DISK PREDIFFUSION IS A RELIABLE METHOD FOR TESTING COLISTIN SUSCEPTIBILITY IN PORCINE *E. COLI* STRAINS

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ABSTRACT

During the last few years, acquired resistance to colistin in *Escherichia coli*, but also in other bacterial species, has been reported. It has been shown that the disk diffusion test is not a reliable method for the detection of this resistance. Therefore, there is a need for a reliable and cheap test to determine colistin susceptibility of pathogenic *E. coli* strains. In the current research, the colistin susceptibility of *E. coli* isolated during the period 2005-2006 from pigs was determined. Results obtained with the Kirby Bauer disk diffusion test (Neosensitabs, Rosco), the disk prediffusion test (Neosensitabs, Rosco) and the E-test (AB Biodisk) were compared with the results of the reference agar dilution assay. The MIC values or inhibition zones showed a bimodal distribution for the results obtained by all test methods, except disk diffusion assay, suggesting acquired resistance in 15 strains (9.6 %). The E-test and disk prediffusion assay generated results within acceptable levels compared to the reference agar dilution assay. The categorical agreement with the results obtained the agar dilution method were good to very good for all tests, except the disk diffusion assay. In conclusion, current results suggest that, in addition to the E-test, the disk prediffusion test is a reliable, alternative agar-based colistin susceptibility method for testing colistin susceptibility of *E. coli* isolates in diagnostic bacteriology.

INTRODUCTION

Colistin was discovered in 1949 (Koyama et al., 1950) and was later recognized to be identical to polymyxin E. Polymyxins are cationic polypeptides that bind to the anionic bacterial outer membrane, leading to membrane disruption, mainly in Gram negative bacteria. Even though colistin is an old antimicrobial substance, its use in human medicine has augmented the last decade, largely due to the appearance of multidrug resistant *Pseudomonas*, *Klebsiella* and *Acinetobacter* spp. (Pasquale and Tan, 2005; Gupta et al., 2009).

In humans, colistin is often parenterally used or by nebulisation for treating pulmonary and systemic infections. Even though parenteral and intramammary administration occasionally occurs in veterinary medicine, colistin is mainly used in oral preparations. Due to its excellent intrinsic activity against *E. coli*, the low prevalence of acquired resistance and the poor absorption after oral administration, colistin is a frequently used antimicrobial agent for the prevention and treatment of neonatal or weaning-associated *E. coli* infections in food producing animals, including pigs (Chauvin et al., 2002; Timmerman et al., 2006).

Even though acquired resistance to colistin in veterinary *E. coli* strains was seen only occasionally in the past, the last few years, this is becoming more common (Bertschinger et al., 1996; Harada et al., 2005; Wang et al., 2008). Mechanisms of acquired colistin resistance have been described in *E. coli* and, more extensively, in the closely related *Salmonella* Typhimurium (Landman et al., 2008).

The disk diffusion test does not seem to be a reliable method for the detection of colistin resistance in several bacterial species (Lo-Ten-Foe et al., 2007; Galani et al., 2008; Landman et al., 2008). Therefore, there is a need for a reliable, fast and cheap test to check colistin susceptibility of pathogenic *E. coli* isolates in routine diagnostics. The objective of the present study was to determine colistin resistance in *E. coli* isolates from diseased pigs, comparing 3 antimicrobial susceptibility tests with the reference agar dilution assay.

MATERIALS AND METHODS

Collection and characterization of strains

One hundred and fifty seven *E. coli* strains were isolated from independent clinically affected pigs (neonatal or postweaning diarrhoea, oedema disease) that were presented at the Animal Health Care Flanders for necropsy during the period of 2005-2006. Faeces, gut samples or mesenteric lymph nodes were inoculated on Columbia agar supplemented with 5% sheep blood (Oxoid, Basingstoke, United Kingdom) and all plates were incubated aerobically at 37°C for approximately 20 hours. The isolates were identified as *E. coli* by colony morphology and standard biochemical methods (Quinn et al. 1994).

Colistin susceptibility tests

Antimicrobial sensitivity testing was carried out using 4 different techniques; agar dilution method (CLSI, 2008); Kirby Bauer disk diffusion test (Neosensitabs, Rosco); 2 + 18 hours disk prediffusion test (Neosensitabs, Rosco) and E-test (AB Biodisk).

As “golden standard”, all strains were tested for susceptibility to colistin through the agar dilution method (CLSI, 2008). The minimum inhibitory concentration (MIC) was determined as the lowest concentration that inhibited isible growth. The strains were considered to have acquired resistance when their MIC higher than the wild type cut-off value (MIC > 2 µg/ml) as described by EUCAST (2009).

The disk diffusion test was performed with Neosensitabs tablets (150 µg, Rosco, Denmark) according to CLSI-guidelines (CLSI, 2008). Growth inhibition zone diameters were measured manually. Interpretative criteria to determine clinical resistance were based upon clinical breakpoints as described by the manufacturer (www.rosco.dk) (sensitive = 20 mm; intermediate 17-19 mm; resistant = 16 mm).

The 2 + 18 disk prediffusion protocol was executed as follows. The colistin containing tablets (10 µg, Rosco, Denmark) were placed on uninoculated Mueller-Hinton plates. After 2 hours incubation at room temperature the disks were removed. The plates were maintained at room temperature for further 18 hours. Subsequently, the plates were inoculated with the different strains using a 0.5 McFarland inoculum as described for the disk diffusion test and thereafter the plates were incubated aerobically overnight at 35 °C. Growth inhibition zone diameters were measured manually. Interpretative criteria to determine clinical resistance were based upon breakpoints described by the manufacturer (www.rosco.dk) (sensitive = 15 mm; intermediate 11-14 mm; resistant = 10 mm).

The E-test with direct reading of the minimal inhibitory concentration (MIC) was performed according to the manufacturers guidelines (AB Biodisk).

E. coli ATCC 25922 was used as an internal control strain in all performed tests (Jones et al., 2005).

Comparative and statistical analysis

Disk diffusion and disk prediffusion inhibition zones d MIC's determined by the E-test were compared with the MIC's determined by the reference agar dilution assay for each strain. Since no clinical colistin breakpoints for *E. coli* are currently provided by the CLSI, for all comparative analyses, interpretative criteria to determine clinical resistance and susceptibility in the agar dilution assay and E-test were based upon the MIC values corresponding to the zone diameter breakpoints for the disk prediffusion test (sensitive = 2 µg/ml; resistant = 8 µg/ml) according to the manufacturer (www.rosco.dk).

A very major error was defined as strains categorised istant by the reference method (agar dilution), but susceptible by the alternative method. A major error was defined strains categorised susceptible by the agar dilution method, but resistant by the alternative method. The categorical interpretation of intermediate for the alternative method, while susceptible or resistant for the agar dilution method was defined as a minor error. Percentages of very major errors exceeding 1.5%, major errors exceeding 3% and minor errors exceeding 10% were regarded as unacceptable.

Categorical agreement was defined as the percentage of strains showing identica categorical susceptible patterns for both methods. Essential agreement was defined as the percentage of strains showing identical MIC values (+/- 1 $\log_2$) for both methods. Finally, the Pearson product-moment correlation coefficient was calculated to estimate the overall correlation between the results of the agar dilution method and the respective alternative method.

120 RESULTS

121 Colistin susceptibility by the reference method

122 Using the results obtained by the agar dilution method, a clear bimodal
123 distribution of MIC values was observed (Table 1) with 15 strains (9.6 %) located in
124 the resistant cluster of the bimodal distribution. These strains showed a MIC value
125 above the wild type cut-off value for colistin in *E. coli* (MIC > 2 µg/ml; EUCAST,
126 2009), indicating acquired resistance towards colistin. Observed MIC_{50} and MIC_{90}
127 values were 0.5 µg/ml and 2 µg/ml, respectively.

128

129 Comparative analysis of susceptibility tests

130 MICs and inhibition zones for the various test methods are shown in Table 1 and 2. A
131 bimodal distribution was observed for the results obtained by all test methods, except the disk
132 diffusion assay.

133 The results of the comparative analyses are summarized in Table 3. The disk diffusion
134 results showed both a low categorical agreement (46.5 %) and a low correlation (-0.09) with
135 the results obtained by the agar dilution method. In addition, the percentages of very major
136 (1.9 %) and minor errors (49.7 %) exceeded the acceptable levels.

137 The results obtained by E-test and disk prediffusion assay generated percentages of
138 minor, major and very major errors beneath the acceptable levels. The categorical agreement
139 with the results obtained by the agar dilution method very good (96.8 %) for both tests.
140 A good correlation with the results obtained by the agar dilution method both tests (> 0.6)
141 and essential agreement (81.5 %) in case of the E-test were obtained.

DISCUSSION

Our current results show that approximately 10 % of the investigated porcine *E. coli* strains showed acquired resistance towards colistin. Even though this is not the first report of colistin resistance in animal associated *E. coli* strains (Bertschinger et al., 1996; Kijima-Tanaka et al., 2003; Wang et al., 2008), manuscripts reporting percentages of acquired resistance exceeding 5% are rare. The emergence of colistin resistance in *E. coli* strains needs further monitoring. Few studies deal with clinical susceptibility of *E. coli* for colistin in animals and until now, no clinical breakpoints for this antibiotic are available for veterinary use. The available human CLSI breakpoints for colistin are for parenteral formulations targeting non-Enterobacteriaceae (CLSI, 2009) and therefore may not predict clinical efficiency of oral formulations in animals for *E. coli* infections. Nevertheless, the clinical breakpoint for resistance used in the current manuscript for statistical purposes (sensitive = 2 µg/ml; resistant = 8 µg/ml) might be close to the actual clinical breakpoint for oral colistin formulations in pigs, since Burch (2007) calculated that for a feed concentration of 66 ppm, colistin reached bactericidal concentrations (AUC/MIC = 100) in the porcine jejunum for strains with a MIC of 8 µg/ml, but not for strains with a MIC of 16 µg/ml.

The present results confirm that the E-test is a reliable method to test colistin susceptibility in *E. coli* isolates, while the disk diffusion test is not (Galani et al., 2008; Landman et al., 2008). Katz et al. (2008) found that the disk prediffusion test was a promising method to discriminate between daptomycin resistant and susceptible *Staphylococcus aureus* (*S. aureus*) strains. To our knowledge, the prediffusion method has not been validated before to determine colistin resistance in *E. coli*. The current results suggest that the 2 + 18 prediffusion protocol, as used in the current setup, provides reliable information on the colistin susceptibility of *E. coli* strains. A clear bimodal distribution of inhibition zones was

seen with 14 of the 15 isolates with acquired resistance, belonging to the population with the smaller inhibition zones. Further improvements as done by Katz et al. (2008), namely using a shorter second incubation period (6 hours instead of 18 hours), may offer a faster and more comfortable protocol. Also an adaptation of the interpretation criteria for inhibition zones may be necessary.

In conclusion, current results suggest that, in addition to the E-test, the prediffusion test can be used as a reliable, alternative agar-based colistin susceptibility testing method for use in *E. coli* strains. As many laboratories still rely on the cheaper disk diffusion test, the emergence of colistin resistance may be missed, as demonstrated in this study. It is clear that this type of resistance needs to be monitored closely, using the appropriate test methods.

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239 Table 1. Distribution of minimal inhibitory concentrations of porcine *E. coli* strains towards colistin, using the E-test and the agar dilution test.

Test	Number of strains with colistin MIC values (µg/ml) of												
	=0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128
E-test*				18	64	37	22	3	8	4	1		
Agar dilution				4	117	20	1	1	11	3			

240 *E-test values were rounded up to the next highest doubling dilution

241 The strains with MIC values higher than the wild type cut-off value (MIC > 2 µg/ml) as described by EUCAST (2009) were considered to have
242 acquired resistance. The clinical breakpoints for susceptibility (MIC = 2 µg/ml) and resistance (MIC = 8 µg/ml) that were used for all
243 comparative analyses are represented by a discontinuous and a solid line respectively.

244 Table 2. Distribution of inhibition zone diameter of regular disk diffusion and disk prediffusion test.

Test	Number of strains with colistin inhibition zone (mm) of																
	= 8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	= 24
Disk diffusion									2	13	30	36	43	17	13	3	
Disk prediffusion	12			1								1				1	142

245 The clinical breakpoints for susceptibility and resistance that were used for all comparative analyses are represented by a solid and a
246 discontinuous line respectively.

Table 3. Discrepancy rates, categorical agreement, essential agreement and correlation between the results obtained by agar dilution assay on the one hand and the results obtained by the regular disk diffusion test, the E-test and the disk prediffusion test on the other hand.

~Agar Dilution	Very major error (< 1.5 %)[§]	Major error (< 3 %)[§]	Minor error (< 10 %)[§]	Categorical agreement[§]	Correlation coëfficient
Disk diffusion	3 (1.9 %)	2 (1.3 %)	78 (49.7 %)	46.5 %	0.09[#]
E-test*	0 (0 %)	1 (0.6 %)	3 (1.9 %)	96.8 %	0.64
Prediffusion	2 (1.3 %)	1 (0.6 %)	1 (0.6 %)	96.8 %	0.80[#]

* E-test values were rounded up to the next highest doubling dilution

[§] Breakpoints used: Disk diffusion: resistant = 16 mm; sensitive = 20 mm; Disk prediffusion: resistant = 10 mm; sensitive = 15 mm; Agar dilution and E-test: sensitive = 2 µg/ml; resistant = 8µg/ml

[#] Actual values are -0.09 and -0.80, since inhibition diameter and MIC values are inversely correlated. For uniformity purposes, the absolute values are shown.

NA: not applicable