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Short communication

Antimicrobial susceptibility patterns of *Haemophilus parasuis* from pigs in the United Kingdom and Spain

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Abstract

A total of 30 British and 30 Spanish *Haemophilus parasuis* isolates were tested for their susceptibility to 19 of the antimicrobials currently used in swine practice with a broth microdilution method in order to know the emergence of resistance against these compounds in this porcine pathogen. All the British isolates were susceptible to penicillin, ceftiofur, erythromycin, tilmicosin, enrofloxacin, and florfenicol, and most of them were susceptible to the remaining antimicrobials (the highest resistance rate found was of 20% to neomycin). In contrast, all the Spanish isolates were susceptible exclusively to florfenicol, and high proportions of resistance were encountered for penicillin, ampicillin, oxytetracycline, erythromycin, tilmicosin, tiamulin and trimethoprim + sulphamethoxazole; in addition, a bimodal or multimodal distribution, or tailing of Spanish isolates over the MIC range was observed for clindamycin, sulphonamides and tylosine tartrate, suggesting the development of acquired resistance. In addition, several multiresistance patterns were found among the Spanish isolates, 23.3% of them being resistant to at least eight antimicrobials, the same rate as that encountered for those being susceptible to all antimicrobials tested. This study showed that in general British *H. parasuis* isolates are susceptible to antimicrobial agents routinely used for treatment of porcine respiratory diseases; however, the Spanish isolates need a more continuous surveillance of their susceptibility patterns.

Keywords: *Haemophilus parasuis*; Glässer's disease; Antimicrobial susceptibility; Clinical Isolates; Pig

1. Introduction

Haemophilus parasuis, a small pleomorphic, nicotinamide adenine dinucleotide (NAD)-dependent Gram-negative rod of the family *Pasteurellaceae*, is an important porcine pathogen and the etiological agent of Glässer's disease. This disease is characterized by fibrinous polyserositis, polyarthritis and meningitis. However, in the acute form it might only be associated with pneumonia and septicemia without polyserositis. Furthermore, some strains of *H. parasuis* are not associated with disease and are considered a commensal of the upper respiratory tract of healthy pigs (Oliveira and Pijoan, 2004). Glässer's disease has historically been considered a sporadic, stress-associated disease of young pigs. However, since the establishment of specific pathogen free herds, increased spread of the disease and increased mortality rates have been described. In such herds, infection may spread as a contagious disease of high morbility, being responsible for increased economic losses in the swine industry worldwide (Smart et al., 1988).

There is evidence that *H. parasuis* is heterogeneous in nature. To date, 15 serovars have been recognized, along with a large number of non-typable isolates. It is unclear if there is a strong relationship between serovar and virulence but it is used as an indicative marker. Thus, serovars 1, 5, 10, and 12-14 may lead to the death of pigs and are considered highly virulent; serovars 2, 4, 8 and 15 are virulent, causing lesions in pigs, but serovars 3, 6, 7, 9 and 11 are considered to be avirulent (Kielstein and Rapp-Gabrielson, 1992). In Spain, the most prevalent serotypes are 4, 5, 7, 10, and 15, whereas in the United Kingdom, the most prevalent serotypes are 1, 2, 5, 7 and 10 (unpublished results). Control of Glässer's disease can be achieved by use of vaccination; however, serovar diversity and the high number of non-typable isolates reported have affected negatively the development of effective cross-protective vaccines (Oliveira and Pijoan, 2004).

For this reason, antibiotic therapy continues to be necessary for the treatment of outbreaks of Glässer's disease, together with other measures that can help to limit outbreaks, such as good animal management including adequate colostral protection. There are few reports on the antimicrobial susceptibility of European *H. parasuis* field isolates (Wissing et al., 2001; Aarestrup et al., 2004; San Millán et al., 2006; Nedbalcova et al., 2006); however, β-lactam-resistant isolates exhibiting β-lactamases have been recently reported in Spain (San Millán et al., 2006). Correct use of antimicrobial agents for treatment of bacterial infections requires knowledge of the susceptibility of the infecting strain to antimicrobial agents, because of the emergence of resistance observed among several organisms, including some of those causing respiratory diseases in pigs, such as *Actinobacillus pleuropneumoniae* (Gutiérrez-Martín et al., 2006) or *Pasteurella multocida* subsp. *multocida* (Vera-Lizarazo et al., 2006). Thus, the purpose of the present work was to determine the antimicrobial susceptibility of a collection of *H. parasuis* strains coming from two geographical origins, the United Kingdom and Spain.

2. Materials and methods

2.1. Bacterial strains

A total of 30 *H. parasuis* isolates supplied by the Veterinary Laboratory Agency (Bury St Edmunds, Suffolk, United Kingdom), which were recovered between 1995 and 2005 from pigs suffering polyseritis, pneumonia or septicemia, as well as 30 other *H. parasuis* isolates, which were recovered from 2002 to 2004 from pigs suffering polyserositis or pneumonia from herds located in central and northwest Spain, were included in this study. Identification of the isolates was carried out by NAD-dependency; absence of hemolysis; urease, oxidase and catalase tests, and using the 16S diagnostic PCR

(Oliveira et al., 2001). The isolates were serotyped by indirect haemagglutination as previously described (del Río et al., 2003).

2.2. Antimicrobial susceptibilities

The antimicrobial susceptibilities of the isolates were determined by a microdilution method using a commercially prepared, dehydrated 96-well microtitre panel recommended for porcine organisms (CMV1ABPF, Sensititre, Trek Diagnostic Systems Inc., United Kingdom). The antimicrobial agents used and their respective dilution ranges were as follows: penicillin (PEN), 0.12-8 µg/ml; ampicillin (AMP), 0.25-16 µg/ml; ceftiofur (XNL), 0.5-8 µg/ml; gentamicin (GEN), 1-8 µg/ml; apramycin (APR) and neomycin (NEO), 4-32 µg/ml; spectinomycin (SPE), 8-64 µg/ml; oxytetracycline (OXY), 0.25-8 µg/ml; clindamycin (CLI), 0.25-2 µg/ml; erythromycin (ERY), 0.25-4 µg/ml; tilmicosin (TYL), 4-32 µg/ml; enrofloxacin (ENRO), 0.12-2 µg/ml; sulphachlorpiridazine (SCP), sulphadimetoxine (SDM) and sulphatiazole (STZ), 32-256 µg/ml; florfenicol (FFC), 0.25-8 µg/ml; tiamulin (TIA), 4-32 µg/ml; tylosin tartrate (TYLT), 2,5-20 µg/ml; and trimethoprim/sulphamethoxazole (COT), 0.5/9.5-2/38 µg/ml. The inocula were prepared from a 24 h chocolate blood agar plate by adjusting to 0.5 McFarland standard and further diluted 1/200 in Veterinary Fastidious Medium (Mueller-Hinton broth -Biolife, Italy- + 2% yeast extract -Biolife, Italy- + 2% lysed horse blood + 2% supplement C -Becton Dickinson Co, MD, USA-) (Clinical and Laboratory Standards Institute -CLSI-, 2002). Fifty microlitres of the adjusted inoculum were deposited in each well of the microplate panel. Microdilution panels were covered with a sterile microwell lid and further incubated at 37º C for 24 h in an atmosphere containing 5% CO_2.

Performance and evaluation of the minimal inhibitory concentration (MIC) determinations followed the recommendations of the CLSI (2002, 2004). The MIC was defined as the lowest concentration at which no visible growth was detectable. Ranges of susceptibility were recorded along with the MIC that inhibited 50% (MIC_{50}) and 90% (MIC_{90}) of the isolates. The breakpoints used for PEN, AMP, XNL, SPE, OXY, ERY, TYL, FFC, TIA and COT were those previously used by Aarestrup et al. (2004) for Danish *H. parasuis* strains. For GEN, NEO and ENRO, breakpoints values used were those recommended by CLSI (2004) for *A. pleuropneumoniae* or *Histophilus somni*. For the remaining antimicrobials, the distribution of strains over the MIC range was considered. The following control strains were included: *A. pleuropneumoniae* ATCC 27090 and *H. somni* ATCC 700025.

2.3. Detection of β-lactamases

The β-lactamase activity was determined by the nitrocefin test (Becton Dickinson Co, MD, USA).

3. Results and discussion

In this study, a microdilution method was used to compare the antimicrobial resistance profiles of British and Spanish *H. parasuis* isolates. The British isolates belonged to seven serovars (1-3, 5, 7, 10 and 12), while the Spanish isolates belonged to ten serovars (2, 4, 5, 7-10, 12, 14, and 15). Correlation between distribution of resistances and serovars could not be established because of the small number of isolates belonging to each serovar. The results of the susceptibility testing as distribution of the MICs values, MIC_{50}, MIC_{90}, and the percentage of resistant strains (when breakpoints are available) are shown in Table 1.

All the British isolates were susceptible to penicillin, which is in accordance to the results obtained in Switzerland using disk diffusion (Wissing et al., 2001). Unlike British strains, only 40% of the Spanish isolates were susceptible to this β-lactam antimicrobial. A high degree of resistance (56.7%) was also recorded for ampicillin among the Spanish isolates; however, only two British isolates (6.7%) had a MIC outside the breakpoint. Similarly, ampicillin-resistant isolates exhibiting β-lactamases have been recently reported in Spain (San Millán et al., 2006). The nitrocefin test was carried out with the 18 isolates having MICs ≥ 4 μg/ml to penicillin, and a positive reaction was observed for 14 isolates, while a clearly negative reaction was yielded by the remaining isolates. These four nitrocefin-negative strains suggest that, in addition to β-lactamases, there are likely other additional mechanisms involved in resistance towards penicillins. All the British strains were susceptible to ceftiofur, but two of the Spanish strains (6.7%) showed a MIC greater than the breakpoint given for this β-lactam. These two isolates also displayed resistance to penicillin and ampicillin. In contrast to our results, 52 Danish *H. parasuis* isolates were fully susceptible to β-lactams, especially to ceftiofur, with all isolates being inhibited by a dose as low as 0.03 μg/ml of this compound (Aarestrup et al., 2004).

With regard to aminoglycosides, a certain degree of resistance was found towards gentamicin, neomycin and spectinomycin, with percentages considerably lower among the British isolates. Resistance to gentamicin has been also reported among Swiss isolates (Wissing et al., 2001). The results obtained for spectinomycin (MIC range of 8-128 μg/ml) differed from those previously reported in Denmark, where all *H. parasuis* isolates were susceptible to 8 μg/ml (Aarestrup et al., 2004). Since no CLSI-breakpoint has been defined for apramycin, the percentage of resistant isolates could not be determined. Nevertheless,

the MIC_{50} and MIC_{90} values of apramycin were quite similar to those observed for neomycin, being identical the dilution ranges tested for both aminoglycosides; consequently, a similar activity to that obtained for neomycin might be deduced for apramycin.

A high rate of resistance to oxytetracycline (40%) was found among the Spanish isolates; however, a substantially lower resistance (6.7%) was observed among the British strains. A considerably lower percentage of resistance to tetracyclines was found in the Czech Republic when compared with the Spanish isolates, with 21.9% of the Czech isolates being resistant (Nedbalcova et al., 2006). Again, Aarestrup et al. (2004) did not report resistance towards tetracyclines among the Danish *H. parasuis* isolates. In addition, *tet*(B) gene associated with two plasmids has been reported in some tetracycline-resistant *H. parasuis* isolates (Lancashire et al., 2005). The high prevalence of resistance ascribed to this antimicrobial group in the present work could be related with its excessive use in therapy for the treatment of infectious diseases caused by respiratory pathogens in Spanish porcine husbandry in recent decades (Gutiérrez et al., 1993), quite different from the more rational use in the United Kingdom.

A high proportion of the Spanish isolates (40%) were resistant to erythromycin and tilmicosin; however, all the British isolates were susceptible to these compounds. The results observed among the British isolates were quite similar to those previously reported in Denmark (Aarestrup et al., 2004), where no resistance was recorded against these antimicrobials. On the contrary, a substantially higher resistance rate (84.4%) was reported to erythromycin among Czech *H. parasuis* isolates (Nedbalcova et al., 2006). Tailing was present for clindamycin among the British *H. parasuis* isolates (with a MIC_{50} of ≤ 0.25

µg/ml), whereas the Spanish isolates seem to form a bimodal population of susceptibility to this compound (with a MIC_{50} of 2 µg/ml), both cases suggesting a possible development of a certain degree of resistance. Tylosine tartrate had MICs of 20 µg/ml against 50% of the Spanish isolates, and of ≤2.5 µg/ml against 50% of the Bristish isolates. Again, the multimodal distribution or the tailing showed respectively by the Spanish or British isolates suggest the development of acquired resistance, especially among the Spanish strains. Tylosine has been used in swine in Spain as growth promoter until it was banned in 1999. It is possible that the high MIC values observed in our investigation arose from long-term exposure of the Spanish isolates to this anitmicrobial agent.

The resistance observed to enrofloxacin among the Spanish isolates (20%) is of particular note, since no resistance was recorded amongst the UK isolates, all of them susceptible to 1 µg/ml of this antimicrobial agent. Similar to this latter result, no resistance has been documented among Swiss isolates (Wissing et al., 2001). Resistance to enrofloxacin has not been described among *H. parasuis* isolates to date; however, in a recent study conducted on 94 field isolates in the Czech Republic using disk diffusion (Nedbalcova et al., 2006), a low resistance (6.3%) was found towards norfloxacin, another fluoroquinolone. On the other hand, florfenicol exhibited an excellent activity against all the 60 isolates tested, regardless of their geographical origin. The investigation performed by Aarestrup et al. (2004) in Denmark also encountered no resistant isolates to florfenicol, with a MIC range even lower than that reported in the present study. Therefore, florfenicol, which was first used in 2000 in the United Kingdom and Spain, remains as useful for treatment of swine with Glässer's disease. Prudent use and guidelines are necessary in order to avoid emergence of resistance to this compound, because its use has increased considerably since then in these countries (J. M. Bollo Bernabé, personal communication).

Similar distributions (bimodal or tailing) of isolates over the MIC range were found for the three sulphonamides tested, independently of the source of the strains, also indicating a supposed resistance mechanism. Resistance to sulphonamides has been previously reported among Swiss isolates using disk diffusion (Wissing et al., 2001). Most Spanish isolates (53.3%) were also resistant to the combination of trimethoprim and sulphamethoxazole, whereas this rate decreased to 10% when testing the British strains. This latter resistance is rather similar to the 3% reported towards trimethoprim + sulphamethoxazole among Danish isolates (Aarestrup et al., 2004).

Finally, a high rate of the Spanish isolates (40%) was found resistant to tiamulin, while all the British isolates were susceptible except one (3.3%). The frequent use of this pleuromutilin derivative for decades in Spain for treating respiratory infectious diseases, mainly porcine pleuropneumonia caused by *A. pleuropneumoniae* (Gutiérrez et al., 1993) could justify the high resistance profile showed by the Spanish *H. parasuis* isolates against tiamulin.

Antimicrobial susceptibility profiles were constructed taking into account only the Spanish isolates and those antimicrobials for which breakpoint was available (Table 2). Only seven isolates (23.3%) were susceptible to all the antimicrobials tested, and several multiresistance patterns were found among the remaining 23 strains. Thus, seventeen isolates (56.7%) were resistant to at least four antimicrobials; seven (23.3%), to at least eight antimicrobials, and only one isolate (3.3%) was resistant to twelve antimicrobials simultaneously. This is the first report showing as many multiresistance patterns for this veterinary pathogen, although one *H. parasuis* isolate being resistant to ten antimicrobial agents has been recently reported in the Czech Republic (Nedbalcova et al., 2006). In

contrast, a total of 24 British isolates (80%) were susceptible to the 19 antimicrobials compared, three isolates were resistant to at least two antimicrobials and only one was resistant simultaneously to GEN, OXY, TIA and COT. Further studies are required with the aim of elucidating the molecular mechanisms involved in these resistances.

Our study demonstrated strongly that the British isolates were much more susceptible to 19 of the antimicrobials routinely used in swine practice than those of Spanish origin. One first explanation for this difference could be related with the sampling period. The recovery period for the British isolates was 10 years, while the Spanish samples were collected only for two years. This fact could have biased the results, so that the isolates collected earlier might be biased a bit towards susceptibility, while the isolates collected later might be biased towards resistance. However, a more likely explanation could be that antimicrobial agents are still excessively used for the treatment or prevention of infectious diseases in pig husbandry in Spain, in a much less rational way than in the United Kingdom. Furthermore, the scarcity of reliable information about the amount of antimicrobial agents used annually in pig practice in Spain (unlike other European countries) does not help to avoid its hurtful use (EMEA, 1999).

In conclusion, our results emphasize the importance of prudent use of antimicrobials in the treatment of Glässer's disease and, in particular, for routine monitoring of the susceptibility patterns of clinical isolates of *H. parasuis* before the administration of a given therapy. Besides, preventive measures such as good hygiene and proper animal handling practices, which limit the use of antimicrobial agents, are essencial to keep the number of Glässer's disease outbreaks low.

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Table 1. MICs for 19 antimicrobial agents of the British and Spanish *Haemophilus parasuis* clinical isolates.

Antimicrobial	Source of isolates	No. of isolates with MIC of (μg/ml)													MIC_{50}	MIC_{90}	% resistance
		0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512			
PEN	UK	22	2	4	1	1									≤0.12	0.5	0
	Spain	4	2	2	2	2	2	2	14						8	>8	60.0
AMP	UK		22	3	1	1	1		1	1					≤0.25	2	6.7
	Spain		6	2	2	2	1	1	2	14					16	>16	56.7
XNL	UK			24	4	2									≤0.5	1	0
	Spain			21	2	3	2	1	1						≤0.5	4	6.7
GEN	UK				16	3	2	6	3						1	8	10.0
	Spain				3	3	6	10	8						8	>8	26.7
APR	UK						11	4	5	6	4				8	>32	---
	Spain						8	6	9	4	3				16	32	---
NEO	UK						10	3	5	6	6				16	>32	20.0
	Spain						5	3	2	10	10				32	>32	33.3
SPE	UK							17	2	6	2	3			4	64	10.0
	Spain							12	1	6	4	7			32	>64	23.3
OXY	UK		13	5	6	2	1	1	2						0.5	4	6.7
	Spain		5	3	3	2	2	3	12						4	>8	40.0
CLI	UK		18	4	4	3	1								≤0.25	2	---
	Spain		5	4	3	4	14								2	>2	---
ERY	UK		6	12	4	8									0.5	2	0
	Spain		8	2	1	2	5	12							4	>4	40.0
TYL	UK						20	10							<4	8	0
	Spain						9	3	6	5	7				16	>32	40.0
ENRO	UK	23	4	2	1										≤0.12	0.25	0
	Spain	12	3	4	5	2	4								0.25	>2	20.0
SCP	UK									10	4	10	3	3	128	256	---
	Spain									5	8	5	4	8	128	>256	---
SDM	UK									6	4	12	6	2	128	256	---
	Spain										3	7	3	17	>256	>256	---
STZ	UK									7	3	6	11	3	128	256	---
	Spain											5	6	19	>256	>256	---
FFC	UK		23	3	2	1	1								≤0.25	1	0
	Spain		12	8	7	2	1								0.5	1	0
TIA	UK						22	2	5	1					≤4	16	3.3
	Spain						12	2	4	7	5				16	>32	40.0

Table 1 (continued)

		No. of isolates with MIC of (μg/ml)							
Antimicrobial	Source of isolates	2.5	5	10	20	40	MIC_{50}	MIC_{90}	% resistance
TYLT	UK	21	4	4		1	≤2.5	10	---
	Spain	11	1	2	1	15	20	>20	---

		No. of isolates (μg/ml) with MIC of						
		0.5/9.5	1/19	2/38	4/76	MIC_{50}	MIC_{90}	% resistance
COT	UK	22	3	2	3	≤0.5/9.5	2/38	10.0
	Spain	6	2	6	16	>2/38	>2/38	53.3

The dilution ranges tested for each antimicrobial agent are those contained within the white area. Values above this range indicate MIC values higher than the highest concentration in the range. Values corresponding to the lowest concentration tested indicated MIC values smaller or equal to the lowest concentration in the range. CLSI resistance breakpoints are indicated with vertical black lines when available.

Abbreviations: PEN, penicillin; AMP, ampicillin; XNL, ceftiofur; GEN, gentamicin; APR, apramycin; NEO, neomycin; SPE, spectinomycin; CTET, chlortetracycline; OXY, oxytetracycline; CLI, clindamycin; ERY, erythromycin; TYL, tilmicosin; ENRO, enrofloxacin; SCP, sulphachlorpiridazine; SDM, sulphadimethoxine; STZ, sulphathiazole; FFC, florfenicol; TIA, tiamulin; TYLT, tylosine tartrate; COT, trimethoprim/sulphamethoxazole.

Table 2. Antimicrobial resistance profiles of the Spanish *Haemophilus parasuis* field isolates in this study

No. of isolates	No. of antimicrobial agents	Resistance to:
7	0	No antimicrobial resistance
1	1	ENRO
1	1	COT
1	2	GEN+COT
1	3	PEN+AMP+SPE
1	3	PEN+AMP+COT
1	3	PEN+AMP+TIA
1	4	PEN+AMP+GEN+NEO
1	4	PEN+ERY+TYL+TIA
1	5	PEN+AMP+NEO+OXY+COT
1	5	GEN+NEO+SPE+ERY+TYL
1	5	NEO+SPE+OXY+ERY+COT
1	6	PEN+AMP+GEN+NEO+TYL+COT
1	6	PEN+AMP+OXY+ERY+TIA+COT
1	7	PEN+AMP+NEO+OXY+ERY+TIA+COT
1	7	PEN+AMP+SPE+OXY+TYL+TIA+COT
1	7	PEN+AMP+OXY+ERY+TYL+TIA+COT
1	8	PEN+AMP+GEN+OXY+ERY+TYL+TIA+COT
1	8	PEN+AMP+NEO+ERY+TYL+ENRO+TIA+COT
1	8	PEN+AMP+SPE+OXY+ERY+TYL+ENRO+COT
1	8	PEN+AMP+OXY+ERY+TYL+ENRO+TIA+COT
1	10	PEN+AMP+XNL+GEN+NEO+OXY+ERY+TYL+TIA+COT
1	10	PEN+AMP+GEN+NEO+SPE+OXY+TYL+ENRO+TIA+COT
1	12	PEN+AMP+XNL+GEN+NEO+SPE+OXY+ERY+TYL+ENRO+TIA+COT

Abbreviations are as defined in the legend of Table 1.