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In vitro susceptibility of *Clostridium perfringens* isolated from farm animals to growth-enhancing antibiotics

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L.A. DEVRIESE, G. DAUBE, J. HOMMEZ AND F. HAESEBROUCK. 1993. Minimal inhibitory concentration (MIC) determinations were carried out with seven growth-enhancing antibiotics against 95 *Clostridium perfringens* field isolates obtained during 1991 and 1992 from poultry, pigs and calves. All were resistant to 64 μg ml^{-1} of the bambermycin antibiotic, flavomycin (flavophospholipol) and susceptible to avoparcin (MIC_{90} 0·25 μg ml^{-1}), avilamycin (MIC_{90} 0·5 μg ml^{-1}) and salinomycin (MIC_{90} $\leq$0·12 μg ml^{-1}). Acquired resistance against bacitracin was detected in some isolates from poultry and bovines and resistance to tylosin and virginiamycin in some strains from all species investigated. Overall, the prevalence of resistance was comparable to the low levels recorded in 1979 in *Cl. perfringens* isolates from the same animal host species.

INTRODUCTION

There is little information about the susceptibility of *Clostridium perfringens* to growth-enhancing antibiotics and the eventual development of resistance has not been adequately monitored. Dutta and Devriese (1980) made a study on the susceptibility of strains isolated in 1979 from pigs, cattle and poultry with the growth-enhancing and therapeutical antibiotics available at that time. More recent information was provided by Benno *et al.* (1988a,b). These authors, however, studied mainly the susceptibility of strains from poultry against some growth-enhancing antibiotics currently available. Stutz and Lawton (1984) determined the *in vitro* activity of several growth-promoting antibiotics against only one reference strain of *Cl. perfringens*.

The present study was undertaken to update our knowledge on susceptibility and resistance of *Cl. perfringens* associated with the most important farm animals, against currently available growth-enhancing antibiotics.

MATERIALS AND METHODS

Strains

Thirty-two strains of *Cl. perfringens* were isolated in 1991 from small intestines of meat type calves up to 3 months of age which were suffering from intestinal disorders. The calves did not receive any food that contained growth-promoting antibiotics. Thirty-one isolates were obtained in 1992 from the caeca of 3–4-week-old broiler chickens sampled during routine checks for coccidial parasites. Another collection of 32 isolates originated from faeces of fattening pigs and from inestines of pigs necropsied during 1992 which did not show signs suggestive of clostridial disease. They were isolated either on Columbia agar (Lab M) containing 5% bovine blood or on the same medium containing polymyxin B sulphate and kanamycin (SR93 supplement, Oxoid), incubated anaerobically. Alpha toxin-positive (haemolytic) colonies and other similar colonies were purified and stored at −20°C in lyophilization medium ('Mist desiccans') until used. Strains were identified as *Cl. perfringens* by their morphology and alpha-toxin reaction and/or by their reactions in lactose, motility, gelatin and nitrate tests.

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Antibacterial compounds and tests

The following antibiotics, manufactured for analytical purposes, were tested: avoparcin (American Cyanamid), flavomycin (Hoechst), virginiamycin (Smith Kline), zinc bacitracin (Apothekernes), salinomycin (Pfizer), tylosin (Elanco) and avilamycin (Elanco). They were dissolved in appropriate solvents, to make stock solutions containing 1000 μg ml^{-1} and then further diluted in sterile distilled water.

MIC tests were carried out on Columbia Agar without added blood, containing doubling dilutions of the antibiotics and used immediately or after storage at 4°C up to 4 d. Virginiamycin-containing plates were used on the day

of preparation. Antibiotic-free agar plates, incubated aerobically and anaerobically, were included as controls. Inocula were prepared by suspending colonies from overnight cultures on blood agar, in buffered saline to a density of 0·5 on the McFarland turbitity scale and diluted 40-fold before inoculation. Plates were seeded with approx. 1×10^5 colony-forming units using a Steers-type inoculum replicator (Mast) and incubated in a H_2–CO_2 atmosphere for approx. 24 h. The lowest concentration of antibiotic which yielded no growth, one discrete colony or a barely visible haze of growth was recorded as the minimal inhibitory concentration (MIC).

Strains resistant to the macrolide antibiotic tylosin and the streptogramin virginiamycin, as well as sensitive control strains, were assayed by DNA–DNA colony hybridization tests with an ErmBP probe as described previously (Daube *et al.* 1992). This probe hybridizes with genes specifying resistance to macrolide, lincosamide and streptogramin B antibiotics, by methylation of bacterial 23S ribosomal rRNA, the target of these antibiotics.

RESULTS

The MIC values of the poultry, pig and cattle strains are shown in Table 1. Results are summarized and compared with feed concentrations permitted in the EEC countries, in Table 2.

All strains were susceptible to avoparcin, avilamycin and salinomycin and resistant to flavomycin (flavophospholipol).

All strains except two avian and three bovine isolates were susceptible to bacitracin. The resistant isolates had MIC levels of 32 μg ml^{-1} or higher.

Two poultry isolates were resistant to virginiamycin and susceptible to the related macrolide antibiotic tylosin. Two bovine, one poultry and two porcine isolates were resistant to tylosin (MIC 8 μg ml^{-1}) and susceptible to virginiamycin. Another pig strain and two calf strains were highly resistant to tylosin (MIC $\geq$64 μg ml^{-1}) and were resistant or showed decreased susceptibility to virginiamycin. Only one of the tylosin-resistant, virginiamycin-sensitive calf strains and one pig strain resistant to both antibiotics, hybridized with the ErmBP probe.

Table 2 Summary of minimal inhibitory concentrations (MIC) of growth-enhancing antibiotics for 95 *Clostridium perfringens* isolates from calves, pigs and chickens compared with permitted feed levels in the EEC

Compound	MIC_{50}*	MIC_{90}*	Feed levels†
Avilamycin	$\leq$0·12	0·5	2·5–20
Avoparcin	0·25	0·25	5–40
Bacitracin	0·25	0·5	5–80
Flavomycin	$\geq$64	$\geq$64	1–25
Salinomycin	$\leq$0·12	$\leq$0·12	15–60
Tylosin	$\leq$0·12	$\leq$0·12	4–40
Virginiamycin	0·25	0·25	5–80

* Indicating that 50%, respectively 90% of isolates investigated were inhibited at the concentrations (μg ml^{-1}) listed.
† The range of permitted feed levels (μg g^{-1} or mg kg^{-1} of complete feedingstuff) of growth-enhancing antibiotics (Anon. 1991).

DISCUSSION

The results obtained with *Cl. perfringens* isolates collected in 1991 and 1992 may be compared with those obtained during 1979 in the same laboratory for strains from similar origins. All isolates in both collections were susceptible to avoparcin and resistant to flavomycin. No evidence of resistance against bacitracin was found among the porcine strains collected in 1979 (Dutta and Devriese 1980) and no virginiamycin resistance was seen among poultry strains. Overall no changes in resistance rates of apparent importance were seen when the 1991–1992 strain collections are compared with those isolated in 1979. The bacitracin MICs on the susceptible strains were about 10-fold lower than the MIC of 3·1 μg ml^{-1} reported by Stutz and Lawton (1984)

Table 1 Minimal inhibitory concentrations (MIC) of growth-enhancing antibiotics against 95 *Clostridium perfringens* isolates of poultry, porcine and bovine origin

	Number of strains with MIC (μg ml^{-1}) of									
Compound	$\leq$0·12	0·25	0·5	1	2	4	8	16	32	$\geq$64
Avilamycin	55	15	25							
Avoparcin	30	64	1							
Bacitracin	19	57	12	2					3	2
Flavomycin										95
Salinomycin	95									
Tylosin	86	1					5			3
Virginiamycin	42	48		2		2	1			

and 10-fold higher in the case of virginiamycin which showed an MIC of 0·02 μg ml^{-1} in their study. Benno *et al.* 1988a) reported high bacitracin resistance rates in Japanese poultry strains, with more than 50% of the strains investigated having MIC of over 100 μg ml^{-1}. Their virginiamycin susceptibility levels ranged from 0·2 to 3·2 μg ml^{-1} with 50% of the strains inhibited at 1·6 μg ml^{-1}.

Results with tylosin may be compared with those of another macrolide, erythromycin, studied in 1979. Here again there is no evidence of increases in resistance rates. Erythromycin, tylosin and virginiamycin belong to the macrolide lincosamide streptogramin (MLS) antibiotic family, therapeutic use of which may select cross-resistant strains. Diverse MLS resistance phenotypes, determined by a complex array of resistance genes, have been described (Arthur *et al.* 1987). Daube *et al.* (1992) used the ErmBP probe (Berryman and Rood 1989) and detected 47% of the isolates expressing resistance to erythromycin present in a collection of bovine, ovine and reference strains. In our study only one of the four macrolide (tylosin)-resistant poultry and porcine strains hybridized with the ErmBP probe. These findings suggest that MLS resistance genes other than *erm*B are present in animal *Cl. perfringens*. Another gene, *erm*Q of the same family, has recently been cloned from *Cl. perfringens* (Rood and Cole 1991). The two poultry strains resistant only to virginiamycin also gave negative results with the ErmBP probe. It is probable that the latter phenotype is not based on methylation of 23S ribosomal rRNA which confers resistance to the streptogramin B component of virginiamycin, as well as to macrolide and lincosamide antibiotics.

In our study no resistance to salinomycin was found (MICs of $\leq$0·12 μg ml^{-1}). Benno *et al.* (1988a) similarly found no resistance in a collection of 80 poultry strains isolated in Japan but the susceptibility levels ranged from 0·2 to 0·8 μg ml^{-1}. In another study the same group found that one of their *Cl. perfringens* strains was resistant to 6 μg ml^{-1} (Benno *et al.* 1988b).

No reports are available about the susceptibility of *Cl. perfringens* to avilamycin.

In our study the terms susceptibility and resistance are used in the microbiological sense, except in the case of flavomycin: strains with decreased susceptibility are termed resistant because they differ distinctly in their susceptibility from other strains that possess the normal susceptibility of the species, as determined by the composition and the build-up of all organisms in this species. In clinical medicine the MICs are also compared with blood and tissue levels to distinguish between susceptible and resistant strains. With growth-enhancing antibiotics this criterion of susceptibility and resistance cannot be used. Most are not absorbed and they are presumably active against bacteria only in the gut. For this purpose it can be assumed that the MIC of a growth-enhancing antibiotic may be compared with the feed levels normally used. Strains with MIC levels that are distinctly higher than the feed levels are less likely or are unlikely to be influenced *in vivo* by the compounds in daily use. As can be seen in Table 2, the MIC_{90} of the *Cl. perfringens* investigated here are well below permitted feed levels, flavomycin excepted. This indicates that six of the seven antibiotics investigated inhibited growth *in vitro* of over 90% of *Cl. perfringens* at concentrations lower than the levels at which these drugs enter the intestines. Stutz and Lawton (1984) found that in the case of intestinal *Cl. perfringens* from poultry, the *in vitro* findings corresponded with the *in vivo* situation, except with the bambermycin antibiotic flavomycin which was inactive *in vitro* but depressed ileal *Cl. perfringens* populations. The reason for this is unknown.

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