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Short communication

Changes in antimicrobial susceptibility of *Actinobacillus pleuropneumoniae* isolated from pigs in Spain during the last decade

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Abstract

A total of 229 Spanish *Actinobacillus pleuropneumoniae* isolates recovered from diseased pigs with pleuropneumonia from 1997 to 2004 was tested for their susceptibility to 11 antimicrobials in a broth microdilution method. All the isolates were susceptible to florfenicol and most of them to cephalothin; however, a high rate of resistance was observed to tetracycline. A bimodal or multimodal distribution of isolates over the MIC range were observed for penicillins, tetracycline, trimethoprim, sulfisoxazole and nalidixic acid, suggesting the development of acquired resistance. Eight resistance patterns were established, and 21.1% of the isolates were resistant to at least two antimicrobials. In addition, a considerable increase in the resistance to tetracyclines was observed during the last decade in Spain, when compared with other *A. pleuropneumoniae* strains isolated during 1987–1988 (Gutiérrez, C.B., Píriz, S., Vadillo, S., Rodríguez Ferri, E.F., 1993. In vitro susceptibility of *Actinobacillus pleuropneumoniae* strains to 42 antimicrobial agents. Am. J. Vet. Res. 54, 546–550); this finding was also observed for gentamicin in minor percentage.

Keywords: *Actinobacillus pleuropneumoniae*; Pleuropneumonia; Antimicrobial susceptibility; Clinical isolates; Pig; Spain

1. Introduction

Actinobacillus pleuropneumoniae is the causative agent of porcine pleuropneumonia, a severe respiratory disease which is a serious problem in pig production worldwide. The acute form of the disease is highly contagious and often fatal, resulting in considerable economic losses for pig producers (Sebunya and Saunders, 1983). Based on NAD requirements, *A. pleuropneumoniae* has been traditionally divided into biovar 1 strains, which are NAD dependent, and biovar 2 strains, which are NAD independent; however, an integration of biovars 1 and

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2 in a single biovar has been proposed (Nielsen et al., 1997). To date, 15 serotypes of *A. pleuropneumoniae* have been reported (Blackall et al., 2002). Although all serotypes are potentially pathogenic, they vary in virulence and their prevalence is related to the geographic region (Sebunya and Saunders, 1983). In Spain, the most prevalent serotypes are 2, 4 and 7, whereas serotypes 1, 3, 5, 6 and 8–12 have been scarcely isolated (Gutiérrez et al., 1995).

Although vaccination and control programmes have been described, antibiotic therapy continues to be necessary for the control of pleuropneumonia outbreaks. Correct use of antimicrobial agents for treatment of infections with *A. pleuropneumoniae* requires knowledge of the susceptibility of the infecting strain to antimicrobial agents, because differences in the resistance patterns have been observed between different countries, serotypes and over-time (Vaillancourt et al., 1988; Kawahara et al., 1989; Asawa et al., 1995; Chang et al., 2002b; Yoshimura et al., 2002). Thus, the purposes of the present work were to determine the antimicrobial susceptibility of a large collection of *A. pleuropneumoniae* strains isolated in Spain during 1997–2004 and to compare it with that obtained approximately a decade ago (Gutiérrez et al., 1993).

2. Materials and methods

2.1. Strains

A total of 229 *A. pleuropneumoniae* isolates, which were recovered from 1997 to 2004 from the lungs of diseased pigs from herds located in central and northwest Spain, were included in this study. The bacteria were isolated on chocolate agar supplemented with PolyVitex (Biomérieux, France) and biochemically identified according to standard procedures (Kilian and Biberstein, 1985). The isolates were serotyped by indirect haemagglutination as previously described (Mittal et al., 1983).

2.2. Antimicrobial susceptibilities

The antimicrobial susceptibilities of the isolates were determined by a microdilution method using commercially prepared, dehydrated 96-well microtitre MIC panels (VAV5 and CMP1ASPV, Sensititre; Trek Diagnostic Systems Inc., England). The antimicrobial agents used and their respective dilution ranges were as follows: penicillin (PEN), 0.12–64 μg/ml; amoxicillin (AMOX), 0.06–32 μg/ml; cephalothin (CEP), 0.5–32 μg/ml; tetracycline (TET), 0.25–128 μg/ml; streptomycin (STR), 1–128 μg/ml; gentamicin (GEN), 0.06–8 μg/ml; erythromycin (ERY), 0.03–4 μg/ml; trimethoprim (TMP), 1–64 μg/ml; nalidixic acid (NAL), 1–16 μg/ml; sulfisoxazole (FIS), 32–512 μg/ml; florfenicol (FFN), 0.12–128 μg/ml.

Performance and evaluation of the MIC determinations followed the recommendations of the NCCLS (2004). The MIC was considered to correspond to the first dilution at which no bacterial strain 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 CEP, TET, GEN, FIS and FFN were those recommended by the NCCLS (2004). For the remaining antimicrobials, the distribution of strains over the MIC range was considered. The following control strains were included: *Escherichia coli* ATCC 25922 and *A. pleuropneumoniae* ATCC 27090.

3. Results and discussion

Serotypes 2 (41.0%) and 4 (40.2%) of *A. pleuropneumoniae* were the most prevalent, followed by serotypes 6 (6.6%) and 7 (5.7%). Serotypes 1, 5, 8 and 11 were isolated in proportions lower than 3%. The results of the susceptibility testing of the 229 clinical isolates as distribution of the MICs values, MIC_{50}, MIC_{90}, and the percentage of resistant strains (when breakpoint is available) are shown in Table 1. The expected MICs values (NCCLS, 2004) for the control strains were observed (*Escherichia coli* ATCC 25922: TET, 0.5 μg/ml; GEN, 0.5 μg/ml; CEP, 4 μg/ml; FIS, <32 μg/ml; FFN, 2 μg/ml; *A. pleuropneumoniae* ATCC 27090: PEN, 0.12 μg/ml; TET, 0.5 μg/ml; GEN, 8 μg/ml; FFN, 0.25 μg/ml).

The two penicillins tested were not interpreted because of the absence of either proposed breakpoints; nevertheless, these antimicrobials showed multimodal distributions indicating a supposed resistance mechanism. In addition, penicillin and amoxicillin had MICs

Table 1
MICs for 11 antimicrobial agents of the *Actinobacillus pleuropneumoniae* clinical strains isolated during 1997–2004

Antimicrobial	No. of isolates with MIC of (μg/ml)																MIC$_{50}$	MIC$_{90}$	% Resistance
	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	>512			
PEN			<3	5	27	129	30			2		3	8>	22			1	64	–
AMOX		<		28	154	10	3			1		3>	30				0.5	64	–
CEP					<184	16	15	8		4	1	1>					0.5	2	0.4
TET				<1	9	23	6	2		19	48	53	67	1>			32	64	73.8
STR						<1				1	38	150	14	1>	24		32	256	–
GEN		<						55	153>	21							8	8	9.2
ERY	<		1				7	177>		44							4	>4	–
TMP						<216	1			1		1>	10				≤1	≤1	–
NAL						<1	180	39		1	1>	7					2	4	–
FIS												<39	108	39	5	3> 35	64	>512	16.6
FFN*			<85	90	32	22									>		0.25	0.5	0

(|): Lines indicate breakpoint for resistance when available; (<): minimum value of concentration used; (>): maximum value of concentration used. Abbreviations: PEN, penicillin; AMOX, amoxicillin; CEP, cephalothin; TET, tetracycline; STR, streptomycin; GEN, gentamicin; ERY, erythromycin; TMP, trimethoprim; NAL, nalidixic acid; FIS, sulfisoxazole; FFN: florfenicol.

of 64 μg/ml against 90% of the isolates tested, being these values considerably lower among the *A. pleuropneumoniae* strains tested in Spain a decade earlier, with MICs$_{90}$ of 4.8 and 1 μg/ml for penicillin and amoxicillin, respectively (Gutiérrez et al., 1993). However, a high degree of activity has been recorded for penicillins in previous reports (Gilbride and Rosendal, 1984; Yoshimura et al., 2002). For cephalothin, which can be used to test all first-generation cephalosporins (NCCLS, 2004), only one isolate of *A. pleuropneumoniae* had a MIC outside the breakpoint; therefore, the frequency of susceptibility would be 99.6%. However, tailing is present for this antimicrobial, thus indicating a possible development of a certain degree of resistance. Chang et al. (2002a) found a MIC$_{90}$ value of 1 μg/ml for cephalothin, just half of that reported in our study. In summary, although the first-generation cephalosporins are widely used in pig practice, the results obtained for cephalothin suggest that they could be still used as routine therapeutic agents for the treatment of swine pleuropneumonia in Spain.

According to NCCLS (2004), tetracycline is the class representative for susceptibility to different tetracyclines. A high rate of resistance to this antimicrobial was found, with 73.8% of the *A. pleuropneumoniae* isolates being resistant. In a previous study carried out using 83 strains isolated in the United States, Canada and Denmark (Salmon et al., 1995), the MIC$_{50}$ and MIC$_{90}$ values obtained for tetracycline (8 and 32 μg/ml, respectively) were quite lower than those in our investigation. It is noticeable that the resistance observed for tetracycline in Spain has raised from the 26.3% (with a MIC$_{50}$ of 1 μg/ml) observed a decade ago (Gutiérrez et al., 1993) to the 73.8% (with a MIC$_{50}$ of 32 μg/ml) obtained 10 years after (Table 1). The high resistance proportion to tetracyclines reported in the present work could be related with the wide use of this antimicrobial agent at sub-therapeutic doses in swine practice during decades. In addition, *tet*(B), *tet*(H), *tet*(L) and *tet*(O) genes associated with plasmids have been found in 46 of the resistant isolates of *A. pleuropneumoniae* tested in this study (Blanco et al., in press).

The 229 *A. pleuropneumoniae* isolates seem to form a monomodal population of susceptibility to streptomycin, although 24 isolates showed MICs of 256 μg/ml. The MIC$_{50}$ and MIC$_{90}$ reported in this

study were higher than those observed for the isolates recovered during 1987–1988 (Gutiérrez et al., 1993). Similarly, the distribution of MICs of gentamicin and erythromycin is clearly unimodal, suggesting that no acquired resistance is present among *A. pleuropneumoniae* isolates. However, using the NCCLS recommendations (2004), a total of 21 isolates (9.2%) had MICs higher than the breakpoint proposed for gentamicin. According to this, the acquired resistance to gentamicin would have increased in Spain during the last decade from the 0% reported for the strains isolated during 1987–1988 (Gutiérrez et al., 1993). No resistant isolates and a MIC_{90} as low as 2 μg/ml have been previously reported for this aminoglycoside in other countries (Chang et al., 2002a; Yoshimura et al., 2002). Similar MIC_{50} and MIC_{90} values to those in our study for erythromycin have been previously found in other American and European countries (Salmon et al., 1995); however, the MIC_{50} value (4 μg/ml) for this macrolide has increased compared to the 1 μg/ml reported for the *A. pleuropneumoniae* strains collected during 1987–1988 (Gutiérrez et al., 1993).

Globally, the isolates belonging to serotype 4 exhibited MICs higher for streptomycin, gentamicin and erythromycin than those of serotype 2. In a similar way, a substantially lower amount of streptomycin-resistant *A. pleuropneumoniae* strains belonging to serotype 2 compared to those of serotype 1 has been isolated in Japan (Asawa et al., 1995), and a considerably lower resistance against other antimicrobial groups (tetracyclines and chloramphenicol) was also found in Japan among isolates of serotype 2 when compared with other serotypes (Yoshimura et al., 2002).

Trimethoprim and nalidixic acid were not interpreted because no breakpoints are available; nevertheless, these antimicrobials seem to show bimodal distributions, thus suggesting the development of acquired resistance in some *A. pleuropneumoniae* isolates. Sulfisoxazole should be used to test for susceptibility to different sulfonamides (NCCLS, 2004). The distribution of MIC of this antimicrobial is also bimodal, with 38 isolates (16.6%) exhibited MICs $\geq$ 512, the breakpoint given for this compound. Finally, resistance to florfenicol was not found (unimodal distribution), and the MIC_{50} and MIC_{90} values were the lowest of those obtained for the 11 antimicrobial agents tested. Other investigations have also encountered no resistant isolates to florfenicol (Yoshimura et al., 2002; Shin et al., 2005). Besides, the ranges of susceptibility showed by this derivative of thiamphenicol in previous reports (0.2–1.56 μg/ml; Ueda and Suenaga (1995) or $\leq$0.12–1.0 μg/ml; Shin et al. (2005)) were closely similar than that obtained in the present study. Because its high activity, florfenicol becomes a valuable alternative to traditional antimicrobials in the prevention and treatment of porcine pleuropneumonia.

Antimicrobial susceptibility profiles were constructed using taking into account cephalothin, tetracycline, gentamicin and sulfisoxazole (Table 2). A total of eight distinct resistance patterns were obtained, and the profile of resistance to tetracyclines exclusively was the most frequently isolated (in 73.7% of the resistant strains), followed by the pattern of resistance to tetracyclines and sulphonamides (in 13.1% of the resistant strains). Thirty-seven isolates (21.1%) were resistant to at least two antimicrobials, and only one isolate (0.6%) was resistant to the four antimicrobials simultaneously. Quite different multiresistance profiles have been previously reported for 60 *A. pleuropneumoniae* strains isolated in Taiwan between 1985 and 1993, which were tested using an agar disk diffusion method (Chang et al., 2002b).

In conclusion, this study demonstrates an increase in resistance for tetracyclines and gentamicin of the porcine *A. pleuropneumoniae* isolates collected during 1997–2004 in Spain compared to those isolated during 1987–1988. This fact, along with the bimodal or multimodal distribution of strains over the MIC range observed for penicillins, trimethoprim, sulfisoxazole and nalidixic acid, suggests the need for a continuous

Table 2
Antimicrobial resistance profiles of the *Actinobacillus pleuropneumoniae* clinical strains isolated during 1997–2004

No. of isolates	No. of antimicrobial agents	Resistance to
129	1	TET
5	1	GEN
4	1	FIS
6	2	TET–GEN
23	2	TET–FIS
2	2	GEN–FIS
5	3	TET–GEN–FIS
1	4	TET–GEN–CEP–FIS

Abbreviations are as defined in the legend of Table 1.

surveillance of the susceptibility pattern of the clinical isolates of this veterinary pathogen. However, cephalothin and especially florfenicol remain as useful for treatment of swine with pleuropneumonia.

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