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# Interactions of virulent and avirulent *Yersinia ruckeri* strains with isolated gill arches and intestinal explants of rainbow trout *Oncorhynchus mykiss*

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ABSTRACT: *Yersinia ruckeri* is the causative agent of enteric redmouth disease leading to significant losses in salmonid aquaculture worldwide. Little information is available on the pathogenesis of this disease. Basic steps in the establishment of an infection include attachment to the epithelium followed by invasion at the portal of entry. In this study, the interactions of *Y. ruckeri* with the gills and the gut of rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792) were studied using standardized perfusion models. Virulent and avirulent *Y. ruckeri* isolates appeared to adhere to and invade both tissues without significant differences. For the first time, the gill and gut perfusion models are shown to be suitable to study bacterial invasiveness.

KEY WORDS: *Yersinia ruckeri* · Adhesion · Invasion · Gill · Gut



## INTRODUCTION

*Yersinia ruckeri* is the causative agent of enteric redmouth disease (ERM) and causes significant losses in salmonid aquaculture worldwide. Despite the importance of ERM, the precise pathogenic mechanisms of *Y. ruckeri* are not well understood. A comprehensive understanding of the pathobiology of the infection is required for the rational design of vaccines and other preventive measures to combat ERM (Haesebrouck et al. 2004). Basic steps in the establishment of an infection include attachment to the epithelium followed by invasion at the portal of entry. Both the gills and intestinal tract have been suggested as possible routes of entry for *Y. ruckeri* (Busch & Lingg 1975, Valtonen et al. 1992, Tobback et al. 2009). Therefore, the interactions of *Y. ruckeri* with the gills and the gut were studied using standardized perfusion models. Virulent and avirulent strains were included to determine whether differences in virulence are related to differences in adherence and invasive capacity.

## MATERIALS AND METHODS

**Bacterial strains.** The *Yersinia ruckeri* isolates used in the present study are listed in Table 1. Stock suspensions were stored at –70°C. After thawing, the bacteria were grown overnight at 20°C on Colombia agar (Oxoid) with 5% sheep blood (blood agar). Colonies were picked from the agar plates and grown in nutrient broth (NB; VWR International) for 24 h at 20°C. A non-invasive *Escherichia coli* strain (DH5α) was included as a negative control in adhesion and invasion experiments (Parthasarathy & Mansfield 2009). This strain was cultured in NB for 24 h at 37°C. The number of colony forming units (CFU) per ml was determined by plating tenfold serial dilutions on blood agar plates.

**Fish.** Twenty-two rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792) of 600 g average weight were obtained from a fish farm (Gérouville, Belgium) with no history of ERM. The fish were maintained in 1000 l tanks in filtered, recirculated tap water. They were fed

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Table 1. *Yersinia ruckeri* strains. Virulence and mortality (no. of dead fish/total no. of fish) were determined in an immersion infection model using juvenile rainbow trout (Tobback et al. 2009). Avirulent: no mortality or persistent infection observed (according to Davies 1991). ERM: enteric redmouth disease. NA: no information available

| *Y. ruckeri* strain | Origin | Biotype | Serotype | Virulence | Mortality |
|---|---|---|---|---|---|
| 5 | *Oncorhynchus mykiss* with ERM, UK, 2001 | 2 | O1a | Virulent | 6/20 |
| 9 | *O. mykiss* with ERM, UK, 1995 | 2 | O1a | Virulent | 4/20 |
| E842-95 | NA | 2 | O1b | Avirulent | 0/20 |
| 17.00(2-1) | *O. mykiss* | 2 | O1a | Avirulent | 0/20 |
| CCUG 14190 | *O. mykiss* with ERM, USA | 2 | O1a | Avirulent | 0/20 |

daily with a commercial diet (Vijver Visvoeder) until 2 d before the start of the experiments. Swabs were taken from the skin, fins and gills of 2 fish, chosen at random, for microscopic evaluation of external parasites upon arrival. The fish were found to be free of external parasitic infestations.

**Gill perfusion model.** The gill perfusion model was used as described by Decostere et al. (2002). Briefly, the fish were euthanized and the first 2 or 3 gill arches on both sides were excised. The smaller dorsal part of each gill arch was cut off to remove the nod to prevent from jeopardizing an efficient perfusion. Thereafter, the afferent (ventral) and efferent (dorsal) arteries of the gill arches were cannulated. Each gill arch was suspended separately in an aerated organ bath surrounded by a water jacket at 20°C and perfused with Cortland solution + dextran 1% (MW 500 000, Sigma) (Decostere et al. 2002). Gill arches were incubated for 1 h at 20°C with a suspension of ~$10^8$ CFU $ml^{-1}$. A non-inoculated perfused gill arch served as negative control. Thereafter, each gill arch was washed in an excess of phosphate buffered saline (PBS) and cut in half. To study the adhesion capacity, a randomly selected sample was homogenized and the number of CFU $g^{-1}$ gill tissue was determined by plating tenfold serial dilutions of the suspensions on MacConkey agar (Oxoid) plates. A second gill sample was fixed in Carnoy's solution for at least 2 h, dehydrated in an alcohol-xylene series, embedded in paraffin wax and further processed for histological and immunohistochemical (IHC) analysis (Tobback et al. 2009). Sections of 5 µm, mounted on glass slides, were stained with haematoxylin and eosin (H&E) and Giemsa. For immunohistochemistry, 5 µm sections on SuperFrost slides (Sigma-Aldrich) were pretreated by the antigen retrieval technique. Thereafter, slides were incubated with 3% $H_2O_2$ in methanol to block endogenous peroxidase and were washed once with PBS. Slides then underwent sequential application of 30% goat serum, primary rabbit anti-*Yersinia ruckeri* 1/800 antibody (Tobback et al. 2009), biotinylated goat anti-rabbit antibody 1/500 (DakoCytomation), StreptABComplex/horseradish peroxidase (DakoCytomation) and finally 3,3′-diamino benzidine tetrahydrochloride (Sigma-Aldrich), with a wash step in between each application. Sections were counterstained by use of an aqueous-based haematoxylin staining and mounted.

To study the invasion capacity, the perfusion fluid at the efferent artery was collected during infection. The number of *Yersinia ruckeri* or *Escherichia coli* present in the collected fluid was determined by plating tenfold serial dilutions on MacConkey agar plates.

**Gut perfusion model.** The gut perfusion model was used as described by Nematollahi et al. (2005). Briefly, the fish were euthanized, the aorta intestinalis ventralis was cannulated after the pyloric caeca and the cranial and caudal ends were severed. Subsequently, the gut was flushed with 10 ml PBS and a ligature was tied around both the cranial and caudal end. The gut was suspended in an aerated organ bath surrounded by a 20°C water jacket and perfused with Cortland solution + dextran 1% (Nematollahi et al. 2005). For inoculation, ~$10^8$ CFU in 0.4 ml PBS were injected inside the lumen of the ligated gut. A gut injected with 0.4 ml PBS served as negative control. The intestines were incubated for 1 h at 20°C and subsequently flushed with 10 ml PBS after removing the ligatures and cut into 3 equal sized pieces. To study the adherence capacity, the proximal, middle and distal gut sections were processed for bacteriological, histological and IHC examination as described for the gill perfusion model.

To determine the invasion capacity, the organ bath fluid was collected following perfusion. The fluid was centrifuged at 1800 × *g* for 10 min, the pellet was resuspended in distilled water and the number of *Yersinia ruckeri* or *Escherichia coli* was determined by plating ten-fold serial dilutions on MacConkey agar plates.

The gill and gut perfusion models were approved by the ethical committee of the Faculty of Veterinary Medicine, Ghent University (EC 2008/117). The experiments were repeated 3 times.

**Statistical analysis.** Differences in adherence and invasion capacity between the 5 *Yersinia ruckeri*

strains were compared for the gill and gut tissue using a one-way ANOVA test. For each strain, differences in adhesion to and invasion of both tissues were examined using the independent-samples t-test. A significance level of 0.05 was accepted.

## RESULTS

### Gill perfusion model

The 5 *Yersinia ruckeri* strains highly adhered to the gill tissue and significant differences were not observed, whereas *Escherichia coli* DH5α showed lower adherence capacity (Table 2). The number of bacterial cells ($\log_{10}$ CFU $ml^{-1}$) in the perfusion fluid was significantly ($p < 0.05$) lower for *Y. ruckeri* CCUG 14190 compared to the virulent strains 5 and 9. *E. coli* DH5α was not recovered from the perfusion fluid.

Histopathological changes were not observed in the gill arches immediately after immersion with any of the 5 *Yersinia ruckeri* strains. Numerous bacteria were found in the mucus of the primary and secondary lamellae after Giemsa and IHC staining of *Y. ruckeri* inoculated gill arches, independent of the strain used for infection. Moreover, these sections often showed bacteria closely associated with the gill epithelium (Fig. 1a) and some invaded the lamina propria (Fig. 1b).

### Gut perfusion model

The 5 *Yersinia ruckeri* strains highly adhered to the gut tissue and significant differences were not observed, whereas *Escherichia coli* DH5α showed lower adhesion (Table 2). The number of bacterial cells ($\log_{10}$ CFU $ml^{-1}$) in the organ bath fluid was lower for strains 17.00(2-1) and CCUG 14190 compared to the other *Y. ruckeri* strains; however, this was not significant ($p > 0.05$). *E. coli* DH5α was not recovered from the organ bath fluid.

Histopathological changes were not observed in the gut segments immediately after infection with any of the 5 *Yersinia ruckeri* strains. IHC staining revealed the presence of *Y. ruckeri* bacteria in the crypts, attached to the villi and within the epithelial layer of gut segments, independent of the strain used for infection (Fig. 2). Few bacteria were also observed in the lamina propria mucosae.

Adhesion to and invasion in gill tissue compared to gut tissue was not significantly different for any *Yersina ruckeri* strain ($p > 0.05$).

## DISCUSSION

This is the first time that both perfusion models were used to study bacterial invasiveness. Only *Yersina ruckeri* strains and not the non-invasive *Escherichia coli* strain were isolated from the perfusion fluid and organ bath fluid in the gill and gut perfusion model, respectively. These findings demonstrate that both perfusion models are suitable to study bacterial invasion.

Using the gill perfusion model, IHC examination revealed the presence of numerous *Yersina ruckeri* adhered to the gill mucus and epithelium and within the lamina propria. Bacteria were also isolated from the perfusion fluid, indicating that they invaded the gill tissue. This is in agreement with previous *in vivo* studies, in which rainbow trout were experimentally infected (Tobback et al. 2009). These findings indicate that the gills are indeed an important portal of entry as has also been reported for other septicaemic fish pathogens such as *Vibrio anguillarum* and *Edwardsiella tarda* (Baudin-Laurencin & Germon 1987, Ling et al. 2001). Gills are highly vascularized, allowing dissemination of the invaded bacteria to other sites of the body.

Table 2. *Oncorhynchus mykiss.* Bacteriological examination results for gill arches and intestines (posterior, middle and anterior gut were pooled) after exposure to *Yersinia ruckeri* or *Escherichia coli* DH5α. Adhesion capacity expressed as number of bacteria (log CFU $g^{-1}$) isolated from gills and intestine. Invasion capacity shown as bacterial titers ($\log_{10}$ CFU $ml^{-1}$) isolated from the perfusion fluid collected at the efferent gill artery and from the organ bath fluid 1 h after infection, in the gill and gut perfusion model, respectively. Data are means ± SE from 3 independent experiments. ND: not detected

| Bacterial strain | Gill perfusion | | Gut perfusion | |
|---|---|---|---|---|
| | Adhesion | Invasion | Adhesion | Invasion |
| *Y. ruckeri* 5 | 6.37 ± 0.38 | 4.37 ± 0.54 | 6.52 ± 0.55 | 3.57 ± 0.37 |
| *Y. ruckeri* 9 | 6.22 ± 0.64 | 5.05 ± 1.03 | 7.58 ± 0.32 | 3.79 ± 0.15 |
| *Y. ruckeri* E842-95 | 6.99 ± 0.54 | 3.49 ± 0.60 | 6.39 ± 0.29 | 3.78 ± 0.37 |
| *Y. ruckeri* 17.00(2-1) | 6.44 ± 0.65 | 2.28 ± 2.02 | 6.69 ± 0.38 | 0.99 ± 1.71 |
| *Y. ruckeri* CCUG 14190 | 5.81 ± 0.34 | 0.77 ± 0.68 | 6.07 ± 0.53 | 1.89 ± 1.74 |
| *E. coli* DH5α | 4.73 | ND | 4.06 | ND |

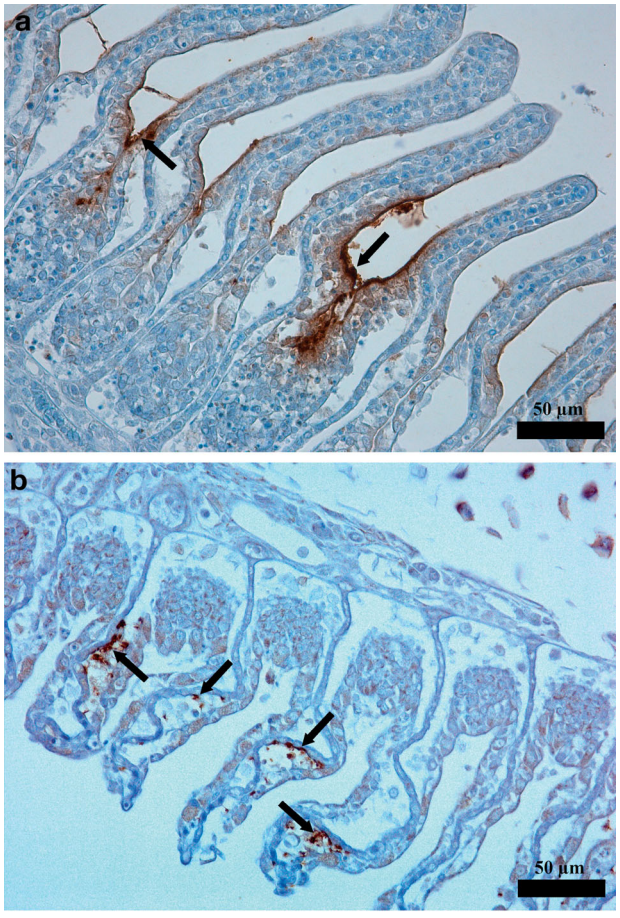


Fig. 1. *Oncorhynchus mykiss*. Gill arch 1 h post challenge with *Yersinia ruckeri* (a) 17.00(2-1) and (b) E842-95. Bacteria either (a) closely associated with the secondary lamellae (arrows) or (b) invaded the lamina propria (arrows). Immunohistochemical staining

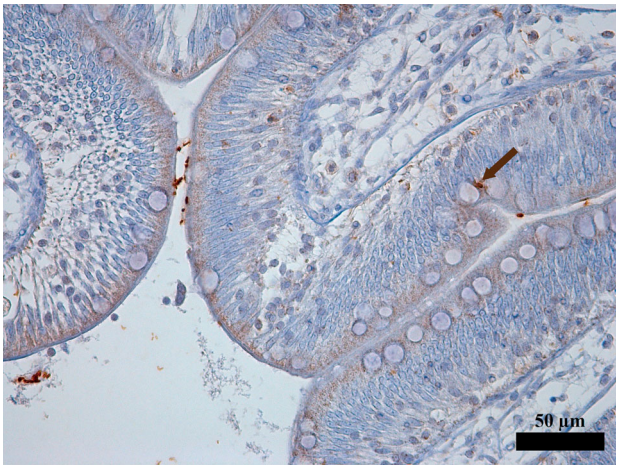


Fig. 2. *Oncorhynchus mykiss*. Gut explant 1 h post challenge with *Yersinia ruckeri* E842-95. Bacteria attached to the mucus and closely associated with the epithelium and within the mucosa (arrow). Immunohistochemical staining

A comparative genomic analysis of different *Yersinia* species by Chen et al. (2010) revealed that the *Y. ruckeri* chromosome does not appear to result from a reductive evolution from the human enteric yersiniae caused by frameshift mutation. Furthermore, *Y. ruckeri* apparently lacks conserved metabolic activity found in other bacteria that colonize the intestinal tract; Chen et al. (2010) concluded that *Y. ruckeri* may use a transmission mode distinct from the enteric pathway. The results of the present and our previous study (Tobback et al. 2009) further support this hypothesis.

Using the gut perfusion model, IHC examination revealed the presence of *Yersinia ruckeri* adhered to the villi and within the mucosa, and the bacteria isolated from the organ bath fluid indicated that they invaded the gut mucosa. Bacterial translocation from the gastrointestinal tract has also been shown for various other fish pathogens including *Vibrio anguillarum* and *V. alginolyticus* (Olsson et al. 1996, Chen et al. 2008).

The ability to adhere or invade gill and gut tissue was not significantly different between virulent and avirulent *Yersinia ruckeri* strains. Likely, other virulence factors such as serum resistance or survival in phagocytes may be important to induce disease (Davies 1991).

Although different perfusion models were applied, both the gill and gut explants were directly exposed to similar numbers of bacteria. Therefore, the results of the gill perfusion model were statistically compared with those of the gut perfusion model. Analysis did not reveal significant differences in adhesion and invasion capacity of any of the *Yersinia ruckeri* strains between gill and gut tissue. This indicates that at least under our experimental conditions both organs are of equal importance in the initial interaction of *Y. ruckeri* with its host. This is in contrast to our previous *in vivo* study where lower numbers of bacteria were isolated from the intestine than from the gills of rainbow trout after bath challenge with *Y. ruckeri* (Tobback et al. 2009). This difference may be due to a different exposure to bacterial cells. Water taken into the mouth of fish is directly forced over the gills, bringing this organ continuously in close contact with the environment. Freshwater fish are reported to drink less than their saltwater counterparts (Bucking & Wood 2006) and, therefore, the gut may possibly be exposed to a lower bacterial number during immersion infection.

In conclusion, *Yersinia ruckeri* was shown to colonize and invade gills and gut tissue of rainbow trout. Significant differences in adherence and invasive capacity were not observed between virulent and avirulent strains.

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