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Colonization and risk factors for *Brachyspira aalborgi* and *Brachyspira pilosicoli* in humans and dogs on tea estates in Assam, India

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SUMMARY

The prevalence of colonization with the anaerobic intestinal spirochaetes *Brachyspira aalborgi* and *Brachyspira pilosicoli* was investigated in humans ($n=316$) and dogs ($n=101$) living on three tea estates in Assam, India. Colonization was detected using PCR on DNA from faeces. Nineteen (6%) human faecal samples contained *B. aalborgi* DNA, 80 (25·3%) contained *B. pilosicoli* DNA, and 10 (3·2%) contained DNA from both species. One canine sample contained DNA from *B. pilosicoli*. Significant factors for *B. aalborgi* colonization in logistic regression were: infection of family members with *B. aalborgi* ($P<0·001$), being a resident of Balipara ($P=0·03$), and use of water treatment ($P=0·03$). For *B. pilosicoli*, significant factors were: other family members being positive for *B. pilosicoli* ($P<0·001$), water obtained from a well ($P=0·006$), water treatment ($P=0·03$), and not having visited a doctor in the previous 12 months ($P=0·03$).

INTRODUCTION

Two species of anaerobic intestinal spirochaetes have been reported to colonize the lower intestinal tract of humans: *Brachyspira aalborgi* [1] and *Brachyspira pilosicoli* [2, 3]. Both species have been associated with a condition called intestinal spirochaetosis (IS), in which a fringe can be seen in histological sections of the colorectal epithelium, caused by end-on attachment of large numbers of spirochaetes. IS has been linked to a number of symptoms, including chronic diarrhoea, rectal bleeding, pseudo-appendicitis, and lower abdominal discomfort [4–7].

Of these two species, *B. pilosicoli* has been isolated and identified in a number of studies. It has been shown to colonize approximately 30% of individuals in developing countries including Oman [8] and Papua New Guinea [9], and, in developed countries, occurs at a similarly high prevalence in Aboriginal people in Australia [10], male homosexuals [3] and patients infected with human immunodeficiency virus (HIV) [11]. By comparison, it is uncommon in the faeces of the general population in developed countries [10, 12, 13]. It is also regularly found in the large intestines of a variety of animal species including pigs [2], chickens [14], water birds [15], and dogs [16]. This spirochaete species also can be invasive, and has been isolated from the bloodstream of immunocompromised individuals in Europe and the United States [17–20].

No similar epidemiological studies have been conducted on *B. aalborgi*, largely because the organism is extremely fastidious, taking up to 3 weeks to grow under anaerobic incubation on specialized media. To date, *B. aalborgi* has only been reported to have been isolated from humans in five studies; in three it was grown from colorectal biopsies [1, 21, 22], and in two it was grown from faeces [23, 24]. In recent studies, DNA has been extracted from colorectal biopsies taken from IS patients in Australia, Scandinavia and the United States, and amplified by polymerase chain

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reaction (PCR) or hybridized with oligonucleotide probes. Somewhat unexpectedly, these studies showed that *B. aalborgi* is much more commonly involved in IS in these developed countries than is *B. pilosicoli* [21, 25–28]. Similar studies have not been conducted on patients in developing countries, where *B. pilosicoli* is known to be common, due to difficulties in accessing suitable samples. Although one histological study in Southern India demonstrated that IS occurs commonly in apparently healthy villagers [29], the spirochaetes present in these samples were not identified. Recently, the PCR techniques used on DNA extracted from biopsies have been adapted for the detection of *B. aalborgi* and *B. pilosicoli* in human faeces [30]. Faecal samples are much easier to obtain than are colorectal biopsies, and hence this technology will facilitate epidemiological studies on these spirochaetes.

The current study used PCR to test faeces collected from individuals in a remote rural community in northeast India, to establish the comparative prevalences of the two spirochaetes. It is the first study to examine the distribution of *B. aalborgi* in a developing country, and the first to examine *B. pilosicoli* in India. The samples examined were collected as part of a separate study investigating gastrointestinal parasites in dogs and human beings [31]. Data from a questionnaire administered with the original survey also were analysed in relation to colonization with intestinal spirochaetes.

METHODS

Collection and processing of faecal samples

The faecal samples examined were from 316 randomly selected people from the three tea-growing estates of Phulbari, Addabarie and Balipara, located in the Indian state of Assam. The estates were situated within a 10 km radius, and had reported populations of 6531, 4839 and 2004 individuals respectively. Faecal samples from 101 dogs from the estates also were collected. All the samples were obtained as part of a separate study into gastrointestinal parasites, and fuller details of the sample origin and sampling methodology have been published elsewhere [31]. To satisfy Australian quarantine requirements, the faeces were stored in 2·5% potassium dichromate solution for transportation to Australia, and were refrigerated at 4 °C upon arrival at Murdoch University. The dichromate solution was washed off prior to DNA extraction. Samples of faeces (0·2 g) were pelleted at 14000 r.p.m. for 1 min, the supernatant discarded and the pellet resuspended in 1 ml sterile phosphate buffered saline (PBS) and centrifuged again. The process was repeated, and the final pellet was used for DNA extraction.

Questionnaire

At the time of sample collection, participants were asked to fill in a questionnaire to gather the following information relating to potential risk factors for infection: gender, age, religion, estate, level of education, occupation, type of toilet used, drinking water source, type of treatment of water, visits to the doctor in the previous 12 months, animal ownership, ownership of dogs, cats, cattle, buffalo, sheep, goats, pigs, birds, horses, donkeys and rabbits. Relevant questions relating to symptoms at time of collection were: whether the individuals were suffering from diarrhoea, abdominal pain or weight loss. Diarrhoea was explained as increased frequency and water content of the stools, above that considered normal.

Control strains

Control strains of *B. aalborgi* and *B. pilosicoli*, obtained from the culture collection at the Reference Centre for Intestinal Spirochaetes at Murdoch University, were used in the PCRs as positive and negative controls. *B. aalborgi* type strain 513^T and *B. pilosicoli* human strain WesB were propagated anaerobically on non-selective Trypticase soy agar containing 5% (v/v) defibrinated sheep blood in an atmosphere of 94% H_2 and 6% CO_2 at 37 °C for 15 days. Viable cells were scraped from the agar and suspended in TE buffer (10 mM Tris–HCl, 1 mM EDTA; pH 8·0) to a concentration of 10^{11} cells/ml.

Seeding of faeces

The sensitivities of the PCRs for detection of the two species were established by seeding two faecal samples selected from the series with known concentrations of cells of the respective species. The faecal samples used were negative for spirochaetes by phase contrast microscopy, and by selective culture and PCR for both spirochaete species. A range of 500 μl serial dilutions of the 10^{11} stocks, from 10^{11} to 10^{1}, were added to 0·2 g of faeces and vortexed until homogenous. Negative controls consisted of 0·2 g faeces with 500 μl sterile PBS.

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DNA extraction

DNA was extracted from the seeded faeces, and the faeces collected in the survey, by use of the QIAamp DNA stool mini kit (Qiagen GmbH, Germany), used according to the manufacturer's instructions. In each case, 0·2 g of faeces was processed through the three main steps in the protocol: lysis of bacterial cells in the stool sample; adsorption and removal of impurities, inhibitors and debris; purification of extracted DNA through QIAamp spin columns. The DNA yield varied between 5 and 100 μg, depending on the sample being processed.

PCRs

The PCRs used were based on those described for detection of the spirochaete species in human colorectal biopsies and faeces [25, 28, 30]. For *B. aalborgi*, a 471-bp section of the 16S rRNA gene equivalent to the base-pair positions 172–675 of the 16S rRNA gene of *Escherichia coli* was targeted for PCR amplification. For *B. pilosicoli*, a 439-bp section of the 16S rRNA gene equivalent to the base-pair positions 204–676 of the 16S rRNA gene of *E. coli* was amplified.

The amplification mixtures contained 25 μl reaction mix of 1 × PCR buffer, 0·55 U of *Tth* plus DNA polymerase, 1·5 mM of $MgCl_2$, 5 nmol of each deoxynucleoside triphosphate (Amersham Pharmacia Biotech AB, Uppsala, Sweden) and 12·5 pmol of each primer. Thermocycling consisted of 4 min 30 s denaturation at 94 °C followed by 33 cycles of denaturation at 94 °C for 30 s, annealing for 30 s at 46 °C for *B. aalborgi* or 51 °C for *B. pilosicoli*, primer extension at 72 °C for 30 s, and a final extension at 72 °C for 5 min. The PCR products were subjected to electrophoresis in 1·5% agarose gels in 1 × TAE buffer (40 mM Tris–acetate, 1 mM EDTA) for 25 min at 110 V, stained by immersion for 10 min in ethidium bromide at a concentration of 0·001 mg/ml in distilled water and viewed over UV light.

Sequencing of PCR products

Amplified products from 16 human faecal samples (eight *B. aalborgi* positive and eight *B. pilosicoli* positive), and the canine faecal sample that was positive for *B. pilosicoli* were purified using a MoBio Ultra Clean PCR clean up DNA purification kit (MoBio Laboratories, Carlsbad, CA, USA), according to the manufacturer's instructions. DNA in the eluent was then sequenced using a ABI PRISM dye terminator cycle sequencing ready reaction kit (ABI Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. The sequence data obtained were aligned and compared with 16S rDNA sequences of the *B. aalborgi* and *B. pilosicoli* type strains using SeqEd version 1.0.3 (ABI Applied Biosystems).

Statistical analysis

The prevalence and 95% confidence interval (CI) were calculated for each species of intestinal spirochaete. Associations between the presence of the spirochaete species and host and environmental factors were initially made using univariate χ^2 tests for independence. Continuous data (age) were analysed using one-way analysis of variance. Logistic regression models were developed for both spirochaete species in relation to potential risk factors for colonization, and for potential clinical signs. Only variables significant at $P<0{\cdot}25$ in the univariate analysis were considered eligible for inclusion in the logistic regression models [32]. Backward elimination was used to determine which factors could be dropped from the model. The likelihood ratio χ^2 statistic was calculated to determine the significance at each step of the model building. The level of significance for a factor to remain in a final model was set at 10%. Statistical comparisons were performed using Statistix for Windows (Analytical Software, Tallahassee, FL, USA), Excel 97 (Microsoft Corporation, USA), and Egret for Windows (version 2.0.3, Cytel Software, Cambridge, MA, USA).

RESULTS

Sensitivity of PCRs on seeded faeces

The lower level of detection for the *B. aalborgi*-specific PCR was between $2{\cdot}5\times10^4$ and $2{\cdot}5\times10^5$ cells/g of faeces, equivalent to $2{\cdot}5\times10^2$ to $2{\cdot}4\times10^3$ cells per PCR reaction. For *B. pilosicoli* the lower limit of detection was between $2{\cdot}5\times10^5$ and $2{\cdot}5\times10^6$ cells/g of faeces or $2{\cdot}5\times10^3$ to $2{\cdot}5\times10^4$ cells per PCR reaction.

Prevalence of disease symptoms

At the time of sampling, 18 people (5·7%) reported having diarrhoea, 29 (9·2%) reported abdominal pain, and 68 (21·5%) recorded that they had suffered weight loss.

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Prevalence of *B. aalborgi*

Nineteen (6%; 95% CI 3·4–8·6) of 316 people tested gave a positive amplification with the *B. aalborgi* PCR. These included 10/145 (6·9%) individuals from Phulbari, 4/122 (3·3%) from Addabarie and 5/49 (10·2%) from Balipara. None of the 101 dogs tested were PCR positive for *B. aalborgi*. Alignment of the 443-bp sequence of products from eight individuals from the three sites confirmed the specificity of the PCR reaction – with the products all having >98·6% sequence homology with the *B. aalborgi*-type strain, and <94·2% homology with the *B. pilosicoli*-type strain.

Risk factors for *B. aalborgi*

Ten individuals (3·2%) were colonized with both *B. aalborgi* and *B. pilosicoli*, and there was a significant association between this dual colonization ($\chi^2 = 7{\cdot}98$; $P = 0{\cdot}005$). There was no significant association between colonization and age. Twenty variables were included in the initial univariate χ^2 analysis, and potential risk factors for colonization with *B. aalborgi* (i.e. those with $P < 0{\cdot}25$) are shown in Table 1. These included other family members being colonized with *B. aalborgi*, estate, religion, visits to a doctor in the preceding 12 months, type of worker, water being treated, owning any type of animal, owning a dog, owning a cat, owning a cow, owning a goat, and owning a pig. The factors remaining significant after logistic regression were: other family members being colonized with *B. aalborgi* ($P < 0{\cdot}001$), being a resident of Balipara ($P = 0{\cdot}03$), and treating drinking water ($P = 0{\cdot}03$) (Table 2).

Table 1. *Factors associated with colonization with* B. aalborgi *having a P value <0·25 in χ^2 analysis, and consequently being included for examination in a logistic regression model*

Factor	Prevalence (%)	*P* value
Other family member infected with *B. aalborgi*	66·7	<0·0001
No other family member infected with *B. aalborgi*	4·8	
Estate		0·19
Phulbari	6·9	
Addabarie	3·3	
Balipara	10·2	
Religion		0·07
Hindu	5·3	
Christian	5·4	
Muslim	0	
Buddhist	23·1	
Staff worker	11·8	0·06
Tea picker	4·9	
Treated drinking water	9·7	0·007
Water not treated	2·5	
Own an animal	6·5	0·24
Don't own an animal	0·0	
Own a dog	7·8	0·13
Don't own a dog	3·7	
Own a cat	13·6	0·12
Don't own a cat	5·5	
Own a cow	7·6	0·24
Don't own a cow	4·5	
Own a goat	7·8	0·24
Don't own a goat	4·6	
Own a pig	0·0	0·18
Don't own a pig	6·6	

Disease association with *B. aalborgi*

None of the three symptoms analysed had a significant association with *B. aalborgi* colonization.

Prevalence of *B. pilosicoli*

Eighty (25·3%; 95% CI 20·5–30·1) of the 316 people tested, and one of the dogs (1%) were PCR positive for *B. pilosicoli*. Positive individuals came from Phulbari, (53/145; 36·6%) Addabarie (23/122; 18·9%) and Balipara (4/49; 8·2%), whilst the positive dog was from Balipara. Sequence analysis of the PCR products from eight positive individuals and the dog confirm that the product was from *B. pilosicoli*, with all having >98·8% sequence homology with the type strain.

Risk factors for *B. pilosicoli*

In univariate analysis the factors found to be significant in relation to colonization with *B. pilosicoli* are listed in Table 3. These included: other family members being colonized with *B. pilosicoli*, estate, religion, not visiting a doctor in the preceding 12 months, type of worker, water source, water treatment, and cat ownership. After logistic regression, remaining significant factors were: other family member being colonized with *B. pilosicoli* ($P < 0{\cdot}001$), water sourced from a well ($P = 0{\cdot}006$), drinking water treatment ($P = 0{\cdot}04$), and not visiting a doctor ($P = 0{\cdot}03$) (Table 4).

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Table 2. *Factors associated with the presence of* B. aalborgi *that remained in the final logistic regression model*

Factor	β	S.E. (β)	Association with infection OR	(95% CI)	*P* value
Constant	−4·08	0·57		—	
Other family members infected with *B. aalborgi*	3·45	0·93	31·4	(5·0–196·4)	<0·001
Resident of Balipara	1·28	0·59	3·6	(1·13–11·4)	0·03
Treatment of water	1·32	0·61	3·8	(1·14–12·4)	0·03

Table 3. *Factors associated with colonization with* B. pilosicoli *having a P value <0·25 in* χ^2 *analysis, and consequently being included for examination in a logistic regression model*

Factor	Prevalence (%)	*P* value
Other family member infected with *B. pilosicoli*	62·1	<0·0001
No other family member infected with *B. pilosicoli*	11·4	
Estate		<0·0001
Phulbari	36·6	
Addabarie	18·9	
Balipara	8·2	
Religion		0·0001
Hindu	22·7	
Christian	25·7	
Muslim	100·0	
Buddhist	46·2	
Visited a doctor in preceding 12 months	23	0·13
Hadn't visited a doctor in preceding 12 months	31·4	
Staff worker	35·3	0·07
Tea picker	23·4	
Drinking water from tap	22·9	0·02
Water from well	39·1	
Treated drinking water	27·9	0·01
Water not treated	22·8	
Own a cat	36·4	0·22
Don't own a cat		

Disease association with *B. pilosicoli*

None of the three health-related factors analysed had a significant association with *B. pilosicoli* colonization.

DISCUSSION

The use of PCR for detection of *B. pilosicoli* in faeces identified a relatively high prevalence of colonization amongst the individuals tested (25·3%). This prevalence was similar to prevalences found in previous studies in developing communities, where selective culture was used to detect the spirochaete. These include studies conducted in Oman (22·7%) [8], Papua New Guinea (22·8%) [9], and in Aboriginals in a community in the remote north of Western Australia (32·6%) [10]. Immigrants to Western Australia from a variety of developing countries have been shown to have a colonization rate of 10·6% on arrival in Australia [13]. The similarities between the prevalences in these studies helps to validate the accuracy of PCR as used here for detection of *B. pilosicoli* in faeces, and suggests that the dichromate treatment of the samples did not have a deleterious effect on the sensitivity of the tests. Although the sensitivity of direct faecal PCR, as assessed in seeded faeces, was 1–2 logs less than that reported for PCR applied to primary growth of *B. pilosicoli* harvested from selective agar plates (i.e. $>5\times10^4$ cells/g faeces), it is approximately equivalent to the sensitivity of detection achieved by culture alone [33]. Clearly, it is probable that there were other individuals who were colonized, but who went undetected because they were only shedding small numbers of spirochaetes in their faeces at the time of sampling.

The overall prevalence of *B. aalborgi* in the study population was 6%, significantly less than the 25% for *B. pilosicoli* ($\chi^2=44{\cdot}57$; $P<0{\cdot}001$). The relatively lower prevalence recorded for *B. aalborgi* was not likely to be a technical artifact, since when used on seeded faeces the PCR was actually more sensitive than was the PCR for *B. pilosicoli* (by 1 log). This is the first study to provide an estimate of the prevalence of *B. aalborgi* in the general population of a developing country. Unfortunately, there have been no studies on the prevalence of *B. aalborgi* in developed countries, apart from in selected groups of patients who have undergone colorectal biopsy for underlying complaints of the large bowel [21, 25–28]. In the

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Table 4. *Factors associated with the presence of* B. pilosicoli *that remained in the final logistic regression model*

Factor	β	S.E. (β)	Association with infection		
			OR	(95% CI)	*P* value
Constant	−2·15	0·36		—	
Other family members infected with *B. pilosicoli*	2·76	0·33	15·8	(8·3–30·0)	<0·001
Water sourced from a well	1·13	0·41	3·1	(1·4–6·9)	0·006
Treatment of water	0·66	0·32	1·9	(1·03–3·6)	0·04
Hadn't visited a doctor in the preceding 12 months	0·73	0·34	2·1	(1·1–4·0)	0·03

absence of this comparative data, it is not possible to determine whether the prevalence of colonization that was detected is unusually high or low, or whether it was influenced by factors peculiar to developing countries.

When considering factors that may increase the risk of human colonization with intestinal spirochaetes, the first interesting feature of the current study was the significantly increased proportion of individuals infected with both spirochaete species. This finding suggests that the two species are likely to share at least some risk factors for colonization. In logistic regression, both spirochaete species showed a highly significant association with the existence of other family members who were colonized with the same species. This result suggests either that the whole family had an increased exposure to a source of infection, and/or that transmission of intestinal spirochaetes within a household is an important means of spread. Exposure to intestinal spirochaetes may be either through direct contact with contaminated faeces, or by indirect contact via contaminated fomites. The people involved in the study lived in relatively crowded conditions, lacking proper hygienic facilities, and under these circumstances transmission is likely to be enhanced. If these are the main means of transmission for both spirochaete species, the difference in prevalence for the two species in the study population suggests that *B. aalborgi* is less infectious than is *B. pilosicoli*. In the laboratory, *B. aalborgi* grows much more slowly than *B. pilosicoli* – and the same may be true *in vivo*. Successful intestinal colonization may need exposure to a greater number of *B. aalborgi* cells than is required for *B. pilosicoli*, and, equally, *B. aalborgi* may be shed in lower numbers in the faeces than is *B. pilosicoli*. Another possible explanation for the difference in prevalence could be that the organisms have different environmental or animal reservoirs, and that comparative exposure to these reservoirs differs amongst the humans examined. This interpretation did not receive any clear support from the results of the analysis of the questionaire; for example, in logistic regression analysis, neither spirochaete species was linked to animal ownership, even though animals are known to be a potential reservoir of *B. pilosicoli*.

The other significant risk factor that applied to both species was treatment of drinking water. As treatment of water is not likely to predispose to infection, it may be that the water sources available to the individuals who treated their water were of inferior quality to those of individuals who did not treat their water. This then suggests a possible link between water quality and infection with the spirochaetes. Less than half of the people performed any type of treatment on their household drinking water before use. The most common method of treatment was boiling, used by 39% of respondents. The remainder used filtration (3·5%), chemical treatment (1·3%), or a combination of filtration and boiling (4·5%). For *B. pilosicoli*, obtaining drinking water from a well was also a significant risk factor that remained in the final logistic regression model. Again this suggests that contaminated water supplies may be a source of infection. Other studies have shown that *B. pilosicoli* may be present in natural water sources, particularly those contaminated with faeces from water birds [15]. Further work is required to determine whether *B. aalborgi* also can survive in water, which would then represent a reservoir for infection.

For *B. aalborgi*, being resident in Balipara was a risk factor, and it would be useful to conduct future studies at this estate, particularly examining water sources for potential contamination. The other significant risk factor for *B. pilosicoli* infection was not visiting a doctor in the last 12 months. All people in the sample set had access to a doctor, provided

through the tea companies for whom they worked, so having the ability to access a doctor in itself was not biasing these results. A possible explanation for the effect is that the individuals who visited the doctor may have received antimicrobial therapy in conjunction with these visits, and this may have removed any colonizing *B. pilosicoli* strains.

Some initial associations with animal ownership were found for *B. aalborgi* infection, but these did not remain in the final regression model. For *B. pilosicoli*, an initial association with cat ownership was found, but again this was discarded in the logistic regression model. Several species of animal are known to be infected with *B. pilosicoli*, but to date the only group of animals known to be infected with *B. aalborgi* are non-human primates [34, 35]. None of the 101 dogs sampled were found to be colonized by *B. aalborgi*, and only one was positive for *B. pilosicoli*. A study in Papua New Guinea found the prevalence of *B. pilosicoli* in village dogs to be 5·3% (4/76 dogs), compared to 22·8% in humans [36]. The low prevalence in dogs in that study, as in the present study, suggests that they are unlikely to be a significant reservoir of *B. pilosicoli* infection for humans in developing communities. Indeed, in such settings, it is likely that dogs become colonized with *B. pilosicoli* following consumption of contaminated human faeces [9, 36].

For both spirochaete species, no significant associations were found with the three indices of disease that were recorded. In part this reflects the relatively low frequency of reported symptoms, and the high likelihood that these could have other causes in a tropical setting. The survey did not elicit information about chronic diarrhoea, and no attempt was made to assess the individual water content of the faecal samples obtained. Most faecal samples appeared loose compared to faeces collected from individuals in developed countries. It was unfortunate that water content was not recorded, since IS has been linked to chronic diarrhoea in humans [5, 6, 37, 38], whilst *B. pilosicoli* infection in animals increases faecal water content directly, and exacerbates diarrhoea due to nutritional and other infectious causes [39, 40]. Colorectal biopsies were not taken in this study, so it is not known whether any of the colonized individuals had colitis or histological evidence of IS. Further work is needed to clarify whether infection with one or other of the two spirochaete species has an impact on health in individuals living in developing communities.

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