

In Vitro Antimicrobial Susceptibility of *Brachyspira pilosicoli* Isolates from Humans

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The in vitro antimicrobial susceptibility of the anaerobic intestinal spirochete *Brachyspira pilosicoli* was investigated by an agar dilution method. Human ($n = 123$) and porcine ($n = 16$) isolates were susceptible to metronidazole, ceftriaxone, meropenem, tetracycline, moxifloxacin, and chloramphenicol; erythromycin and ciprofloxacin were not active. Resistance to amoxicillin and clindamycin varied. Amoxicillin susceptibility was restored by clavulanic acid.

Brachyspira (previously *Serpulina*) *pilosicoli* is an anaerobic intestinal spirochete which colonizes the large intestines of pigs, numerous other farmed and wild animals, and humans (25). In humans, *B. pilosicoli* is one cause of intestinal spirochetosis, a condition characterized by end-on attachment of the organism to colorectal epithelial cells (9, 16, 21). In several reports, intestinal spirochetosis was the only abnormal finding for patients with abdominal pain, rectal bleeding, and diarrhea, and many of these symptoms were long-term or recurrent (4, 12, 16).

B. pilosicoli has been isolated at a prevalence of 10 to 30% from the feces of a range of different human population groups, including Aboriginal Australians (3, 15), individuals from developing countries (2, 3, 23), homosexual males, and human immunodeficiency virus-positive individuals (21). Carriage of these organisms was associated with gastrointestinal symptoms, particularly diarrhea; however *B. pilosicoli* was also isolated from healthy individuals in these populations. *B. pilosicoli* has also been isolated from the bloodstream of individuals in France, the United States, and Greece (6, 10, 13, 24). All of the individuals in the latter group were immunocompromised and suffered other debilitating illnesses, and six subsequently died.

The in vitro susceptibility of *B. pilosicoli* isolates from humans has received little attention (20), and currently there are no approved standards for antimicrobial testing of *Brachyspira* species (17). Thus, the aim of this study was to test the in vitro antimicrobial susceptibility of isolates of *B. pilosicoli* according to NCCLS guidelines for testing anaerobes. Human isolates obtained from a number of geographic locations were tested together with a small collection of porcine isolates included to provide comparative data. It was hypothesized that isolates from different sources would show different patterns of antimicrobial susceptibility.

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The 139 *B. pilosicoli* isolates studied included 123 from humans and 16 from pigs. Human isolates were obtained from Papua New Guinean (PNG) villagers ($n = 29$) (23), Aborigines from Western Australian communities ($n = 32$) (3, 15), migrants to Western Australia ($n = 24$) (3), homosexual males ($n = 14$), and individuals from Oman ($n = 10$) (2) and Italy ($n = 7$). Seven human isolates from blood samples (6, 24) were tested also. Porcine isolates were from Australia ($n = 9$), North America ($n = 6$) (5), and the United Kingdom (the type strain P43/6/78 [ATCC 51139^T] (25)). All *B. pilosicoli* isolates were obtained from the culture collection held at the Reference Centre for Intestinal Spirochetes at Murdoch University. The reference organisms for control purposes included the facultative anaerobes *Staphylococcus aureus* ATCC 29213, *S. aureus* ATCC 25923, and *Enterococcus faecalis* ATCC 29212, and the anaerobes *Bacteroides fragilis* ATCC 25285, *Bacteroides thetaiotaomicron* ATCC 29741, and *Clostridium perfringens* ATCC 13124. With the exception of *B. thetaiotaomicron*, which was obtained from Judy Holdsworth, Fremantle Hospital Microbiology Section, the control organisms were obtained from the Division of Microbiology and Infectious Diseases, Western Australian Centre for Pathology and Medical Research. All organisms were stored at -70°C in 1% brain heart infusion broth–10% glycerol–50% horse serum.

The antimicrobial agents tested were amoxicillin (CSL, Parkville, Australia), ceftriaxone (Roche Products, Dee Why, Australia), chloramphenicol and tetracycline (Sigma Aldrich, Castle Hill, Australia), ciprofloxacin and moxifloxacin (Bayer, Leverkusen, Germany), clindamycin and gentamicin (Pharmacia and Upjohn, Kalamazoo, Mich.), erythromycin (Blaschim, Milan, Italy), and meropenem (Zeneca, Wilmington, Del.). The β -lactamase inhibitor clavulanic acid (GlaxoSmithKline, Boronia, Australia) was tested in combination with amoxicillin. Agents were dissolved in appropriate solvents and stored at -70°C at concentrations of 2,560 or 5,120 $\mu\text{g/ml}$ until required.

The antimicrobial susceptibility of *B. pilosicoli* isolates was determined by using an agar dilution method based on NCCLS

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TABLE 1. In vitro activities of 12 antimicrobial agents against 139 *B. pilosicoli* isolates

Antimicrobial agent	No. of isolates inhibited at MIC ($\mu\text{g/ml}$) of:															MIC ₅₀	MIC ₉₀	% Sus ^a	
	≤ 0.016	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256				512
Amoxicillin					4	26	34	5	7	5	24	18	12	4			4	64	54.7
Amoxicillin-clavulanic acid ^b					4	35	39	35	26								1, 0.5	4, 2	100
Ceftriaxone				19	85	23	11	1									0.25	1	100
Chloramphenicol						4	95	40									1	2	100
Ciprofloxacin							4	51	84								4	4	39.6
Clindamycin	3	0	8	13	21	32	23	14	6	8	10	1				0.5	8	86.3	
Erythromycin									1	0	1	8	2	32	66	0	256	>512	NA
Gentamicin						3	4	14	48	55	13	2				4	16	NA	
Meropenem				16	115	8										0.25	0.25	100	
Metronidazole				19	110	10										0.25	0.25	100	
Moxifloxacin							4	113	2							2	2	NA	
Tetracycline				66	47	0	10	9	7							0.25	2	100	

^a Sus, susceptible. The following breakpoints for resistance were used: for amoxicillin, $\geq 8 \mu\text{g/ml}$; for amoxicillin-clavulanic acid, ≥ 16 and $8 \mu\text{g/ml}$, respectively; for ceftriaxone, $\geq 64 \mu\text{g/ml}$; for chloramphenicol, $\geq 32 \mu\text{g/ml}$; for clindamycin, $\geq 8 \mu\text{g/ml}$; for meropenem, $\geq 16 \mu\text{g/ml}$; for metronidazole, $\geq 32 \mu\text{g/ml}$; and for tetracycline, $\geq 16 \mu\text{g/ml}$ (17). A breakpoint of $\geq 4 \mu\text{g/ml}$ was used for ciprofloxacin (18). NA, not available.

^b Separate MIC₅₀s and MIC₉₀s are given for amoxicillin and clavulanic acid.

guidelines for susceptibility testing of anaerobic bacteria (17). Briefly, spirochete stocks were thawed and passaged once on Columbia base agar with 6% horse blood agar at 37°C for 5 days in an anaerobic chamber (Don Whitley Scientific, Ltd., Shipley, Yorkshire, England) in an atmosphere of 80% N₂, 10% H₂, and 10% CO₂. In preliminary experiments, Wilkins-Chalgren agar and brucella blood agar for susceptibility testing (19) did not support the growth of *B. pilosicoli*. Therefore, Wilkins-Chalgren agar containing 5% horse serum plus the appropriate concentration of antimicrobial agent was used. The agar was made less than 24 h before inoculation and stored at 4°C until required. The plates containing amoxicillin were made on the day of use. Facultative control organisms were inoculated onto Mueller-Hinton agar without horse serum. Growth was resuspended in brucella broth using a sterile cotton-tipped swab at a concentration of 3×10^7 to 3×10^8 spirochetes/ml, in accordance with McFarland standards. The suspension was added to the wells of a sterile multipoint inoculator tray and used to inoculate the agar by means of a Denley multipoint inoculator (Mast Labs, Liverpool, England), which delivered 3×10^4 to 3×10^5 organisms per spot. Trays contained the test organisms plus anaerobe controls, while facultative controls were added to another tray and inoculated and incubated separately. The trays were incubated immediately after inoculation for 96 h, with anaerobic cultures in the anaerobic chamber and facultative controls at 37°C aerobically.

Cultures were compared to a growth control plate containing no antibiotic, and the MIC of an agent was determined as the lowest concentration of antimicrobial yielding no growth, a haze, one discrete colony, or multiple tiny colonies (19). Tests for each organism were performed at least in triplicate, and the MIC of each agent was determined as the modal value. An organism was defined as resistant to a particular agent if the MIC obtained was above the published breakpoint for anaerobic bacteria (17).

As a control for antibacterial activity, and to detect variations in MICs due to medium-antibiotic interactions, MICs of agents for anaerobic reference organisms were also determined in triplicate on the same media under the same test conditions in accordance with NCCLS standard protocols. Where an agent did not have published breakpoint values for

anaerobes, aerobic reference organisms inoculated on Mueller-Hinton agar were included for comparison.

β -Lactamase production in all strains was assessed by using BBL Cefinase nitrocefin disks (Becton Dickinson). Each disk was moistened with sterile distilled water, and a loopful of spirochete growth was smeared onto the disk surface. The disk was protected from drying and examined for a color change reaction (from colorless to red) within 10 min and then at 30 min. *S. aureus* ATCC 29213 and *S. aureus* ATCC 25923 were included as positive and negative controls, respectively.

Isolates were categorized on the basis of source, and proportions of resistant isolates and of isolates for which the MICs were elevated were calculated. Statistical analysis was carried out using the chi-square or Fisher's exact test to compare groups.

The numbers of isolates inhibited at various MICs, the MICs at which 50% of the isolates were inhibited (MIC₅₀s), and the MIC₉₀s of the 12 antimicrobials tested against *B. pilosicoli*, with proportions of isolates susceptible at published breakpoints for anaerobic bacteria, are presented in Table 1. In general, *B. pilosicoli* isolates were susceptible to the agents tested, with MIC₉₀s below the breakpoints. Exceptions to this were amoxicillin and clindamycin, which had MIC₉₀s of 64 and 8 $\mu\text{g/ml}$, respectively. Over 50% of the isolates were nitrocefin positive, which, for all except eight isolates, corresponded to an amoxicillin MIC above the published breakpoint.

A bimodal distribution of MICs was recorded for amoxicillin, erythromycin, and tetracycline but not for clindamycin or gentamicin. For amoxicillin, the trough in the distribution corresponded to the published breakpoint; however, the tetracycline MIC for all isolates was below the breakpoint. Amoxicillin susceptibility was restored by clavulanic acid in all instances (MIC₉₀, 4 $\mu\text{g/ml}$), suggesting that β -lactamase activity was involved in the initial lack of susceptibility. Similar findings were reported by Tompkins et al. (20), who examined 19 spirochete isolates from Asians and homosexual males living in the United Kingdom. Activity against penicillin and ampicillin was demonstrated for four spirochetes (which were almost certainly *B. pilosicoli*). Furthermore, the β -lactamases were found to be membrane bound and noninducible.

Based on NCCLS breakpoints for anaerobes, isolates of *B. pilosicoli* were susceptible to ceftriaxone, chloramphenicol,

TABLE 2. Percentages of *B. pilosicoli* isolates which were nitrocefin positive and resistant to amoxicillin and clindamycin and for which the MICs of erythromycin, gentamicin, tetracycline, and three or more of these five antimicrobials were raised

Source	No. of isolates	% of <i>B. pilosicoli</i> isolates ^a						
		Nitrocefin positive	Amoxicillin MIC, ≥ 8 $\mu\text{g/ml}$	Clindamycin MIC, ≥ 8 $\mu\text{g/ml}$	Erythromycin MIC, >512 $\mu\text{g/ml}$	Gentamicin MIC, ≥ 16 $\mu\text{g/ml}$	Tetracycline MIC, ≥ 1 $\mu\text{g/ml}$	Raised MICs of ≥ 3 antibiotics
Aboriginal Australians	32	93.8*	81.3*	12.5	28.1	0.0†	3.1†	12.5
Migrants to WA ^b	24	58.3	45.8	12.5	20.8	4.2	4.2†	4.2
Homosexual males	14	50.0	50.0	28.6	35.7	21.4	85.7*	42.9*
Omani nationals	10	60.0	60.0	0.0	0.0	10.0	0.0	0.0
Blood isolates	7	28.6	14.3	42.9	42.9	28.6	42.9	28.6
Italian patients	7	28.6	28.6	0.0	0.0	14.3	28.6	14.3
PNG villagers	29	0.0†	0.0†	0.0†	0.0†	0.0†	0.0†	0.0†
Swine	16	62.5	62.5	31.3*	43.8*	43.8*	43.8*	37.5*
Total	139	51.1	45.3	13.7	20.9	10.8	18.7	15.1

^a * Denotes percentage values which are significantly higher than the percentage obtained for all isolates; † denotes percentage values which are significantly lower than the percentage obtained for all isolates.

^b WA, Western Australia.

meropenem, metronidazole, and tetracycline. Erythromycin (MIC₉₀, >512 $\mu\text{g/ml}$) was not active against these organisms. Breakpoints are currently unavailable for ciprofloxacin, which was included as it is often used to treat diarrheal illness; however, when a breakpoint of ≥ 4 $\mu\text{g/ml}$ was used (18), 60% of the isolates were resistant, which is consistent with the finding that the early quinolones are not very active against anaerobes (1). Moxifloxacin (MIC₉₀, 2 $\mu\text{g/ml}$) demonstrated better activity, although its MICs were slightly higher for *B. pilosicoli* than for other susceptible anaerobes (7). Clindamycin and erythromycin showed some cross-resistance: the MICs of erythromycin for all isolates ($n = 19$) resistant to clindamycin were >512 $\mu\text{g/ml}$; however, 10 other isolates for which the erythromycin MICs were >512 $\mu\text{g/ml}$ were not resistant to clindamycin.

Comparisons between the MICs for isolates from the various sources are presented in Table 2. The MICs of amoxicillin, clindamycin, erythromycin, gentamicin, and tetracycline varied with the source of the isolate. All isolates from PNG villagers were susceptible to these antibiotics; they also were negative for β -lactamase production. In all instances, proportions of resistant isolates were significantly lower than for other population groups (P values were <0.001 , except with gentamicin, with which P was 0.024). The PNG villagers had very basic living conditions and were originally chosen for study because of their lack of exposure to antibiotics (22). These isolates may well represent a naive population of *B. pilosicoli*.

Significantly higher proportions of isolates from Australian Aboriginals than from other groups were resistant to amoxicillin ($P < 0.001$) or were β -lactamase producers ($P < 0.001$). However, for significantly lower proportions of these isolates, the MICs of gentamicin ($P = 0.015$) and tetracycline ($P = 0.021$) were raised. The high proportions of β -lactamase production most likely reflected exposure of this group to antibiotics. Respiratory disease and otitis media are significant health problems in the Aboriginal Australian population (14, 26). Pneumococci are the biggest cause of pneumonia and otitis media in this population, and recommended therapies are penicillin G and amoxicillin, respectively (8, 26).

Tetracycline MICs for over 85% of isolates from homosexual males were elevated ($P < 0.001$), but these isolates were not resistant to tetracycline as assessed with NCCLS breakpoints. This phenomenon was also recorded by Tompkins and

colleagues for isolates from three homosexual males (20). The proportions of isolates for which the tetracycline MICs were elevated were also significantly higher for pigs ($P = 0.013$). The MICs of all five antibiotics for the porcine isolates were raised, and those of clindamycin ($P = 0.046$), erythromycin ($P = 0.025$), and gentamicin ($P < 0.001$), as well as tetracycline, were significantly higher. All groups except those from PNG and Oman contained isolates for which the MICs of three or more antibiotics were elevated; the MICs for isolates only from pigs ($P = 0.013$) and homosexual males ($P = 0.006$) were significantly higher, while those for isolates from PNG villagers ($P = 0.0062$) were significantly lower.

The higher MICs of β -lactam and lincosamide antibiotics for pig isolates may be due to the use of antibiotics as growth promoters and therapeutic agents in veterinary settings. The prominent use in swine farming of tylosin, another macrolide, and lincomycin, another lincosamide, has led to reports of a high frequency of resistance of intestinal spirochetes to these antibiotics (11). Penicillins are also used frequently as therapy for other swine infections. Gentamicin MICs were raised only for isolates from pigs. For pig isolates, Duhamel et al. (5) recommended gentamicin breakpoints of ≤ 1 $\mu\text{g/ml}$ as susceptible, 5 $\mu\text{g/ml}$ as intermediate, and ≥ 10 $\mu\text{g/ml}$ as resistant; however, the bimodal distribution they described was not seen in the present study.

Previous studies of the antimicrobial susceptibility of intestinal spirochetes have tended not to adhere to standard methods. Although agar dilution susceptibility methods have used Trypticase soy agar, parameters such as inoculum concentration, duration of incubation, and criteria for determination of MIC have differed. Reproducible susceptibility testing of *Brachyspira hyodysenteriae* isolates has been achieved by broth microdilution with brain heart infusion broth plus 10% fetal calf serum and specialized microplates (11); however, these are manufactured in Sweden and are not commercially available. In addition, the methods still do not conform to NCCLS recommendations.

The results obtained in this study indicate that antimicrobial susceptibility testing of *B. pilosicoli* is possible by the NCCLS agar dilution method. The only modifications made were the addition of 5% horse serum to the Wilkins-Chalgren agar, a recommendation supported by the *Wadsworth Anaerobic Bac-*

teriology Manual (19), and an increase in the incubation time for these slowly growing organisms. The addition of horse serum permitted consistent growth of all isolates tested while allowing reference anaerobes to remain within their accepted susceptibility ranges. The method was highly reproducible and easily adapted for testing *B. pilosicoli* and may be suitable for testing other *Brachyspira* spp. The data generated in this study may be useful in selecting a therapeutic regimen for infections with *B. pilosicoli*.

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