β -Lactamase Production by Intestinal Spirochaetes

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β -Lactamase production was demonstrated in four of nineteen strains of intestinal spirochaetes isolated from human subjects. The enzyme was preferentially active against penicillins and was inhibited by clavulanic acid; it was membrane bound and non-inducible. No plasmids were detected in the intestinal spirochaetes and the β -lactamase-production characteristic was not transferable to non-producing strains.

INTRODUCTION

Spirochaetes are present in the intestinal tract of man and other animals; *Treponema hyodysenteriae*, the causative agent of swine dysentery, is a proven pathogen, but similar spirochaetes occur as part of the normal porcine intestinal flora (Harris & Kinyon, 1974; Lemcke & Burrows, 1981). A selective medium used initially for the isolation of *T. hyodysenteriae* has also been used to isolate intestinal spirochaetes from human subjects (Tompkins *et al.*, 1981; Sanna *et al.*, 1982). Spirochaetes are found more commonly in the intestinal tracts of homosexuals, residents of tropical areas and Asian subjects resident in Britain than in other populations (Tompkins *et al.*, 1986; Cooper *et al.*, 1986).

In a recent survey, intestinal spirochaetes were isolated from 23 of 1527 subjects resident in West Yorkshire; sixteen isolates were from Asians and seven from homosexuals (Tompkins *et al.*, 1986). All sixteen isolates from Asians and three of those from homosexuals had a close phenotypic similarity to porcine intestinal spirochaetes. In this study, we investigated the relationship between resistance to β -lactam agents and production of β -lactamase by four of the nineteen human isolates tested.

METHODS

Isolation of spirochaetes. Intestinal spirochaetes were isolated from faeces samples or rectal swabs on a selective medium cultured anaerobically for at least 5 d as described previously (Tompkins *et al.*, 1981). Sensitivity to antibiotics was tested by disc diffusion on blood agar incubated anaerobically for 48 h using the Oxford staphylococcus (NCTC 6571) and *Clostridium perfringens* (NCTC 8347) as controls (Stokes' method). Minimal inhibitory concentrations (MICs) were determined by incorporation of antibiotic solutions in blood agar plates, using a modification of the method of Kitai *et al.* (1979). Tryptic Soy Agar (Difco) was used as the basal medium with 5% (v/v) defibrinated horse blood, and 10 ml medium was used per plate; growth was detected easily by production of clear zones of haemolysis as well as by observation of surface growth. Plates were used on the day of preparation to minimize the effect of degradation of the β -lactam agents. A suspension of each organism was made in Tryptic Soy Broth (Difco) equivalent in turbidity to McFarland Standard no. 5, giving an inoculum of 10^8 – 10^9 c.f.u. ml⁻¹, and was applied with a multipoint inoculator delivering 1 μ l per spot. A heavy inoculum was used to give a readable result with these slow-growing organisms after 48 h anaerobic incubation at 37 °C.

β -Lactamase production. This was detected by two methods. Inhibition by cross-streaked spirochaetes of the sensitivity of the Oxford staphylococcus (NCTC 6571) to a 10 μ g ampicillin disc was tested on blood agar incubated anaerobically for 48 h (the 'clover-leaf' method: McGhie *et al.*, 1977). Suspensions of spirochaetes (10^{10} – 10^{12} c.f.u. ml⁻¹) in phosphate-buffered saline (PBS) (NaCl 8.0 g l⁻¹, KCl 0.2 g l⁻¹, Na₂HPO₄ 1.15 g l⁻¹, KH₂PO₄ 0.2 g l⁻¹; pH 7.3) were added to an equal volume of a solution of nitrocefin (chromogenic cephalosporin, 87/312, Glaxo) at a concentration of 200 mg l⁻¹ in the wells of a micro-titre plate, and incubated at room temperature for up to 1 h (Sykes & Matthew, 1979).

Cell-free extracts prepared by repeated freeze-thawing (Bidwell & Reeves, 1980) or by sonication followed by centrifugation were examined by isoelectric focusing using agarose gels stained with nitrocefin (Vecoli *et al.*, 1983).

The rates of hydrolysis of penicillin, ampicillin and cephaloridine were measured by a microbiological method (Sykes & Matthew, 1979). Spirochaetes were cultured on a selective medium, blood agar with vancomycin (10 mg l⁻¹) amphotericin B (2 mg l⁻¹) and colistin sulphate (6 mg l⁻¹), to suppress plate contaminants. After anaerobic culture for 72 h, surface growth from seven plates was harvested into 2 ml PBS pH 7.3 to give 10¹⁰–10¹² c.f.u. ml⁻¹, and stored at -20 °C. Thawed spirochaete suspension (0.05 ml) was added to 0.95 ml antibiotic substrate in PBS and incubated at 37 °C. The reaction was stopped with iodine reagent and residual antibiotic was assayed by plate diffusion using the Oxford staphylococcus (NCTC 6571), after 2, 4 and 6 h incubation.

Induction of β -lactamase production was determined as follows. Spirochaetes were incubated anaerobically for 4 d in tryptic soy broth (Difco) containing 10% (v/v) foetal calf serum, 0.5% (w/v) glucose and 0.5% (w/v) cysteine hydrochloride, plus a range of subinhibitory concentrations of methicillin (1–10 mg l⁻¹). β -Lactamase activity was then measured semi-quantitatively. A 10 ml volume of broth culture was centrifuged at 800 *g* for 15 min; the deposit was re-suspended in 0.5 ml PBS pH 7.3, added to 0.5 ml nitrocefin solution (200 mg l⁻¹) and incubated at room temperature. The time at which a colour change was detected was recorded.

Tests for the presence of plasmids. Strains were examined for the presence of plasmid DNA by the method of Bennett *et al.* (1986), and transfer of the β -lactamase characteristic from positive to negative strains was attempted by the method of Avila *et al.* (1984).

RESULTS AND DISCUSSION

On disc sensitivity testing, four of the nineteen strains of spirochaete were resistant to penicillin (1 unit) and ampicillin (10 μ g). All strains were sensitive to cephaloridine (30 μ g), cephadrine (30 μ g), cefotaxime (30 μ g), cefoxitin (30 μ g), tetracycline (10 μ g), chloramphenicol (25 μ g) and metronidazole (2.5 μ g). All strains were resistant to vancomycin (25 μ g) and colistin sulphate (25 μ g).

The four penicillin-resistant strains gave positive results with the nitrocefin solution; two strains (B and 11) produced a colour change from yellow to red within 10 min, but two (4 and 9) gave a change to an orange colour only after 1 h at room temperature. After repeated freeze-thawing or sonication and centrifugation, nitrocefin activity remained in the cell envelope pellet but was not demonstrable in the supernatant and no bands were demonstrated on IEF, which suggests that the enzyme activity was membrane-bound. All four strains gave positive 'clover-leaf' tests (Fig. 1). The penicillin-sensitive isolates gave negative nitrocefin and 'clover-leaf' tests.

MICs are shown in Table 1; results were reproducible when using the method described. Variation of inoculum size and duration of incubation caused variation in the MICs to penicillin of sensitive strains from 0.125 to 1 mg l⁻¹, and of β -lactamase producers from 4 to 32 mg l⁻¹. In all experiments the four β -lactamase-producing strains had MICs to penicillin within one dilution of each other and, as a group, were separated by four or more dilutions from the MICs of sensitive strains. Similar results were obtained with ampicillin. The results of one experiment using samples of one solution of each agent are given in Table 1 to show that a subinhibitory concentration of clavulanic acid lowered the MICs of penicillin and ampicillin for the β -lactamase-producing strains. The three strains with MICs of tetracycline of >1 mg l⁻¹ were isolated from homosexuals.

Substrate-concentration-dependent inhibition of β -lactamase activity was demonstrated by strain B; with a cell concentration of 1.2×10^{11} c.f.u. ml⁻¹ there was no hydrolysis of penicillin in concentrations exceeding 0.25 mM. The results of hydrolysis of 0.125 mM-solutions of penicillin, ampicillin and cephaloridine by the four β -lactamase-producing strains and a control non-producer are given in Table 2, which shows mean values of one experiment performed in duplicate. In this and separate experiments with strain B, reproducibility was within the acceptable limits of a microbiological assay. The two strains, B and 11, which hydrolysed penicillin and ampicillin more rapidly also produced a more rapid colour change with nitrocefin than strains 4 and 9. No residual penicillin or ampicillin was detectable by our assay method after 6 h incubation with strains B and 11. Control antibiotic solutions, incubated without spirochaetes, showed a low level of spontaneous breakdown of penicillin and ampicillin, and

Fig. 1. β -Lactamase from spirochaete strain B inhibiting the sensitivity of the Oxford staphylococcus (NCTC 6571) to ampicillin (the 'clover-leaf' test).

Table 1. *Susceptibility of 19 intestinal spirochaetes to antimicrobial agents*

Antibiotic	No. of strains*									
	MIC (mg l ⁻¹):									
	<0.12	0.25	0.5	1	2	4	8	16	32	64
Penicillin†	2	4	9					4		
Penicillin + clavulanic acid (2 mg l ⁻¹)†	4	5	6	2	2					
Ampicillin†			5	9	1			3	1	
Ampicillin + clavulanic acid (2 mg l ⁻¹)†		2	4	7	2					
Clavulanic acid†				1	3					
Methicillin					1	5	8		8	7
Cephadrine							1	1	1	3
Cefotaxime							2	8	2	
Cefoxitin								4	5	
Chloramphenicol				4	8	3				
Tetracycline	9	4		3	1					
Metronidazole	3				1	1				
	4	4	7							
		2	2							

* The numbers of β -lactamase-producing strains are shown in *italic* type.

† Results of one experiment. The values for the other antibiotics are median results from three experiments.

Table 2. *β -Lactamase activity of intestinal spirochaetes*

The rates of hydrolysis of penicillin and ampicillin were similar at 2 h and 4 h, and mean values are given. No hydrolysis of cephaloridine was detected for any of these strains.

Strain	β -Lactamase activity of 10^{11} cells ($\mu\text{mol h}^{-1}$)	
	Penicillin	Ampicillin
C (control)	ND	ND
B	8.1	10.5
9	4.4	5.5
4	0.4	0.1
11	12.2	12.0

ND, No hydrolysis detected.

appropriate adjustments were made in the calculations of the rates of hydrolysis. Amounts of enzyme produced were small and varied between producing strains but were sufficient to confer the demonstrable differences in MICs compared with those of non β -lactamase-producing strains in these slow-growing organisms. There was no detectable hydrolysis of cephaloridine after incubation for 6 h, and reduction of the concentration of cephaloridine to <1.0 mM with the same concentration of cells did not result in any detectable hydrolysis. No increase of β -lactamase activity was detected by a semi-quantitative method following incubation of broth cultures containing a range of methicillin concentrations. MICs of methicillin for the β -lactamase-producing strains ranged from 8 to 32 mg l^{-1} and for non-producing strains from 2 to 32 mg l^{-1} .

No plasmids were detected in the four β -lactamase-positive and four β -lactamase-negative strains, and the β -lactamase characteristic was not transferred to a non-producer strain in mixed culture. The amounts of RNA and chromosomal DNA present in preparations from spirochaete cultures were similar to those extracted from staphylococci and *Escherichia coli*.

We are not aware of any previous reports of β -lactamase production by spirochaetes. *T. hyodysenteriae* is moderately sensitive to penicillin (MIC $0.5\text{--}1.0 \text{ ml l}^{-1}$) (Kitai *et al.*, 1979) and no plasmids have been demonstrated in this or other similar large spirochaetes. Two pathogenic spirochaetes, *Treponema pallidum* and *Borrelia burgdorferi* (the causative agent of Lyme disease), contain plasmids (Norgard & Miller, 1981; Hyde & Johnson, 1984). These have not been associated with antibiotic resistance, but the potential exists and has given rise to concern (Stapleton *et al.*, 1985).

In our experience, the presence of large spirochaetes in the human intestinal tract is rarely associated with disease (Tompkins *et al.*, 1986). Intestinal spirochaetes are more common in homosexual males than in control populations (Cooper *et al.*, 1986; Tompkins *et al.*, 1986), and close association with *T. pallidum* on the rectal mucosa would afford an ideal environment for exchange of genetic material.

There has been one reported bacteraemia with these organisms in an alcoholic patient with bloody diarrhoea (Lambert & Goursot, 1982). This patient responded initially to penicillin therapy, but later died in cardiac failure. All 19 isolates in our study were sensitive to metronidazole, which may be the agent of choice for the therapy of genuine infection with large intestinal spirochaetes.

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