forms of experimental shock (Haglund *et al.,* 1984). Although the pathogenesis of this damage is still controversial, tissue hypoxia is thought to play a major role in the occurrence of lesions in hypotensive states (Redors *et al.,* 1984). Since blood pressure of dually TGEV and ETEC infected piglets significantly decreases during the diarrheal disease (Chapter III.B.5.), lesions could have been due to tissue hypoxia. However, this does not explain why the severe degenerative changes mainly occurred in the duodenum. It has been shown that luminal factors such as pancreatic proteases and bile acids can aggravate the extent of damage and that regurgitation, as observed in our model, may play a role in the development of duodenal lesions (Bounous, 1982). Regardless of cause, damage results in a marked decrease in net fluid and electrolyte absorption (Redfors *et al.,* 1984).

In conclusion, chloramphenicol treatment followed by a dual TGEV-ETEC infection induces a profuse diarrhoea. This diarrhoea is in part the result of a severely impaired absorption due to TGEV infection. Additional factors such as rotavirus and perhaps chloramphenicol may aggravate this malabsorption. In most piglets with serious dehydration colonization of villi with ETEC strains occurred, suggesting an increased intestinal fluid loss due to the ETEC strains. Severe watery diarrhoea can lead to hypotension which in turn can cause intestinal mucosal damage, so decreasing the intestinal absorption and causing a fatal diarrhoea.

III.B.5. PATHOPHYSIOLOGY OF DIARRHOEA INDUCED BY A COMBINED INFECTION WITH TRANSMISSIBLE GASTROENTERITIS VIRUS AND ENTEROTOXIGENIC *ESCHERICHIA COLI* IN NEWLY WEANED PIGLETS*

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ABSTRACT

Pretreatment of newly-weaned 3-4 week old piglets (n = 21) with chloramphenicol followed by inoculation with transmissible gastroenteritis virus and enterotoxigenic *E. coli* strains (O149:K91:K88ac; LT, STa and STb enterotoxin positive) induced diarrhoea (100%) and vomiting (65%). Piglets with severe watery diarrhoea shed the inoculated *E. coli* in high number, excreted TGEV and had a faecal osmotic gap (measured osmolality - 2 x (Na^+ + K^+concentrations)) intermediate between the faecal osmotic gap of a pure secretory and a pure osmotic diarrhoea, suggesting that the diarrhoea was a result of a malabsorption (TGEV) and a hypersecretion (ETEC). The fluid loss resulted in death of 71 % of the piglets within 5 days following inoculation. During this period the piglets had decreases in body weight, arterial pressure and leucocyte count and increases in heart rate and in total plasma protein concentration. The plasma pH and lactic acid concentration decreased, whereas the values for PO_2, PCO_2 and frequency of respiration did not change significantly. No significant changes in the serum concentrations of potassium, chloride or calcium were observed, whereas sodium concentration revealed a transient increase. In shocked and dying piglets a significant increase in haematocrit was observed, whereas base excess and bicarbonate concentration decreased.

INTRODUCTION

Enterotoxigenic *Escherichia coli* (ETEC) are involved in the post-weaning diarrhoea syndrome in pigs. However, experiments performed in our laboratory to induce hyper secretion diarrhoea by inoculation of newly-weaned piglets with ETEC, possessing fimbrial adhesins and producing heat-labile (LT) and heat-stable (STa and STb) enterotoxins, were not always successful

*The results of these study were partly published in: Cox, E., Cools, V. and Houvenaghel, A.1988. Pathophysiology of diarrhoea induced by a combined infection with transmissible gastroenteritis virus and enterotoxigenic *Escherichia coli* in newly weaned piglets and the effect of flurbiprofen treatment. Veterinary Research Communications, 12 (4-5) 383-399.

(Cox *et al.*, 1986a). Post-weaning diarrhoea has a multifactorial etiology. The genetic susceptibility of the host to adhesion of ETEC as well as nutritional, environmental and immunological factors and the presence of a rotavirus infection can probably seriously influence the occurrence and the severity of the diarrhoea (Tzipori *et al.*, 1980; Lecce *et al.*, 1982; Lecce, 1983; Sellwood, 1983; 1984; Bijlsma *et al.*, 1985).

When the experimental ETEC infection was preceded by oral chloramphenicol treatment we observed an enhanced occurrence, severity and duration of diarrhoea (Cox *et al.*, 1986a). This 'colonization-resistance-suppressing' antibiotic probably makes the gut more susceptible to ETEC (Kaufman, 1984). The induced diarrhoea, however, was still insufficiently pronounced to result in significant hypovolaemia and shock.

We therefore introduced a combined viral-bacterial infection into our piglet diarrhoea model. Since rotavirus is enzootic in Belgian piggeries and most pigs are infected before the age of 6 to 7 weeks (Debouck, 1984), we inoculated piglets with transmissible gastroenteritis (TGE) virus, a coronavirus, prior to ETEC infection. This combined infection resulted in pronounced diarrhoea and hypovolaemia and in 100% mortality within 5 days of TGE virus inoculation (Cox *et al.*, 1986b).

The purpose of the present study was to evaluate the pathological changes induced by the combined TGE virus and ETEC inoculation in newly-weaned piglets.

MATERIALS AND METHODS

The experiments were performed on 21 newly-weaned, 3-4 week old female piglets, weighing 3.2 to 7.2 kg, from primiparous sows purchased from the same commercial farm. The piglets were of mixed breeding (Pietrain x Belgian Landrace). They were individually housed at 27°C and allowed to drink UHT sterilized whole cow's milk ad libitum. On day 1 of the experiment the piglets were anaesthetized with 15 mg/kg methomidate (Hypnodil ®, Janssen) and 2 mg/kg azaperone (Stressnil ®, Janssen) and a Silastic catheter (Dow Corning) was implanted in the left carotid artery for daily blood sampling and pressure recording. To enhance ETEC colonization of the small intestine, the piglets were pretreated on days 1, 2 and 3 with chloramphenicol (Chloromycetin ®, Parke-Davis; 1,875 mg/l milk). After a starvation period during the first three hours of day 4, the piglets were orally inoculated with TGE virus (1.66×10^6 pig infective dose/ animal). Twenty-four hours later (day 5) the piglets were again starved for 3 hours, after which 62 ml of a 1.4% $NaHC0_3$ solution was given intragastrically to prevent ETEC destruction. Fifteen minutes later they were each intragastrically inoculated with 10 ml of a suspension of two ETEC strains. Both strains, 0149 K91 K88ac, LT, STa and STb enterotoxin positive, were grown on brain heart infusion agar (Oxoid) at 37°C for 24 hours. The bacteria were suspended and diluted in sterile physiological saline to an A620 of 0.4, approximately 1.2×10^9 bacteria/ml, as determined by viable count..

Weight change, milk intake, body temperature, appearance and severity of diarrhoea, and clinical symptoms were registered daily. From day 3 onwards the following parameters were also evaluated: survival rate; frequency of respiration; arterial blood pressure (Pa); heart rate (HR);

haematocrit (Hct); plasma protein (Merck kit), glucose (Merck kit) and lactic acid (Boehringer kit) concentrations; arterial pH, pO_2 and $pC0_2$ (Astrup, Radiometer); base excess (BE) and bicarbonate (porcine acid-base alignment nomogram for arterial blood; Hannon, 1984); leucocyte count; serum concentrations of sodium, potassium (flame photometry), chloride (chloridometer) and calcium (atomic absorption); sodium and potassium concentrations and osmolality (cryoscopy) of faecal water, obtained as the supernatant after centrifugation of freshly collected faeces at 6,000 g for 5 min; faecal ETEC excretion; and the faecal shedding of TGE virus, rotalike- and rotavirus (enzyme-linked immunosorbent assay).

The severity of diarrhoea was evaluated by arbitrarily scoring the consistency of the faeces (O = normal; 1 = pasty; 2 = semiliquid; 3 = watery). Scoring was always performed by the same person. The osmotic gap was calculated from the faecal water values as the difference between the measured osmolality and the estimated osmolality, based on twice the sum of the sodium and potassium concentrations (Shiau *et al.*, 1985). Secretory diarrhoea is commonly associated with a negative osmotic gap, whereas osmotic diarrhoea is associated with a positive osmotic gap of greater than 160 mosmol/l, at least in man (Shiau *et al.*, 1985). Faecal ETEC excretion was evaluated as the approximate percentage of haemolytic *E. coli* among the total number of aerobic bacteria in rectal swabs inoculated onto blood agar plates at 37°C for 24 hours and was also arbitrarily scored (O = 0%; 1 = 0-25%; 2 = 25-50%; 3 = 50-75%; 4 = 75-100%).

The carotid arterial pressure was registered with a saline-filled polyethylene catheter connected to a miniature pressure transducer (Gaeltec). The electrocardiogram (ECG), which was used to measure HR, was monitored with five ECG electrodes fixed with rubber bands cranial to the left and right shoulder joint, above the seventh vertebral spine and on the abdomen cranial to the left and right stifle joint. Pa and ECG were recorded on a Beckman multichannel recorder.

Shocked piglets died spontaneously or were killed with an overdose of methomidate. The other piglets were euthanatized with methomidate 10 days after the start of the experiment. All piglets were examined post mortem.

The genetic susceptibility of the piglets to K88ac-ETEC adhesion to their small intestinal villi was determined by the *in vitro* technique described by Girardeau (1980).

The daily diarrhoea score and faecal ETEC score are expressed as medians and ranges or individual values and were statistically analysed using the Mann-Whitney U or Wilcoxon tests. All other parameters are given as means ± SEM. A one-way analysis of variance and simple contrasts were used to assess the statistical significance between the values of the different parameters registered after the experimental infection and the values measured before infection on day 3.

RESULTS

The post mortem *in vitro* adhesion assay revealed that the villi of all piglets were susceptible to adhesion by K88ac-ETEC to their enterocytes.

Chloramphenicol pretreatment followed by experimental infection with TGE virus and ETEC resulted in a pronounced mortality, starting from day 5 and reaching maximal values on

days 5 to 6 (Table 1). The mortality of 71 % of the piglets was provoked by a severe watery diarrhoea, which occurred in all inoculated animals (Table 1), and by vomiting which was observed in 65 % of the animals. Piglets with watery diarrhoea showed a severe depression and obtained cyanosis of their extremities. In the terminal stage some pigs lay in lateral decubitus with the eyes retracted in the orbits, the flanks sunken and a prominent pelvis as a result of the severe dehydration.

Diarrhoea already occurred in 60 % of the piglets during the chloramphenicol pretreatment (Table 1; day 3) and seriously worsened following inoculation with TGE virus and ETEC ($P < 0.01$). The osmotic gap of the faeces significantly increased from -14 ± 11.6 mosmol/l on day 3 to 53.6 ± 15.8 mosmol/l on day 5 ($P < 0.01$) and 95 .6 ± 27.5 mosmol/l on day 7 ($P < 0.001$).

Table 1. Survival rate, diarrhoea score (median and ranges), faecal ETEC excretion (median and ranges) and weight-change (means ± SEM) following a combined infection of newly-weaned piglets with TGE virus and ETEC.

Time in days	Survival (%)	Diarrhoea score[a]	Faecal ETEC[b] exretion	Weight-change[c] (g/day)
1	100	0	0	- 92 ± 40
2	100	0 (0 - 3)	0	+101 ± 40
3	100	2 (0 - 3)	0	+119 ± 40
4	100	2 (0 - 3)	0 (0 - 3)	- 78 ± 80**
5	86	3 (1 - 3)**	4.0 (3 - 4)***	- 306 ± 80***
6	62	3 (1 - 3)	2.5 (1 - 4) **	- 197 ± 70***
7	43	3 (0 - 3)	1.0 (0 - 4) **	- 135 ± 90**
8	33	2 (0 - 3)	0.0 (0 - 1)	+ 69 ± 80
9	29	1 (0 - 3)	0.5 (0 - 1)	+ 54 ± 80
10	29	0 (0 - 2)	0.5 (0 - 1)	+178 ± 80

[a]Diarrhoea scores: 0 = normal; 1 = pasty; 2 = semiliquid; 3 = watery; [b]Faecal ETEC excretion: 0 = 0%; 1 = 0-25%; 2 = 25-50%; 3 = 50 -75%; 4 = 75-100% haemolytic *E. coli* among the total number of aerobic bacteria in rectal swabs; [c] + = increase, - = decrease; Significant differences from the values prior to infection (day 3) are indicated by * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

A chloramphenicol resistant *E. coli* was isolated from the faeces of one piglet. From about 20 hours following ETEC-infection a substantial number of haemolytic *E. coli* were shed in the faeces (Table 1). In 80 percent of the expiring piglets, between 50 and 100% haemolytic *E. coli* among the total number of aerobic bacteria were shed in the faeces at the moment of dead, whereas in surviving piglets the percentage haemolytic *E. coli* rapidly decreased from between 50 and100 % on day 5 to between 25 and 50 % on day 6.

TGE virus was demonstrated in the faeces of all the piglets following inoculation. Rota- and rotalikevirus, were each only detected in the faeces of one pig.

Daily milk intake revealed a non-significant decrease from 0.944 ± 0.106 liter on day 3 to 0.744 ± 79 liter on day 5 followed by an increase to 1.447 ± 0.351 liter on day 10. The pronounced and sustained diarrhoea, vomiting and loss of appetite resulted in a significant decrease in body weight (Table 1).

The body temperature showed initially a decrease on day 5 to 7 whereafter it significantly increased on day 10 (Table 2). The decrease in body temperature was significant in shocked piglets (from 39.6 ± 0.2 °C on day 3 to 38.8 ± 0.4 on day 6 ($p < 0.01$) and 38.6 ± 0.2 on day 7 ($p < 0.01$)).

Table 2. Changes in haematocrit, in arterial pressure, leucocyte counts, body temperature following a combined infection of newly-weaned piglets with TGE virus and ETEC. The values presented as means ± SEM.

Time in days	Haematocrit (%)	Arterial pressure (mm Hg)	Leucocyte count (x 10^3/mm^3 blood)	Body temperature (° C)
3	38 ± 1	94.8 ± 2.1	12.1 ± 0.7	39.6 ± 0.1
4	38 ± 1	92.1 ± 1.9	8.7 ± 0.6**	39.5 ± 0.1
5	41 ± 2	87.4 ± 2.0*	9.4 ± 0.8**	39.1 ± 0.2
6	40 ± 2	89.4 ± 3.3	12.8 ± 1.4	39.2 ± 0.4
7	36 ± 2	88.4 ± 5.0	11.2 ± 0.5	39.1 ± 0.2
8	35 ± 2	NG	NG	39.7 ± 0.2
9	33 ± 2	NG	NG	39.5 ± 0.2
10	33 ± 2*	98.1 ± 4.5	15.1 ± 1.6*	40.0 ± 0.3*

Significant differences from the values prior to infection (day 3) are indicated by * $P < 0.05$; * * $P < 0.01$; * * * $P < 0.001$.

The mean Hct for all piglets initially increased and then gradually decreased (Table 2). In surviving piglets, the Hct gradually and significantly decreased from 38 ± 1 % on day 3 to 33 ± 2 % on day 10. In shocked and expiring piglets, however, a significant increase in Hct occurred from 38 ± 1 % on day 3 to 44 ± 1% just before death; $P < 0.05$. Significant increases were also observed in the total plasma protein concentration, from 4.6 ± 0.2 g/dl on day 3 to 5.7 ± 0.2 g/dl on day 6 ($P < 0.001$) and in heart rate, from 151 ± 6 beats/min on day 3 to 184 ± 9 beats/min on day 5 ($P < 0.01$) 12.9 ± 1.6 mg/dl on day 5 ($P < 0.01$) and a significant decrease was observed in Pa (Table 2).

Arterial pH revealed a significant decrease from 7.48 ± 0.03 on day 3 to 7.32 ± 0.14 on

day 7 ($P < 0.05$) and the lactic acid concentration also significantly decreased from 21.7 ± 2.2 mg/dl on day 3 to 12.9 ± 1.6 mg/dl on day 5 ($P < 0.01$). After an initial non significant increase from 94.1 ± 6.1 mg/dl on day 3 to 97.9 ± 97.9 mg/dl on day 6, the plasma glucose concentration decreased 76.7 ± 4.0 mg/dl on day 10 ($P < 0.05$). The values for PO_2 (control value on day 3: 90.0 ± 3.4 mm Hg), pCO_2 (control value: 29.6 ± 1 mm Hg) and the frequency of respiration (control value: 26.3 ± 2.8/min) did not change significantly.

In shocked and expiring piglets, BE decreased from -6.8 ± 2.4 mEq/l on day 3 to -24.6 ± 3.70 mEq/l on day 5 ($P< 0.001$) and bicarbonate decreased from 23.3 ± 1.9 mEq/l on day 3 to 8.6 ± 1.9 mEq/l on day 5 ($P< 0.001$). The corresponding value for pH on day 5 was 7.28 ± 0.05 ($P < 0.01$). No such changes occurred in the surviving piglets.

There were no significant changes in the serum concentrations of potassium (control value on day 3: 4.1 ± 0.1 mEq/l), chloride (control value: 97.1 ± 1.8 mEq/l) or calcium (5.1 ± 0.2 mEq/l). Sodium concentration revealed a significant increase from 130 ± 2.5 mEq/l on day 3 to 137 ± 1.5 mEq/l on day 6 ($P< 0.01$), followed by a return to near the control value on day 10 in surviving piglets.

Following the TGE virus and ETEC inoculation, the leucocyte count significantly decreased (Table 2). In surviving piglets this parameter increased to give a significant leucocytosis on day 10 (Table 2).

Necropsy of shocked piglets revealed erosions and congestion of the mucosa of the fundic region of the stomach, a congested, thin-walled small intestine, especially the ileum, with a watery content, and sometimes also congestion of the colon wall.

DISCUSSION

Since the turn of the century *E. coli* has been implicated in the aetiology of porcine weaning diarrhoea (colibacillosis). The causal strains are usually haemolytic, possess fimbriae which mediate specific adherence of the *E. coli* to receptors on villous enterocytes and produce enterotoxins (Lecce, 1983). The enterotoxins LT and STa induce fluid secretion by the crypt cells, mediated by cyclic AMP and cyclic GMP respectively, whereas LT also inhibits absorption by villous cells. Although STb can induce villous atrophy, the exact mechanism through which it increases intestinal fluid loss is still unclear (Whipp *et al.*, 1987).

Recently rotavirus has been shown to be causally associated with weaning diarrhoea of piglets. A concurrent infection of ETEC and rotavirus have been reported in piglets with post-weaning diarrhoea (Tzipori *et al.*, 1980b) and experimental infection of newly-weaned piglets with rotavirus and ETEC caused a more severe diarrhoea than an infection with either pathogen alone (Tzipori *et al.*, 1980a; Lecce *et al.*, 1982). Rotavirus multiplies in and destroys the absorptive cells of the upperpart of small intestinal villi, leading to malabsorption, dehydration and death in very young piglets (Lecce, 1983). In our model TGE virus, another enteropathogenic virus, was selected since rotavirus is enzootic in Belgian piggeries (Debouck, 1984), since the effects of this coronavirus in swine are well characterized and since it, like rotavirus, infects mature villous epithelial cells and does not infect the undifferentiated crypt cells. TGE virus consistently induces

villous atrophy and crypt hyperplasia. The disease is most acute and almost invariably fatal in the very young piglet, in which vomiting and profuse diarrhoea can lead rapidly to dehydration and death. Young pigs over 3 weeks old, as in our study, experience a debilitating diarrhoea of 10 to 14 days duration and usually survive (Aitken, 1983; Whipp *et al.*, 1985).

In the present study, the dually-infected piglets experience profuse diarrhoea, vomiting, dehydration, weight loss and hypovolaemic shock, causing the death of 71% of the animals. The clinical signs in dying piglets in our study agree with the observations on *E. coli* infection in gnotobiotic pigs and the post mortem findings correspond to those observed in piglets which have died from post-weaning diarrhoea.(Kenworthy and de G. Mitchell, 1976). The experimentally induced diarrhoea is probably the result of a small intestinal malabsorption and hypersecretion.

Indeed, a significant villous atrophy has previously been demonstrated in our model 2 to 3 days following the TGE virus inoculation (1 to 2 days following ETEC inoculation) (chapter III.B.3). This villous atrophy is mainly induced by the TGE virus infection, since TGE virus was seen by transmission electron microscopy in villous enterocytes of some piglets with atrophic villi (chapter III.B.4) and since TGE virus was excreted in the faeces of all piglets in the present study. Villous atrophy results in a decrease in the intestinal absorptive surface and thus in a malabsorption. The subsequent accumulation of non-absorbable nutrients in the intestinal lumen, and bacterial fermentation of these nutrients, promotes a rise in the luminal osmotic pressure, encouraging fluid movement from blood to the gut lumen. This osmotic effect is considered to be the major component of TGE virus diarrhoea (Aitken, 1983). The TGEV-induced malabsorption could have been aggravated by a rotavirus- or pararotavirus-induced villous atrophy, as observed in 2 piglets and/or by villous atrophy induced by the STb enterotoxin which is produced by both ETEC strains used to inoculate the piglets (Lecce, 1983; Whipp *et al.*, 1987).

The occurrence of an intestinal malabsorption following the experimental inoculation was further substantiated by the increase of the osmotic gap from negative before (day 3) to positive after the inoculation (day 5 to 7). The osmotic gap is a measure for the amount of osmotically active particles other then sodium and potassium salts. The rise in the osmotic gap indicates an increased amount of such particles. Pure secretory diarrhoea is associated with a negative osmotic gap, whereas a pure osmotic diarrhoea is associated with a positive osmotic gap of greater than 160 mosmol/l (Shiau *et al.*, 1985).Upon the combined TGEV-ETEC infection, the osmotic gap remained below 96 mosmol/l, suggesting that the induced diarrhoea was a combination of an osmotic and a secretory diarrhoea. Most likely, hypersecretion is induced by the inoculated K88ac-ETEC strains since K88ac positive *E. coli* were isolated in high number from the faeces of piglets with severe watery diarrhoea and since colonization of atrophic villi with *E. coli* was observed by electron microscopy in dually inoculated piglets (chapter III.B.4.).

Sixty percent of the piglets showed diarrhoea during the chloramphenicol pretreatment (days 1 to 3). In the present study, a chloramphenicol-resistant K88ac$^+$ *E. coli* strain was isolated from the faeces of 1 on 21 piglets. Selection for chloramphenicol-resistant ETEC was thus not the mechanism by which diarrhoea occurred in most piglets following antibiotic administration. Oral chloramphenicol treatment in calves has been observed to reduce villous height and crypt depth and thus to induce malabsorption (Mero *et al.*, 1985). The faecal osmotic gap of piglets with diarrhoea following chloramphenicol-treatment, however, was negative, indicating a secretory diarrhoea. There is no explanation for this. Chloramphenicol has been

mentioned to suppress the anaerobic gut flora resulting in an increased susceptiblility to infection by potentially pathogenic microorganisms (Kaufman, 1984). This effect can explain the more severe diarrhoea after ETEC inoculation as observed in Chapter III.B.2, but not the diarrhoea during the chloramphenicol administration.

The high control values for arterial pH (day 3) in piglets of both groups in the present study agree with the observation on conscious immature pigs of 2 to 3 months old. Pigs of this age, however, reveal a higher $pC0_2$ value, a higher $HC0_3^-$ concentration, a lower $P0_2$ value and a lower Hct (Hannon, 1983). Differences in our results can probably partly be attributed to the diarrhoea occurring on day 3. The decrease in Hct in surviving pigs of our study can probably be explained by a haemodilution effect, resulting from the daily extraction of blood.

When piglets developed hypovolaemia and shock some characteristic changes occurred within a few hours: an abrupt rise in Hct, a pronounced decrease in BE and a concomitant decrease in bicarbonate resulting in an acidosis. Since lactic acid did not increase but significantly decreased, other acid metabolites must be responsible for this metabolic acidosis. In experiments on ETEC-induced and spontaneous diarrhoea in neonatal piglets a metabolic acidosis with low pH and an abrupt decrease in BE and an increase in Hct was also reported in dying animals (Andren and Persson, 1983). Kutas and Szabo (1971) likewise reported low pH and BE values in diarrhoeic piglets, resulting from experimental *E. coli* infection. In shocked animals in our study, the BE values were lower than - 10 mEq/l, a critical limit below which the prognosis should be considered poor, at least in neonatal piglets (Andren and Persson, 1983).

In experiments on gnotobiotic pigs, 27 to 34 days old when infected with ETEC-strains, animals developed a severe diarrhoea resulting in dehydration and death between 16 and 36 hours after infection. As in our model, pigs revealed a neutropenia soon after infection and an abrupt increase in Hct (Kenworthy and de G. Mitchell, 1976). Polymorphonuclear cell infiltration in the intestinal mucosa is observed in pigs following infection with TGE virus or with STb producing ETEC strains (Pensaert *et al.*, 1970;Rose and Moon, 1985). This can probably partly explain the severe neutropenia in the dually inoculated piglets in the present study.

In conclusion, the chloramphenicol pretreatment, followed by a TGE virus and ETEC inoculation induced a reproducible diarrhoea and dehydration. The diarrhoea is most likely the result of small intestinal malabsorption (TGE virus) and hypersecretion (ETEC) and resulted in hypovolemic shock in 71 % of the piglets.

III.C. EFFECT OF ANTISECRETORY DRUGS IN AN EXPERIMENTAL DIARRHOEA MODEL IN NEWLY-WEANED PIGLETS

III.C.1. INTRODUCTION

In the past seven years there has been steady progress on potentially useful drugs which can reduce fluid loss in secretory diarrhoeas. These drugs act by enhancing absorption and/or decreasing secretion of fluid and electrolytes by enterocytes. They are called antisecretory drugs. Several drugs have been mentioned to exert an antisecretory effect such as neuroleptics, calcium channel blockers, non-steroidal antiinflammatory agents, α_2-adrenergic agonists and ß-antagonists (Chapter I: chapter I.D.). Only some of these antisecretory drugs have been tested *in vivo* in animal models with beneficial results such as chlorpromazine which has been shown to effectively reverse hypersecretion during colibacillary diarrhoea in neonatal piglets (Lönnroth *et al.*, 1979)

An advantage of using antisecretory drugs for treatment of diarrhoea is that they immediately inhibit fluid loss in the small intestine and thus have a direct beneficial effect on diarrhoea. Furthermore, most of the antisecretory drugs do not interfere with the development of an active immunity against the bacteria which induce the secretory diarrhoea since bacterial growth is not inhibited.

Post-weaning diarrhoea is the result of small intestinal malabsorption and hypersecretion (Chapter I: chapter I.C.). It is therefore interesting to evaluate the effect of drugs which enhance the intestinal absorption or decrease hypersecretion.

In order to evaluate the effect of antisecretory drugs on diarrhoea with subacute dehydration in newly-weaned piglets, a reproducible diarrhoea model was developed which mimiced the post-weaning diarrhoea syndrome in piglets. Piglets were pretreated during 3 days with chloramphenicol, subsequently inoculated with TGE virus and one day later with 2 enterotoxigenic *E. coli* (ETEC) strains. So, a diarrhoea was induced which was most likely the result of a malabsorption (TGE virus) and hypersecretion (ETEC). On this model antisecretory drugs were tested . Drugs were chosen which act via different mechanisms on electrolyte transport processes by enterocytes.

As has been mentioned in Chapter I (chapter I.A.), 3 important intracellular mediators are involved in the regulation of intestinal secretion and absorption of electrolytes by enterocytes namely calcium (Ca^{++}), cAMP and cGMP. The intracellular concentration of calcium can be increased by enhancing the influx into the cell or by releasing calcium from intracellular stores. The effects of Ca^{++} are dependent on the binding of Ca^{++} to the receptor protein, calmodulin. cAMP and cGMP are produced by adenylate cyclase and guanylate cyclase respectively. In this study the effects of chlorpromazine, clonidine, propranolol, verapamil and flurbiprofen were evaluated. Chlorpromazine has been observed to exert a membrane-stabilizing effect by which it is thought to decrease the activity of adenylate cyclase and furthermore chlorpromazine has an inhibitory effect on calmodulin. Clonidine has been observed to decrease the entry of calcium into

the cells and to decrease the adenylate cyclase activity by acting on α_2-adrenergic receptors on enterocytes. Propranolol, a ß-adrenergic antagonist, inhibits the activation of adenylate cyclase by ß-adrenergic agonists. Verapamil, a calcium-channel blocker, inhibits the secretion which is the result of an increased entry of calcium into the enterocytes. Flurbiprofen is a non-steroidal antiinflammatory drug (NSAID). NSAID's inhibit the production of prostaglandins which can activate adenylate cyclase. Furthermore, the antisecretory activity of NSAID's has been attributed to an increased absorption, to an inhibition of the effect of cholera toxin on the cAMP-adenylate cyclase system and to an inhibition of the cAMP protein kinase activity (chapter I; section I.D.).

Veterinary Research Communications, 13 (1989)159-170
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III.C.2. EFFECT OF ANTISECRETORY DRUGS ON EXPERIMENTALLY INDUCED WEANLING DIARRHOEA IN PIGLETS

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ABSTRACT

In 45 newly-weaned 3 to 4-week-old piglets, diarrhoea was induced by a combined infection with transmissible gastroenteritis (TGE) virus and enterotoxigenic *E. coli* (ETEC) strains. In untreated control animals this dual inoculation resulted in profuse diarrhoea, vomiting, hypovolaemic shock and death of 77% of the animals within five days of TGE virus inoculation. Antisecretory drugs were administered intramuscularly for three consecutive days after experimental infection. The neurolepticum chlorpromazine, at 2 mg/kg/24 h, resulted in a significant inhibition of diarrhoea and vomiting, and in an increase in weight gain and survival. Sedation and hypothermia, however, were serious side-effects. The α_2-agonist clonidine, at 80 µg/kg/12 h, induced a significant antidiarrhoeal effect and a reduction in mortality. The drug, however, provoked decreased activity excitation and incoordination. The ß-adrenergic antagonist propranolol, at 0.33 mg/kg/8 h, and the calcium-channel blocker verapamil, at 2 mg/kg/8 h, had no beneficial effect on the experimentally induced diarrhoea.

INTRODUCTION

With improved knowledge of the mechanisms of action of specific diarrhoea producing organisms, many therapeutic agents have been examined which might exert inhibitory effects at one or another level of the diarrhoeic pathways.

Enterotoxigenic *E. coli* (ETEC) strains adhere with fimbriae to villous epithelial cells, colonize the small intestinal surface and elaborate heat-labile (LT) and/or heat-stable (ST, type a and/or b) enterotoxins, which stimulate cyclic adenosine monophosphate (cAMP)-and/or cyclic guanosine monophosphate (cGMP)-mediated enteric fluid loss by inducing small intestinal hypersecretion. Treatment of secretory diarrhoea could be performed using drugs which inhibit this

hypersecretion and/or stimulate intestinal absorption (Donowitz, Wicks and Sharp, 1986; Powell, 1986). Neuroleptics, e.g. chlorpromazine, are reported to induce inhibition of cAMP mediated fluid secretion (Donowitz, Wicks and Sharp, 1986; Greenough and Rabbani, 1986). Adrenergic receptor agonists, especially the α_2-agonists, are another promising group of potentially antidiarrhoeal drugs, acting primarily by increasing sodium and chloride absorption and by inhibiting bicarbonate secretion (Donowitz, Wicks and Sharp, 1986; Powell, 1986). The ß-adrenergic antagonist, propranolol, has been shown to be antisecretory for cholera toxin and dibutyryl cAMP in rabbit and rat intestine (Donowitz, Wicks and Sharp, 1986). In animal studies verapamil, a well-known calcium channel blocker, increases basal ileal absorption of water, sodium and chloride. Some calcium channel blockers have also been shown to prevent secretion induced by cholera toxin and by ST-enterotoxin of *E. coli* (Donowitz, Wicks and Sharp, 1986).

In pigs post-weaning diarrhoea often results from a combined infection with *E. coli* and a viral enteropathogen (mostly rotaviruses, sometimes coronaviruses) (Tzipori, Chandler, Makin and Smith, 1980; Lecce, 1983; Wilson and Francis, 1986). The enteropathogenic viruses damage the small intestinal villi, resulting in villous atrophy and subsequently in small intestinal malabsorption (Moon, 1978). The dual infection induces a more severe diarrhoea than the infection with ETEC alone (Tzipori, Chandler, Makin and Smith, 1980).

We therefore established a combined hypersecretion-malabsorption diarrhoea model in newly-weaned piglets by inoculating them with transmissible gastroenteritis (TGE) virus, an enteropathogenic coronavirus, and ETEC. In preliminary experiments it has been shown that this combined viral-bacterial infection induces profuse diarrhoea and hypovolaemic shock, which provokes 100% mortality within five days (Cox, Cools, Charlier, Schrauwen and Houvenaghel, 1986).

In the present study the effects of chlorpromazine, clonidine, propranolol and verapamil were evaluated in this model.

MATERIALS AND METHODS

The experiments were performed on 45 newly-weaned, 3 to 4-week-old, female piglets, weighing 4.2 to 7.2 kg, from primiparous sows purchased from the same commercial farm. The piglets were of mixed breed (Pietrain x Belgian Landrace). They were individually housed at 27 ± 2°C and allowed to drink UHT sterilized whole cow's milk ad libitum. To enhance ETEC colonization of the small intestine, the piglets were orally pre-treated on days 1, 2 and 3 with chloramphenicol (Chloromycetin, Parke-Davis; 1875 mg/L milk). This pre-treatment with chloramphenicol renders the dual infection more severe (Cox, Schrauwen and Houvenaghel, 1986), probably by making the gut more susceptible to ETEC (Kaufman, 1984). After starvation for the first three hours of day 4, the piglets were orally inoculated with TGE virus (1.66×10^6 pig infective dose/animal). Twenty-four hours later (day 5) the piglets were starved again for three hours, after which 62 ml of a 1.4% $NaHC0_3$ solution was given intragastrically to neutralize the gastric acidity and so prevent ETEC destruction. Fifteen minutes later they were intragastrically inoculated with 10 ml of a bacterial suspension of two ETEC strains. Both strains, 0149 K91

K88ac; LT, STa and STb enterotoxin positive respectively, were grown on brain heart infusion agar (Oxoid) at 37°C for 24 h. Bacteria were suspended and diluted in sterile physiological saline to an A620 of 0.4, approximately 1.2 x 10^9 bacteria/ml as determined by viable count.

Antisecretory drug treatment was started 5 h after the ETEC inoculation and was continued for three consecutive days (Table 1). Six piglets were injected with chlorpromazine hydrochloride (Rhone-Poulenc) at a dose rate of 1 mg/kg/24 h and six others were injected at a dose rate of 2 mg/kg/24 h. Another group of six piglets was injected with clonidine hydrochloride (Boehringer Ingelheim) at 0.04 mg/kg/12 h, whereas six animals received 0.08 mg/kg/12 h. A further six piglets were injected with propranolol hydrochloride (ICI) at a dose rate of 0.33 mg/kg/8 h. Finally, six piglets were treated with verapamil hydrochloride (Knoll AG) at a dose rate of 2 mg/kg/8 h. To reduce side-effects the first or last dosage was lower for several of the drugs (Table 1). All the drugs were dissolved in distilled water and injected intramuscularly. The other nine piglets were kept as untreated controls.

During the experiment clinical signs, severity of diarrhoea, milk intake, weight change, body temperature and survival were recorded daily. The severity of the diarrhoea was evaluated by arbitrarily scoring the consistency of the faeces (0 = normal; 1 = pasty; 2 = semi-liquid; 3 = watery). Scoring was always performed by the same person.

Shocked piglets died spontaneously or were killed with an overdose of methomidate (Hypnodil, Janssen). The other piglets were killed with methomidate 10 days after the start of the experiment. All piglets were examined post mortem.

The genetic susceptibility of the piglets to K88ac -ETEC adhesion to their small intestinal villi was determined by the *in vitro* technique described by Girardeau (1980).

The daily diarrhoea score was expressed as median and ranges or individual values and statistically analysed using the Mann-Whitney U or Wilcoxon tests. The one-way analysis of variance and simple contrasts were used to assess the statistical significance between the values of the different parameters after experimental infection and those before the infection on day 3. The Mann-Whitney U test was used to demonstrate significant differences for time-matched values between control and treatment groups. The differences in survival time between the treatment groups and the control group were evaluated using the Mantel modification of the Gehan generalized Wilcoxon test (Knapp and Wise, 1985).

RESULTS

The *in vitro* adhesion assay performed immediately post mortem revealed that the villi of seven piglets were genetically resistant to adhesion of K88ac-ETEC to their enterocytes (Table 1). Data from these piglets are not included in the results.

As previously described (Cox, Charlier and Houvenaghel, 1988), piglets developed diarrhoeic faeces during the chloramphenicol pretreatment period (Figure 1, day 3).

TABLE I
Experimental groups, dose and frequency of drugs studied, total number of piglets in each group (N1), number of piglets susceptible to K_{88ac}-adhesion (N2), percentage of piglets vomiting, percentage survival and mean survival time in shocked piglets

Group	Dosage mg/kg	Doses/day	N1	N2	Vomiting (%)	Survival (%)	Mean survival time of shocked pigs (days after TGE virus)
Control	–	–	9	6	50	33	3.3 ± 0.5
Chlorpromazine	1	1	6	5	80	0	4.1 ± 0.7
Chlorpromazine	2[a]	1	6	5	0	80	3.0
Clonidine	0.04[b]	2	6	5	40	40	3.8 ± 0.5
Clonidine	0.08[c]	2	6	6	50	83 *	6.0*
Propranolol	0.33	3	6	5	100	60	3.5 ± 0.5
Verapamil	2	3	6	6	50	33	2.9 ± 0.5

[a] Third injection, 1.5 mg/kg; [b] first injection, 0.03 mg/kg; [c] first injection, 0.04 mg/kg
* Significant difference from the control group, $p < 0.05$

In the control group diarrhoea seriously worsened after inoculation with TGE virus and ETEC ($p < 0.05$). Daily milk intake decreased (day 3, 1117 ± 132 ml: day 6, 630 ± 184 ml; $p > 0.05$), vomiting occurred in 50% of the animals (Table I) and there was a significant weight loss (Figure 2) and decrease in body temperature (Table II). Piglets became dehydrated as shown by bilateral retraction of the eyeballs and a decrease in skin turgor. Cyanosis of the extremities and depression occurred, and 77% of the animals developed hypovolaemic shock (Table I). In the terminal stage some animals lay in lateral decubitus, with sunken flanks and a prominent pelvis.

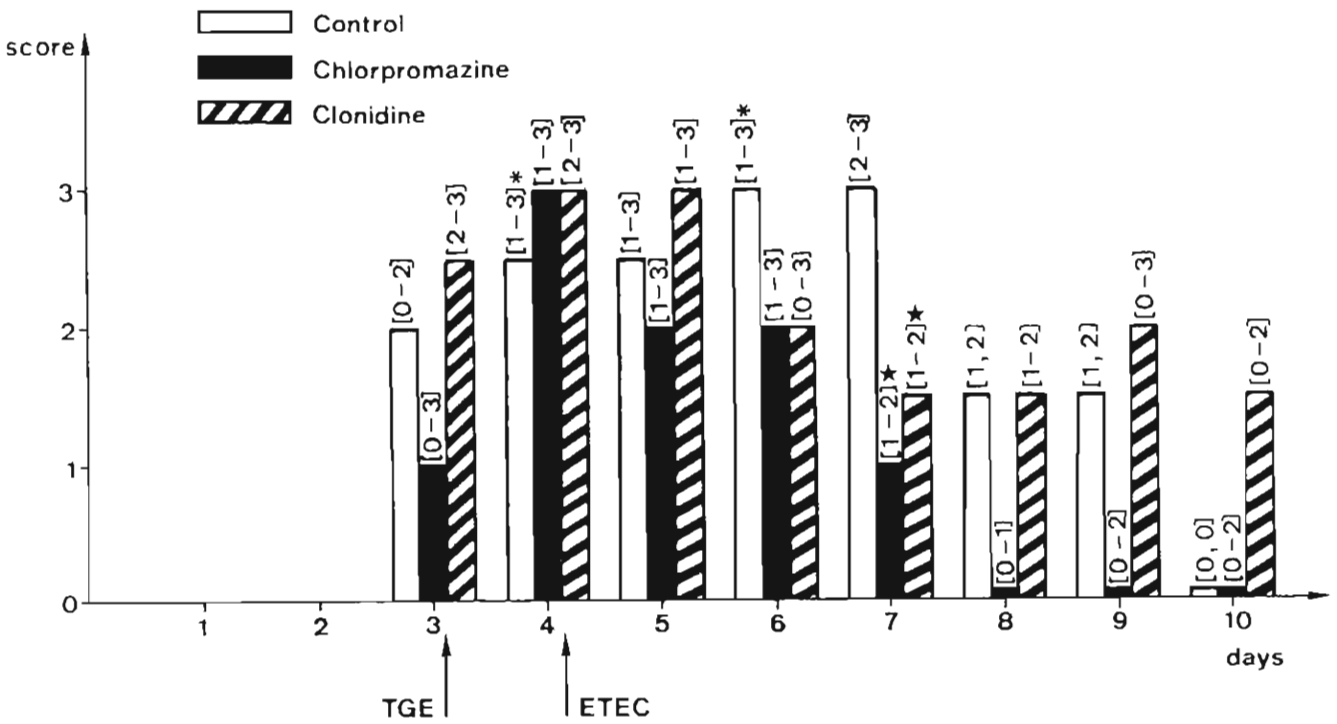


Figure 1. Diarrhoea scores (0 = normal; 1 = pasty; 2 = semi-liquid; 3 = watery) after a combined infection of newly-weaned piglets with TGE virus and ETEC (infected control group; n = 6) and the effect of treatment with chlorpromazine at 2 mg/kg/24 h (chlorpromazine group; n = 5) or with clonidine at 80 μg/kg/12 h (clonidine group; n = 6). Bars represent medians; ranges or individual values are given in brackets. Significant differences from the values prior to infection (day 3) are indicated by *($p < 0.05$). Significant differences from the values of the control group are indicated by ★($p < 0.05$)

TABLE II
Daily body temperature in °C (mean ± SEM or individual values) in the control and treatment groups

Groups (n)	Time (days) 3	4	5	6	7	8	9	10
Control (6)	39.3 ± 0.1	39.1 ± 0.1	38.6 ± 0.4**	38.8 ± 0.3*	38.9 ± 0.2	39 & 39.2	38.9 & 39	38.6 & 39.3
Chlorpromazine 1 mg (5)	39.7 ± 0.1†††	39.6 ± 0.1††	38.6 ± 0.2***	36.9 ± 0.7	38.4 & 41.1	36.1 & 39.8	41.7	–
Chlorpromazine 2 mg (5)	39.7 ± 0.1††	39.4 ± 0.2	38.9 ± 0.2	38.0 ± 0.6†	38.8 ± 0.3	39.0 ± 0.2	–	38.9 ± 0.4
Clonidine 0.04 mg (5)	39.4 ± 0.1	39.9 ± 0.2††	39.9 ± 0.2††	38.1 ± 0.5	38.6 ± 0.5	39.4 & 39.7	38.9 & 39.2	38.7 & 39.4
Clonidine 0.08 mg (6)	39.1 ± 0.1	38.7 ± 0.1†	38.9 ± 0.1	39.1 ± 0.2	39.8 ± 0.2†	40.0 ± 0.3	39.7 ± 0.2	39.5 ± 0.3
Propranolol (5)	39.2 ± 0.2	39.3 ± 0.2	39.5 ± 0.2††	40.0 ± 0.3†	39.4 ± 0.2	39.8 ± 0.1	39.4 ± 0.3	39.1 ± 0.4
Verapamil 2 mg (6)	39.5 ± 0.1	39.1 ± 0.1	39.3 ± 0.3	38.8 ± 0.5	38.3 ± 1.0	39.5 & 39.8	38.9 & 39.2	38.7 & 39.1

n = Number of piglets susceptible to K_{88ac}-adhesion in each group

Significant differences from the values prior to infection (day 3 values) are indicated by *($p < 0.05$); **($p < 0.01$); ***($p < 0.001$)

Significant differences from the control group are indicated by †($p < 0.05$); ††($p < 0.01$); †††($p < 0.001$)

– = Not determined

Mean survival time of shocked piglets was 3.3 ± 0.5 days after TGE virus inoculation. The gross changes at necropsy were cyanosis of the extremities, a filled stomach, severe congestion of the gastric mucosa, watery contents in and congestion of the small intestine and sometimes of the colon, and petechial haemorrhages in the kidneys.

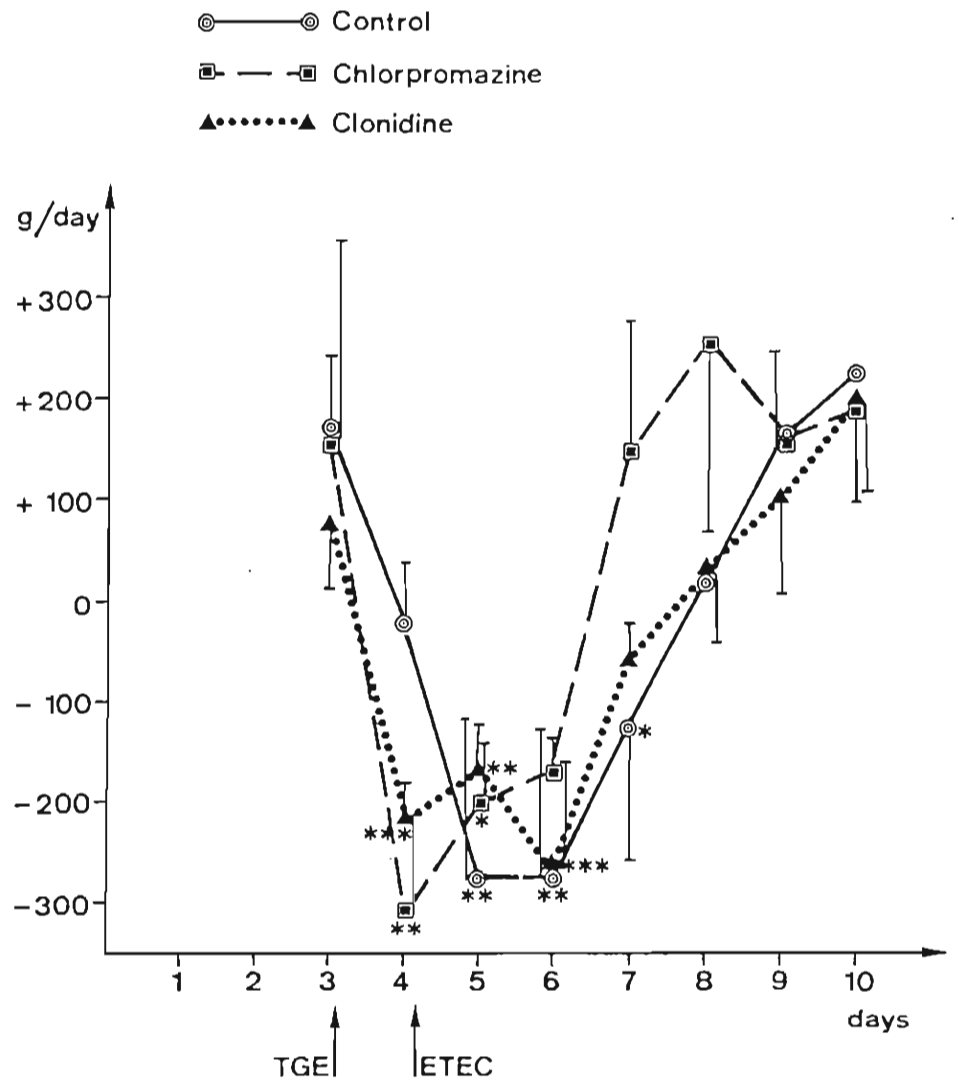


Figure 2. Weight change after a combined infection of newly-weaned piglets with TGE virus and ETEC (infected control group; n = 6) and effect of treatment with chlorpromazine at 2 mg/kg/24 h (chlorpromazine group; n = 5) or with clonidine at 80 µg/kg/12 h (clonidine group; n = 6). The values represent means ± SEM. Significant differences from the values prior to infection (day 3) are indicated by * ($p < 0.05$); **($p < 0.01$) and ***($p < 0.001$)

Treatment with the low dose of chlorpromazine at 1 mg/kg/24 h was without beneficial effect on diarrhoea score, vomiting or survival (Table I). However, the larger dose of this neurolepticum, 2 mg/kg/24 h, resulted in a significant decrease in the diarrhoea score on day 7 (Figure 1), a complete inhibition of vomiting (Table I), a less pronounced ($p > 0.05$) weight loss (Figure 2) and an increase, although not a significant one, in percentage survival (Table I). Chlorpromazine administration, especially in the larger doses, also provoked marked sedation and a resulting further reduction in daily milk intake (340 ± 118 ml on day 6, as against 630 ± 184 ml in the control group; $p > 0.05$), and a significantly more pronounced decrease in body temperature on day 6 (Table II) as compared to the control piglets. The decrease in body temperature was observed within one hour of the first administration of the neurolepticum and became more pronounced after the second. On the third day we therefore injected 1.5 mg/kg instead of 2 mg/kg.

Administration of clonidine at the low dosage, 0.04 mg/kg/12 h, was without influence on the parameters studied, whereas at a dose rate of 0.08 mg/kg/12 h there was a decrease in diarrhoea score (Figure l; day 7 values) and a significant reduction in mortality (Table I). Vomiting (Table I), daily milk intake and weight change (Figure 2) were not significantly influenced by this α_2-adrenoceptor agonist, whereas body temperature did not undergo the significant decrease after experimental infection observed in the control pigs (Table II). In both clonidine groups serious side-effects were observed. Administration of the low dose induced decreased activity, whereas with the higher dose, although activity was usually decreased, there was sometimes excitation or even incoordination. No analgesic effects were noted 15 min or 1 h after clonidine injection as evaluated by gently pinching the coronets and the skin of the feet and thorax.

Propranolol at 0.33 mg/kg/8 h and verapamil at 2 mg/kg/8 h were without significant beneficial effect on diarrhoea score, vomiting, weight change or survival rate (Table I). The decrease in body temperature observed in control piglets after TGE virus and ETEC inoculation was not influenced by the calcium antagonist, whereas administration of the ß-adrenergic antagonist resulted in an increase in temperature (Table II).

DISCUSSION

ETEC strains are involved in the multifactorial post-weaning diarrhoea syndrome in piglets, stimulating electrolyte and fluid secretion by the crypt cells (LT and STa enterotoxins) or diminishing absorption by the villous cells (LT enterotoxin). The mechanism of action of STb is still unclear. Recently rotavirus has also been shown to exert a role in the decrease of villous absorption (Tzipori, Chandler, Makin and Smith, 1980; Lecce, 1983). Since rotavirus is enzootic in Belgian piggeries (Debouck, 1984) and the experiments were performed on conventionally bred piglets, TGE virus, another enteropathogenic virus, was selected to induce a combined viral-bacterial enteritis. Whereas rotavirus only infects the upper part of the villi, TGE virus multiplies in and destroys the major part of the villous epithelium (Moon, 1978), resulting in more profuse diarrhoea and vomiting. In the very young piglets TGE is almost invariably fatal, whereas animals over three weeks old usually survive the infection (Aitken, 1983). In the present study most dually

infected piglets in the control group and also in some of the treatment groups developed hypovolaemic shock as a result of vomiting, induced by the TGE virus, and diarrhoea, probably induced by a combination of malabsorption (TGE virus) and hypersecretion (ETEC) (Cox, Charlier and Houvenaghel, 1988). The diarrhoea occurring during the chloramphenicol pre-treatment can be explained by the change in diet from sow's to cow's milk, by the loss of maternal immunity, by environmental changes (Tzipori, Chandler, Makin and Smith, 1980; Lecce, 1983) and perhaps also by a direct effect of the antibiotic on the intestine. It has been shown that chloramphenicol suppresses the anaerobic gut flora thus probably increasing the intestinal susceptibility to potentially pathogenic micro-organisms (Kaufman, 1984), whereas in calves a malabsorption diarrhoea has been reported following oral treatment with chloramphenicol (Mero, Rollin and Phillips, 1985). We recently evaluated the effect of the chloramphenicol pre-treatment on our diarrhoea model. Four piglets were treated with chloramphenicol for three days and were subsequently not inoculated with TGE virus and ETEC strains. Faeces became pasty to semi-liquid in two animals during treatment. After treatment, however, diarrhoea resolved within one day (Cox, Charlier and Houvenaghel, 1988).

The clinical signs in the markedly shocked piglets in our study agree with earlier observations on *E. coli* infections in gnotobiotic piglets (Kenworthy and de G. Mitchell, 1976), whereas our necropsy findings correspond to those observed in piglets which have died from post-weanling diarrhoea.

In the last decade there has been steady progress in research on potentially useful drugs which can reduce fluid loss in secretory diarrhoea (Greenough and Rabbani, 1986; Powell, 1986). These drugs, however, have never been tested in a combined secretion-malabsorption diarrhoea.

In animals chlorpromazine inhibits cAMP-induced hypersecretion provoked by the LT enterotoxin of *E. coli* and Vibrio cholerae, prostaglandins and dibutyryl cAMP, as well as cGMP-mediated hypersecretion due to the ST enterotoxin of *E. coli* (Lönnroth and Jennische, 1982; Donowitz, Wicks and Sharp, 1986). This neurolepticum probably also stimulates intestinal absorption of sodium and chloride, as demonstrated for promethazine in the rabbit ileum (Cohen, Sharp and Donowitz, 1985). In human medicine the beneficial effect of chlorpromazine as a therapeutic antisecretory drug was first reported in patients with severe diarrhoea due to cholera enterotoxin (Greenough and Rabbani, 1986). However, sedation was a serious disadvantage in its clinical use. In newborn piglets with experimentally ETEC-induced diarrhoea, a single intramuscular administration of chlorpromazine at 5 mg/kg one hour after the onset of diarrhoea completely normalized the intestinal fluid content within four hours but seriously sedated the animals, whereas at a dosage of 1-2 mg/kg there were no side-effects but an obvious antisecretory effect still occurred (Lönnroth, Andrén, Lange, Martinsson and Holmgren, 1979). These authors confirmed their findings in a field trial in which the duration of diarrhoea in a spontaneous outbreak due to ETEC was significantly reduced by a single intramuscular injection of chlorpromazine at 1 mg/kg in addition to oral electrolyte solution and standard antimicrobial agents. In our present study, in which no additional therapy was performed, we only observed significant antidiarrhoeic and antiemetic effects resulting in increased survival following the administration of 2 mg/kg/24 h of the neurolepticum for three consecutive days. However, it must be mentioned that the piglets in

the present study were newly weaned and had been inoculated with an enteropathogenic virus prior to ETEC infection, resulting in a more pronounced small intestinal fluid loss. Sedation and suppression of vomiting are well-known pharmacological properties of neuroleptic agents (Booth, 1977; Baldessarini, 1980). These drugs also induce hypothermia (Booth, 1977; Baldessarini, 1980), which explains the significantly more pronounced decrease in body temperature in the chlorpromazine (2 mg/kg) group as compared to the control group. Despite important side-effects such as sedation, decreased daily milk intake and hypothermia, chlorpromazine at 2 mg/kg/24 h would probably be useful in the treatment of severe diarrhoea of mixed viral-ETEC origin in weanling piglets.

Clonidine has been reported to inhibit castor oil-induced secretion diarrhoea in rats (Lal, Shearman and Ursillo, 1981; Spraggs and Bunce, 1983). In ligated jejunal loops in 2-week-old piglets this drug reduces the net secretion of water and electrolytes induced by the ST enterotoxin of *E. coli* (Ahrens and Zhu, 1982; Zhu and Ahrens, 1983). The α_2-adrenergic agonist, however, was without effect on the ST-induced increase in chloride efflux from isolated porcine enterocytes (Ahrens and Panichkriangkrai, 1985). In a study on its antidiarrhoeal action in man, Schiller, Santa Ana, Morawski and Fordtran (1985) postulated that clonidine has two actions which might contribute to this effect. Firstly, the drug directly stimulates the rate of intestinal water and electrolyte absorption both *in vitro* and *in vivo* by means of an α_2-adrenergic receptor. Secondly, clonidine has been found to inhibit intestinal motility. In the present porcine study, administration of clonidine at 80 µg/kg/12 h for three consecutive days resulted in beneficial effects on diarrhoea and survival rate. However, the drug also induced important side-effects such as decreased activity or excitation and incoordination, thus compromising its use as an antidiarrhoeal drug in practice. The sedative effects are similar to those of chlorpromazine (Blaschke and Melmon, 1980). Our observations do not confirm the analgesic effects of clonidine previously reported in pigs, horses and man especially after epidural administration (Wintzer, Krause, Siedentopf and Frey, 1985; Gordh, Feuk and Norlen, 1986).

The ß-adrenergic antagonist, propranolol, has a species-specific action. It has been reported to be antisecretory for cholera toxin and dibutyryl cAMP in rat and rabbit but not in mice small intestine (Larsen, 1982; Donowitz, Wicks and Sharp, 1986). In human medicine, an increase in body temperature in response to propranolol is an infrequent side-effect, reflecting an allergic reaction (Weiner, 1980). The calcium antagonist, verapamil, has been observed to increase basal ileal absorption of sodium, chloride and water and to block jejunal secretory response to the ST enterotoxin of *E. coli* (Forsyth, Wong and Maenz, 1985; Donowitz, Wicks and Sharp, 1986). The results from our porcine study, however, failed to reveal any beneficial effects of verapamil or propranolol, at least in the dosage studied, on the experimentally induced diarrhoea.

Our results suggest that, of the drugs studied, only chlorpromazine and clonidine are likely to be of value in the treatment of severe diarrhoea of mixed viral-ETEC nature in newly-weaned piglets. Their observed side-effects are, however, a serious disadvantage for their practical use.

J. Vet. Med. A 35, 744—754 (1988)
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ISSN 0931—184X

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III.C.3. EFFECT OF AN ALPHA2-ADRENOCEPTOR AGONIST ON EXPERIMENTALLY INDUCED DIARRHOEA IN NEWLY-WEANED PIGLETS

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ABSTRACT

In newly-weaned piglets (n = 27; 3—4 weeks) diarrhoea and vomiting were provoked by inoculation with transmissible gastroenteritis virus and enterotoxigenic Escherichia coli strains, producing LT, STa and STb enterotoxins. This combined infection resulted in a hypovolaemic shock and in a mortality of 71% within 7 days. Animals revealed decrease in body weight, in arterial pressure, leucocyte count, plasma pH and plasma lactic acid concentration, and an increase in heart rate and in total plasma protein concentration. In shocked and expiring animals an increase in haematocrit and a decrease in base excess and actual bicarbonate were noted.

Clonidine, administered intramuscularly to 6 animals on 3 successive days following the dual infection in a dosage of 80 μg/kg/12 h, was without beneficial effects on the diarrhoea and survival rate. Moreover, the decrease in arterial pressure resulting from the hypovolaemia was aggravated by the hypotensive and bradycardiac effects of the α_2-adrenoceptor agonist.

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INTRODUCTION

Experiments performed in our laboratory to induce hypersecretion diarrhoea by inoculating newly-weaned piglets with enterotoxigenic *Escherichia coli* (ETEC), possessing fimbrial adhesions and producing heat-labile (LT) and heat-stable (STa and STb) enterotoxins were not successful (Cox *et al.*, 1986a). When the experimental ETEC infection was preceded by an oral chloramphenicol treatment an enhanced occurrence, severity and duration of diarrhoea was observed (Cox *et al.*, 1986a). This "colonization-resistance-suppressing" antibiotic makes the gut more susceptible to ETEC (Kaufman, 1984), and/or can induce a malabsorption as recently shown in calves (Mezo *et al.*, 1985). The resulting diarrhoea, however, was still ineffective in less producing a significant hypovolaemia and shock. ETEC infection in newly-weaned piglets is often combined with rotavirus infection, which can seriously influence the occurrence and severity of diarrhoea (Lecce *et al.*, 1982; Tzipori *et al.*, 1980a).

We therefore introduced a viral infection into our piglet diarrhoea model. Since rotavirus is enzootic in Belgian piggeries (Debouck, 1984), piglets were inoculated with transmissible gastroenteritis (TGE) virus— an enteropathogenic coronavirus which can also be isolated from piglets with weaning diarrhoea (Pritchard, 1987)—prior to ETEC infection (Cox *et al.*, 1986c).

Adrenergic receptor agonists are a promising group of potentially antidiarrhoeal drugs. They appear to act primarily by increasing NaCI absorption and inhibiting $HC0_3$ secretion. Potentially the most useful of these drugs are the α_2-adrenergic agonists, which work at the lowest concentrations (Donowitz *et al.*, 1986; Powell, 1986). In *E.coli* ST-enterotoxin-exposed ligated jejunal loops of 2-week-old pigs, clonidine, an imidazoline derivative with highly effective antihypertensive properties (Arndts *et al.*, 1983), reduced the net secretion of water and electrolytes induced by the toxin (Arhens and Zhu, 1982). This α_2-adrenergic agonist, however, was without effect on ST induced increased chloride efflux from isolated porcine enterocytes (Arhens and Panichkriangkrai, 1985).

In the present study the effect of clonidine treatment was evaluated in our combined TGE virus-ETEC diarrhoea model in newly-weaned piglets.

MATERIAL AND METHODS

The experiments were performed on newly-weaned, female piglets (n = 27; 3- to 4-week-old; 3.2 to 7.2 kg) from primiparous sows, purchased from the same commercial farm. Piglets were of mixed breeding (Pietrain x Belgian Landrace). They were individually housed at 27 ± 2 °C and allowed to drink UHT sterilized whole cow's milk ad libitum. On day 1 of the experiment piglets were anaesthetized with I5 mg/kg methomidate (Hypnodil ®, Janssen) and 2 mg/kg azaperone (Stressnil ®, Janssen), and a silastic catheter (Dow Corning) was implanted in the left carotid artery for daily blood sampling and pressure recording. Piglets were pretreated on days 1, 2 and 3 with chloramphenicol (Chloromycetin ®, Parke-Davis; 1,875 mg/l milk), which probably enhances ETEC colonization of the small intestine. After a starvation period for the first three hours of day 4, piglets were orally inoculated with TGE virus (1.66×10^6 pig infective dose/animal).

Twenty-four hours later (day 5) piglets were starved again for 3 h, after which 62 ml of a 1.4% $NaHCO_3$ solution was given intragastrically to neutralize gastric acidity (prevention of ETEC destruction). Fifteen minutes later they were intragastrically inoculated with a bacterial suspension of two ETEC strains, 10 ml/strain per animal. Both strains, O149K91K88ac; LT, STa and STb enterotoxin positive; were grown on brain heart infusion agar (Oxoid) at 37°C for 24 h. Bacteria were suspended and diluted in sterile physiological saline until an A 620 of 0.4, approximately 1.2 x 10^9 bacteria/ml, as determined by viable count.

Clonidine treatment was started 5 h following the ETEC inoculation and was performed for 3 consecutive days. Six piglets (clonidine-group) were intramuscularly injected with clonidine-hydrochloride (Boehringer Ingelheim), dissolved in distilled water, in a dose of 0.08 mg/kg/ 12 h; the first administration was 0.04 mg/kg. The other piglets (n=21) were kept as untreated controls.

During the experiment several parameters were recorded daily, such as weight change; milk intake; body temperature; appearance and severity of diarrhoea; clinical symptoms; survival rate; frequency of respiration; arterial blood pressure (Pa); heart rate (HR); haematocrit (Hct); plasma protein (Merck kit), glucose (Merck kit), and lactic acid (Boehringer kit) concentrations; arterial pH, $p0_2$ and $pC0_2$ (Astrup, Radiometer); base excess (BE) and actual bicarbonate (porcine acid-base alignment nomogram for arterial blood) (Hannon, 1983); leucocyte count; serum concentrations of sodium, potassium (flame photometry), chloride (chloridometer), and calcium (atomic absorption); faecal shedding of TGE virus, rota-like- and rotavirus (enzyme-linked immunosorbent assay) and faecal ETEC excretion. Faecal ETEC excretion was evaluated as the approximate percentage of haemolytic E. coli among the total number of aerobic bacteria in rectal swabs inoculated on blood agar plates at 37°C for 24 h.

The severity of diarrhoea was evaluated by arbitrarily scoring the consistency of the faeces (0 = normal; 1 = pasty; 2 = semiliquid; 3 = watery). Scoring was always performed by the same person.

The carotid arterial pressure was recorded with a saline-filled silastic catheter connected to a miniature pressure transducer (Gaeltec). The electrocardiogram (ECG) was monitored to measure HR. Pa and ECG were recorded on a Beckman multi-channel recorder.

Dehydrated and shocked piglets died spontaneously or were killed with an overdose of methomidate. The other piglets were killed with methomidate 10 days after the start of the experiment. All piglets were examined post-mortem.

The genetic susceptibility of the piglets to K88ac-ETEC adhesion to their small intestinal villi was determined by the in vitro technique, described by Girardeau (1980).

The daily diarrhoea score is expressed as median and ranges or individual values and was statistically analysed using the Mann-Whitney U test. All other parameters are given as means ± SEM. A one-way analysis of variance and simple contrasts were used to assess the statistical significance between the values of the different parameters recorded after the experimental infection and the values measured before infection (values on day 3). The Mann-Whitney U test was used to demonstrate significant differences for time-matched values of the clonidine group and the control group. The percentage of survival in both groups was analysed by the Mantel modification of the

Gehan generalized Wilcoxon test (Knapp and Wise, 1985).

RESULTS

The post-mortem *in vitro* adhesion assay revealed that the villi of all piglets from the control and the clonidine group were susceptible to adhesion of K88ac-ETEC to their enterocytes.

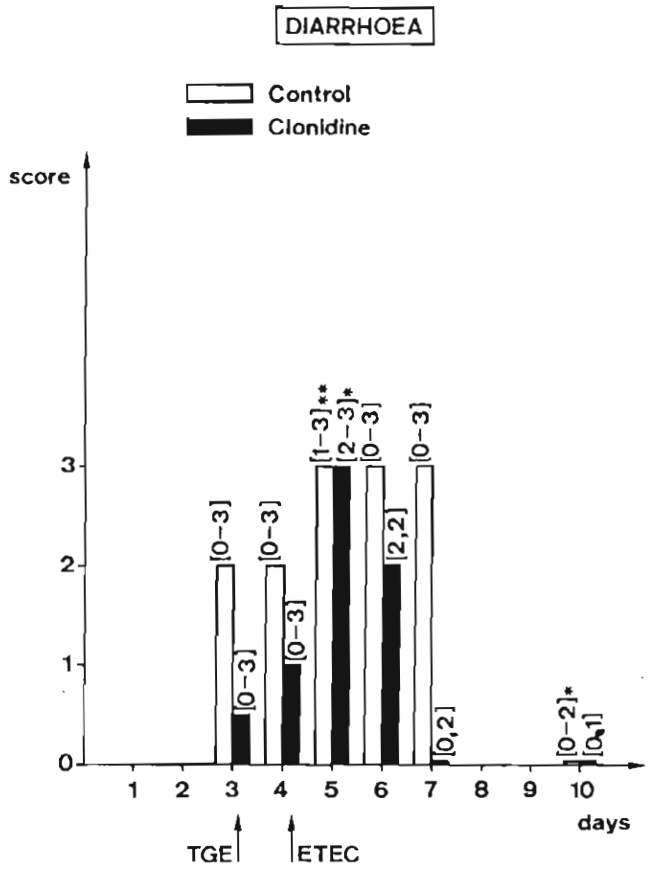


Fig. 1. Diarrhoea score (0 = normal; 1 = pasty; 2 = semiliquid; 3 = watery) following a combined infection of newly-weaned piglets with TGE virus and ETEC (control group; n = 21) and effect of treatment with clonidine (n=6). Bars represent medians; ranges or individual values are given in brackets. Significant difference from the value prior to infection (day 3) is indicated by * $p < 0.05$; ** $p < 0.01$.

In both groups chloramphenicol pre-treatment followed by TGE virus and ETEC inoculation induced a profuse watery diarrhoea (Fig. 1), resulting in hypovolaemia, shock and death of 71% of the animals in the control group and 67 % of the clonidine group (Fig.2; $p>0.05$) within the 10-day observation period (mean survival time of shocked piglets: 2.5 ± 0.5 days after TGE infection for the clonidine group and 2.8 ± 0.5 days for the control group; $p > 0.05$). Pigs already showed diarrhoeic faeces during the chloramphenicol pre-treatment period (Fig.1; day 3).

Table 1. Daily values (means ± SEM) for milk intake, for concentrations of plasma protein, lactic acid, serum potassium and chloride, and for leucocyte count in the control (n=21) and the clonidine treated group (n = 6). Values for days 8 and 9 were not determined.

Time	Milk intake (ml/day)		Plasma protein conc. (g/dl)		Lactic acid conc. (mg/dl)	
(days)	Control	Clonidine	Control	Clonidine	Control	Clonidine
3	944 ± 110	1,368 ± 230	4.6 ± 0.2	3.6 ± 0.2††	21 .7 ± 2.2	32.0 ± 5.5†
4	986 ± 110	598 ± 130***	5.2 ± 0.1*	4.0 ± 0.1†††	15.9 ± 2.8*	23.1 ± 3.3†
5	744 ± 80	517 ± 50***†	5.5 ± 0.2**	5.2 ± 0.9***	12.9 ± 1.6**	18.2 ± 4.7*
6	1,044 ± 170	690 (600; 780)	5.7 ± 0.2***	5.6 (5.4; 5.7)	13.1 ± 1.8**	21.9 (16.5; 27.3)
7	1,161 ± 300	840 (830; 850)	5.5 ± 0.4**	4.9 (4.7; 5.1)	16.1 ± 4.7	15.3 (9.8; 20.8)
10	1,447 ± 360	970 (950;990)	4.7 ± 0.4	4.9 (4.7;5.1)	12.9 ± 2.5	11.6 (9.7; 13.5)

	Potassium conc. (mmol/l)		Chloride conc. (mmol/l)		Leucocyte count (WBC/mm^2)	
	Control	Clonidine	Control	Clonidine	Control	Clonidine
3	4.1 ± 0.1	3.8 ± 0.3	97.1 ± 1.8	91.0 ± 1.0†	12,097 ± 727	9,667 ± 1,157
4	4.0 ± 0.2	3.9 ± 0.2	93.2 ± 1.4	94.0 ± 2.0	8,692 ± 605**	7,333 ± 424
5	4.3 ± 0.2	4.7 ± 0.5*	97.8 ± 2.0	100 ± 1.6***	9,395 ± 789**	9,600 ± 1 058
6	5.1 ± 0.8	3.8 (3.4; 4.1)	101.3 ± 2.9	105 (104; 105.5)	12,844 ± 1,381	10,850(10,250; 11,450)
7	4.8 ± 0.5	3.9 (3.7; 4.1)	96.6 ± 3.9	101 (96.5; 105.5)	11,175 ± 531	15,200(12,200; 18,200)
10	3.8 ± 0.2	4.6 (4.5; 4.6)	91.1 ± 3.0	88 (86; 90)	15,083 ± 1,618*	19,850(12,450;27,250)

Significant differences from the values prior to infection (day 3) are indicated by :*$p < 0.05$; ** $p < 0.01$; ***$p < 0.001$. Significant differences between groups are indicated by † $p < 0.05$; †† $p < 0.01$; ††† $p < 0.001$

Diarrhoea, however, was seriously aggravated in both groups following inoculation with TGE virus and ETEC ($p < 0.05$). No significant difference in diarrhoea score was observed between the two both groups (Fig. 1). Twenty hours after ETEC inoculation 75—100 % of the total number of aerobic bacteria shed in the faeces of pigs in both groups were haemolytic *E. coli* of the same serotype as the inoculum. In piglets with severe diarrhoea, shedding remained high (> 50 %) until death. In all piglets of both groups TGE virus was identified in the faeces. Rota- and rota-like virus were each only detected in the faeces of one pig of the control group, and rotavirus also in one pig of the clonidine group.

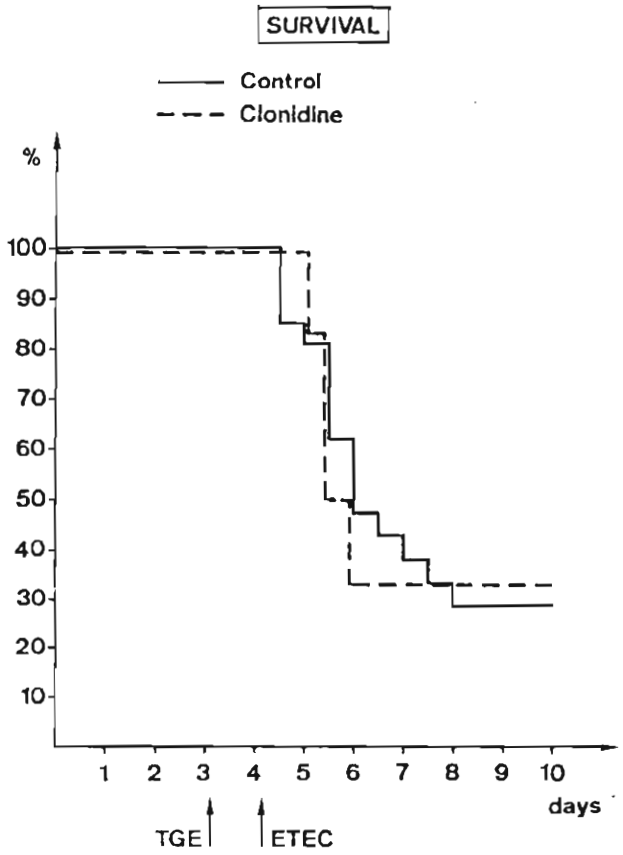


Fig. 2. Survival rate following a combined infection of newly-weaned piglets with TGE virus and ETEC (control group; n=21) and effect of treatment with clonidine (n=6)

Vomiting was noted in 65 % of the piglets from the control group and 80 % from the clonidine group. Fifteen min and 1 h after clonidine administration, no analgesic effects were observed as evaluated by gently pinching the coronets and the skin of feet and thorax. Shocked piglets treated with clonidine demonstrated excitation and incoordination. Surviving animals of this group showed a decreased activity. Lethargy was also noted in shocked piglets from the control group. In the control group, daily milk intake did not change significantly during the experiment, whereas in the clonidine group a significant decrease was observed (Table 1). However, due to the vomiting this parameter was difficult to measure exactly. As a result of the vomiting and the pronounced and sustained diarrhoea, pigs from both groups revealed severe weight loss (Fig. 3). Dehydrated piglets gradually developed cyanosis of the ear margins, tail and coronets. In the terminal stage some animals lay in lateral decubitus with retracted eyes and sunken flanks.

In both groups Hct revealed no significant changes during the experiment (day 3 values: 38 ± 1% for the control group; 33 ± 2 % for the clonidine group). In shocked and expiring piglets of both groups, however, a significant increase in Hct was observed (to 44 ± 1%; $p < 0.05$). A significant increase in total plasma protein concentration was observed in both groups (Table 1).

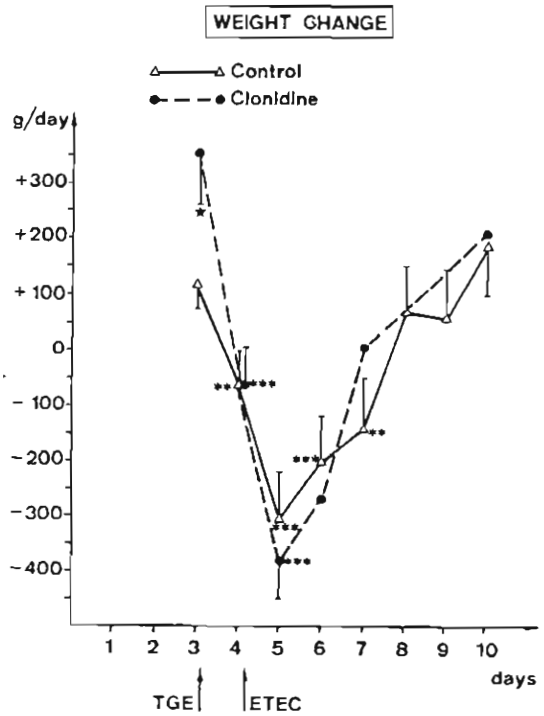


Fig. 3. Weight change following a combined infection of newly-weaned piglets with TGE virus and ETEC (control group; n=21) and effect of treatment with clonidine (n=6). The values represent means ± SEM. Significant differences from the values prior to infection (day 3) are indicated by ** $p < 0.01$; *** $p < 0.001$. Significant difference between groups is indicated by $^{\star}p < 0.05$

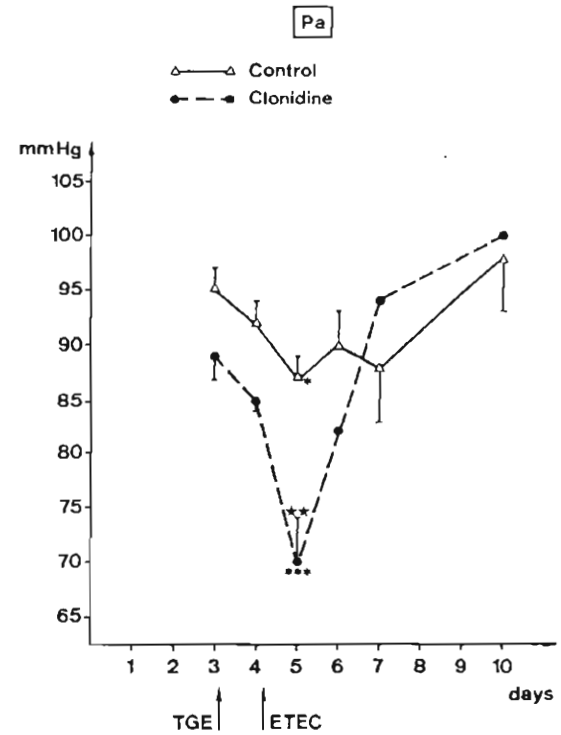


Fig. 4. Decrease in arterial pressure (Pa) following a combined infection of newly-weaned piglets with TGE virus and ETEC (control group; n=21) and effect of treatment with clonidine (n=6). The values represent means ± SEM. Significant differences from the values prior to infection (day 3) are indicated by * $p < 0.05$; ** $p < 0.001$. Significant difference between groups is indicated by $^{**}p < 0.01$

In control as well as in clonidine-treated pigs, hypovolaemia resulted in a significant decrease in Pa, which was significantly more pronounced on day 5 for the clonidine group (Fig.4). In the control group this hypotension was accompanied by an increase in HR, whereas in the clonidine group a significant bradycardia was observed on day 5 (Fig. 5).

Arterial pH decreased in both groups (Fig.6), and lactic acid concentration also showed a decrease (Table 1). After an initial, not significant increase on day 6, plasma glucose concentration decreased in the control group (day 3 vs. day 10 values: 94.1 ± 6.1 and 76.7± .0 mg/dl; $p<0.05$). In the clonidine group, however, a significant increase occurred on day 5 (day 3 vs. day 5 values: 77.2 ± 8.9 and 119.5 ± 2.4 mg/dl; $p < 0.001$). In the control group values for arterial P02 did not change significantly during the course of the experiment (control value on day 3: 90.0 ± 3.4 mm Hg), whereas in the clonidine group a significant decrease was observed (day 3 vs. day 5 values: 97.0 ± 1.8 and 85.8 ± 3.3 mm Hg; $p < 0.05$). Values for pC02 and frequency of respiration showed no significant changes within both groups (day 3 values for control and clonidine group, respectively: for .$pC0_2$: 29.6 ± 1.0 and 32.8 ± 2.0 mm Hg; for respiration: 26 ± 3 and 34 ± 5/min).

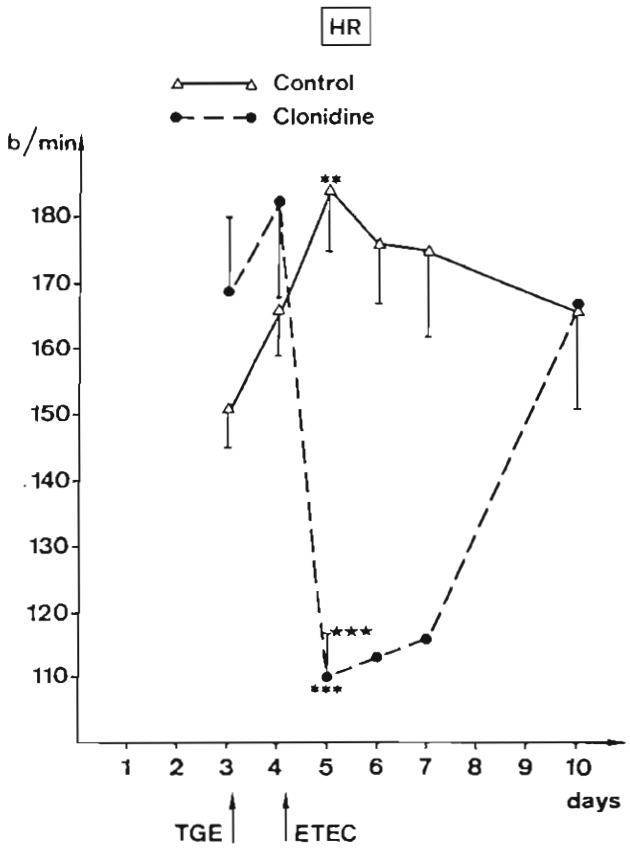


Fig. 5. Changes in heart rate (HR), in beats (b) per min, following a combined infection of newly weaned piglets with TGE virus and ETEC (control group; n=21) and effect of treatment with clonidine (n = 6). The values represent means ± SEM. Significant differences from the values prior to infection (day 3) are indicated by: ** $p < 0.01$; *** $p < 0.001$. Significant difference between groups is indicated by ***$p < 0.001$.

In shocked and expiring piglets of both groups arterial values for BE and actual bicarbonate decreased (day 3 vs. day 5 values: for BE: - 6.8 ± 2.4 and - 24.6 ± 3.7 mmol/l, $p < 0.001$, for the control group, and - 2.0 ± 2.2 and - 14.1 ± 3.7 mmol/l, $p < 0.01$, for the clonidine group- for HC03: 23.3 ± 1.9 and 8.6 ± 1.0 mmol/l, $p < 0.001$, for the control group, and 28.4 ± 2.4 and 17.6 ± 3.3 mmol/l, $p < 0.01$, for the clonidine group). Despite the pronounced dehydration, no significant changes occurred in serum osmolality (day 3 values: 274 ± 5 and 277 ± 10 mosmol/l for control and clonidine group, respectively), or in calcium concentration (day 3 values: 2.5 ± 0.1 and 2.7 ± 0.05 mmol/l, respectively) in the two groups. Control pigs revealed a transient but significant increase in sodium concentration (from 130.0 ± 2.5 mmol/l on day 3 to 137.0 ± 1.5 mmol/l on day 6, $p < 0.01$), followed by a return to near control value on day 7 in surviving piglets, whereas within the clonidine group no significant changes in sodium concentration were noted (control value on day 3: 130.0 ± 1.0 mmol/l). For the control group potassium concentration did not change significantly during the experimental period, whereas the value increased significantly within the clonidine group (Table 1). Values for chloride concentration revealed no significant changes in the control group, whereas in the clonidine group a significant increase was noted on day 5 (Table 1).

Following TGE virus and ETEC inoculation, the leucocyte count decreased in both groups, but the decrease was significant only for the control group (Table 1). This transient decrease was followed by a return to near control value and by a secondary increase (Table 1).

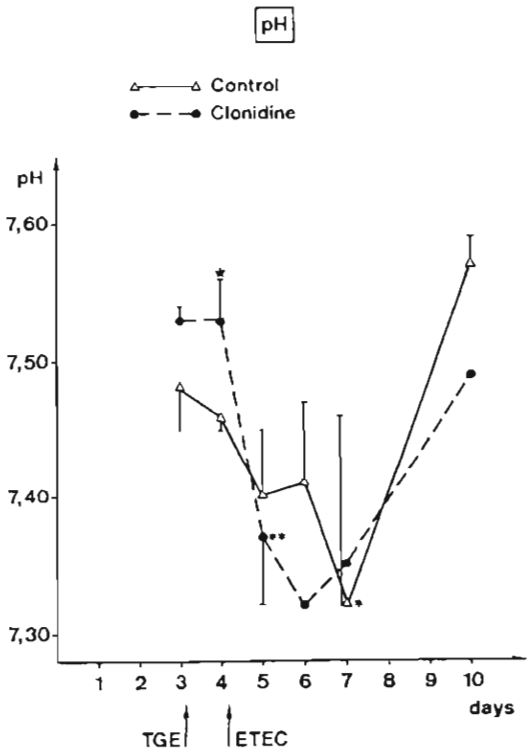


Fig. 6. Decrease in pH following a combined infection of newly-weaned piglets with TGE virus and ETEC (control group; n=21) and effect of treatment with clonidine (n=6). The values represent means ± SEM. Significant differences from the values prior to infection (day 3) are indicated by : * $p < 0.05$; ** $p < 0.01$. Significant difference between groups is indicated by *$p < 0.05$.

Body temperature revealed a non-significant decrease in both groups (control values on day 3 for the control and clonidine group, respectively: 39.6 ± 0.1 and 39.4 ± 0.1 °C). This decrease was followed by a return to near control value and by a secondary increase (40.0 ± 0.3 °C on day 10 for the surviving pigs in the control group; $p < 0.05$).

Necropsy in expiring piglets of both groups revealed an over-filled stomach with congestion of the mucosa of the fundic region, congested thin-walled small intestine, especially the ileum, with watery contents, and sometimes also congestion of the colon wall.

DISCUSSION

In the present study most dually infected piglets of both groups revealed a hypovolaemic shock as a result of vomiting and diarrhoea probably induced by a combination of malabsorption (TGE virus) and hypersecretion (ETEC).

The diarrhoea occurring during the chloramphenicol pre-treatment could be explained by the change in diet from sow's to cow's milk, by the loss of maternal immunity, by environmental changes (Lecce, 1983), and by an effect of the drug on the intestine. It has been reported that chloramphenicol can suppress the anaerobic gut flora, thus probably increasing the susceptibility to potentially pathogenic microorganisms (Kaufman, 1984). In calves, however, following oral treatment with chloramphenicol, a malabsorption diarrhoea has been reported (Mero *et al.*, 1985).

The more pronounced hypotension in the clonidine-treated piglets as compared to control animals is probably provoked by a central action of the α_2-adrenergic drug. It is generally accepted that the hypotensive action of clonidine is due to the stimulation of presynaptic α_2-adrenoceptors within the caudal brain stem centres (medulla), resulting in sympatho-inhibition and vagal facilitation and in hypotension, bradycardia and decreased cardiac output (Bentley *et al.*, 1986). The pronounced bradycardia in the clonidine-treated pigs of the present study corresponds with this theory.

Clonidine has previously been reported to inhibit castor oil-induced diarrhoea in rats (Lai *et al.*, 1981). Much larger doses of the adrenergic drug seem to be required for anti-diarrhoeic activity as for cardiovascular effects (Spraggs and Bunce, 1983). In a study of the anti-diarrhoeal action in man, Schiller *et al.* (1985) postulate that clonidine has two actions that might contribute to this effect. First, clonidine has been reported to directly stimulate the rate of intestinal water and electrolyte absorption, both *in vitro* and *in vivo*, in animals by means of an α_2 -adrenergic receptor. Second, clonidine has been reported to alter intestinal motility in animals. In a recent study clonidine has been found to inhibit the migrating myoelectric complexes of the small intestine in rats (Thollander *et al.*, 1987).

The high control value (day 3) for arterial pH in pigs of both groups agrees with that observed in conscious immature 2- to 3-month-old pigs (Hannon, 1983). The metabolic acidosis and abrupt increase in Hct in shocked piglets were also observed in dying neonatal piglets following ETEC-induced or spontaneous diarrhoea (Andrén and Persson, 1983). Lactic acid, however, did not increase in our pigs, suggesting that other acid metabolites are responsible for the

acidosis, which is not of respiratory nature since $pC0_2$ was not significantly increased. The decrease in $p0_2$ in the clonidine group can probably partly be explained by the more pronounced hypotension in piglets of this group than in animals of the control group. The hyperglycaemia in the clonidine-treated piglets is probably the result of the well known metabolic effect of adrenergic drugs.

In a study on gnotobiotic pigs, 27 to 34 days old when infected with ETEC strains, animals also developed a severe diarrhoea resulting in dehydration and death between 16 and 36 h following infection (Kenworthy and de Mitchell, 1976). As in piglets of both groups of the present study no elevation of body temperature was corded during this period. Piglets also revealed a neutropenia (Kenworthy and de Mitchell, 1976).

The clinical symptoms in the seriously ill piglets of both groups in our study agree with these reported in the above-mentioned *E. coli* infection experiments in gnotobiotic pigs (Kenworthy and de Mitchell, 1976). Observations in our study do not confirm the analgesic effects of clonidine, as previously reported in pigs, horses and man (Gordh *et al.*, 1986), especially after epidural administration.

Pharmacokinetic studies with clonidine in man revealed an elimination half-life of 20—25 h following oral or intravenous administration in a dosage of 1—5 µg/kg (Arndts *et al.*, 1983). We administered the adrenergic drug twice daily in a dosage of 80 µg/kg. This dosage was chosen since the dosage, 40 µg/kg 12 h, was ineffective in a previous study on non-catheterized diarrhoeic pigs, whereas a dosage of 80 µg/kg 12 h, resulted in a significant improvement of diarrhoea and survival rate (Cox *et al.*, 1987).

In the present study on catheterized animals, however, this dosage did not result in beneficial effects. Due to the pronounced hypotension and bradycardia induced by the dose, the study of eventual anti-diarrhoeic effects of larger doses of clonidine is not appropriate.

In the present model on catheterized piglets the induced diarrhoea is more severe than in non-catheterized animals (Cox *et al.*, 1986b), probably resulting in a more pronounced dehydration. The more severe hypovolaemia could have made piglets more susceptible to the hypotensive action of clonidine. In this way the drug could have interacted with the normal compensatory mechanisms occurring during excessive fluid loss (Michell, 1985), compromising the beneficial effects of clonidine in our piglet diarrhoeic model.

Despite the promising results in ligated porcine jejunal loops exposed to *E. coli* ST enterotoxin (Arhens and Zhu, 1982; Zhu and Arhens, 1983), and our previous observations on non-catheterized diarrhoeic piglets (Cox *et al.*, 1987), clonidine must be excluded as a useful drug for treatment of severe acute diarrhoea from combined viral-bacterial origin in piglets.

J. Vet. Med. A 36, 142—151 (1989)
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ISSN 0931—184X

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III.C.4. EFFECT OF CHLORPROMAZINE ON EXPERIMENTAL DIARRHOEA IN JUST-WEANED PIGLETS

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ABSTRACT

In just-weaned piglets (n=30, 3—4 weeks) diarrhoea (100%) and vomiting (66%) were provoked by inoculation with transmissible gastroenteritis virus and enterotoxigenic E. coli strains (0149: K91: K88ac; LT, STa and STb enterotoxin positive). This combined infection resulted in a mortality of 71% within 7 days. During this period animals revealed a decrease in body weight, in arterial pressure, in leukocyte count, in plasma pH and in plasma lactic acid concentrations, and an increase in heart rate and in total plasma protein concentration. In shocked and expiring piglets an increase in haematocrit and a decrease in base excess and actual bicarbonate were observed.

Chlorpromazine, administered intramuscularly on 3 successive days following the dual infection in 8 K88ac susceptible pigs, in a dosage of 2 and 1.5 mg/kg 24 h, somewhat retarded the appearance of severe diarrhoea and suppressed vomiting. These beneficial effects, however, did not result in an increased survival.

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INTRODUCTION

Enterotoxigenic Escherichia coli (ETEC) strains are involved in the multifactorial post-weaning diarrhoea syndrome in piglets occurring within the first weeks after weaning. These strains adhere with fimbriae to receptor sites on villous epithelial cells, colonize the small intestinal villous surface and produce heat-labile (LT) and/or heat-stable (type a, STa) and/or type b (STb) enterotoxins, which stimulate intestinal fluid loss. The effects of LT and STa are mediated by a toxin-induced increase in cAMP (LT) and cGMP (STa), resulting in electrolyte and fluid secretion by the crypt cells (LT and STa) and in a diminished absorption by the villous cells (LT). The mechanism of action of STb is still unclear.

Several studies, both in vitro and in vivo, demonstrated that neuroleptics induce an inhibition of cAMP-mediated fluid secretion (see e.g. Donowitz *et al.*, 1986, Lönnrothand Munck, 1980). One of them — chlorpromazine (CPZ) — effectively reverses hypersecretion in cholera patients (Rabbani *et al.*, 1979) as well as in piglets infected with ETEC (Lönnroth *et al.*, 1979).

ETEC infection in just-weaned piglets is often combined with rotavirus infection, which can seriously influence the occurrence and the severity of diarrhoea (Tzipori *et al.*, 1980a). Since rotavirus is enzootic in Belgian piggeries we inoculated piglets with transmissible gastroenteritis (TGE) virus, an enteropathogenic coronavirus which can also be isolated from piglets with weaning diarrhoea (Pritchard, 1987), prior to ETEC infection. Moreover, piglets were pretreated with chloramphenicol, a "colonization-resistance suppressing" antibiotic, which probably makes the gut more susceptible to ETEC infection (Kaufman, 1984).

In the present study the effect of CPZ treatment was evaluated in a combined TGE virus-ETEC diarrhoea model in just-weaned piglets.

MATERIAL AND METHODS

The experiments were performed on just-weaned, female piglets (n = 30; 3 to 4 week-old; 3.2 to 7.2kg) from primiparous sows, purchased from the same commercial farm. Piglets were of mixed breed (Pietrain x Belgian Landrace). They were individually housed at 27 ± 2 °C and allowed to drink UHT sterilized whole cow's milk ad libitum. On day 1 of the experiment piglets were anesthetized (10 mg/kg methomidate, Hypnodil ®, Janssen; and 2mg/kg azaperone, Stressnil®, Janssen) and a Silastic catheter (Dow Corning) was implanted in the left carotid artery for daily blood sampling and pressure recording. Piglets were pretreated on days 1, 2 and 3 with chloramphenicol (Chloromycetin ®, Parke-Davis; 1,875mg/l milk), which probably enhances ETEC colonization of the small intestine. After a starvation period for the first three hours of day 4, piglets were orally inoculated with TGE virus (1.66×10^6 pig infective dose/animal). Twenty-four hours later (day 5) piglets were starved again for 3 h, after which 62 ml of a 1.4 % $NaHCO_3$ solution was given intragastrically to prevent ETEC destruction. Fifteen minutes later they were intragastrically inoculated with a bacterial suspension of two ETEC strains, 10ml/strain/animal. Both strains (O149K91K88ac; LT, STa and STb enterotoxin positive) were grown on brain heart infusion agar (Oxoid) at 37 °C for 24 h. Bacteria were suspended and diluted in sterile

physiological saline until an A620 of 0.4, approximately 1.2 x 10^9 bacteria/ml, as determined by viable count.

CPZ treatment was started 5 h after the ETEC inoculation and was continued for 3 consecutive days. Nine piglets (CPZ group) were intramuscularly injected with chlorpromazine hydrochloride (Rhone Poulenc), dissolved in distilled water, in a dose of 2 mg/kg 24 h (first injection) and 1.5 mg/ kg 24h (second and third injection). The other piglets (n = 21) were kept as untreated controls (control group).

During the experiment several parameters were studied daily, such as weight change, milk intake, body temperature, appearance and severity of diarrhoea, clinical signs, survival rate, frequency of respiration, arterial blood pressure (Pa), heart rate (HR), haematocrit (Hct), plasma protein (Merck kit), glucose (Merck kit), and lactic acid (Boehringer kit) concentration; arterial pH, pO2 and pC02 (Atrup, Radiometer); base excess (BE) and actual bicarbonate (porcine acid-base alignment nomogram for arterial blood) (Hannon, 1984); leucocyte count; serum concentration of sodium, potassium (flame photometry), chloride (chloridometer), and calcium (atomic absorption); faecal ETEC excretion and faecal shedding of TGE virus, rotavirus and pararotavirus (enzyme-linked immunosorbent assay). Faecal ETEC excretion was evaluated as the approximate % of haemolytic E. coli among the total number of aerobic bacteria in rectal swabs inoculated on blood agar plates at 37 °C for 24 h.

The severity of diarrhoea was evaluated by arbitrarily scoring the consistency of the faeces (0 = normal; 1 = pasty; 2 = semiliquid; 3 = watery). Scoring was always performed by the same person.

The carotid arterial pressure (Pa) was recorded with a saline-filled polyethylene catheter connected to a miniature pressure transducer (Gaeltec). The electrocardiogram (ECG) was monitored to measure HR. Pa and ECG were recorded on a Beckman multichannel recorder.

Dehydrated and shocked piglets died spontaneously or were killed with an overdose of methomidate. The other piglets were euthanatized with methomidate 10 days after the start of the experiment. All piglets were examined post mortem.

The genetic susceptibility of the piglets to K88ac-ETEC adhesion to their small intestinal villi was determined by the in vitro technique, described by Girardeau (1980).

The daily diarrhoea score is expressed as median and ranges or individual values and is statistically analysed using the Mann-Whitney U or Wilcoxon tests. All other parameters are given as means ± SEM. The one-way analysis of variance and simple contrasts were used to assess the statistical significance between the values of the different parameters recorded after the experimental infection and the values measured before infection (values on day 3). The Mann-Whitney U test was used to demonstrate significant differences for time-matched values of the CPZ group and the control group. Vomiting was analyzed by the Chi-square test and the percentage of survival in both groups by the Mantel modification of the Gehan generalized Wilcoxon test (Knapp and Wise, 1985).

RESULTS

The post mortem in vitro adhesion assay revealed that the villi of all piglets from the control and 8 of the 9 CPZ-treated piglets were susceptible to adhesion of K88ac-ETEC to their enterocytes. The K88ac resistant piglet survived the experimental infection. K88ac negative piglets are more resistant to ETEC exposure. Data from this piglet were therefore not included in the results.

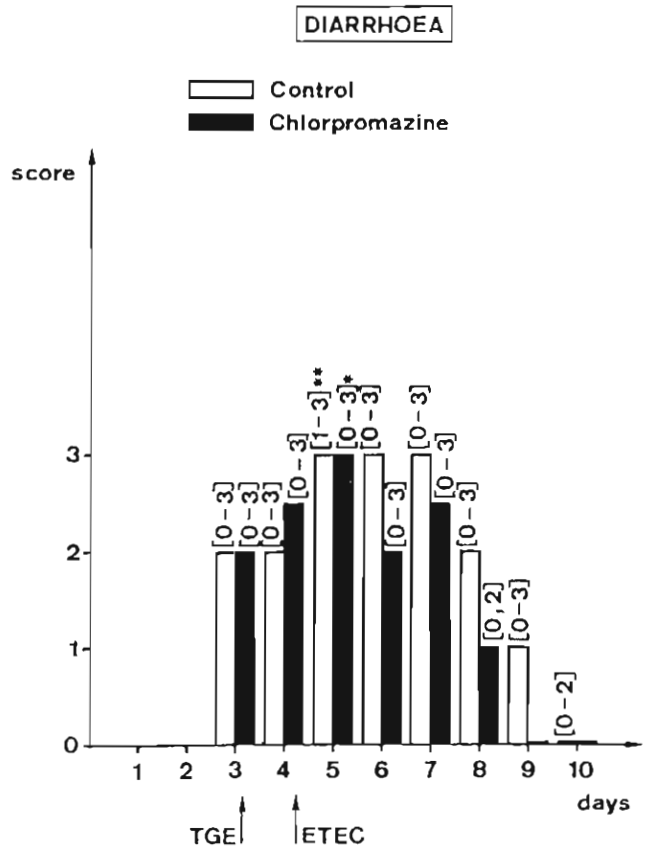


Fig. 1. Diarrhoea score (0 = normal, 1= pasty, 2 = semiliquid, 3 = watery) following a combined infection of just-weaned piglets with TGE virus and ETEC (control group n = 21) and effect of treatment with chlorpromazine (n = 8). Bars represent medians; ranges or individual values are given in brackets. Significant difference from the value prior to infection (day 3) is indicated by * $P < 0.05$, ** $P < 0.01$.

In both groups chloramphenicol pretreatment followed by TGE virus and ETEC infection induced a profuse watery diarrhoea (Fig. 1), resulting in hypovolemia, shock and death of 71% of the animals in the control group and 87.0 % of the CPZ group (Fig. 2; $p > 0,05$) within the 10 days observation period. Although hypovolemic shock was apperently delayed in the CPZ group, the survival rate has not increased at the end of the experiment (Fig.2; $p< 0.05$) within the 10 days observation period. Altough the hypovolemic shock was apparently delayed in the CPZ group the survival rate has not increeased at the end of the experiment (Fig. 2) (mean survival time of shocked piglets/ 3.6 ± 0.5 days in the CPZ group; 2.8 ± 0.5 days in the control group). Pigs

already showed diarrhoeic faeces during the chloramphenicol pretreatment period (Fig. 1; day 3). Diarrhoea, however, was seriously aggravated in both groups following inoculation with TGE virus and ETEC ($p < 0.01$). No significant difference in the diarrhoea score was observed between the two groups (Fig. 1). Twenty hours after ETEC inoculation 75—100 % of the total number of aerobic bacteria shed in the faeces of pigs in both groups were haemolytic E. coli of the same serotype as the inoculum, and in piglets with severe diarrhoea shedding remained high (> 50 %) until death. In all piglets of both groups TGE virus was identified in the faeces. Rota- or pararotavirus were detected in the faeces of two pigs of the control group, and of 7/8 K88ac

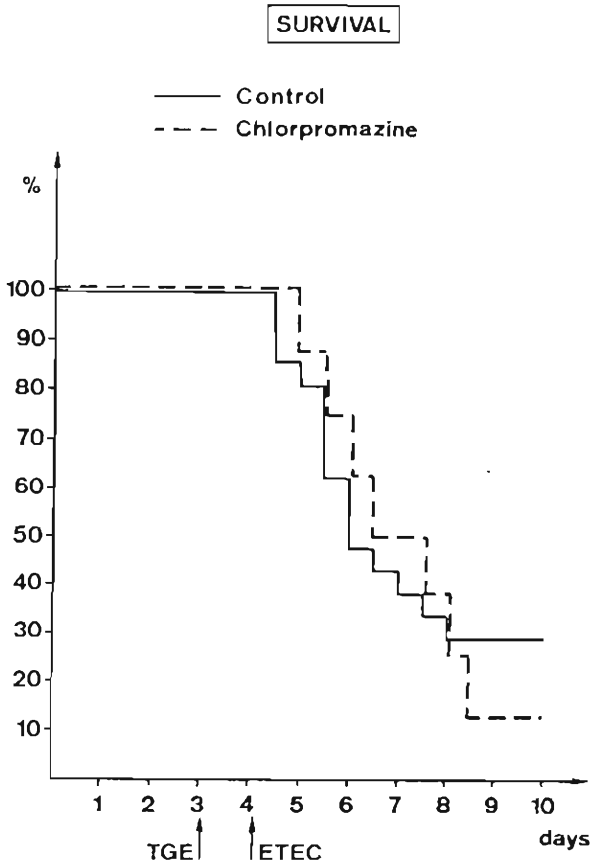


Fig. 2. Survival rate following a combined Infection of just-weaned piglets with TGE virus and ETEC (control group; n = 21) and effect of treatment with chlorpromazine (n = 8)

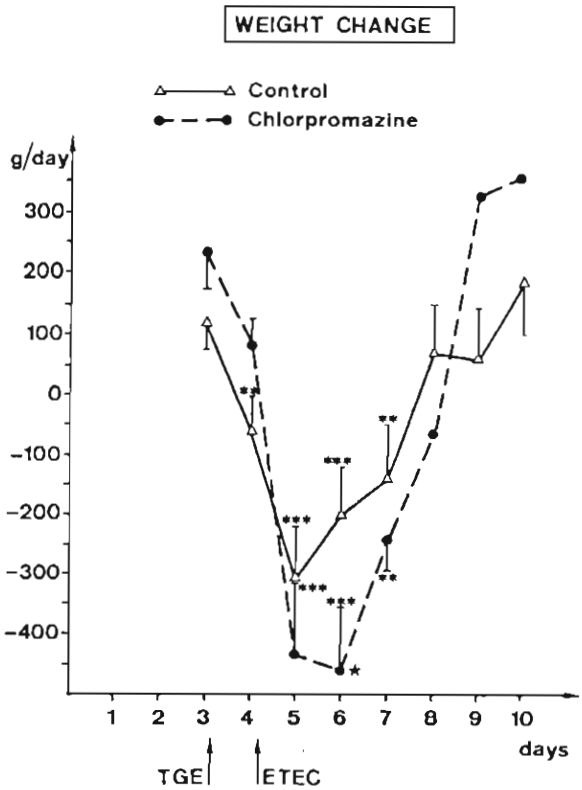


Fig. 3 Weight change following a combined infection of just-weaned piglets with TGE virus and ETEC (control group; n = 21) and effect of treatment with chlorpromazine (n = 8). The values represent means ± SEM . Significant differences from the values prior to infection (day 3) are indicated by : * $p < 0.01$, ***: $p < 0.001$. Significant difference between groups is indicated by *$p < 0.05$.

susceptible animals of the CPZ group. Vomiting was noted in 66 % of the piglets from the control group and in 37.5% from the CPZ group ($P > 0.05$). Dehydrated pigs from both groups revealed depression, poor appetite, and weight loss which was more pronounced on day 6 in the CPZ pigs than in the control pigs (Fig. 3). Piglets gradually developed cyanosis of the ear margins, tail and coronets. In the terminal stage some animals lay in lateral decubitus with retracted eyes and sunken flanks. During CPZ treatment animals were markedly sedated. In both groups daily milk intake did not change significantly (day 3 values: 0.944 ± 0.146 and 1.132 ± 0.1631 for control and CPZ group, respectively).

In control as well as in CPZ pigs hypovolaemia resulted in a significant decrease in blood pressure (Fig. 4) and in a significant increase in heart rate (Table 1). Following the first CPZ administration this tachycardia was significantly more pronounced than at the time-matched value in the control group.

In both groups the Hct revealed no significant changes (day 3 values: 0.380 ± 0.010 /1/1 for the control group; 0.378 ± 0.012 /1/1 for the CPZ group). In shocked and expiring piglets of both groups, however, a significant increase in Hct was observed (to 0.439 ± 0.009 /1/1; $P < 0.05$). A significant increase in total plasma protein concentration was observed in both groups (Table 1).

Arterial pH revealed a decrease in both groups (Table 1) and lactic acid also showed a

decrease (Table 1). After an initial, non-significant increase on day 6, plasma glucose concentration decreased from 5.22 ± 0.34 mmol/l on day 3 to 4.26 ± 0.22 mmol/l on day 10 ($P < 0.05$) in the control group. In the CPZ group, however, a significant increase occurred on day 6 in comparison with day 3 (5.24 ± 0.44 vs. 11.25 ± 4.31 mmol/l; $p < 0.05$). During the course of the experiment, values for arterial $P0_2$, $pC0_2$, and frequency of respiration did not change significantly in either group (day 3 values for control and CPZ group,

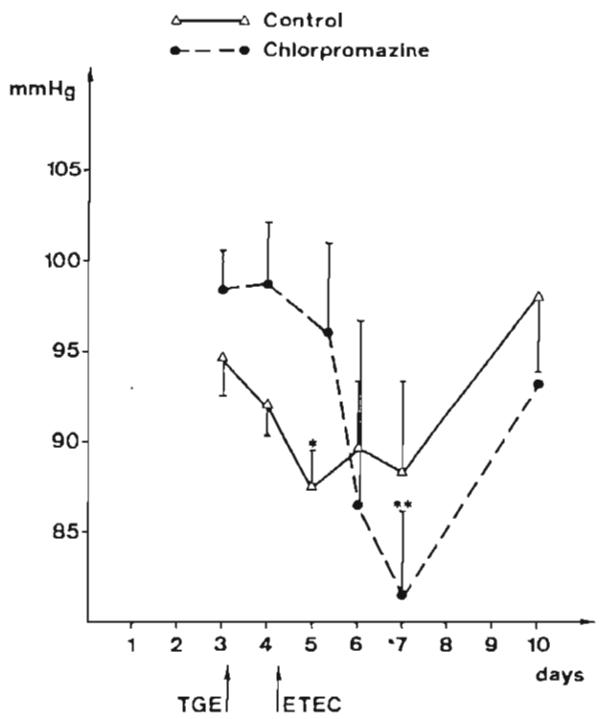


Fig. 4. Decrease in arterial pressure (Pa) following a combined infection of just-weaned piglets with TGE virus and ETEC (control group; n=21) and effect of treatment with chlorpromazine (n = 8). The values represent means ± SEM. Significant differences from the values prior to infection (day 3) are indicated by * $p < 0.05$, ** $p < 0.01$.

respectively: for $P0_2$: 90 ± 3.4 and 97.0 ± 6.3 mm Hg; for $pC0_2$: 29.6 ± 1.0 and 28.8 ± 2.5 mm Hg; for respiration: 26 ± 3 and 25 ± 4 / min). The day 7 value for pO_2 in the CPZ group (92.8 ± 4.8 mm Hg) was higher than the corresponding value in the control group (81.3 ± 1.1 mm Hg; $p < 0.05$), whereas day 6 value for the frequency of respiration (18 ± 2 / min) was lower than the time-matched value for the control group (28 ± 4/min; $p < 0.05$).

In shocked and expiring piglets of both groups arterial values for BE and actual bicarbonate decreased (day 3 vs. day 6: for BE: -6.8 ± 2.4 and -20.9 ±1.7 mEq/l, $p < 0.01$, for control group, and - 5.0 ± 3.6 and - 13.8 ± 5.5 mEq/l, $p > 0.05$, for CPZ group; for $HC0_3^-$: 23.3 ± 1.9 and 12.4 ± 1.6 mEq/l, $p < 0.05$, for control group, and 25.1 ± 9.6 and 17.2 ± 4.3 mEq/l, $p>0.05$, for CPZ group). CPZ treatment apparently retarded these decreases.

Table 1. Daily values (means ± SEM) for heart rate, plasma protein, leucocyte count, arterial pH, serum potassium and lactic acid concentration in the control (n=21) and chlorpromazine (CPZ) treated (n = 8) group. Values for days 8 and 9 were not determined

Time (days)	Heart rate (beats/min)		Plasma protein (g/dl)		Leucocyte count (WBC/mm^3)	
	Control	CPZ	Control	CPZ	Control	CPZ
3	151.3 ± 5.9	149.4 ± 6.6	4.6 ± 0.2	4.6 ± 0.2	12,097 ± 727	9,778 ± 634*
4	166.2 ± 6.8	148.2 ± 10.9	5.2 ± 0.1†	4.8 ± 0.2*	8,692 ± 605††	7,236 ± 936
5	183.9 ± 8.5††	220.0 ± 10.9†††**	5.5 ± 0.2††	4.6 ± 0.5	9,395 ± 789††	8,884 ± 1,278
6	176.3 ± 9.3†	202.6 ± 9.0††	5.7 ± 0.2†††	6.3 ± 0.4†††	12,844 ± 1,381	16,217 ± 2,155
7	174.6 ± 12.7	199.5 ± 15.8††	5.5 ± 0.4††	7.1 ± 0.8†††	11,175 ± 531	17,767 ± 4,288
0	166.2 ± 15.4	122.4	4.7 ± 0.4	4.2	15,083 ± 1,618†	17,500

	pH		Potassium conc. (mmol/l)		Lactic acid (mmol/l)	
	Control	CPZ	Control	CPZ	Control	CPZ
3	7.48 ± 0.03	7.57 ± 0.04	4.1 ± 0.1	4.1 ± 0.3	2.41 ± 0.24	2.85 ± 0.56
4	7.46 ± 0.01	7.51 ± 0.02*	4.0 ± 0.2	4.1 ± 0.1	1.76 ± 0.31†	2.30 ± 0.36
5	7.40 ± 0.05	7.52 ± 0.05	4.3 ± 0.2	3.9 ± 0.2	1.43 ± 0.18††	1.93 ± 0.44
6	7.41 ± 0.06	7.42 ± 0.07	5.1 ± 0.8	4.9 ± 0.4†	1.45 ± 0.20††	1.95 ± 0.40
7	7.32 ± 0.14†	7.34 ± 0.16†	4.8 ± 0.5	5.7 ± 0.4††	1.79 ± 0.52	1.51 ± 0.3
10	7.57 ± 0.02	7.67	3.8 ± 0.2	5.6	1.43 ± 0.28	2.91

Significant differences from the values prior to infection (day 3 values) are indicated by † $p < 0.05$, †† $p < 0.01$, ††† $p < 0.001$. Significant differences between groups are indicated by * $p < 0.05$, ** $p < 0.01$.

Despite the pronounced dehydration, no significant changes in serum osmolality (day 3 values: 298.0 ± 3.75 and 281.6 ± 9.4 mosmol/l for control and CPZ group, respectively), and in concentrations of chloride (day 3 values: 97.1 ± 1.8 and 100.1 ± 2.3 mmol/l, respectively) and calcium (day 3 values: 2.55 ± 0.09 and 2.70 ± 0.08 mmol/l, respectively) were observed within both groups. Control pigs revealed a transient but significant increase in sodium concentration (from 130.0 ± 2.5 mmol/l on day 3 to 137.0 ± 1.5 mmol/l on day 6, $p < 0.01$), followed by a return to near control value on day 7 in surviving piglets, whereas within the CPZ group no significant changes in sodium concentration were noted. For the control group the potassium concentration did not change significantly during the experimental period, whereas the value increased significantly within the CPZ group (Table 1).

Following TGE virus and ETEC inoculation the leucocyte count decreased in both groups, this decrease being significant only for the control group (Table 1). This transient decrease was followed by a return to near control values and by a secondary increase (Table 1).

Body temperature revealed a decrease, which was only significant for the CPZ group (day 3 vs. day 6: 39.6 ± 0.1 and 39.2 ± 0.4°C, $p > 0.05$, for the control group; 39.6 ± 0.1 and 38.5 ± 0.7 °C, $p < 0.01$, for the CPZ group). This decrease was followed by a return to near control values and by a secondary increase (40.0 ± 0.3°C on day 10 for the control group, $p < 0.05$).

Necropsy in piglets of both groups revealed congestion of the mucosa of the fundic region of the stomach, congested thin-walled small intestine, especially the ileum, with watery contents, and sometimes also congestion of the colon wall.

DISCUSSION

Enterotoxigenic E. coli strains are implicated in the aetiology of porcine weaning diarrhoea (Lecce, 1983; Tzipori *et al.*, 1980). Recently, rotavirus has also been shown to exert a role in this disease (Lecce, 1983; Tzipori *et al.*, 1980a). In our model TGE virus, an enteropathogenic coronavirus, was selected since rotavirus is enzootic in Belgian piggeries (Debouck, 1984). TGE virus consistently induces intestinal villous atrophy and crypt hyperplasia, resulting in a profuse diarrhoea and vomiting, which are almost invariably fatal in very young piglets, whereas animals over 3 weeks old usually survive (Aitken, 1983).

In the present study most dually infected piglets revealed a hypovolaemic shock as a result of vomiting and of diarrhoea probably induced by a combination of malabsorption (TGE virus) and hypersecretion (ETEC).

The clinical signs in the seriously ill piglets of our study agree with the observations in E. coli infections in gnotobiotic pigs (Kenworthy and de Mitchell, 1976). The diarrhoea occurring during the chloramphenicol pretreatment can be explained by the change in diet from sow's to cow's milk, by the loss of maternal immunity, by environmental changes (Lecce, 1983; Tzipori *et al.*, 1980a), and by an effect of the antibiotic on the intestine. It has been shown that chloramphenicol can suppress the anaerobic gut flora, so probably increasing the susceptibility to potentially pathogenic microorganisms (Kaufman, 1984), whereas in calves, following oral treatment with chloramphenicol a malabsorption diarrhoea occurred (Mero *et al.*, 1985).

The high control value (day 3) for arterial pH agrees with that observed in conscious immature 2 to 3-month-old pigs (Hannon, 1983). The acidosis and abrupt increase in Hct in shocked piglets were also observed in dying neonatal piglets, following ETEC-induced or spontaneous diarrhoea (Andrén and Persson, 1983). Lactic acid, however, did not increase in our study, suggesting that other acid metabolites are probably responsible for the acidosis, which is not of respiratory nature since frequency of respiration and pCO_2 did not change significantly. The loss of bicarbonate ions as a result of the severe diarrhoea may also be important.

In experiments on gnotobiotic pigs, 27 to 34 days old, when infected with ETEC strains, the animals developed a severe diarrhoea resulting in dehydration and death between 16 and 36 h after infection. As in piglets of our study no elevation of body temperature was recorded during the corresponding period. Pigs also revealed a neutropenia soon after infection and an abrupt increase in Hct (Kenworthy and de Mitchell, 1976). The leucopenia we noted was already apparent after

TGE virus inoculation and could have been partly due to the migration of polymorphonuclear cells to the damaged intestinal mucosa (Pensaert *et al.*, 1970a).

The post mortem findings in piglets of our study correspond to those observed in piglets that died from post-weaning diarrhoea.

In human medicine the role of CPZ as a therapeutic antisecretory agent was first found in cholera patients based on experimental work in mice by Holmgren *et al.* (1978) who showed that CPZ has an inhibitory action against the adenylate cyclase-cAMP system, the mediator of LT enterotoxin-induced hypersecretion. Results of the clinical trials with CPZ (1 or 4 mg/kg per os or I. M.) in man with severe diarrhoea due to cholera demonstrated beneficial effects (Greenough and Rabbani, 1986). However, CPZ-associated sedation was a constraint on its clinical use. In animals CPZ inhibits cAMP-induced secretion, due to LT E. coli and Vibrio cholera enterotoxin, prostaglandins, and dibutyryl cAMP, as well as cGMP mediated secretion, due to ST E. coli enterotoxin (Donowitz *et al.*, 1986; Lönnroth and Jennische, 1982), and probably also stimulates intestinal NaCI absorption, as recently demonstrated for promethazine in the rabbit ileum (Cohen *et al.*, 1985). In the rat ligated intestinal loop model the CPZ-induced reduction of fluid accumulation provoked by cholera toxin was probably mediated by a cAMP-independent enhancement of fluid absorption in the jejunum, and an inhibition of the cAMP-dependent hypersecretion in the jejunum as well as the ileum (Jennische and Lönnroth, 1982).

In newborn piglets with experimentally ETEC-induced diarrhoea the I. M. administration of CPZ, 5 mg/kg /h after the onset of diarrhoea, completely normalized the intestinal fluid content, but seriously sedated the animals, whereas in a dosage of 1 to 2 mg sedation did not pose problems and yet a strong antisecretory effect occurred (Lönnroth *et al.*, 1979). Subsequently these authors confirmed these results in a field trial in which the duration of diarrhoea in a spontaneous outbreak due to ETEC was significantly reduced when piglets were treated with CPZ, 1 mg/kg I. M., in addition to oral electrolyte solution and standard antimicrobial agents.

We recently reported some beneficial effects of CPZ treatment, 2 and 1.5 mg/kg 24 h I. M. during 3 successive days, in non-catheterized, and also dually (TGE virus and ETEC) infected piglets. Survival rate on day 10 amounted to 80 % as compared to 33 % for non treated piglets. The severity of diarrhoea evaluated by scoring as in the present study was significantly reduced and vomiting was completely inhibited (Cox *et al.*, 1987). Although administration of CPZ, 1 mg/kg, was without beneficial effect, the results were interesting since they indicated that CPZ could be therapeutically used in ETEC diarrhoea complicated by viral infection (malabsorption), as often seems to occur post-weaning (Nabuurs *et al.*, 1986). In the present study on catheterized piglets, however, these promising results could not be reproduced. Instead, some serious side effects such as sedation and decrease in body temperature and arterial blood pressure were noticed, thus compromising the potential therapeutic value of CPZ in severe diarrhoea.

Sedation, suppression of vomiting, hypothermia, hypotension and hyperglycaemia are well known pharmacological properties of neuroleptic agents (Baldessarini, 1980; Booth, 1977). The more pronounced tachycardia observed in the CPZ group following the first administration of the neuroleptic as compared to time-matched control piglets is probably a reflexogenic compensation of its hypotensive action (Baldessarini, 1980).

In our model on catheterized piglets the induced diarrhoea was more severe than in non-catheterized animals (Cox *et al.*, 1986b), probably resulting in a more pronounced dehydration. The more severe hypovolaemia could have made piglets more susceptible to the hypotensive action of the drug. In this way CPZ could have interacted with the normal compensatory mechanisms occurring during excessive fluid loss (Michell, 1985), compromising the beneficial effects of CPZ in our piglet diarrhoeic model.

III.C.5. EFFECT OF FLURBIPROFEN ON DIARRHOEA INDUCED BY A COMBINED INFECTION WITH TRANSMISSIBLE GASTROENTERITIS VIRUS AND ENTEROTOXIGENIC *ESCHERICHIA COLI* IN NEWLY WEANED PIGLETS*

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ABSTRACT

In newly-weaned 3-4 week old piglets (n = 29) diarrhoea (100%) and vomiting (65%) were induced by inoculation with transmissible gastroenteritis virus and enterotoxigenic *E. coli* strains (O149:K91:K88ac; LT, STa and STb enterotoxin positive). This combined infection resulted in a mortality within 7 days. During this period the piglets had decreases in body weight, arterial pressure and leucocyte count and increases in heart rate and in total plasma protein concentration. The plasma pH and lactic acid concentration decreased. In shocked and dying piglets an increase in haematocrit was observed, whereas base excess and bicarbonate concentration decreased.

Flurbiprofen, a potent non-steroidal anti-inflammatory drug, administered intramuscularly on 3 successive days following the combined infection at a dosage of 1 mg/kg/12 h induced a temporary non-significant faecal fluid loss but did not reduce mortality.

INTRODUCTION

Enterotoxigenic *Escherichia coli* (ETEC) are involved in the post-weaning diarrhoea syndrome in pigs. However, experiments performed in our laboratory to induce hypersecretion diarrhoea by inoculation of newly-weaned piglets with ETEC, possessing fimbrial adhesins and producing heat-labile (LT) and heat-stable (STa and STb) enterotoxins, were not always successful (Cox *et al.*, 1986a). Post-weaning diarrhoea has a multifactorial etiology. The genetic susceptibility of the host to adhesion of ETEC as well as nutritional, environmental and immunological factors

*The results of this study were partly published in: Cox, E., Cools, V. and Houvenaghel, A.1988. Pathophysiology of diarrhoea induced by a combined infection with transmissible gastroenteritis virus and enterotoxigenic *Escherichia coli* in newly weaned piglets and the effect of flurbiprofen treatment. Veterinary Research Communications, 12 (4-5) 383-399

and the presence of a rotavirus infection can probably seriously influence the occurrence and the severity of the diarrhoea (Tzipori *et al.*, 1980a; Lecce *et al.*, 1982; Lecce, 1983; Sellwood, 1983; 1984; Bijlsma *et al.*, 1985a).

When the experimental ETEC infection was preceded by oral chloramphenicol treatment we observed an enhanced occurrence, severity and duration of diarrhoea (Cox *et al.*, 1986a). This 'colonization-resistance-suppressing' antibiotic probably makes the gut more susceptible to ETEC (Kaufman, 1984). The induced diarrhoea, however, was still insufficiently pronounced to result in significant hypovolaemia and shock.

We therefore introduced a combined viral-bacterial infection into our piglet diarrhoea model. Since rotavirus is enzootic in Belgian piggeries and most pigs are infected before the age of 6 to 7 weeks (Debouck, 1984), we inoculated piglets with transmissible gastroenteritis (TGE) virus, a coronavirus, prior to ETEC infection. This combined infection resulted in pronounced diarrhoea and hypovolaemia and in 100% mortality within 5 days of TGE virus inoculation (Cox *et al.*, 1986b).

We recently reported the effect of the antisecretory drugs chlorpromazine, verapamil, clonidine and propranolol on diarrhoea and survival in our piglet diarrhoea model (Cox *et al.*, 1987; Cools *et al.*, 1987). Several studies suggest the involvement of prostaglandins as important messengers in the secretory response of the enterocytes to bacterial enterotoxins (Bennett, 1971; Wise *et al.*, 1983; Powell, 1986). Non-steroidal anti-inflammatory drugs (NSAID's), which inhibit prostaglandin synthesis by blocking the cyclooxygenase pathway, have been shown to reduce cholera or ETEC-induced hypersecretion in several species (Willard, 1985; Greenough and Rabbani, 1986). However, in clinical trials in man, aspirin and indomethacin were ineffective in the treatment of cholera-induced hypersecretion diarrhoea (Greenough and Rabbani, 1986).
The purpose of the present study was to evaluate the effects of flurbiprofen, a potent NSAID of the arylacetic acid class, on this diarrhoea model.

MATERIALS AND METHODS

The experiments were performed on 29 newly-weaned, 3-4 week old female piglets, weighing 3.2 to 7.2 kg, from primiparous sows purchased from the same commercial farm. The piglets were of mixed breeding (Pietrain x Belgian Landrace). They were individually housed at 27°C and allowed to drink UHT sterilized whole cow's milk ad libitum. On day 1 of the experiment the piglets were anaesthetized with 15 mg/kg methomidate (Hypnodil ®, Janssen) and 2 mg/kg azaperone (Stressnil ®, Janssen) and a Silastic catheter (Dow Corning) was implanted in the left carotid artery for daily blood sampling and pressure recording. To enhance ETEC colonization of the small intestine, the piglets were pretreated on days 1, 2 and 3 with chloramphenicol (Chloromycetin ®, Parke-Davis; 1,875 mg/l milk). After a starvation period during the first three hours of day 4, the piglets were orally inoculated with TGE virus (1.66×10^6 pig infective dose/ animal). Twenty-four hours later (day 5) the piglets were again starved for 3 hours, after which 62 ml of a 1.4% $NaHC0_3$ solution was given intragastrically to prevent ETEC destruction. Fifteen minutes later they were each intragastrically inoculated with 10 ml of a

suspension of two ETEC strains. Both strains, 0149 K91 K88ac, LT, STa and STb enterotoxin positive, were grown on brain heart infusion agar (Oxoid) at 37°C for 24 hours. The bacteria were suspended and diluted in sterile physiological saline to an A620 of 0.4, approximately 1.2 x 10^9 bacteria/ml, as determined by viable count.

Flurbiprofen treatment was started 5 hours after the ETEC inoculation and was performed for 3 consecutive days. Eight piglets (flurbiprofen group) were intramuscularly injected with flurbiprofen (Boots) at a dose of 1 mg/kg/12 h. Flurbiprofen was dissolved in 0.1 M NaOH (10 mg/ml) and the solution was neutralized to pH 7.4 with 0.1 M HCl. Xylocaine (2 mg) was added to reduce the pain induced by the intramuscular injection of flurbiprofen.

The other piglets (n = 21) were kept as untreated controls (control group).

Weight change, milk intake, body temperature, appearance and severity of diarrhoea, and clinical symptoms were registered daily. From day 3 onwards the following parameters were also evaluated: survival rate; frequency of respiration; arterial blood pressure (Pa); heart rate (HR); haematocrit (Hct); plasma protein (Merck kit), glucose (Merck kit) and lactic acid (Boehringer kit) concentrations; arterial pH, pO_2 and $pC0_2$ (Astrup, Radiometer); base excess (BE) and bicarbonate (porcine acid-base alignment nomogram for arterial blood; Hannon, 1984); leucocyte count; serum concentrations of sodium, potassium (flame photometry), chloride (chloridometer) and calcium (atomic absorption); sodium and potassium concentrations and osmolality (cryoscopy) of faecal water, obtained as the supernatant after centrifugation of freshly collected faeces at 6,000 g for 5 min; faecal ETEC excretion; and the faecal shedding of TGE virus, rotalike- and rotavirus (enzyme-linked immunosorbent assay).

The severity of diarrhoea was evaluated by arbitrarily scoring the consistency of the faeces (O = normal; 1 = pasty; 2 = semiliquid; 3 = watery). Scoring was always performed by the same person. The osmotic gap was calculated from the faecal water values as the difference between the measured osmolality and the estimated osmolality, based on twice the sum of the sodium and potassium concentrations (Shiau *et al.*, 1985). Secretory diarrhoea is commonly associated with a negative osmotic gap, whereas osmotic diarrhoea is associated with a positive osmotic gap of greater than 160 mosmol/l, at least in man (Shiau *et al.*, 1985). Faecal ETEC excretion was evaluated as the approximate percentage of haemolytic *E. coli* among the total number of aerobic bacteria in rectal swabs inoculated onto blood agar plates at 37°C for 24 hours and was also arbitrarily scored (O = 0%; 1 = 0-25%; 2 = 25-50%; 3 = 50-75%; 4 = 75-100%).

The carotid arterial pressure was registered with a saline-filled polyethylene catheter connected to a miniature pressure transducer (Gaeltec). The electrocardiogram (ECG), which was used to measure HR, was monitored with five ECG electrodes fixed with rubber bands cranial to the left and right shoulder joint, above the seventh vertebral spine and on the abdomen cranial to the left and right stifle joint. Pa and ECG were recorded on a Beckman multichannel recorder.

Shocked piglets died spontaneously or were killed with an overdose of methomidate. The other piglets were euthanatized with methomidate 10 days after the start of the experiment. All piglets were examined post mortem.

The genetic susceptibility of the piglets to K88ac-ETEC adhesion to their small intestinal

villi was determined by the *in vitro* technique described by Girardeau (1980).

The daily diarrhoea score and faecal ETEC score are expressed as medians and ranges or individual values and were statistically analysed using the Mann-Whitney U or Wilcoxon tests. All other parameters are given as means ± SEM. A one-way analysis of variance and simple contrasts were used to assess the statistical significance between the values of the different parameters registered after the experimental infection and the values measured before infection on day 3. The Mann-Whitney U test was used to demonstrate significant differences for time-matched values of the flurbiprofen group and the control group. The percentage of survival in both groups was statistically evaluated using the Mantel modification of the Gehan generalized Wilcoxon test.

RESULTS

The post mortem *in vitro* adhesion assay revealed that the villi of all piglets from the control and the flurbiprofen group were susceptible to adhesion by K88ac-ETEC to their enterocytes.

Control group

Experimental infection with TGE virus and ETEC resulted in a pronounced mortality (K88ac-ETEC of the animals), starting from day 5 and reaching maximal values on days 5 to 6 (Figure 1). Mortality was provoked by the pronounced watery diarrhoea, resulting in a hypovolaemic shock (Figure 2). Vomiting was observed in 65 % of the animals. Hypovolaemic piglets had a poor appetite, severe depression and cyanosis of the extremities.

In the terminal stage some pigs lay in lateral decubitus with the eyes retracted in the orbits, the flanks sunken and a prominent pelvis as a result of the severe dehydration. The piglets developed diarrhoeic faeces during the chloramphenicol pretreatment (Figure 2; day 3). Diarrhoea, however, seriously worsened following inoculation with TGE virus and ETEC ($P< 0.01$). The faecal osmotic gap significantly increased from -14 ± 11.6 mosmol/l on day 3 to 53.6 ± 15.8 mosmol/l on day 5 ($P< 0.01$) and 95 .6 ± 27.5 mosmol/l on day 7 ($P< 0.001$).

From about 20 hours following ETEC-infection a substantial number of haemolytic *E. coli* were shed in the faeces (Figure 3). TGE virus were demonstrated in the faeces of all the piglets. Rota- and rotalikevirus, were each only detected in the faeces of one pig.

Daily milk intake revealed a non-significant decrease (control value on day 3: 0.944 ± 0.106 liter), followed by an increase to 1.447 ± 0.351 liter on day 10. The pronounced and sustained diarrhoea, vomiting and loss of appetite resulted in a significant decrease in body weight (Figure 4).

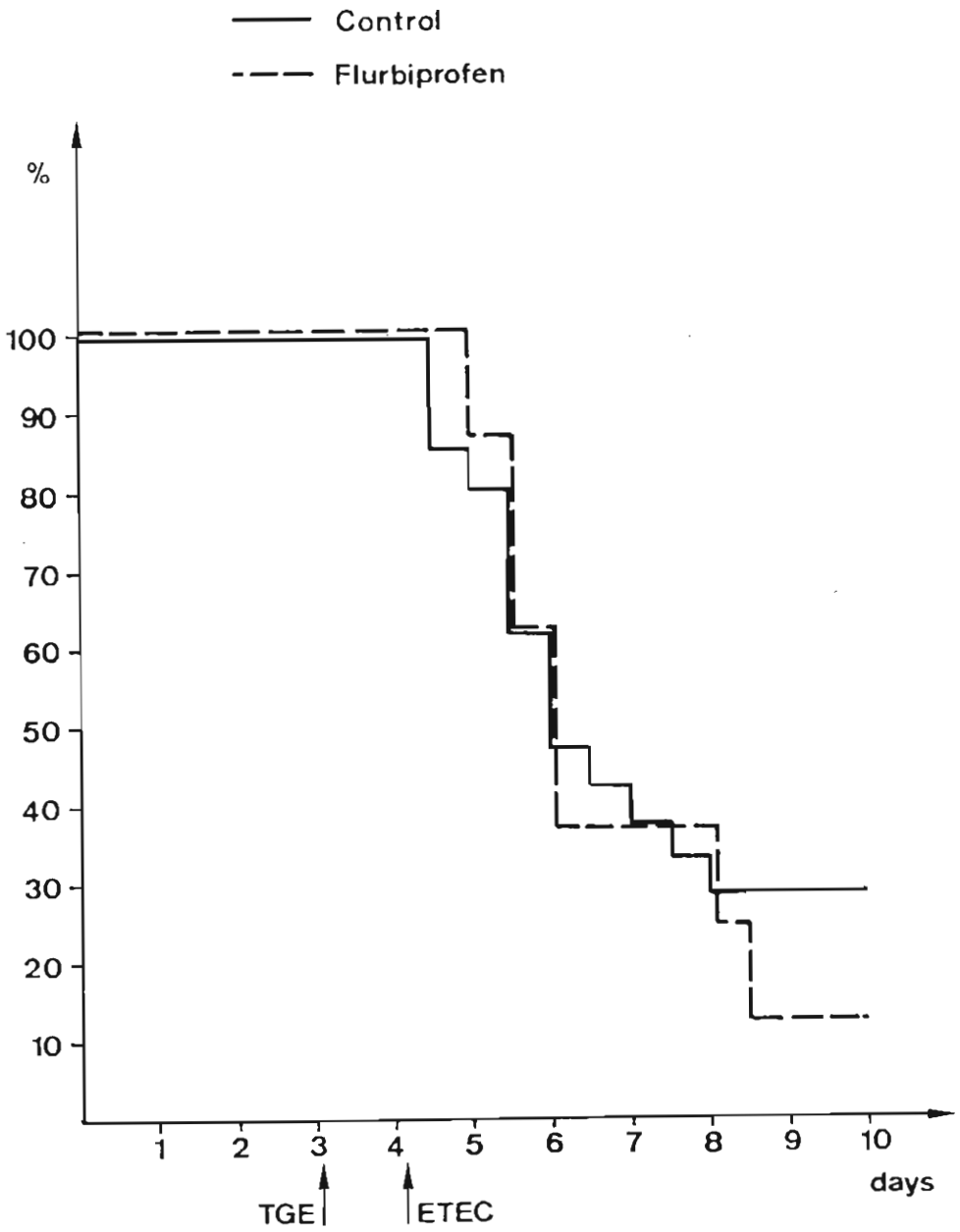


Figure 1. Survival rate following a combined infection of newly-weaned piglets with TGE virus and ETEC (control group; n = 21) and the effect of treatment with flurbiprofen (n = 8).

Hct initially increased and then gradually decreased (Figure 5). In shocked and expiring piglets, however, a significant increase in Hct was observed, from 38.1 ± 0.8 % on day 3 to 43.9 ± 0.9% just before death ($P < 0.05$). Dehydration and hypovolaemia also resulted in a significant increase in the total plasma protein concentration from 4.6 ± 0.2 g/dl on day 3 to 5.7 ± 0.2 g/dl on day 6 ($P < 0.001$) and also in HR, from 151 ± 6 beats/min on day 3 to 184 ± 9 beats/min on day 5 ($P< 0.01$), and in a significant decrease in Pa (Figure 6).

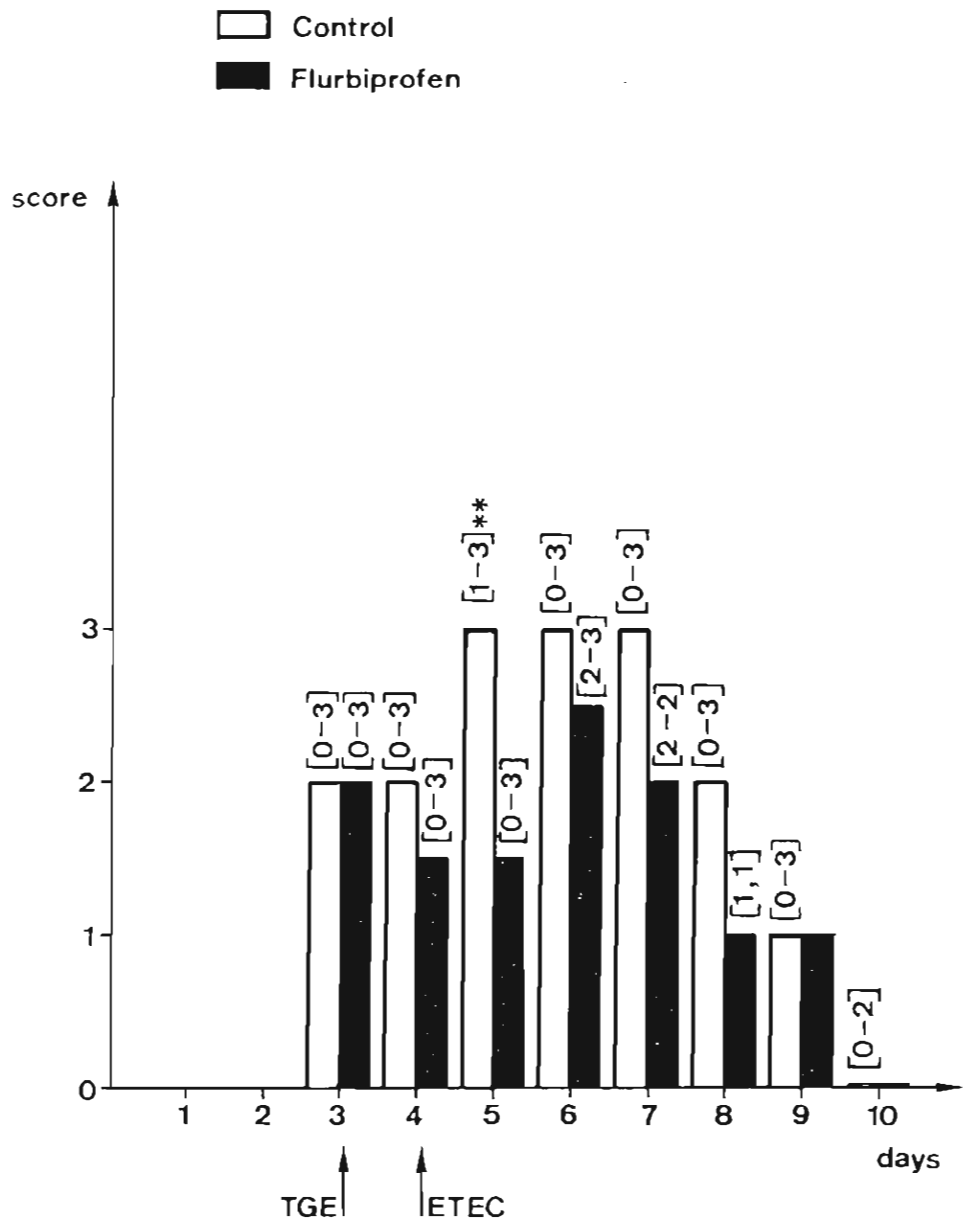


Figure 2. Diarrhoea scores (0 = normal; 1 = pasty; 2 = semiliquid; 3 = watery) following a combined infection of newly-weaned piglets with TGE virus and ETEC (control group; n = 21) and the effect of treatment with flurbiprofen (n = 8). Bars represent medians; ranges or individual values are given in brackets. Significant difference from the value prior to infection (day 3) is indicated by ** $P < 0.01$.

Arterial pH revealed a significant decrease from 7.48 ± 0.03 on day 3 to 7.32 ± 0.14 on day 7 ($P < 0.05$) and the lactic acid concentration also significantly decreased, from 21.7 ± 2.2 mg/dl on day 3 to 12.9 ± 1.6 mg/dl on day 5 ($P < 0.01$). After an initial non significant increase on day 6, the plasma glucose concentration decreased from 94.1 ± 6.1 mg/dl on day 3 to 76.7 ± 4.0 mg/dl on day 10 ($P < 0.05$). The values for PO_2 (control value on day 3: 90.0 ± 3.4 mm Hg),

pCO_2 (control value: 29.6 ± 1 mm Hg) and the frequency of respiration (control value: 26.3 ± 2.8/min) did not change significantly.

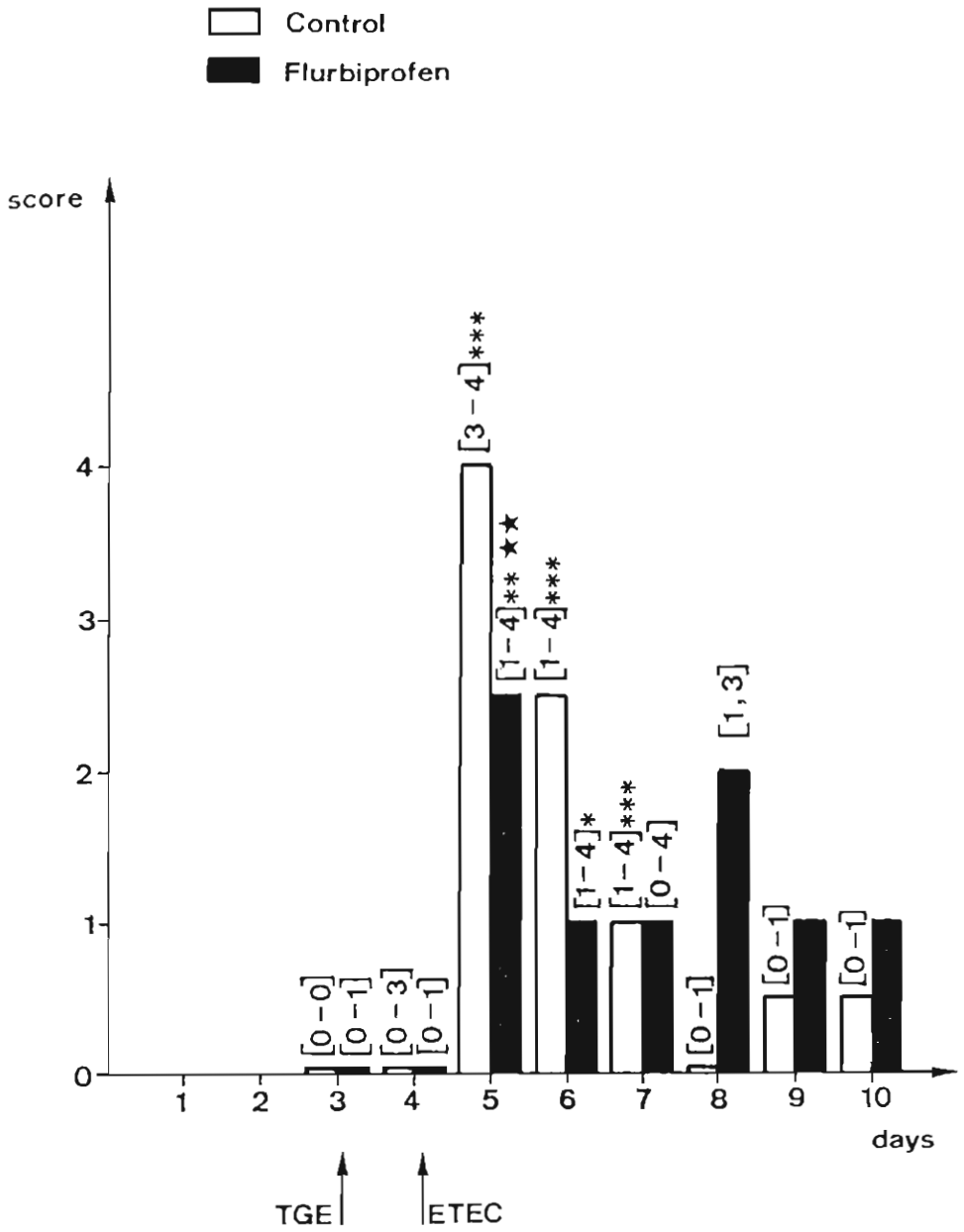


Figure 3. Faecal ETEC excretion, evaluated as the approximate percentage of haemolytic *E. coli* among the total number of aerobic bacteria in rectal swabs (0 = 0%; 1 = 0-25%; 2 = 25-50%; 3 = 50 -75%; 4 = 75-100%), following a combined infection of newly-weaned piglets with TGE virus and ETEC (control group; n = 21) and the effect of treatment with flurbiprofen (n = 8). Bars represent medians; ranges or individual values are given in brackets. Significant differences from the values prior to infection (day 3) are indicated by * $P < 0.05$; * * $P < 0.01$; * * * $P < 0.001$. A significant difference between groups is indicated by ** $P < 0.01$.

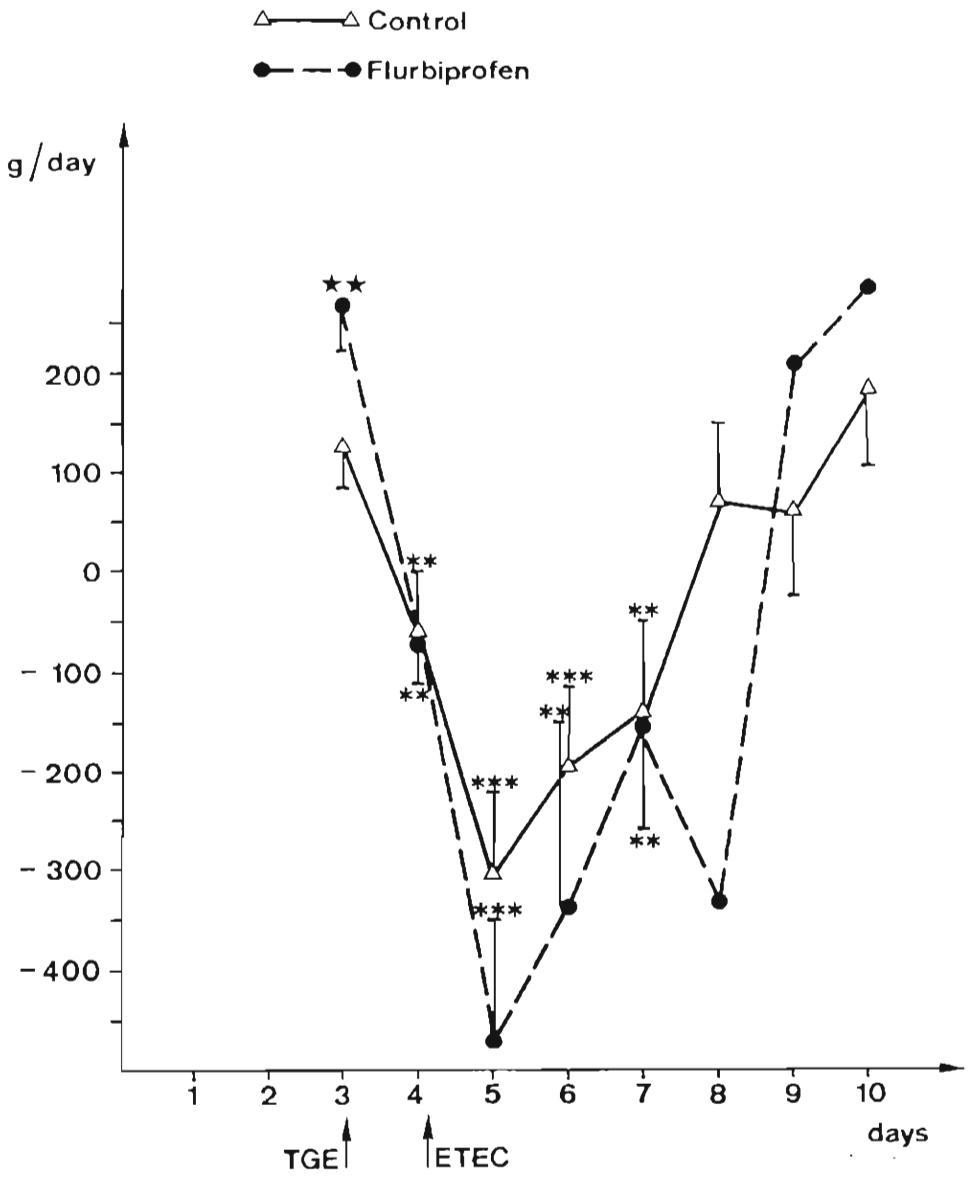


Figure 4. Weight changes following a combined infection of newly-weaned piglets with TGE virus and ETEC (control group; n = 21) and effect of treatment with flurbiprofen (n = 8). The values represent means ± SEM. Significant differences from the values prior to infection (day 3) are indicated by ** P< 0.01; *** P< 0.001. A significant difference between groups is indicated by**P< 0.01.

In shocked and expiring piglets BE decreased from -6.8 ± 2.4 mEq/l on day 3 to -24.6 ± 3.70 mEq/l on day 5 (P< 0.001) and bicarbonate decreased from 23.3 ± 1.9 mEq/l on day 3 to 8.6 ± 1.9 mEq/l on day 5 (P< 0.001). The corresponding value for pH on day 5 was 7.28 ± 0.05 (P < 0.01).

Despite the pronounced dehydration there were no significant changes in the serum concentrations of potassium (control value on day 3: 4.1 ± 0.1 mEq/l), chloride (control value: 97.1 ± 1.8 mEq/l) or calcium (5.1 ± 0.2 mEq/l). Sodium concentration revealed a significant

increase from 130 ± 2.5 mEq/l on day 3 to 137 ± 1.5 mEq/l on day 6 (P< 0.01), followed by a return to near the control value on day 10 in surviving piglets.

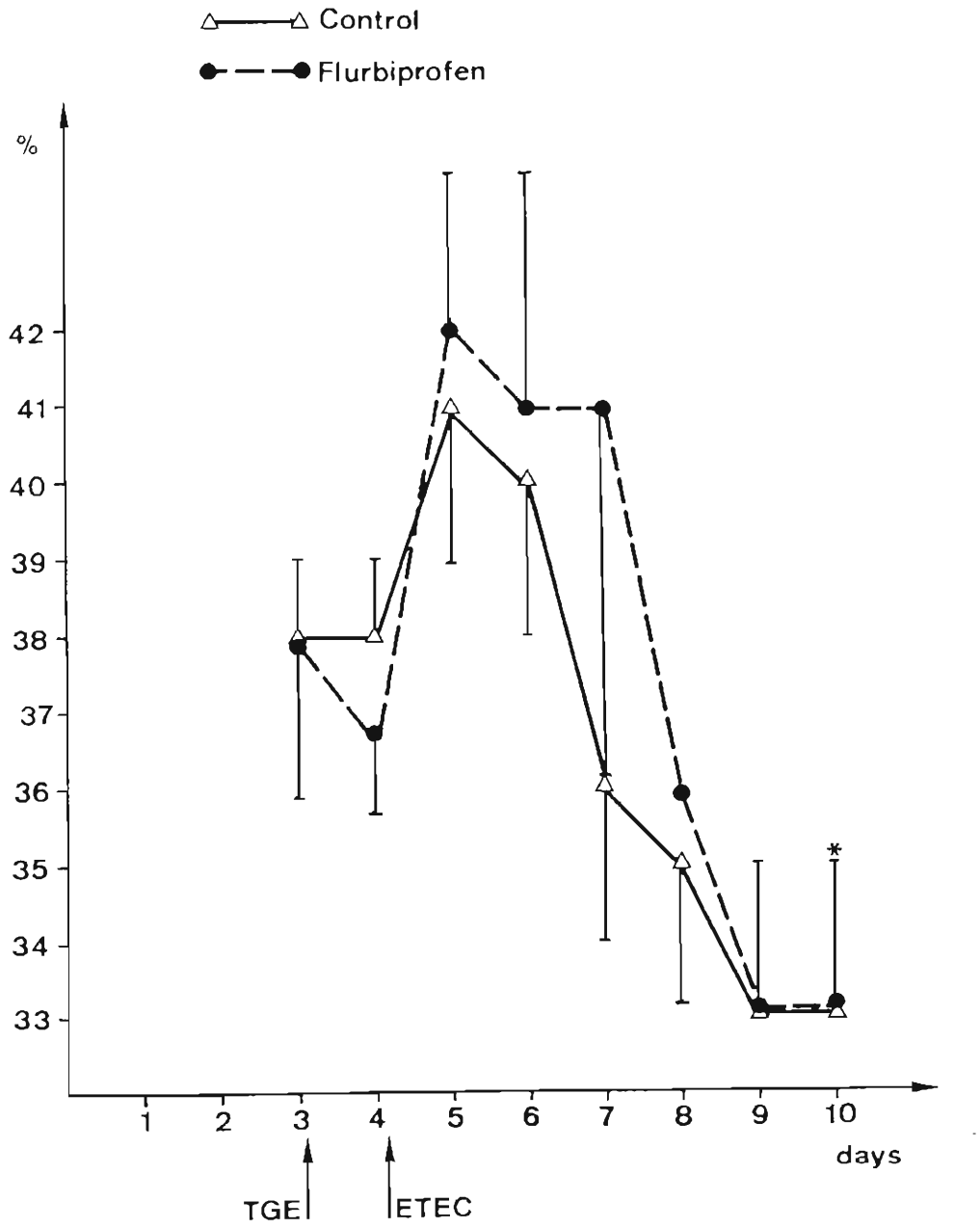


Figure 5. Changes in haematocrit (Hct) following a combined infection of newly-weaned piglets with TGE virus and ETEC (control group; n = 21) and the effect of treatment with flurbiprofen (n = 8). The values represent means ± SEM. A significant difference from the value prior to infection (day 3) is indicated by * P<0.05.

Following the TGE virus and ETEC inoculation, the leucocyte count significantly decreased (Figure 7). In surviving piglets this parameter increased to give a significant leucocytosis on day 10 (Figure 7). After an initial, non-significant decrease the body temperature returned to

near the control value and was significantly increased on day 10 in surviving animals (Figure 8).

Necropsy revealed erosions and congestion of the mucosa of the fundic region of the stomach, a congested, thin-walled small intestine, especially the ileum, with a watery content, and sometimes also congestion of the colon wall.

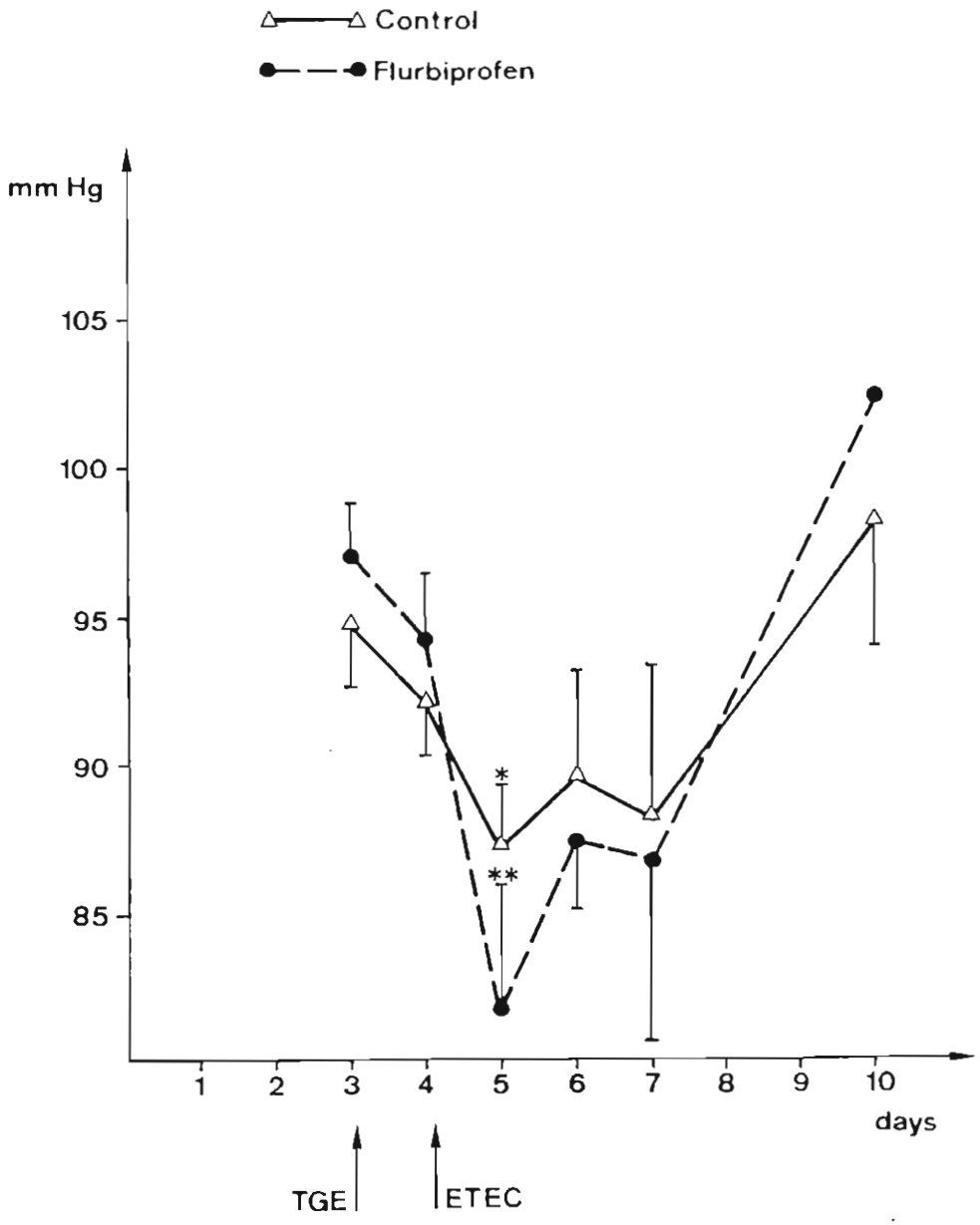


Figure 6. Changes in arterial pressure (Pa) following a combined infection of newly-weaned piglets with TGE virus and ETEC (control group; n = 21) and the effect of treatment with flurbiprofen (n = 8). The values represent means ± SEM. Significant differences from the values prior to infection (day 3) are indicated by * $P< 0.05$; ** $P< 0.01$.

Flurbiprofen group

Flurbiprofen treatment was without beneficial effects on the mortality induced by the combined TGE virus and ETEC inoculation (Figure 1; $P > 0.05$). As in the control group, the pigs developed severe diarrhoea during the chloramphenicol pretreatment period (Figure 2, day 3). The diarrhoea score for this group did not significantly differ from the values in the control group during the whole experimental period (Figure 2). The first administration of flurbiprofen on day 5 was followed by a temporary non-significant reduction in faecal fluid loss (Figure 2), a significant

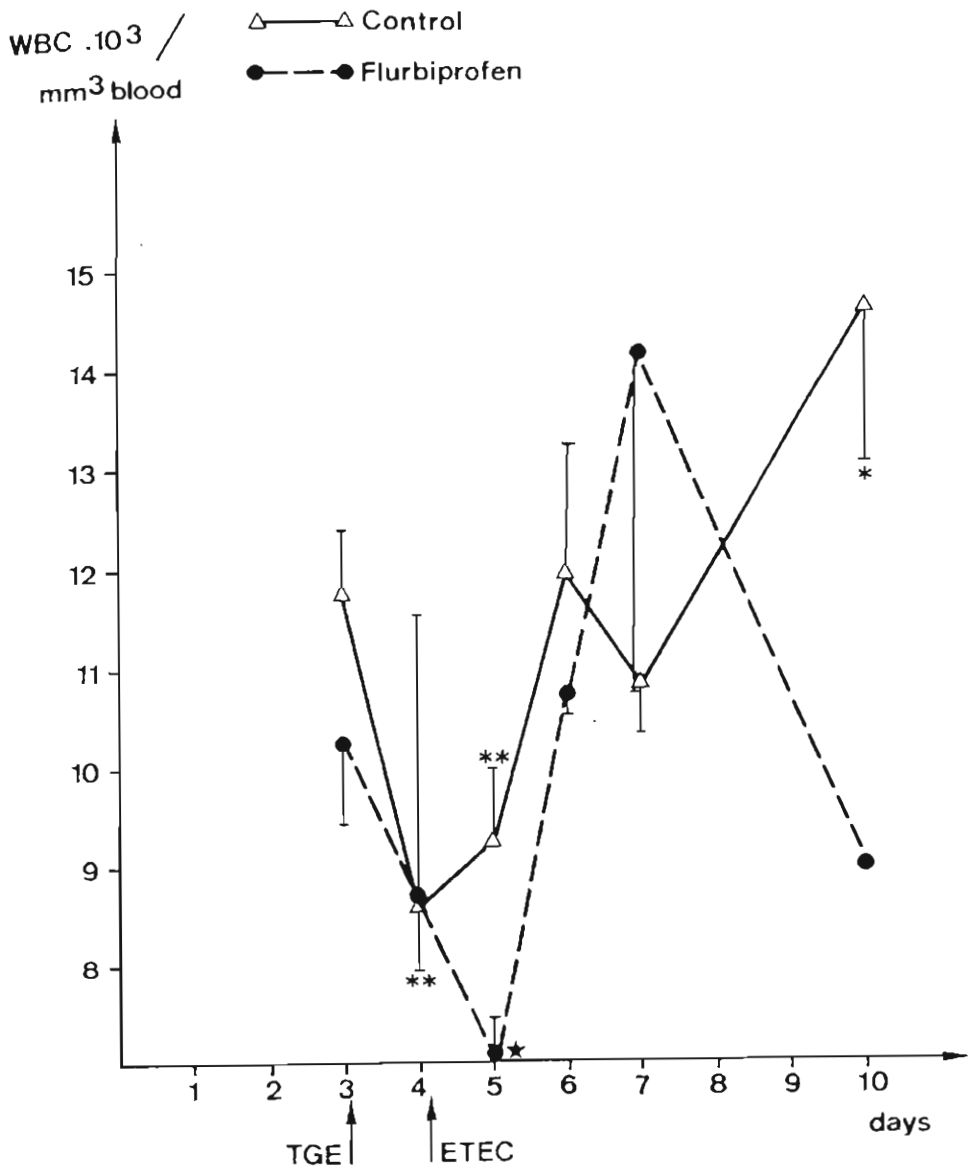


Figure 7. Changes in leucocyte counts following a combined infection of newly-weaned piglets with TGE virus and ETEC (control group; n = 21) and the effect of treatment with flurbiprofen (n = 8). The values represent means ± SEM. Significant differences from the values prior to infection (day 3) are indicated by * $P < 0.05$; * * $P < 0.01$. A significant difference between groups is indicated by * $P < 0.05$.

reduction in faecal shedding of haemolytic *E. coli* (Figure 3), a significantly more pronounced decrease in leucocyte count (Figure 7) and a temporary normalization of body temperature (Figure 8). After the later administrations, however, the evolution of all the parameters studied was comparable to that of the control group (Figures 3-8). Except for a decreased activity in the flurbiprofen group there were no significant differences between the groups for any parameter not shown in the Figures.

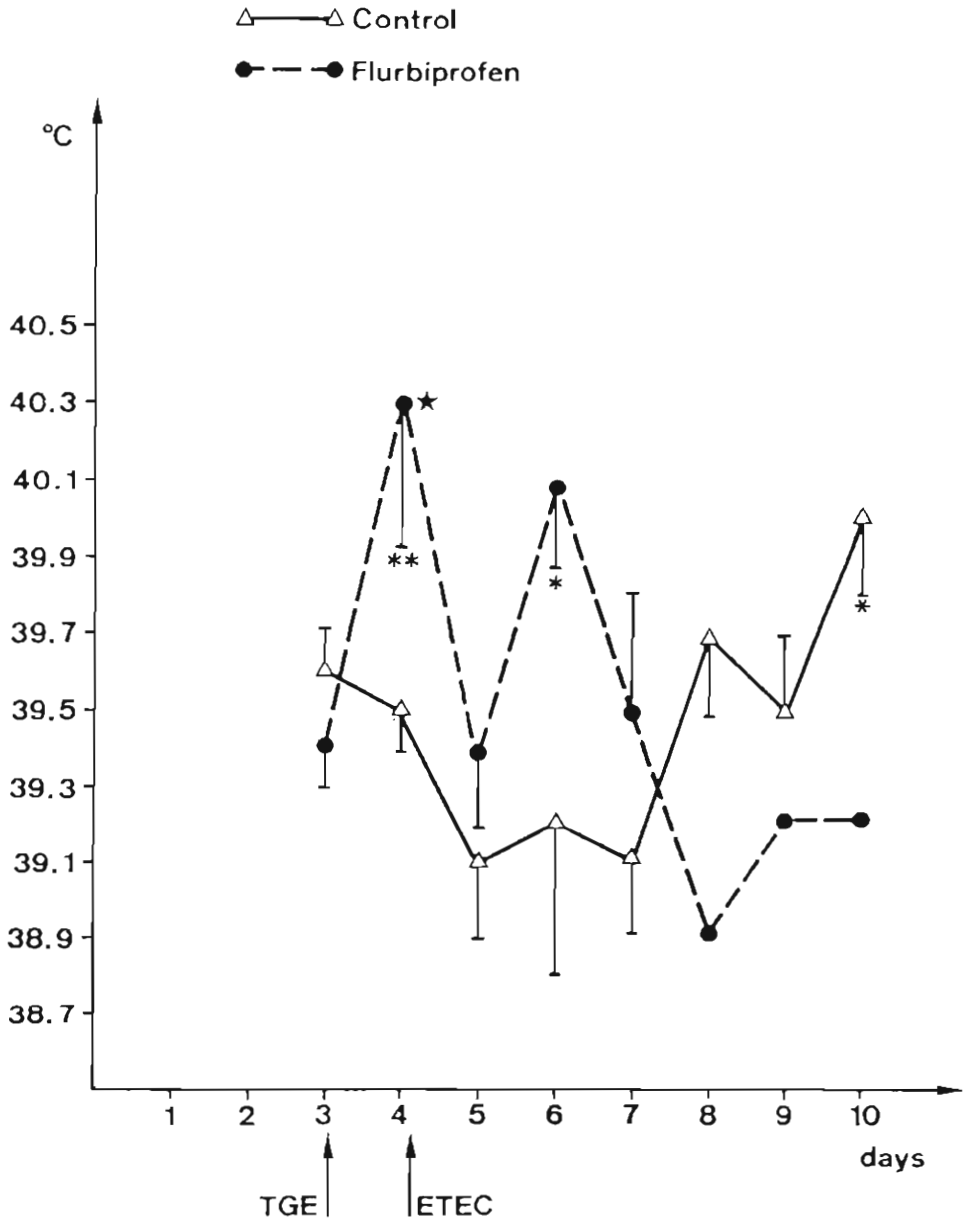


Figure 8. Changes in body temperature following a combined infection of newly-weaned piglets with TGE virus and ETEC (control group; n = 21) and the effect of treatment with flurbiprofen (n = 8). The values represent means ± SEM.Significant differences from the values prior to infection (day 3) are indicated by * $P < 0.05$; * * $P < 0.01$. A significant difference between groups is indicated by *$P< 0.05$.

DISCUSSION

Since the turn of the century enterotoxigenic *E. coli* have been implicated in the aetiology of porcine weaning diarrhoea (colibacillosis). The causal strains possess fimbriae which mediate specific adherence of the *E. coli* to receptors on villous enterocytes and produce enterotoxins which induce the fluid loss (Lecce, 1983). The enterotoxins LT and STa induce fluid secretion by the crypt cells, mediated by cyclic AMP and cyclic GMP respectively, whereas LT also inhibits absorption by villous cells. Although STb can induce villous atrophy, the exact mechanism through which it increases intestinal fluid loss is still unclear (Whipp *et al.*, 1987). Recently rotavirus has been shown to be causally associated with weaning diarrhoea of pigs. This virus multiplies in and destroys the absorptive cells of the villi of the small intestine, leading to malabsorption, dehydration and death in very young piglets (Lecce, 1983). In our model TGE virus, another enteropathogenic virus, was selected since rotavirus is enzootic in Belgian piggeries (Debouck, 1984). TGE virus consistently induces villous atrophy and crypt hyperplasia. The disease is most acute and almost invariably fatal in the very young piglet, in which vomiting and profuse diarrhoea can lead rapidly to dehydration and death. Young pigs over 3 weeks old, as in our study, experience a debilitating diarrhoea of 10 to 14 days duration but usually survive (Aitken, 1983; Whipp *et al.*, 1985).

In our model, chloramphenicol pretreatment followed by an infection with TGE virus and ETEC induced a profuse diarrhoea, vomiting, dehydration, weight loss and hypovolaemic shock, causing the death of 71% of the animals. The induced diarrhoea is probably a combination of malabsorption (TGE virus) and hypersecretion (ETEC), since the combined infection induces a more severe diarrhoea then the infection with either enteropathogen alone (chapter III.B.2), since piglets developed severe villous atrophy (chapters III.B.3 and III.B.4.) and atrophic villi were mostly colonized by coliform bacteria following inoculation (chapter III.B.4.), since piglets with watery diarrhoea excreted TGE virus and shed high amounts of K88ac *E. coli* in their faeces and since the osmotic gap of the faecal water became between 53 and 96 mosmol/l following the experimental inoculation (chapter III.B.5). A negative osmotic gap has been associated with a secretory diarrhoea , whereas an osmotic gap of ≥ 160 mosmol/l is associated with a pure osmotic diarrhoea (Shiau *et al.*, 1985). The measured osmotic gap thus suggest that the diarrhoea is partly osmotic and partly secretory.

The high control values for arterial pH (day 3) in piglets of both groups in the present study agree with the observation on conscious immature pigs of 2 to 3 months old. Pigs of this age, however, reveal a higher $pC0_2$ value, a higher $HC0_3^-$ concentration, a lower $P0_2$ value and a lower Hct (Hannon, 1983). Differences in our results can probably partly be attributed to the diarrhoea occurring on day 3. The decrease in Hct, following the initial increase, in surviving pigs of our study can probably be explained by a haemodilution effect, resulting from the daily extraction of blood.

When piglets developed hypovolaemia and shock some characteristic changes occurred within a few hours: an abrupt rise in Hct, a pronounced decrease in BE and a concomitant decrease

in bicarbonate resulting in an acidosis. In experiments on ETEC-induced and spontaneous diarrhoea in neonatal piglets a metabolic acidosis with low pH and an abrupt decrease in BE and an increase in Hct was also reported in dying animals (Andren and Persson, 1983). Kutas and Szabo (1971) likewise reported low pH and BE values in diarrhoeic piglets, resulting from experimental *E. coli* infection. In shocked animals in our study, the BE values were lower than - 10 mEq/l, a critical limit below which the prognosis should be considered poor, at least in neonatal piglets (Andren and Persson, 1983).

In experiments on gnotobiotic pigs, 27 to 34 days old when infected with ETEC-strains, animals developed a severe diarrhoea resulting in dehydration and death between 16 and 36 hours after infection. As in the piglets in our control group, no elevation of body temperature was recorded during this period. Pigs also revealed a neutropenia soon after infection and an abrupt increase in Hct (Kenworthy and de G. Mitchell, 1976). Polymorphonuclear cell infiltration in the intestinal mucosa was observed in pigs following infection with TGE virus or with STb producing ETEC strains (Pensaert *et al.*, 1970a; Rose and Moon, 1985). This observation can probably partly explain the severe neutropenia observed in our study.

Several studies suggest the involvement of prostaglandins in the LT and ST enterotoxin-induced hypersecretion (Berridge, 1984; Petzinger, 1984; Donowitz *et al.*, 1986). Results of studies on the effects of aspirin, indomethacin and other NSAID's are, however, somewhat inconsistent. *In vivo*, indomethacin inhibits secretion caused by *Vibrio cholerae*, cholera toxin and ST enterotoxin of *E. coli*. *In vitro*, this agent decreases the effects of cAMP and of drugs that stimulate the adenylate cyclase-cAMP system. *In vivo*, aspirin inhibits small intestinal secretion provoked by cholera toxin and *E. coli* LT enterotoxin but not *E. coli* ST enterotoxin (Donowitz *et al.*, 1986). In ligated calf's jejunal loops, salicylates reduced *E. coli* ST induced hypersecretion (Wise *et al.*, 1983). In the porcine jejunal loop, however, this NSAID was ineffective in reducing *E. coli* ST induced hypersecretion (Ahrens and Zhu, 1982). The inhibitory effect of the NSAID's appears to be due to increased absorption, although it is associated with some inhibition of activation of the cyclic AMP-system by LT enterotoxin of *Vibrio cholerae* and *E. coli*. (Donowitz *et al.*, 1986).

In the present experiments we studied the effect of flurbiprofen, a potent NSAID, on diarrhoea induced by combined TGE virus and ETEC infection in newly-weaned piglets. The dosage, 1 mg/kg/12 h, was derived from the oral dose in humans and from the plasma mean half-life value of approximately 7 hours after oral dosage in man (Szpunar *et al.*, 1987). In our model flurbiprofen was without significant beneficial effect on lethality. Only the first administration of this NSAID resulted in a temporary normalization of body temperature and a temporary non-significant reduction in faecal fluid loss. This could indicate that prostaglandines are involved in the pathophysiology in our model, but that other pathophysiological mechanisms are more important in the induced diarrhoea and hypovolemic shock. Furthermore, the treatment induced a decreased activity.

The effects of various NSAID's on cholera toxin-induced intestinal secretion in a number of animal species have generally shown them to be effective when given before but not after toxin administration (Wise *et al.*, 1983). In our experiments flurbiprofen was administered following the

combined viral-bacterial inoculation. It recently appears that arachidonic acid metabolites may play an important role in invasive, inflammatory diarrhoea, but not in secretory diarrhoea (Powell, 1986; Turnberg, 1986). In our model the dual infection induced inflammatory intestinal changes (unpublished observation), as consistently occur following TGE virus and STb enterotoxin exposure (Pensaert *et al.*, 1970; Rose and Moon, 1985). This could perhaps explain the beneficial effects of the flurbiprofen treatment in the present study. Perhaps treatment with NSAID's would be more appropriate in diarrhoeic conditions caused by enteroinvasive bacterial strains (Petzinger, 1984).

Chapter IV. GENERAL DISCUSSION AND CONCLUSIONS

The diarrhoea syndrome occurring within the first weeks after weaning has a complex and multifactorial etiology. Enterotoxigenic *Escherichia coli* (ETEC) play an important role in this diarrhoea. ETEC possess two important virulence factors nl. fimbriae and enterotoxins. The fimbriae allow the ETEC strains to adhere to receptors or receptor sites on the brush border of villous enterocytes and so to resist and circumvent the flushing and clearing mechanisms in the small intestine. Subsequently colonisation of the small intestine can occur. Adhesion is thus an essential step in ETEC-induced diarrhoea.

K88 (F4), K99 (F5), P987 (F6) and F41 fimbriae can be identified on ETEC strains isolated from piglets with neonatal colibacillary diarrhoea, but $K88^+$ strains are most frequently identified from the faeces of piglets with post-weaning diarrhoea (Francis et al., 1982; Wilson and Francis, 1986; Broes et al, 1988 and Söderlind et al., 1988. In Chapter III.A.2., the adhesion of *E. coli* producing one or more of these fimbriae was compared to villi of 4- to 5-week-old piglets using an *in vitro* adhesion assay. Results showed that the $K88^+$ strains adhere significantly more to the villi of most piglets than strains producing one or more of the other fimbriae. The strong adhesion of the $K88^+$ strains is in accordance with the fact that these strains are most frequently isolated from piglets with post-weaning diarrhoea. Of the 3 known K88 variant fimbriae, namely K88ab, K88ac and K88ad, the K88ac variant is most often identified on strains isolated from piglets with weanling diarrhoea. This does not seem to be due to differences in adhesion between K88ac and both other K88 variant$^+$ strains since K88ab$^+$ and K88ac$^+$ *E. coli* adhered equally well to villi of the piglets, and even though K88ad$^+$ *E. coli* adhered significantly less to the villi than both other K88 variant$^+$ strians, the adhesion was still too pronounced to explain its low appearance. Results of the comparative study were performed *in vitro*. These results do not exclude that the enhanced prevalence of K88ac as an etiology of colibacillary diarrhoea reflects a mechanism which only occurs *in vivo*. Differences between the *in vitro* adhesion assay and *in vivo adhesion* have untill now not been demonstrated for $K88^+$ strains, however, $P987^+$ strains adhere *in vitro* but not *in vivo* to villi of older piglets (Dean et al., 1989).

In the comparative study, not all piglets were highly susceptible to adhesion of the K88 variant$^+$ strains. In some piglets the adhesion of one or more of the K88 variant$^+$ strains was weak or even absent. It has been shown that presence or absence of K88 receptors for one or more of the K88 variant$^+$ strains is genetically determined (Bijlsma *et al.*, 1982; Sellwood, 1984; Bijlsma and Bouw, 1985). Absence or a decreased number of K88 receptors results in no or a mild diarrhoea upon infection of piglets with K88 variant$^+$ ETEC (Sellwood, 1983, 1984).

Thus, when experimentally inoculating piglets with $K88^+$ ETEC to induce colibacillary diarrhoea, the expression of K88 receptors on brush border of their villous enterocytes has to be determined. This can be done using villi, villous enterocytes or the brush borders of these enterocytes in an *in vitro* adhesion assay. The intestinal villi can be collected post mortem or in alive piglets by surgery. In Chapter III.A.3, it was shown that the presently used *in vitro* adhesion assays

can not be replaced by *in vitro* adhesion to buccal epithelial cells or by haemagglutination. Bijlsma *et al.* (1985) also demonstrated that haemagglutination could not be used for phenotyping piglets for receptors for K88 fimbriae. Valpotic *et al.* (1989) furthermore excluded Peyer's patch lymphocytes, peripheral blood lymphocytes and rectal epithelial cells as cells to determine phenotypic susceptibility of swine to K88-mediated adhesion.

It is possible to select for K88 resistant swine (Bijlsma and Bouw, 1985; Sellwood, 1983). The prevalence of piglets with receptors for K88$^+$ *E. coli* was determined in a Belgian piggery (Chapter III.A.4). Only 3.6 % of the piglets were totally resistant to K88 adhesion. Since the studied piglets were purebred Belgian Landrace or crossbred Belgian Landrace x Piétrain, it can be assumed that K88ab, K88ac and K88ad receptors are frequent in both Belgian breeds. Thus selection for a K88 resistant population will not be easy in Belgium. During the study, the percentage of piglets genetically susceptible to adhesion of the K88ac$^+$ *E. coli* strains increased from 78,4 during the first 7 months to 94,3 % during the last seven months. Simultaneously with this increase, post-weaning diarrhoea occurred. K88ac$^+$ *E. coli* were isolated from diarrhoeal faeces. This indicates that in the evaluation of farms with weanling diarrhoea, the genetic susceptibility of the piglets to adhesion of K88$^+$ ETEC has to be taken into account.

Besides colonizing the small intestinal mucosa, the ETEC strains elaborate heat-stable (ST, type a and/or type b) and/or heat-labile enterotoxins (LT) which induce intestinal fluid loss, resulting in a diarrhoea which can lead to a subacute dehydration. STa acts by increasing the intracellular level of cyclic guanosine monophosphate (cGMP). cGMP subsequently decreases the absorption by villous enterocytes (Dreyfus *et al.*, 1984). STb induces a slight reduction in villous length so decreasing the absorptive surface (Whipp *et al.*, 1987) and also has been observed to stimulate the active secretion of an anion which is not chloride (Weikel *et al.*, 1986). LT increases the intracellular concentration of cyclic adenosine monophosphate (cAMP). cAMP increases in crypt enterocytes the secretion and decreases in villous enterocytes the absorption of water and electrolytes (Field, 1979; Berridge, 1984). LT, furthermore, induces the release of serotonin by enterochromaffin cells (Cassuto *et al.*, 1981). Serotonin increases the influx of calcium into the cells (Berridge, 1984) and can induce via reflex arcs the release of the neurotransmitter VIP (Cassuto *et al.*, 1983). VIP can stimulate adenylate cyclase (Simon *et al.*, 1981). The fluid loss induced by enterotoxigenic *E. coli* is thus the resultant of the effect of several secretagogues which act via different mechanisms on the intestinal electrolyte transport. This will be one of the reasons why the administration of either cholera toxin, heat-labile enterotoxin, the serotonin precursor DL 5-hydroxytryptophan (HTP), or a combination of castor oil and HTP only caused a mild diarrhoea (Chapter III.B.2). Furthermore, the induced diarrhoea will have resulted in a quick clearance of the intragastrically administered secretagogues from the intestinal tract so that the secretagogues have to be given continuously to mimic the continuous release of enterotoxins by ETEC during colibacillary diarrhoea.

It was tried to induce diarrhoea and dehydration in newly-weaned, 3- to 4-week-old, piglets by inoculating them with K88ac$^+$ ETEC (O149 K91 K88ac, LT, STb and STa positive). It was demonstrated in this thesis, that inoculating piglets only with K88ac$^+$ETEC did not consistently induce diarrhoea. However, when several factors were introduced which have been mentioned to be

involved in post-weaning diarrhoea, a reproducible diarrhoea and dehydration was obtained. This is in accordance with previous reports which show that post-weaning diarrhoea appears to have a multifactorial etiology (Smith and Hall, 1968; Arbuckle, 1970; Sarmiento *et al.*, 1988). A reproducible diarrhoea (93 % of the piglets) and dehydration (hypovolaemic shock in 80 % of the piglets) was induced by orally pretreating K88ac susceptible piglets with chloramphenicol during 3 days, by inoculating them on day 4 with transmissible gastroenteritis virus (TGEV) and on day 5 with K88ac ETEC strains (Chapter III.B.2).

The chloramphenicol pretreatment seems to enhance diarrhoea via several ways. Approximatively 70 % of the piglets had already diarrhoea on the third day of the chloramphenicol pretreatment (Chapters III.B.2, III.B.3, III.B.5 and III.C.5). It has been mentioned that oral administration of antibiotics can cause colibacillary diarrhoea by selecting for antibiotic-resistant ETEC strains (Hinton *et al.*, 1985).Only in 2 piglets, chloramphenicol-resistant K88ac$^+$ *E. coli* were isolated from the faeces at the end of the chloramphenicol pretreatment, so that the diarrhoea in most piglets was not induced by a selection for antibiotic-resistant ETEC (Chapter III.C.5). In calves, a 5 day oral chloramphenicol treatment induced a small intestinal malabsorption (Mero *et al.*, 1985). In our study, scanning electron microscopy revealed atrophic villi, in duodenum but not in caudal jejunum and ileum in 4 piglets following treatment with chloramphenicol (Chapter III.B.3). These 4 piglets were excreting rotavirus, so that the observed villous atrophy in these piglets could have been due to the rotavirus infection. Furthermore, piglets with diarrhoea on day 3 of the chloramphenicol treatment had a negative faecal osmotic gap (Chapter III.C.5). A negative faecal osmotic gap has been associated with a secretory and not with a malabsorption diarrhoea (Shiau *et al.*, 1985), indicating that small intestinal malabsorption was not the mechanism via which chloramphenicol induced diarrhoea in the piglets.The negative osmotic gap could be explained by a suppressing effect of chloramphenicol on the gut flora. Normally the bacterial flora in cecum and colon ferments undigested particles into more osmotically smaller molecules, whereas short-chain fatty acids, formed by the bacterial flora, can stimulate the colonic absorption of sodium (Hildman *et al.*, 1980). A suppression of the flora will than result in a decreased formation of short-chain fatty acids and subsequently in an increased loss via the faeces of Na^+. Furthermore, suppression of bacterial fermentation will decrease the formation of osmotic active particles so that less osmotic active particles will be found in the faeces. The osmotic gap will then become negative (osmotic gap = osmolality - 2 x (Na^++ K^+)). Suppression of the anaerobic gut flora has been mentioned to result in an increased susceptibility to infection by potentially pathogenic microorganisms (Kaufman, 1984). Such an effect could be suggested in our model since the pretreatment enhanced the occurrence, severity and duration of diarrhoea following ETEC inoculation and since coliform bacteria were observed between 5 and 7 days following ETEC inoculation on villi of 4 on 7 piglets pretreated with chloramphenicol in comparison with only 1 on 5 piglets not pretreated with chloramphenicol (Chapter III.B.2).

The diarrhoea occurring following the TGEV- ETEC inoculation was the result of a TGEV-induced small intestinal malabsorption and an ETEC-induced hypersecretion since: (1) diarrhoea was more severe in piglets inoculated with TGEV and ETEC than with either enteropathogen alone; (2) in

10 on 11 of the piglets with severe diarrhoea colonization of atrophic villi with coliform bacteria was observed by electron microscopy (Chapter III.B.4); (3) all piglets with severe diarrhoea and dehydration excreted TGEV and intensely shed the inoculated ETEC strains in the faeces untill they died or were euthanatized (Chapter III.B.4 and III.B.5); (4) the faecal osmotic gap was between 50 and 96 mosmol/l upon the combined TGEV-ETEC infection, suggesting that the induced diarrhoea was a combination of an osmotic (TGEV) and a secretory (ETEC) diarrhoea (Chapter III.B.5). A pure secretory diarrhoea is associated with a negative osmotic gap, whereas a pure osmotic diarrhoea is associated with a positive osmotic gap of greater than 160 mosmol/l (Shiau *et al.*, 1985).

Similarly to our findings, it has been demonstrated that a combined infection of newly-weaned piglets with rotavirus and ETEC caused a more severe diarrhoea than an infection with either pathogen alone (Tzipori *et al.*, 1980b; Kyriakis, 1982; Lecce *et al.*, 1982). Concurrent infections of ETEC and rotavirus or TGEV have been reported in piglets with weanling diarrhoea (Tzipori *et al.*, 1980b; Wilson and Francis, 1986; Pritchard, 1987). Rotavirus and TGEV both replicate in and destroy villous enterocytes so causing villous atrophy and subsequently small intestinal malabsorption (Moon, 1978). The mechanism through which enteropathogenic viruses enhance the occurrence of colibacillary post-weaning diarrhoea is not completely determined. Lecce *et al.* (1982) postulated that a virus infection can change membrane nature of villous enterocytes, so that the adhesion of ETEC to villous enterocytes becomes facilitated. In the TGEV-ETEC model a severe villous atrophy was observed in piglets euthanatized 2 to 3 days after the TGEV infection. Using an *in vitro* adhesion assay, it was demonstrated in Chapter III.B.3. that the atrophic villi possessed a decreased number of K88ac receptors. These results do not support the hypothesis that the changes in membrane nature of the enterocytes caused by an infection with an enteropathogenic virus result in an enhanced adhesion of ETEC to the brush border of the enterocytes.

Even though the virus-induced villous atrophy resulted in a decreased number of receptors for K88ac$^+$ETEC, colonization of the atrophic villi with coliform bacteria could still be demonstrated *in vivo* by electron microscopy in all except one piglet with severe diarrhoea and dehydration (Chapter III.B.4). It has been postulated that the virus-induced villous atrophy and malabsorption could enhance the proliferation of *E.coli* in the gut (Lecce *et al.*, 1983). Tzipori *et al.* (1984) observed that a change from a milk diet to less digestible dry food followed by inoculation with K88$^+$ETEC resulted in severe villous atrophy and an increased colonization of the small intestine with the ETEC strain. Recently, however, Deprez (1988) demonstrated that even in the absence of villous atrophy, malabsorption and maldigestion an important proliferation of hemolytic *E. coli* occurred post-weaning. This indicates that malabsorption itself is not the most important factor leading to proliferation and subsequently to colonization of *E. coli*.

There was a difference in clinical response following pretreatment with chloramphenicol and a dual TGEV and ETEC inoculation between catheterized and non-catherized piglets. Whereas all catherized piglets developed diarrhoea leading to hypovolemic shock in 70 % of the piglets (Chapter III.B.5), one of the non-catheterized piglets did not develop diarrhoea and mortality was 57 % (Chapter III.B.2). Itis known that surgery leads to reticuloendothelial cell depression, so making the host more susceptible to infection (Altura, 1983). This perhaps can explain the difference in

clinical response.

The presence of K88ac receptors on villous enterocytes of piglets seemed to be necessary to induce hypovolemic shock following the TGEV-K88ac$^+$ETEC inoculation since 4 on 7 non-catherized piglets with K88ac receptors died, whereas 3 piglets without K88ac receptors survived (Chapter III.B.2).

In this diarrhoea model, some of the factors which are mentioned to play a role in post-weaning diarrhoea were included such as a change in diet, the cessation of lacteal immunity, stress resulting from a change in environment, antibiotics in the feed, a decrease in the gastrical pH, a susceptibility to adhesion of K88ac$^+$ ETEC, an infection with K88ac$^+$ETEC and a concurrent infection with an enteropathogenic virus (Prohaszka and Baron, 1980; Tzipori *et al.*, 1980a; Bijlsma *et al.*, 1982; Madec and Josse, 1983; Hinton *et al.*, 1985; Deprez, 1988). This experimental diarrhoea model mimics the multifactorial aspect of postweaning diarrhoea occurring on farms and could thus be used to evaluate the impact of several factors on the occurrence, severity and course of the diarrhoea.

In 21 piglets in which a catheter was implanted in the left a. carotis, the chloramphenicol pretreatment and TGEV-K88ac$^+$ETEC inoculation induced diarrhoea in all K88ac-susceptible piglets. The diarrhoea induced weight-loss and increases in the Hct and plasma protein concentration. Initially, no changes in the plasma electrolyte concentrations were observed, indicating that the diarrhoea resulted in an isotonic dehydration. When piglets developed severe hypovolemia some characteristic changes occurred within a few hours: an abrupt rise in Hct, a severe metabolic acidosis, a decrease in arterial pressure, an increase in heart rate, an increase in the potassium concentration. Hypovolemic shock occurred in 71 % of the inoculated piglets (Chapter III.B.5). Similar observations have been done in ETEC-induced diarrhoea in neonatal piglets (Andren and Persson, 1983). The abrupt rise in Hct, indicates the presence of an acute fluid loss.The degeneration of villous enterocytes which was observed by transmission electron microscopy in severely dehydrated piglets, will have resulted in an increased permeability of the intestinal mucosa and in an increased fluid loss (Chapter III.B.4). The metabolic acidosis was not the result of an increased formation of lactic acid, but could have been due to a renal dysfunction. Such a dysfuntion can occur following severe hypotension.

Antisecretory drugs were tested on the diarrhoea model which act differently on electrolyte transport processes by enterocytes nl. chlorpromazine, clonidine, propranolol, verapamil and flurbiprofen (Chapter III.C). Chlorpromazine has been observed to exert a membrane-stabilizing effect by which it is thought to decrease the activity of adenylate cyclase and furthermore chlorpromazine has an inhibitory effect on calmodulin. Clonidine has been observed to decrease the entry of calcium into the cells and to decrease the adenylate cyclase activity by acting on α_2-adrenergic receptors on enterocytes. Propranolol, a ß-adrenergic antagonist, inhibits the activation of adenylate cyclase by ß-adrenergic agonists. Verapamil, a calcium-channel blocker, inhibits secretion as a result of an increased entry of calcium into the enterocytes. Non-steroidal antiinflammatory agent, such as flurbiprofen, inhibit the production of prostaglandins which then can activate adenylate cyclase and probably also are antisecretory by inducing an increased absorption, by

inhibiting adenylate cyclase and cAMP protein kinase activity (Chapter I: section I.D.).

Chlorpromazine and clonidine were observed to decrease the severity of the diarrhoea and to reduce mortality in non-catheterized piglets (Chapter III.C.2). Chlorpromazine was also antiemetic. One of the reasons why chlorpromazine and clonidine exerted beneficial effects, whereas propranolol and verapamil were without effect, could perhaps be that chlorpromazine and clonidine both can inhibit the LT-enterotoxin-induced cAMP production and the fluid loss via an increase in the intracellular calcium concentration. So, reducing more efficiently the ETEC-induced fluid loss than verapamil which only inhibits the increased calcium influx into the cell and than propranol which only inhibits the increased cAMP production. Propranolol has been observed to have a species-specific action which could explain the absence of a beneficial effect (Donowitz *et al.*, 1986). Otherwise the dosage of propranol and verapamil could have been to low to induce antisecretory effects. However, increasing the dosage would most likely have induced systemic effects.

On catheterized piglets, the beneficial effects of clonidine and chlorpromazine could not be reproduced (Chapters III.C.3 and III.C.4). Instead, some serious side-effects were observed. Both drugs have an hypotensive effect (Baldessarini, 1980; Arndts *et al.*, 1983). In our model, catheterized piglets showed a more severe diarrhoea and probably dehydration than non-catheterized piglets. Piglets with hypovolemia will develop hypotension which they try to compensate by peripheral vasoconstriction and increasing their heart rate. Drugs which decrease heart rate and blood pressure and induce vasodilatation interfere with the normal compensatory mechanisms during excessive fluid loss. This can even enhance the occurrence of shock as observed in the clonidine-treated piglets.

Flurbiprofen treatment of catheterized piglets in which diarrhoea was induced caused a transient non-significant decrease in the severity of the diarrhoea and a significant decrease in the excretion of hemolytic *E. coli*. The effect on the ETEC excretion can not be explained. Since flurbiprofen inhibits prostaglandin formation, the decrease in the diarrhoea could suggest that prostaglandines play a role in the experimental malabsorption-hypersecretion diarrhoea. Flurbiprofen treatment, however, did not result in an increased survival and can thus not be used to treat piglets with a combined malabsorption-hypersecretion diarrhoea.

The results of the studies with the antisecretory drugs show that some drugs can significanlty inhibit fluid loss due to a combined malabsorption-hypersecretion diarrhoea. However, these drugs also have systemic effects which can cause serious side-effects in hypovolemic pigs and therefore, excludes them for treatment of severe acute diarrhoea. Results indicate that research should be directed on antisecretory drugs which act more specifically on the intestine. Such drugs can become important therapeutics in treatment of post-weaning diarrhoea in piglets.

Chapter V: SUMMARY

In Chapter I a review is given on mechanisms of water and electrolyte transport in the normal intestinal tract, on pathophysiological mechanisms of diarrhoea, on post-weaning diarrhoea in piglets and on antisecretory drugs and their use in treatment of diarrhoea.

The aim of the present studies is given in Chapter II. Diarrhoea which occurs the first weeks after weaning is an important cause of weight loss and sometimes of mortality. This post-weaning diarrhoea has a multifactorial etiology and is, therefore, difficult to treat. The aim of the present studies was to evaluate the effect of antisecretory drugs in newly-weaned piglets with diarrhoea.

Enterotoxigenic *Escherichia coli* (ETEC) play an important role in the postweaning diarrhoea syndrome. ETEC possess 2 important virulence factors namely fimbriae and enterotoxins. Fimbriae allow ETEC to adhere to receptors on the brush border of villous enterocytes, so facilitating colonization of the small intestinal villous mucosa with ETEC. K88 (F4) , K99 (F5), P987 (F6) and F41 fimbriae have been identified on ETEC strains isolated from piglets with neonatal colibacillary diarrhoea, whereas $K88^+$ ETEC strains are playing an important role in post-weaning diarrhoea.

Besides possessing fimbriae, ETEC produce heat-labile (LT) and/or heat-stable (ST type a and/or type b) enterotoxins. These enterotoxins induce diarrhoea by increasing the secretion and/or decreasing the absorption of water and electrolytes in the small intestine.

Besides ETEC, environmental, immunological and nutritional factors, the presence of K88 receptors on villous enterocytes and an infection with rotavirus or with transmissible gastroenteritis virus (TGEV) have been mentioned to play a role in post-weaning diarrhoea. Several of these factors provoke a small intestinal villous atrophy and thus decrease the absorptive surface. This can lead to a malabsorption. It has been mentioned that malabsorption enhances the proliferation of *E. coli* in the small gut and so the colonization of the villous mucosa with ETEC.

From a pathophysiological point of view, post-weaning diarrhoea can thus be seen as a combination of small intestinal malabsorption and hypersecretion. Treatment could, therefore, be performed by stimulating the absorption and/or decreasing the secretion of water and electrolytes. Several drugs have been identified which have these effects. They are called antisecretory drugs. To evaluate the effect of antisecretory drugs in newly-weaned piglets with diarrhoea, a reproducible diarrhoea model had to be developed.

The experimental studies are presented in Chapter III. Firstly, the *in vitro* adhesion of fimbriae producing ETEC strains to villi was tested. It was also assayed wether the *in vitro* villous adhesion assay could be replaced by a test which could easier be performed in alive piglets. Secondly, a reproducible diarrhoea model was developed in newly-weaned piglets and the pathophysiogy of this diarrhoea was studied. In the third part of the experimental studies, the therapeutic effect of some antisecretory drugs was evaluated on this diarrhoea model.

In chapter III.A.2., it is shown, using an *in vitro* villous adhesion assay, that K88ab and $K88ac^+$ *E. coli* adhered significantly more to villi of most 4- to 5-week old piglets than K88ad,

K99, F41 and P987$^+$ *E. coli* so that K88ab or K88ac$^+$ ETEC are most indicated to be used in an experimental diarrhoea model.

In some piglets, K88$^+$ *E. coli* adhered weak or did not adhere to their villi. The susceptibility of pigs for adhesion of K88$^+$ *E. coli* to their villous enterocytes is genetically determined. The presence of receptors for K88ac fimbriae can be tested using the *in vitro* villous adhesion assay on small intestinal villi collected either in alive piglets by surgery or post mortem. It is difficult to use surgery on a large scale. It was therefore determined whether the *in vitro* villous adhesion assay could be replaced by K88 mediated *in vitro* adhesion to buccal epithelial cells or by hemagglutination. Buccal epithelial cells and erythrocytes are easy to obtain in alive pigs (Chapter III.A.3). There was no correlation between adhesion to villi and adhesion to buccal epithelial cells or hemagglutination.

The results of the *in vitro* adhesion of K88$^+$ *E. coli* to villi of 140 piglets of the same piggery are given in Chapter III.A.4. Adhesion of all 3 K88 variants (K88ab, K88ac and K88ad) was observed in 80.7 % of the piglets, whereas only 3,6 % were resistant to adhesion of all 3 K88 variant strains. Since the piglets were purebred Belgian Landrace or crossbred Belgian Landrace x Piétrain, it can be assumed that K88 receptors appear frequently in both Belgian pig breeds. Breeding of pigs without K88 receptors could result in a direct decrease of the diarrhoea induced by K88$^+$ ETEC. However, due to the high prevalence of pigs with K88 receptors, selection for K88 resistant piglets will not be easy.

In Chapter III.B., a diarrhoea model was developed in newly-weaned piglets and the pathophysiology of the diarrhoea was studied.

In Chapter III.B.2, it was shown that an experimental infection of 3- to 4-week-old piglets with K88ac$^+$ ETEC (O149:K91:K88ac; LT:STa:STb) alone, or an oral pretreatment with chloramphenicol during 3 consecutive days, followed by the ETEC infection, did not induce a reproducible diarrhoea and dehydration. However, when the chloramphenicol pretreatment was followed by an inoculation with TGEV on day 4 and an inoculation with ETEC on day 5, than a severe diarrhoea developed resulting in weight loss, dehydration and a high mortality. This diarrhoea model combines malabsorption, induced by TGEV and an infection with K88ac$^+$ ETEC. Diarrhoea and dehydration were more severe in piglets in which a catheter was implanted in the left a. carotis than in non-cathterized piglets.

In Chapter III.B.3, it was demonstrated, using an *in vitro* villous adhesion assay, that K88$^+$ *E. coli* adhered significantly less to atrophic villi of piglets infected with TGEV, than to villi of non-infected piglets or of piglets recovered from the infection. The results of this study do not support the hypothesis that changes in membrane nature of enterocytes as a result of a viral infection can facilitate the adhesion of ETEC to the villi.

In Chapter III.B.4., the morphological and ultrastructural changes of the small intestinal mucosa were studied in newly-weaned piglets dually inoculated with TGEV and ETEC and changes were related to possible mechanisms of diarrhoea. Scanning electron microscopy of the small intestinal mucosa of severely dehydrated piglets (n=11) showed severe villous atrophy and colonization of villous-sites and -base of the atrophic villi with coliform bacteria. These piglets all

excreted K88ac$^+$ *E. coli* in high number in their faeces. Transmission electron microscopy revealed severely degenerated cells in the duodenum of 5 hypovolaemic animals. The results of this study show that chloramphenicol treatment followed by a dual TGEV-ETEC infection induces a profuse diarrhoea which is in part the result of a severely impaired absorption due to the TGEV infection. In piglets with serious dehydration colonization of villi with ETEC strains occurred, suggesting an increased intestinal fluid loss due to the ETEC strains. Severe watery diarrhoea can lead to hypotension which in turn can cause intestinal mucosal damage, so decreasing the intestinal absorption and causing hypovolaemic shock and death.

In Chapter III.B.5, the pathophysiology of diarrhoea induced by the combined infection with TGEV and ETEC was studied in newly-weaned piglets with a catheter was implanted in the left a. carotis. Clinical, hematological, biochemical and hemodynamic parameters were registered daily. The combined infection induced diarrhoea in all (n=21) and vomiting in 65 % of the piglets. Diarrhoea and vomiting resulted in weight loss, increase in the haematocrit (Hct), and in a metabolic acidosis. When the Hct increased to about 42 %, piglets became severely ill, and shock and mortality (71 %) rapidly occurred. In these piglets the abrupt rise in Hct was accompanied with a significant increase in total plasma protein concentration, in heart rate and a significant decrease in the arterial blood pressure, arterial pH, bicarbonate concentration and the body temperature. No significant changes in the serum concentration of Cl^- and Na^+ were observed even though piglets had a profuse waterhy diarrhoea. In surviving piglets (29 %), only a significant increase of the body temperature was observed.

In Chapter III.C., the effects of several antisecretory drugs were evaluated in the post-weaning diarrhoea model.

In Chapter III.C.2., results are described of treatment of pigs with chlorpromazine (1 or 2 mg/kg/24 h), verapamil (2 mg/kg/8 h), clonidine (0.04 or 0.08 mg/kg/12 h) or propranolol (0.33 mg/kg/12 h). The treatment was started 5 hours after the ETEC inoculation and was continued for 3 consecutive days. The drugs were injected intramuscularly. Only chlorpromazine and clonidine in the highest administered dosage showed beneficial effects. The administration of the neurolepticum chlorpromazine resulted in a significant inhibition of diarrhoea and vomiting and in an increase in weight gain and survival. The α_2-agonist, clonidine, induced a significant antidiarrhoeal effect and a reduction in mortality.

The therapeutic effects of clonidine and chlorpromazine were studied more thoroughly in piglets in which a catheter was implanted in the left carotid artery (Chapters III.C.3 and III.C.4). The beneficial effects observed following treatment of non-catheterized piglets, could not be reproduced on catheterized piglets. Instead, severe side-effects were seen. Clonidine induced in hypovolaemic piglets excitation, incoordination and a significant hypotension and decrease in heart rate. Chlorpromazine caused sedation, and significant decreases in body temperature and in arterial pressure. In our model on catheterized piglets the induced diarrhoea was more severe than in non-catheterized animals, probably resulting in a more pronounced dehydration. The more severe hypovolaemia could have made the piglets more susceptible to the hypotensive action of both drugs. In this way these drugs could have interacted with the normal compensatory mechanisms

occurring during excessive fluid loss, so compromising their beneficial effects.

In Chapter III.C.5, the effect of flurbiprofen, a potent non-steroidal anti-inflammatory drug, was examined in the post-weaning diarrhoea model. Flurbiprofen was administered intramuscularly on 3 successive days following the combined infection at a dosage of 1 mg/kg/12 h. Flurbiprofen induced a temporary, non-significant decrease in fluid loss, but did not inhibit the occurrence of hypovolemic shock. Flurbiprofen is not effective for treatment of a combined malabsorption-hypersecretion diarrhoea.

The results of the studies with the antisecretory drugs show that some drugs can significantly inhibit diarrhoea. However, the drugs tested have systemic effects which cause serious side-effects in hypovolaemic pigs and, therefore, excludes them for treatment of severe acute diarrhoea. The results nevertheless indicate that antisecretory drugs, which will be shown to act only on the gut, can become important therapeutics in treatment of diarrhoea.

Chapter VI: SAMENVATTING EN CONCLUSIES

Het *eerste hoofdstuk* is een literatuuroverzicht. In dit overzicht worden de fysiologische mechanismen besproken die een rol spelen in de normale beweging van water en electrolieten over het maagdarmkanaal nl. de intestinale motiliteit, permeabiliteit, absorptie en secretie. Vervolgens worden de belangrijkste pathofysiologische mechanismen van diarree beschreven: veranderingen in motiliteit, een toegenomen permeabiliteit van de darmmucosa , een verminderde vertering van voedingsstoffen, een verminderde absorptie van voedingsstoffen, water en electrolieten en een verhoogde secretie van water en electrolieten. De pathofysiologie en etiologie van speendiarree wordt in een apart hoofdstuk beschreven. Het laatste deel van het overzicht is gewijd aan antisecretorische farmaca voor de behandeling van secretorische diarree.

In het *tweede hoofdstuk* wordt het doel van het experimenteel deel van deze studie uiteengezet. Dit doel was de therapeutische bruikbaarheid na te gaan van antisecretorische farmaca bij de behandeling van diarree met subacute deshydratatie bij biggen.

De diarree die bij biggen de eerste weken na het spenen optreedt is een belangrijke oorzaak van gewichtsverlies en soms ook van sterfte. Ze kent meestal een subacuut verloop. Therapeutische behandeling van speendiarree gebeurt voornamelijk door orale vochttherapie (electrolieten en glucose) samen met een antibiotica-behandeling. Speendiarree heeft een multifactoriële oorzaak. Benevens enterotoxigene *Escherichia coli* (ETEC) spelen andere factoren een rol in het ontstaan van dit diarree-syndroom. De ingestelde behandeling is daardoor niet steeds effectief. Behandeling met antibiotica kan daarenboven leiden tot het ontstaan van een antibiotica resistentie van pathogene bacteriën en kan de normale opbouw van een intestinale mucosale immuniteit tegenover de enteropathogene bacteïen remmen. Deze immuniteit ontstaat immers enkel indien er een normale vermeerdering van bacteriën plaatsgrijpt. Er is een behoefte aan andere therapeutica voor de behandeling van speendiarree.

Zoals vermeld spelen ETEC een voorname rol in het speendiarree-complex. ETEC bezitten 2 kenmerken die er voor zorgen dat ze diarree kunnen induceren nl. fimbriae en enterotoxinen. De fimbriae zijn fijne draadvormige structuren aan de oppervlakte van de *E. coli* bacteriën. Hiermee kunnen ze zich in de dunne darm vasthechten aan receptoren ter hoogte van de borstelzoom van villusenterocyten, waarna kolonisatie van de dunne darmmucosa kan optreden. Een tweede virulentiekenmerk van ETEC zijn thermolabiele en/of thermostabiele enterotoxinen door de ETEC geproduceerd. Het zijn deze enterotoxinen die diarree veroorzaken doordat ze in de dunne darm de secretie van water en electrolieten stimuleren en/of de absorptie verminderen.

Ofschoon ETEC een belangrijke rol spelen in het speendiarree-complex resulteert een inoculatie van biggen op speenleeftijd met ETEC niet steeds in diarree. Er zijn predisposerende factoren nodig zoals een wijziging in de voeding van nat (melk) naar droog voeder, de aanwezigheid van vreemde antigenen (voedereiwitten) in het droge voeder en een infectie met rotavirus of andere enteropathogene microorganismen. Verschillende van deze factoren veroorzaken een verkorting van de dunne darmvilli. Deze villusatrofie zou de kolonisatie van de villi met ETEC in de hand werken.

Daarenboven veroorzaakt villusatrofie een vermindering van de absorberende oppervlak in de dunne darm wat resulteert in een verminderde absorptie van voedingsstoffen (malabsorptie).

Aangezien speendiarree pathofysiologisch kan gezien worden als een combinatie van een intestinale malabsorptie (villusatrofie) en een hypersecretie (ETEC) zou behandeling kunnen gebeuren door de absorptie van water en electrolieten ter hoogte van de darmmucosa te stimuleren en/of de secretie te remmen. Antisecretorische farmaca zouden deze effecten hebben. Een antisecretorische therapie zou daardoor tot een directe remming van het vochtverlies ter hoogte van de darm kunnen leiden. Daarenboven zou door stimulatie van de absorptie van water en electrolieten het effect van een ingestelde vochttherapie kunnen worden versneld. Dergelijke therapeutica zouden bovendien het voordeel hebben dat ETEC niet worden geïnhibeerd in hun groei zodat de biggen een actieve immuniteit kunnen opbouwen en beschermd zijn tegenover een infectie met dezelfde fimbriae en/of toxinen producerende ETEC stammen.

Om het effect van antisecretorische farmaca te kunnen nagaan op speendiarree moest een reproduceerbaar speendiarree model worden ontwikkeld dat toeliet klinische, pathologische, hematologische, biochemische en hemodynamische veranderingen te bestuderen tijdens diarree bij pasgespeende biggen zodat het effect van antisecretorische farmaca op sommige van deze parameters kon worden geëvalueerd. In dit speendiarree model werden pasgespeende biggen geïnoculeerd met een combinatie van transmissible gastroenteritis virus (TGEV) en K88ac$^+$ ETEC. Dus het model combineerde hoogstwaarschijnlijk malabsorptie ten gevolge van villusatrofie veroorzaakt door het enteropathogene TGEV met een infectie met secretie-inducerende ETEC.

Ofschoon ETEC stammen geïsoleerd bij biggen met colibacillaire diarree, K88ab, K88ac, K88ad, K99, P987, F41, K99 of een combinatie van sommige van deze fimbrae kunnen produceren, worden bij speendiarree bijna uitsluitend K88ac fimbriae positieve ETEC geïsoleerd. Uitzonderlijk worden K88ab$^+$, K88ad$^+$, K99$^+$ of P987$^+$ *E. coli* geïsoleerd. Daarom werd de adhesie nagegaan van verschillende fimbriae producerende *E. coli* stammen aan dunne darmvilli van pasgespeende biggen, gebruikmakende van een *in vitro* adhesietest. De aanwezigheid van receptoren voor K88$^+$ *E. coli* ter hoogte van de villus enterocyten is genetisch bepaald. *In vivo* kan de aanwezigheid van deze receptoren enkel aangetoond worden gebruikmakende van chirurgisch verzamelde dunne darmmucosa en een *in vitro* adhesietest. Er werd daarom naar een test gezocht die gemakkelijk kan worden uitgevoerd bij levende varkens. Tevens werd het voorkomen van varkens met K88 receptoren onderzocht. Immers indien een groot deel van de Belgische varkens geen receptoren voor K88 fimbriae bezitten, dan zou het interessant kunnen zijn foktechnisch in te grijpen door te selecteren naar K88 negatieve varkens.

Benevens ETEC speelt villusatrofie een belangrijke rol bij speendiarree. In dit proefschrif werd de hypothese getoetst dat er door villusatrofie veranderingen zouden ontstaan ter hoogte van de borstelzoom van villusenterocyten die resulteren in een toename van het aantal bindingsplaatsen voor ETEC.

In het *derde hoofdstuk* van het proefschrift worden de experimentele studies beschreven. In deel III.A van dit hoofdstuk wordt de *in vitro* adhesie van ETEC aan de dunne darmvilli van 4 tot 5 weken oude biggen beschreven. In deel III.B worden studies in verband met een experimenteel

diarree model beschreven. In deel III.C. wordt het effect van antisecretorische farmaca in dit model beschreven.

Bij biggen met colibacillaire diarree worden K88ab, K88ac, K88ad, P987, K99 of F41 of fimbriae positieve ETEC stammen geïsoleerd. Er werd kwantitatief nagegaan welk van deze fimbriae het meest kunnen adheren aan de villi van 4 tot 5 weken oude biggen (deel III.A.2). Dit werd nagegaan op villi van 33 biggen gebruikmakende van een *in vitro* villus adhesietest. Het aantal bacteriën dat adheerde aan de darmvilli was meestal het hoogst voor K88ab$^+$ en K88ac$^+$ *E. coli* (55 ± 5 tot 81± 4 *E. coli* per 250 µm villuslengte) en nam af in de volgende volgorde: K88ad > P987 > K99/F41 > K99 > F41(2 ± 1 tot 4 ± 1 E. coli per 250 µm villuslengte). Aangezien er significant meer receptoren voor K88ab$^+$ en K88ac$^+$ETEC stammen aanwezig zijn ter hoogte van de borstelzoom van villus-enterocyten van 4 tot 5 weken oude biggen, dan voor de andere fimbriae zijn deze beide het meest aangewezen zijn om te gebruiken in een speendiarree model. Omdat in België vooral K88ac$^+$ ETEC stammen worden geïsoleerd bij biggen met colibacillaire diarree, werden deze gekozen om te gebruiken in inoculatie-experimenten. Bij sommige biggen was er slechts een zwakke of geen adhesie van één of meer K88 variant$^+$ ETEC aan hun villi. Net zoals in varkenspopulaties in andere landen komen in België dus meedere adhesieve phenotypes voor bij biggen. Aan- of afwezigheid van receptoren voor K88$^+$ ETEC is genetisch bepaald.

Het feit dat biggen resistent kunnen zijn aan adhesie van K88ac$^+$ ETEC stammen, maakt dat in inoculatie-experimenten de aanwezigheid van K88-receptoren steeds moet bepaald worden. Dit kan *in vitro* worden gedaan op villi die post mortem worden afgeschraapt van de darmmucosa. Dezelfde test kan echter ook bij levende biggen worden uitgevoerd op villi afgeschraapt van stukjes darm die operatief door middel van een darmbiopsie of een darmresectie worden verkregen. Een operatieve ingreep is echter moeilijk op grote schaal uit te voeren. Daarom werd onderzocht of de *in vitro* villus adhesietest niet kon vervangen worden door een *in vitro* adhesietest aan epitheelcellen van de wangmucosa of door een hemagglutinatietest (deel III.A.3). Zowel wangepitheelcellen als rode bloedcellen kunnen gemakkelijk verzameld worden bij levende biggen. Er kon echter geen verband worden aangetoond tussen adhesie van de K88$^+$ bacteriën aan darmvilli en adhesie aan wangepitheelcellen of tussen adhesie aan villi en hemagglutinatie.

In deel III.A.4. worden de resultaten weergegeven van de *in vitro* adhesie van K88ab$^+$, K88ac$^+$ en K88ad$^+$ *E. coli* aan dunne darmvilli van 140, 4 tot 5 weken oude biggen, afkomstig van één Kempisch fokbedrijf. Adhesie van de 3 K88 varianten (K88ab, K88ac en K88ad) werd gezien bij 80,7 % van de biggen, adhesie van 2 varianten (K88ab en K88ac of K88ab en K88ad) bij 12,1 %, adhesie van 1 variant (K88ad) bij 3,6 % en geen adhesie van K88$^+$ *E. coli* bij 3,6 % van de biggen. Biggen waren afkomstig uit kruisingen van 43 eerstelingszeugen en 15 verschillende beren. Het waren kruisingen van raszuivere Belgische Landvarkens of van Landvarken en Piétrain zodat mag aangenomen worden dat gevoeligheid voor adhesie van K88$^+$ ETEC sterk verspreid is onder beide Belgische rassen.

Deel III.B.2. is gewijd aan het op punt stellen van een reproduceerbaar diarree model in 3 tot 4 weken oude pasgespeende biggen. Eerst werd geprobeerd diarree en deshydratatie te induceren door de biggen farmaca of toxines toe te dienen die de secretie ter hoogte van de dunne darm

stimuleren nl.één of tweemaal LT-enterotoxine, cholera toxine, ricinusolie, DL-5-hydroxytryptofaan (een precursor van serotonine), of 2-maal daags gedurende 3 opeenvolgende dagen een combinatie van ricinusolie en DL-5-hydroxytryptofaan. Dit resulteerde in een milde diarree zonder deshydratatie. Waarschijnlijk moeten secretie-stimulerende farmaca of toxinen continu toegediend worden om erge diarree met deshydratatie te induceren zoals gebeurt tijdens colibacillaire diarree waar de ETEC voortdurend enterotoxinen vrijstellen. Daarom werd geprobeerd colibacillaire diarree te reproduceren bij gespeende biggen door ze te inoculeren met K88ac$^+$ ETEC. Biggen werden op het einde van het experiment steeds getest voor aanwezigheid van K88ac-receptoren. Indien deze receptoren niet aanwezig waren werden de biggen niet in de resultaten opgenomen. Noch inoculatie van 3 tot 4 weken oude biggen met K88ac$^+$ ETEC, noch een 3 dagen durende voorbehandeling met chloramphenicol (onderdrukken van de kolonisatie-resistentie in de dunne darm) gevolgd door een ETEC-inoculatie induceerde een reproduceerbare diarree en deshydratatie. Wanneer biggen daarentegen (n=21) na de chloramphenicol behandeling, op dag 4 werden geïnoculeerd met het transmissible gastroenteritis virus (TGEV) en op dag 5 met 2 ETEC stammen (beide O149:K91:K88ac;LT:STa:STb), dan trad er bij 93 % van de biggen zeer erge diarree, deshydratatie en gewichtsverlies op resulterend in sterfte van 80 % van de biggen. Om een reproduceerbare diarree te kunnen induceren bij pasgespeende biggen zijn dus meerdere factoren nodig. De ergheid van de diarree en de graad van diarree neemt toe naarmate meer factoren aan het diarree model worden toegevoegd.

Het werd gepostuleerd dat de villusatrofie die wordt veroorzaakt door een rotavirus infectie misschien aanleiding zou geven tot veranderingen ter hoogte van de borstelzoom van de villusenterocyten, waardoor meer bindingsplaatsen zouden kunnen ter beschikking komen voor ETEC. In ons experimenteel speendiarree model werd nagegaan wat het effect was van TGEV-geïnduceerde villusatrofie op adhesie van K88ac$^+$ *E. coli* (deel III.B.3). Hiertoe werd de *in vitro* villus adhesie-test uitgevoerd op villi van biggen die op verschillende tijdstippen na de TGEV-inoculatie geëuthanasieerd waren. De resultaten werden vergeleken met deze van de *in vitro* adhesie aan villi van niet-geïnoculeerde biggen. De resultaten tonen aan dat er significant minder bacteriën kunnen adheren aan de sterk atrofische villi verkregen van biggen geëuthanasieerd 2 tot 3 dagen na de TGEV-inoculatie dan aan villi van niet-geïnfecteerde biggen of aan villi die terug volledig geregenereerd zij na de infectie (7 dagen na de TGEV-inoculatie). De resultaten van deze studie konden de hypothese niet ondersteunen dat villusatrofie zou gepaard gaan met een toegenomen adhesie van ETEC.

Er stelde zich de vraag wat er *in vivo* in de darm gebeurde tijdens de TGEV-ETEC infectie. Daarom werden de morfologische en ultrastructurele veranderingen ter hoogte van de dunne darmmucosa in het diarree model bestudeerd met de electronenmicroscoop (deel III.B.4). Deze studie liet toe een inzicht te verkrijgen in het mechanisme van de geïnduceerde diarree. De met TGEV en ETEC geïnoculeerde biggen werden geëuthanaseerd op het ogenblik dat erge deshydratie optrad (tussen 1 en 5 dagen na de TGEV-inoculatie) en er niet meer werd verwacht dat de biggen zouden herstellen, ofwel werden ze 7 dagen na de TGEV-inoculatie (6 dagen na de ETEC inoculatie) geëuthanasieerd. Bij biggen met zeer erge diarree en deshydratatie (n=11) werd met scanning electronenmicroscopie (SEM) sterke villusatrofie, versmelting van villi en kolonisatie van de

zijkanten en basis van de verkorte villi met coliforme bacteriën gezien. Al deze biggen excreteerden de geïnoculeerde ETEC in hun faeces. Met transmissie electronenmicroscopie (TEM) werden in het duodenum bij 5 van de 11 hypovolemische biggen gedegenereerde epitheelcellen gezien. Deze celdegeneratie was niet gecorreleerd met de aanwezigheid van coliforme bacteriën op of van virus in deze cellen. Bij de biggen waarbij de diarree veel minder erg was en die geëuthanasieerd werden 7 dagen na de TGEV-inoculatie (n=4) waren de villi evenlang als bij niet-geïnfecteerde biggen. Slechts bij één van deze biggen werden met SEM enkele coliforme bacteriën gezien op de villi. Met TEM werd geen degeneratie van epitheelcellen gezien. De bevindingen van deze studie tonen aan dat de experimentele TGEV-ETEC infectie een erge diarree induceert die gedeeltelijk het gevolg is van een malabsorptie door TGEV veroorzaakt. Bij biggen met zeer erge deshydratatie wordt een kolonizatie van de villi met ETEC gezien, suggererend dat er bij deze biggen een verhoogd verlies is van water en electrolieten door de ETEC-kolonizatie. De degeneratie van enterocyten die gezien wordt bij de sterk gedeshydrateerde biggen is niet direct veroorzaakt door de infectie. Erge deshydratatie resulteert in een bloeddrukdaling waardoor een verminderde zuurstof aanvoer ter hoogte van darmvilli kan ontstaan. Deze hypoxie kan in combinatie met intraluminale stoffen (galzouten en pancreasenzymen) aanleiding geven tot celdegeneratie. Deze degeneratie verergert de malabsorptie en versnelt het optreden van hypovolemische shock en vervolgens van sterfte.

De pathofysiologie van de diarree in dit model werd eveneens bestudeerd met behulp van klinische, hematologische, biochemische en hemodynamische parameters (deel III.B.5). Bij de biggen werd voorafgaand aan de experimentele inoculatie een catheter geïmplanteerd in de linker a. carotis. Hierlangs werden dagelijks bloedstalen verzameld en werd de arteriële bloeddruk gemeten. De gecombineerde infectie induceerde diarree bij alle biggen (n=21). Braken werd bij 65 % van de biggen vastgesteld. Diarree en braken resulteerden in een significant gewichtsverlies, een niet-significante stijging van de hematocriet (Hct), significante stijgingen van de totale plasma proteïnconcentratie en van het hartritme, in een metabole acidose (daling van pH, terwijl pO_2, pCO_2 en ademhalingsritme niet significant veranderden) en in een significante bloeddrukdaling. Wanneer de Hct een waarde van ongeveer 42 % bereikte, verslechterde de toestand van de biggen zeer snel en trad er shock en sterfte (71 %) op. Bij biggen die shock ontwikkelden werden ook een significante stijging van Hct, en significante dalingen van HCO_3^-, base excess en van de lichaamstemperatuur geregistreerd. Ondanks de erge diarree werden geen veranderingen van kalium-, chloor- en calcium-concentraties in het bloed vastgesteld, terwijl er wel een tijdelijke stijging werd gezien van de natriumconcentratie. Bij overlevende biggen (29 %) werden behalve een stijging van de lichaamstemperatuur geen significante veranderingen waargenomen.

In deel III.C.2 werd het effect van antisecretorische farmaca nagegaan bij biggen waarbij geen catheter in de linker a. carotis was geïmplanteerd. Er werd geopteerd voor farmaca die op verschillende wijze inwerken op de secretie- en absorptiemechanismen van darmenterocyten. Er werden dagelijks slechts een beperkt aantal parameters geregistreerd nl. ergheid en duur van de diarree, braken, gewichtsverandering, voedselopname, lichaamstemperatuur en sterfte. Zo kon snel een aantal farmaca getest worden in ons model. Biggen werden na 3 dagen chloramphenicol voorbehandeling geïnoculeerd met TGEV op dag 4 en vervolgens met ETEC op dag 5. Vanaf 5 uur

na de ETEC inoculatie werden de volgende antisecretorische farmaca intramusculair toegediend gedurende 3 opeenvolgende dagen: chlorpromazine (1 of 2 mg/kg/24 h), verapamil (2 mg/kg/8 h), clonidine (0.04 of 0.08 mg/kg/12 h) of propranolol (0.33 mg/kg/12 h). Chlorpromazine is een neurolepticum dat de intestinale secretie zou remmen door zijn membraanstabilizerende eigenschappen en door een inhibitie van calmoduline. Verapamil remt intestinale secretie door inhibitie van de calcium influx in de cel (calcium-channel blokker). Clonidine is een α_2-adrenoreceptor agonist en propranolol een ß-adrenoreceptor antagonist die via verschillende receptoren het adenylaatcyclase enzym complex inhiberen en zo het secretiemechanisme remmen. Verapamil en propranolol induceerden geen significante verbeteringen, terwijl chlorpromazine en clonidine in de hoogst toegediende dosis wel gunstige effecten hadden. Chlorpromazine behandeling had een antiemetische werking (volledige remming van het braken), verminderde de diarree significant en resulteerde in een toegenomen overleving. Clonidine verminderde de diarree en verhoogde de overleving significant. Beide producten vertoonden echter ook nevenwerkingen. Clonidine verminderde de activiteit van de biggen en enkele biggen vertoonden excitatie en incoördinatie. Chlorpromazine sedeerde de biggen en induceerde een daling van de lichaamstemperatuur.

In deel III.C.3 werd het effect van clonidine bestudeerd bij gecatheterizeerde biggen (n=6) waarbij experimenteel diarree werd geïnduceerd. Clonidine werd intramusculair toegediend aan de dosis (0.08 mg/kg/12h gedurende 3 dagen) die bij de niet-gecatheterizeerde biggen een duidelijk therapeutisch effect had. Ook hier werden de eerder waargenomen gunstige effecten, nl. verminderde diarree en toegenomen overleving, niet meer gezien en waren de neveneffecten veel duidelijker. Clonidine veroorzaakte bij biggen met erge deshydratatie steeds een sterk uitgesproken excitatie en incoördinatie, een significante daling van de arteriële bloeddruk en van het hartritme. Deze systemische effecten waren verantwoordelijk voor de sterfte van sommige biggen. Clonidine heeft een hypotensieve werking die een gevolg is van een stimulatie van presynaptische α_2-adrenoceptoren in het centraal zenuwstelsel. De uitgesproken deshydratatie bij de gecatheterizeerde biggen maakt ze hoogstwaarschijnlijk gevoeliger voor de hypotensieve werking van clonidine.

Het effect van chlorpromazine werd in deel III.C.4 bestudeerd bij biggen (n=8) waarbij een catheter werd geïmplanteerd in de linker a. carotis. De behandeling gebeurde zoals in het vorige experiment aan de dosis die een duidelijk therapeutisch effect had (intramusculair 2 mg/kg24 h gedurende 3 dagen). De gunstige resultaten die werden gezien bij behandeling van niet-gecatheterizeerde biggen, werden niet meer of minder duidelijk gezien bij de gecatheterizeerde biggen. Er was geen toegenomen overleving meer en er was nog wel een antiemetisch effect, maar dit was minder uitgesproken. Daarentegen waren er duidelijke neveneffecten. Chlorpromazine behandeling veroorzaakte sedatie, een significante bloeddrukdaling en een stijging van het hartritme. Chlorpromazine heeft een perifeer vasodilaterende werking. Indien biggen slechts licht gedeshydrateerd zijn kan een geringe perifere vasodilatatie gunstig zijn. Bij erg vochtverlies is vasodilatatie nadelig en zal dit het optreden van shock versnellen.

In deel III.C.5 werd het effect beschreven van een intramusculaire toediening van flurbiprofen (1 mg/kg/12 h) aan biggen (n= 8) die geïnoculeerd waren met TGEV en ETEC. Bij deze biggen was een catheter geïmplanteerd in de a. carotis. Flurbiprofen is een niet-steroid

antiinflammatoir farmacon dat de prostaglandine-vorming remt en dat op die manier een antisecretorisch effect zou kunnen uitoefenen. Enkel de eerste dag van de behandeling werden gunstige veranderingen gezien ten opzichte van de controle groep. Er was minder diarree en significant minder excretie van $K88^+$ *E. coli*. Daarenboven induceerde flurbiprofen een leucopenie en had het een antipyretische werking. Deze effecten waren niet meer aanwezig tijdens de 2de behandelingsdag en de flurbiprofen behandeling had dan ook geen gunstig effect op de diarree, deshydratatie, gewichtsverlies en sterfte. Flurbiprofen behandeling ging gepaard met een verminderde activiteit van de biggen. Er mag geconcludeerd worden dat flurbiprofen aan de geteste dosis niet kan gebruikt worden voor behandeling van een gecombineerde malabsorptie en hypersecretie diarree.

Zowel in de controle groep als in de behandelingsgroepen vertoonden gecatheterizeerde biggen meer diarree en sterfte dan niet gecatheterizeerde biggen. Hoogstwaarschijnlijk verhoogde de operatie en het dagelijks manipuleren (bloedname, bloeddrukmeting) de gevoeligheid van de biggen voor de experimentele infectie, resulterend in een meer uitgesproken deshydratatie.

De resultaten van de behandelingen met antisecretorische farmaca tonen aan dat chlorpromazine en clonidine de diarree significant kunnen verbeteren, doch dat beide producten systemische effecten bezitten die onder bepaalde omstandigheden nadelig kunnen zijn, nl. bij biggen met een uitgesproken deshydratatie. De resultaten van deze studies indiceren tevens dat antisecretorische farmaca waarvan zal blijken dat ze vooral ter hoogte van de darm aktief zijn, belangrijke therapeutica kunnen worden bij de behandeling van secretorische maar ook van een gemengd secretorische-malabsorptie diarree, zoals voorkomt na het spenen bij biggen.

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DANKWOORD

Een proefschrift is het werk van vele mensen. Bij het beëindigen ervan wens ik dan ook mijn dank te betuigen aan ieder die heeft bijgedragen in het tot stand komen ervan.

Mijn dank gaat in de eerste plaats naar mijn promotor Prof. Dr. Haesebrouck en copromotor Prof. Dr. A. Houvenaghel. Prof. Haesebrouck dank ik omdat hij het promotorschap heeft willen aanvaarden en voor zijn begeleiding tijdens het schrijven van mijn proefschrift. Prof. Houvenaghel, in uw labo is dit werk begonnen. Dank zij uw inspiratie, inzet en wetenschappelijke begeleiding kwam dit proefschrift tot stand. Ik dank U daarvoor en voor het feit dat U steeds tijd vond om mij raad te geven.

Tevens ben ik de leden van de leescommissie erkentelijk voor de beoordeling van dit werk.

Naar Prof. Pensaert gaat mijn dank uit voor het verkrijgen van het TGE virus, voor zijn raad en voor zijn steun bij het voltooien van dit proefschrift.

Voorts dank ik mijn collega's van het Laboratorium voor Fysiologie van de Huisdieren te Antwerpen, Dr. V. Cools, Dr. E. Schrauwen en Dr. L. Wechsung, voor hun hulp en suggesties bij het uitvoeren van de proeven. Mijn collega's Dr. P. Callebaut, Dr. K. De Smet, Dr. H. Nauwynck en Dr. K. Van Deun van het laboratorium voor Virologie en Immunologie van de Huisdieren dank ik omdat ze bereid waren te helpen bij het uitvoeren van het diagnostische werk op de ogenblikken dat het schrijven van dit proefschrift mij aan mijn bureau kluisterde.

Prof. Dr. J. Hoorens, Dr. H. Thoonen, Dr. G. Charlier dank ik voor hun enthousiaste medewerking bij sommige studies uit dit proefschrift. Dr. G. Charlier dank ik tevens voor de prachtige electronenmicroscopische opnamen.

Voorts dank ik Dr. I.G.W. Bijlsma, Dr. P. Deprez, Dr. J.G. Mainil, Dr. L. Ockerman, Dr. L. Ooms, Dr. P. Pohl en Dr. A. Weyns voor hun raad en hulp.

Bij het uitvoeren van de experimenten en het typen van de teksten werd deskundige hulp verleend door de Heer J. Bollé, Mevr. M. Carpentier, Mevr. C. De Schepper-Van Hoecke en Mevr. R. Hens-Sleeckx, waarvoor mijn oprechte dank.

Verscheidene van de gebruikte substanties werden geschonken door The Boots Company (flurbiprofen), Smith-Kline-RIT (LT-enterotoxin en GIS 25), Rhone-Poulenc (chlorpromazine), Boehringer Ingelheim (clonidine), ICI-Pharma (propranolol) and Knoll AG (verapamil).

Dit werk werd financieel gesteund door het Instituut voor Wetenschappelijk Onderzoek in Nijverheid en Landbouw (IWONL, Brussel).

Voorts wil ik Janssen Pharmaceutica bedanken voor het copiëren en inbinden van het proefschrift.