The occurrence and epidemiology of intestinal spirochaetes in humans in Western Australia

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SUMMARY

The intestinal spirochaetes *Brachyspira aalborgi* and *Brachyspira pilosicoli* colonise the large intestine of humans, whilst *B. pilosicoli* also colonises a number of animal species. Both organisms have been detected in human intestinal spirochaetosis (HIS), a syndrome where the spirochaetes attach end-on to the colorectal epithelium, forming a characteristic "false brush border". Colonisation by *B. pilosicoli* occurs at >30% in Australian Aborigines (Lee & Hampson, 1992), homosexual males (Trivett-Moore *et al.*, 1998), human immunodeficiency virus (HIV)-positive individuals and individuals from developing countries (Barrett, 1990; Trott *et al.*, 1997a). Studies on *B. aalborgi* have focussed mainly on its detection from Caucasians.

Clinically, colonisation with *Brachyspira* spp. has been linked to gastrointestinal disorders, including chronic diarrhoea, abdominal pain and rectal bleeding. More seriously, invasive colitis and hepatitis associated with intestinal spirochaetes have been recorded, and *B. pilosicoli* has been isolated from the blood of nine patients. However, the pathogenic significance of *Brachyspira* spp. remains uncertain, due to an inconsistent clinical picture, carriage by healthy individuals and the absence of accurate spirochaete identification in many studies.

Identification of the organisms by culture and microscopy has been difficult, due to morphological similarities and biochemical heterogeneity of the *Brachyspira* spp.. In addition, *B. pilosicoli* is the easier spirochaete to culture, and so has predominated in studies assessing faecal carriage. The recent development of molecular methods has allowed unequivocal speciation of the *Brachyspira* spp.. Thus the main aim of this thesis was to examine the prevalence and epidemiology of *B. pilosicoli* and *B. aalborgi* in Australia, using this new methodology.

Intestinal carriage of both species was examined in faecal specimens using culture, microscopy and two species-specific 16S rRNA PCRs. Carriage of *Brachyspira* spp. was determined in individuals from rural areas of Western Australia (WA) with gastrointestinal symptoms, (including 151 Aboriginal and 142 non-Aboriginal Australians), 227 migrants to Australia, and 90 healthy control individuals from Perth, Western Australia. When samples were assessed by culture, *B. pilosicoli* was isolated from the faeces of 10.6% of migrant and 9.9% of Aboriginal Australian individuals. *B. pilosicoli* carriage was significantly higher in Aboriginal children aged 2 - 4 years ($P = 0.0027$) and migrants of African, Middle Eastern or Asian origin ($P = 0.0034$). Carriage in each population was associated with the detection of a number of intestinal parasites. It was not clear whether an association existed between *B. pilosicoli* isolation and disease, as similar proportions were found in sick Aboriginal patients and presumably healthy migrant individuals. *B. pilosicoli* may have caused disease in Aboriginal children where other pathogens were not present, or may have contributed to disease with known or suspected pathogens. In particular, colonisation was significantly more frequent in

individuals with chronic diarrhoea or failure to thrive (FTT) (P = 0.016), and was also increased in elderly patients. The association of *B. pilosicoli* with parasites may have been due to behavioural factors, or an as yet unknown relationship between the organisms.

B. aalborgi was not isolated from the specimens of rural individuals or migrants, as culture media and conditions for this organism were not optimal, but was thought to have been the spirochaetes seen by microscopy in culture negative samples. As a result, a more appropriate isolation medium for *B. aalborgi* was developed, containing brain heart infusion agar with 10% bovine blood, spectinomycin and polymyxin B. One isolate of *B. aalborgi* was obtained on this medium, from a control individual with self-reported chronic diarrhoea. The colonial, phase contrast and electron microscopic morphologies of two cell types present in the sample were described, and partial 16S rRNA sequences obtained. However, even with the modified media, other samples in this population contained spirochaetes that could not be cultured.

PCR conducted on DNA extracted by the Qiagen QIAmp column method was more suitable for determining the prevalence of *Brachyspira* spp. in these specimens. *B. pilosicoli* was detected in higher proportions by PCR, in 15.0% of migrants and 14.5% of Aboriginals, but again not in symptomatic or healthy non-Aboriginal Australians. Detection of *B. pilosicoli* by PCR changed the identification of risk factors only a little; detection of protozoa in migrants, and protozoa, *G. intestinalis*, *H. nana* in Aboriginals were no longer associated with *B. pilosicoli* detection. *B. aalborgi* DNA was detected in 7.9% of migrants, 6.9% of Aboriginals, 5.6% of non-Aboriginal patients, and 5.6% of controls. Approximately equal proportions were obtained from migrant individuals from African, Middle Eastern and Eastern European countries. Both spirochaete species were detected in migrants (5.6%) and Aboriginal Australians (4.1%), and carriage of one spirochaete was significantly associated with carriage of the other spirochaete in both populations (P <0.001). These data suggest that the two *Brachyspira* spp. have a different epidemiology, and that *B. aalborgi* may be distributed at a similar prevalence in many human populations.

Carriage of *B. aalborgi* was associated with larger family size in migrants (P <0.001), and if a family member was positive, it was significantly more likely that other family members would be colonised (P = 0.008). Two colonised control individuals also came from the same family, all suggesting that transmission of *B. aalborgi* can occur between family members. Carriage of *B. aalborgi* was significantly higher in Aboriginal patients aged 2 - 18 y, and in non-Aboriginal patients aged >18 y and between 60 - 69 y. Detection was associated with various parasites in Aboriginals, but only protozoans in migrants (P = 0.014). Detection of *B. aalborgi* was associated with diarrhoea in non-Aboriginals (P = 0.048), with an absence of diarrhoea (P = 0.016) amongst Aboriginals, and with eosinophilia, weight loss, being underweight and suspected worms in this group also (P <0.001). Whether *B. aalborgi* was a cause of these symptoms, or was excreted as a result of the symptoms caused by some other source, was unknown. The pathogenic significance of

B. aalborgi also could not be resolved in this investigation, as similar proportions were detected in ill and healthy individuals, and detection was associated with different reasons for investigation in the two ill population groups. *B. aalborgi* could be a commensal in the human gastrointestinal tract, or may have the capacity to cause disease under certain circumstances, such as in individuals with poor immune status, or in a conducive gut microenvironment.

PCR products were sequenced to attempt to differentiate between *B. aalborgi* strains. All except five strains were identical to the type strain, three had nucleotide changes that placed them in cluster 3 described by Pettersson *et al*. (2000), another strain shared similarity with cluster 2 or 3 strains and the final strain remained in cluster 1. More complete sequencing of this gene, or the use of another sequence based typing method, such as multi-locus sequence typing, would be useful to further investigate the molecular epidemiology of *B. aalborgi* carriage in human populations.

A comparison between techniques for intestinal spirochaete detection was made. PCR was the most successful method for detecting spirochaetes in faeces, with a lower limit of detection of *B. pilosicoli* in seeded faeces at 1 x 10^6 organism/g and 1 x 10^5 organism/g for *B. aalborgi*. Microscopy was inadequate for identifying the spirochaetes, and lacked sensitivity, with only 1 x 10^7 - 1 x 10^5 organisms/g of seeded faeces visible in this way. Culture was adequate for *B. pilosicoli*, with isolation possible from seeded faeces containing 1 x 10^2 – 2 x 10^3 cfu/g. Culture was inadequate for *B. aalborgi* detection, despite isolation from seeded faeces at 2 x 10^4 cfu/g, and advances made to isolation media for this spirochaete in the course of this investigation.

There were concerns that *B. pilosicoli* spirochaetaemia might be occurring in Western Australian populations with a high rate of intestinal carriage, but going undiagnosed. An evaluation of the blood culture systems commonly used in Australia indicated that the automated BACTEC system using the BACTEC plus Anaerobic/F bottle was best for the detection of *B. pilosicoli* in seeded blood. A protocol longer than the 5 d normally utilised by clinical laboratories was required to detect the organism when seeded at low concentrations. Both manual Hémoline and Septi-Chek media were as reliable as the BACTEC for *B. pilosicoli* growth, however detection in these systems required frequent subculture, and was thus time-consuming and laborious. The automated BacT/Alert system performed poorly both for detection and recovery of *B. pilosicoli*, and medium and algorithmic components were probable contributing factors.

BACTEC Plus Anaerobic/F bottles inoculated with blood from 801 patients thought to be at risk for blood infection with *B. pilosicoli* were cultured for 14 d, or 21 d after transport from rural centres. No isolate was obtained from these individuals, but there were some problems inherent in the investigation. However, while bacteraemia with *B. pilosicoli* is clearly a rare medical event, it should be considered as a differential diagnosis in

immunocompromised individuals with gastrointestinal symptoms, and possibly in groups with high rates of intestinal carriage of *B. pilosicoli*.

Pulsed-field gel electrophoresis, utilising *Mlu*I and *Sma*I restriction enzymes, was used to examine the relationships between *B. pilosicoli* isolates collected from Aboriginal and migrant individuals, and some pre-existing strains. In migrants, isolates were distributed throughout the dendrogram created after analysis with *Mlu*I; however, the migrants had originated from 10 countries around the world. Two *B. pilosicoli* isolates from two individuals from the same family were closely related, indicating that exposure from a common source or transmission from one individual to another might have occurred. As no other isolate from migrants were related, even among members of other families, transmission between family members did not appear to be common.

Strains from Australian Aboriginals were also distributed throughout the *Mlu*I dendrogram, but the majority (89.7%) were clustered in one of six clonal groups. Isolates were obtained from different locations and years of sampling, supporting previous observations that *B. pilosicoli* colonising Aboriginal people have an epidemic population structure (Trott *et al.*, 1998). An explanation for the presence of clonal groups was not clear. Increased virulence of isolates may have contributed to the spread of *B. pilosicoli* clones in WA Aboriginals. All isolates from hospitalised individuals, and those obtained from samples where no other pathogen was detected were members of clonal groups, however, no isolate from healthy Aboriginals was included in the investigation. Resistance to one or more antimicrobial also may have favoured selection for and spread of clonal isolates in the Aboriginal population.

The antimicrobial susceptibility of 139 *B. pilosicoli* isolates was obtained by a procedure modified from the NCCLS agar dilution method (NCCLS, 1990). Strains were generally sensitive to agents tested, but variably resistant to clindamycin and amoxicillin, depending on the source of strains. Over 50% of strains contained a β-lactamase that was inhibited by clavulanic acid. Significantly higher proportions of isolates from Aboriginal Australians ($P < 0.001$) contained a β-lactamase than other isolates, while no isolate from PNG natives did ($P < 0.001$), nor was any isolate from this population resistant to any other antimicrobial. Isolates from pigs and homosexuals demonstrated raised MICs for tetracycline and resistance to amoxicillin and clindamycin. All Aboriginal isolates in clonal groups contained a β-lactamase, as did other isolates not classified within clonal groups. Further analysis of the virulence characteristics and antibiotic susceptibility of the isolates is required.

Based on susceptibility results and success in previous studies, metronidazole was a potential choice for treatment of HIS, and to remove intestinal carriage of *B. pilosicoli*. For therapy in spirochaetaemia, intravenous ceftriaxone or Timentin would be preferred over penicillin antibiotics in view of the frequent occurrence of resistance. Carriage of spirochaetes is currently going unrecognised in Australian laboratories, as current protocols do not specifically target their detection, and current medical opinion remains ambivalent about their significance. Detection by any of culture, microscopy, PCR is not difficult, and could be implemented to examine faecal specimens during investigations of individuals with chronic diarrhoea of unknown aetiology, or in children who are failing to thrive.

In conclusion, the work presented in this thesis examined a range of issues relating to the epidemiology of intestinal spirochaetes in humans. The prevalence of *B. aalborgi* and *B. pilosicoli* was determined in faeces by microscopy, culture and PCR. The spirochaetes were present in both healthy and ill individuals, at around 15% for *B. pilosicoli* and 6% for *B. aalborgi.* Each organism was associated with specific risk factors in the populations assessed. The prevalence of bacteraemia with *B. pilosicoli* was examined, but no episode was identified in the study. The molecular epidemiology of *B. pilosicoli* was analysed using PFGE. MIC data were determined for *B. pilosicoli* and based on these, therapies for infections with this organism were suggested. Similar studies on *B. aalborgi* are now required to provide a more complete understanding of the epidemiology of this organism in humans.

DECLARATION

I hereby declare that unless otherwise stated, all work in this thesis was carried out by myself, and that it has not previously been submitted for a degree at any tertiary institution.

……..

Celia Josephine Brooke

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PUBLICATIONS AND CONFERENCE PROCEEDINGS

As a result of the work carried out in this thesis, the following papers, abstracts and conference proceedings were published:

Journal Articles

Brooke CJ, Margawani KR, Pearson AK, Riley TV, Robertson ID and Hampson DJ, 2000. Evaluation of blood culture systems for detection of the intestinal spirochete *Serpulina pilosicoli* in human blood. Journal of Medical Microbiology. **49:** 1031-1036.

Mikosza ASJ, La T, Margawani KR, **Brooke CJ** and Hampson DJ, 2001. PCR detection of *Brachyspira aalborgi* and *Brachyspira pilosicoli* in human feces. FEMS Microbiology Letters. **197:** 167-170.

Brooke CJ, Clair AN, Mikosza ASJ, Riley TV and Hampson DJ, 2001. Carriage of intestinal spirochaetes by humans: epidemiological data from Western Australia. Epidemiology and Infection. **127:** 369-374.

Brooke CJ, Riley TV, Lum G, and Hampson DJ, 2001. Failure to detect *Brachyspira pilosicoli* in bloodstream of Australian patients. Journal of Clinical Microbiology. **39:** 4219.

Brooke CJ, Riley TV and Hampson DJ, 2002. In vitro antimicrobial susceptibilities of human strains of *Brachyspira pilosicoli*. (Abstract) Anaerobe. **8:** 147-148.

Brooke CJ, Riley TV and Hampson DJ, 2003. Evaluation of selective media for the isolation of *Brachyspira aalborgi* from human faeces. Journal of Medical Microbiology. **52:** 509-513.

Brooke CJ, Hampson DJ and Riley TV, 2003. In vitro antimicrobial susceptibility of human isolates of *Brachyspira pilosicoli*. Antimicrobial Agents and Chemotherapy. **47:** 2354-2375.

Abstracts and Conference Proceedings

Brooke CJ, Riley TV and Hampson DJ, 1999. Detection of *Serpulina pilosicoli* in automated and manual blood culture systems. Program and Abstracts of the Australian Society for Microbiology Tri-state Meeting "Spirochaetes in the Spinifex", Alice Springs, Australia. Abstr. S2.18.

Brooke CJ, Riley TV and Hampson DJ, 1999. Automated detection of *Serpulina pilosicoli* in two blood culture systems: a comparative trial. Program and Abstracts of the International Union of Microbiological Societies IX International Congress of Bacteriology and Applied Microbiology, Sydney, Australia. Abstr. BPO23.01, p. 137.

Brooke CJ, Mikosza AJ, Riley TV and Hampson DJ, 2000. Faecal carriage of intestinal spirochaetes in Western Australia. Program and Abstracts of the Australian Society for Microbiology 2000 Joint Scientific Meeting and Exhibition, Cairns, Australia. Abstr. P3.04.

Brooke CJ, Clair AN, Riley TV, and Hampson, DJ, 2001. Molecular epidemiology of *Brachyspira pilosicoli* in Western Australia. Program and Abstracts of the Australian Society for Microbiology 2001 Annual Scientific Meeting and Exhibition, Perth, September Australia. Abstr. PP5.2.

Brooke CJ, Riley TV and Hampson DJ, 2002. In vitro antimicrobial susceptibilities of human strains of *Brachyspira pilosicoli*. Abstracts of Anaerobe Olympiad 2002. The 6th Biennial Congress of the Anaerobe Society of the Americas, Park City, UT USA. Abstr. SP4.

Brooke CJ, Riley TV and Hampson DJ, 2002. In vitro antimicrobial susceptibilities of human strains of *Brachyspira pilosicoli*. *In:* Programme and Abstracts of the Eighth Western Pacific Congress on Chemotherapy and Infectious Diseases. Perth, Australia. Abstr. 179.

Brooke CJ, Riley TV and Hampson DJ, 2003. The prevalence of intestinal spirochaetes in Western Australians. *In*: Programme and Abstracts of the Second International Conference on Colonic Spirochaetal Infections in Animals and Humans, Edinburgh, Scotland. Abstr. 7.

ABBREVIATIONS AND SYMBOLS

AA01	Aboriginal Australian sample 01
ABA	anaerobe basal agar
AFLP	amplified fragment length polymorphism
AIDS	acquired immune deficiency syndrome
ANGIS	Australian National Genomic Information Service
ANOVA	analysis of variance
ATCC	American Type Culture Collection
BA	blood agar
BB	bovine blood
BHI	brain heart infusion
BHIA	brain heart infusion agar
BHIB	brain heart infusion broth
BHIB/G	brain heart infusion broth with glycerol
bp	base pairs
BP	British Pharmacopoeia
BrB	brucella broth
BrBAB	brucella blood agar base
BrBAS	brucella blood agar for susceptibility testing
BrBAS+H	brucella blood agar for susceptibility testing plus hemin
°C	degrees Celsius
C	colistin
C01	control sample 01
CBA	columbia base agar
cfu	colony forming units
CO_2	carbon dioxide
d	days
Da	Dalton
DepC	diethylpyrocarbonate treated water
DEX	diatomaceous earth extraction
DIW	deionised water
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphatases
EDTA	ethylenediaminetetraacetic acid, disodium salt
EM	electron microscopy
ET	electrophoretic type
FCS	foetal calf serum
FISH	fluorescent *in situ* hybridisation
FOS	Fastidious Organism Supplement
FTT	failure to thrive
g	force of gravity
g	grams
G+C content	guanine plus cytosine content
GI	gastrointestinal
Gr.	Greek
h	hours
HB	human blood
HIS	human intestinal spirochaetosis
HIV	human immunodeficiency virus

HPW	high pure water
HS	horse serum
IFAT	indirect immunofluorescent antibody test
IS	intestinal spirochaetosis
l	litres
i.u.	international units
kDa (bp)	kiloDalton (base pairs)
L.	Latin
m	metres
M	Molar
M01	migrant sample 01
mol%	mole percent
mAb	monoclonal antibody
MLEE	multilocus enzyme electrophoresis
µg (l, m, M)	micrograms (litres, metres, Molar)
mg (l, m, M)	milligrams (litres, metres, Molar)
MH	Mueller Hinton
MIC	minimum inhibitory concentration
min	minutes
n	number
N	Normal
NA01	non-Aboriginal sample 01
NCCLS	National Committee for Clinical Laboratory Standards
nox	NADH oxidase
OB	ovine blood
OR	odds ratio
P	polymyxin B
PathCentre	Western Australian Centre for Pathology and Medical Research
PB	phosphate buffer
PBS	phosphate buffered saline
ρmol	picomoles
PCR	polymerase chain reaction
PET	paraffin embedded tissue
PFGE	pulsed-field gel electrophoresis
PNG	Papua New Guinea
PIS	porcine intestinal spirochaetosis
R	rifampicin
RBC	red blood cells
RE	restriction enzyme
REA	restriction enzyme analysis
RFLP	restriction fragment length polymorphism
rRNA	ribosomal ribonucleic acid
RT	room temperature
s	seconds
S	spectinomycin
SD	standard deviation
Sd	swine dysentery
SDW	sterile deionised water
SEM	scanning electron microscopy
SN	supernatant

S medium	trypticase soy agar medium containing spectinomycin (400µgml)
SP medium	trypticase soy agar medium containing spectinomycin (400µgml) and polymycin B (5µgml)
spp.	species
SPS	sodium polyanetholsulfonate
TAE	tris acetate EDTA
TBE	tris borate EDTA
TE	tris EDTA
TEM	transmission electron microscopy
TES	tris EDTA sarcosine buffer
TSA	trypticase soy agar
TSB	trypticase soy broth
U	units
UHPW	ultra high pure water
UK	United Kingdom
USA	United States of America
UV	ultraviolet
V	vancomycin
WA	Western Australia
WBHIS	weakly β-haemolytic intestinal spirochaete
WCA	Wilkins Chalgren agar
WCA+HS	Wilkins Chalgren agar plus horse serum
WHP01	Western Australian human *B. pilosicoli* isolate 01
w/v	weight for volume

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