An unexpectedly high prevalence of colonization with the intestinal spirochaete *Brachyspira aalborgi* amongst residents of the Indonesian island of Bali

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PCR assays designed to amplify DNA from the anaerobic intestinal spirochaete *Brachyspira aalborgi* were conducted on DNA extracted from 938 faecal samples from 469 residents on the Indonesian island of Bali. The individuals tested were sampled twice in one year and were from four rural villages, one peri-urban centre and the capital city, Denpasar. Overall, an unexpectedly high prevalence of colonization (24.7 %) was found, with prevalence rates at different locations varying from a low of 15.6 % at one village to 41.5 % in the peri-urban centre. Comparison of prevalence rates at the two sampling times suggested that, in many individuals, colonization was likely to be prolonged (>3 months) and/or that reinfection was occurring frequently in these people. Analysis of a questionnaire administered to the individuals who were sampled identified specific risk factors for colonization as location, co-colonization with the related intestinal spirochaete *Brachyspira pilosicoli* and use of drinking water obtained from wells rather than from taps. No specific associations with clinical symptoms were identified.

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INTRODUCTION

Brachyspira aalborgi and *Brachyspira pilosicoli* are anaerobic spirochaetes that colonize the human large intestine, where they attach by one cell end to the colorectal epithelium to form a characteristic 'false brush border' (Hovind-Hougen *et al.*, 1982; Mikosza & Hampson, 2001). The potential role of the two species as pathogens of humans has remained somewhat controversial, although it is thought that colonization with these spirochaetes can be associated with a variety of non-specific clinical problems, including chronic diarrhoea, abdominal discomfort and failure to thrive in children (Brooke *et al.*, 2006; Körner & Gebbers, 2003; Marthinsen *et al.*, 2002; Weisheit *et al.*, 2007). Besides humans, *B. aalborgi* has been detected in non-human primates (Duhamel *et al.*, 1997; Munshi *et al.*, 2003). In comparison, *B. pilosicoli* colonizes a variety of species of animals and birds, in which it is a recognized cause of mild colitis and diarrhoea (Hampson *et al.*, 2006).

Many aspects of the epidemiology of these two spirochaete species remain unclear. *B. aalborgi* in particular has remained enigmatic, mainly because of its extremely slow growth rate and the fact that few isolates are available for study (Brooke *et al.*, 2003; Calderaro *et al.*, 2003; Hovind-Hougen *et al.*, 1982; Kraaz *et al.*, 2000). To overcome difficulties with isolation, PCR assays have been developed for these two spirochaete species using DNA extracted from colorectal biopsies (Mikosza *et al.*, 1999) or faeces

(Mikosza *et al.*, 2001). Based on differences in 16S rRNA gene sequences in DNA extracted from fixed biopsy tissues, at least three clusters or 'lineages' of *B. aalborgi* have been described (Jensen *et al.*, 2004; Mikosza *et al.*, 2004; Pettersson *et al.*, 2000), but to date all isolates that have been cultured have belonged to cluster one. Using PCR-based methods, *B. aalborgi* has been detected at a consistent prevalence of around 6 % in individuals from four different human population groups in Australia (Brooke *et al.*, 2006), in villagers in Assam, India (Munshi *et al.*, 2004), and in patients in northern Italy (Calderaro *et al.*, 2007).

In a previous study, selective anaerobic culture was used to study the prevalence of *B. pilosicoli* in individuals from the island of Bali, Indonesia (Margawani *et al.*, 2004). Prevalence rates of 3.3–23.4 % were found depending on location, with the higher values found being similar to those found for *B. pilosicoli* in rural Australian Aborigines (Lee & Hampson, 1992) and in villagers in India (Munshi *et al.*, 2004) and in Papua New Guinea (Trott *et al.*, 1997).

The aim of this study was to examine the prevalence and potential risk factors for human colonization with *B. aalborgi* in Bali. The faecal samples used were a subset of those previously collected to determine the prevalence of *B. pilosicoli* by selective anaerobic culture and PCR, and the original questionnaire responses were also used to determine potential risk factors for colonization (Margawani *et al.*, 2004).

Abbreviations: CI, confidence interval; OR, odds ratio.

METHODS

Approval. This study was conducted with the approval of the Murdoch University Human Ethics Committee. The subjects involved gave their informed consent to participate in the study.

Source of faecal samples. Faecal samples were obtained from 469 people living in four traditional Balinese villages, one semi-urban location and Denpasar, the capital city. The numbers of people sampled from the six locations are shown in Table 1. Participants were sampled twice in one year (in August, the dry season, and December, the wet season), giving 938 samples in total. The four rural farming villages were well-run by the local communities, and there was a strong appreciation for the need to preserve a clean environment and safe water supply. The residents of urban Denpasar were generally settled, well-housed and had a reasonably high standard of living. In comparison, the peri-urban centre of Sesetan was crowded, had a large transient population and had a generally poor environment (Margawani *et al.*, 2004). Samples were transported to the laboratory at 4 °C and maintained at this temperature until processed.

DNA extraction and PCR. The methodology used for DNA extraction and PCR analysis has been described previously (Munshi *et al.*, 2004). Briefly, over a 1-month period, DNA was extracted from batches of stored faecal samples using a QIAamp DNA Stool Mini kit (Qiagen). The primers used were designed to amplify a 471 bp section of the 16S rRNA gene of *B. aalborgi*. In previous experiments, the authors showed that this PCR had a sensitivity of detection of between 2.5×10^4 and 2.5×10^5 cells of *B. aalborgi* (g faeces)⁻¹ (Munshi *et al.*, 2004).

DNA sequencing. Twenty-two randomly selected PCR products were sequenced with a commercially available cycle sequencing kit (ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit; Applied Biosystems), according to the manufacturer's instructions. The sequence data obtained were aligned and compared with 16S rRNA gene sequences of *B. aalborgi* type strain 513A^T (GenBank accession no. Z22781) and *B. pilosicoli* type strain P43/6/78^T (GenBank accession no. U23032) using SeqEd, version 1.0.3 (Applied Biosystems).

Statistical analysis. One-way analysis of variance was used for analysis of continuous data, whilst either a χ^2 test for independence or Fisher's exact test was used for categorical data. Odds ratios (ORs) and 95 % confidence intervals (CIs) were calculated. Comparisons were made with previous results for *B. pilosicoli* from the same samples (Margawani *et al.*, 2004).

RESULTS AND DISCUSSION

The 22 PCR products that were sequenced had 98.6–100 % nucleotide sequence identity with the product from *B. aalborgi* type strain 513A^T, confirming the specificity of the PCR for *B. aalborgi*. The sequences were consistent with them coming from lineage one, although only a small region of the 16S rRNA gene was sequenced.

The number of samples that were positive for *B. aalborgi* using the PCR at the six locations at both sampling times is shown in Table 1. Overall, 118 (25.2 %) of the 469 August samples and 104 (22.2 %) samples from December were PCR-positive. The prevalence at different locations and times was highly variable, ranging from 15.6 to 41.5 %. Combining the results for the four traditional villages gave 85/378 (22.5 %) positive individuals in August and 74/378 (19.6 %) in December. For Sesetan (peri-urban), the corresponding figures were 41.5 and 38.5 %, respectively, and for Denpasar (the capital city) they were 23.1 and 19.2 %, respectively.

The relationship between location and colonization was significant ($P_{\text{AUG}}=0.019$ and $P_{\text{DEC}}=0.001$). Living in Sesetan was an important risk factor (OR 2.4, CI 1.4–4.2 in August; and OR 2.6, CI 1.5–4.5 in December). Presumably in Sesetan there is an increased opportunity for *B. aalborgi* to be transmitted or to persist amongst people in the crowded and poorer environment. In our previous study, colonization rates for *B. pilosicoli* were also high at Sesetan (Margawani *et al.*, 2004).

Although water source was not a significant risk factor for August ($P=0.148$), it was for December ($P=0.029$), with people drinking well water being more likely to be colonized than those drinking tap water (OR 1.6, CI 1.1–2.6). Conversely, a significant negative relationship existed for tap water (OR 0.6, CI 0.4–0.9), suggesting a protective effect. Water from wells is more likely to be contaminated from environmental sources, including human faeces, than tap water. The significant effect in the wet season (December) would be consistent with transmission associated with local flooding of septic tanks located near many

Table 1. Number of people positive for *B. aalborgi* amongst 469 individuals sampled twice in the same year at six locations in Bali
August, dry season; December, wet season.

Location	Description	No. of people sampled	No. positive in August (%)	No. positive in December (%)	No. positive at both samplings (%)	No. newly positive in December (%)
Melinggih	Village	122	26 (21.3)	19 (15.6)	16 (13.1)	3 (2.5)
Karang Suwung	Village	94	27 (28.7)	25 (26.6)	14 (14.9)	11 (11.7)
Payangan Desa	Village	89	17 (19.1)	18 (20.2)	9 (10.1)	9 (10.1)
Badung	Village	73	15 (20.5)	12 (16.4)	12 (16.4)	0
Sesetan	Peri-urban	65	27 (41.5)	25 (38.5)	20 (30.8)	5 (7.7)
Denpasar	Urban	26	6 (23.1)	5 (19.2)	4 (15.4)	1 (3.8)
Total		469	118 (25.2)	104 (22.2)	75 (16.0)	29 (6.2)

of the wells, particularly in Sesetan. Despite this possibility, the overall prevalence did not show a consistent seasonal difference. Furthermore, colonization with *B. pilosicoli* was more common in people who used well water in August but not in December (Margawani *et al.*, 2004). The reason for these apparent differences in seasonally associated risks of water supply for the two spirochaete species is unclear, but presumably reflects some differences in the biology of the spirochaetes.

The previously described prevalence for *B. pilosicoli* in the same individuals who were sampled here was 9.8 % (46/469) in August and 11.9 % (56/469) in December (Margawani *et al.*, 2004). By comparing these previous data on *B. pilosicoli* with the current data, a total of 27/469 (5.8 %) and 24/469 (5.1 %) individuals were identified as being colonized with both spirochaete species at the two sampling times. On both occasions, infection with one spirochaete was a significant risk factor for colonization with the other ($P=0.000$ and 0.000 , respectively). Similar associations between these two spirochaete species have been recorded elsewhere (Brooke *et al.*, 2006; Munshi *et al.*, 2004) and suggest the existence of some common risk factors or predisposition for colonization by the two species.

There was no significant association of age, gender, occupation, animal contact, faecal consistency or health status with colonization by *B. aalborgi*. The lack of association with the presence of clinical symptoms is at odds with results from Australia, where colonization with *B. aalborgi* amongst Aboriginal patients was associated with chronic diarrhoea, failure to thrive and being underweight (Brooke *et al.*, 2006). Such differences could perhaps be associated with the presence of different 'lineages' or strains of *B. aalborgi* in the different countries, but this possibility was not investigated.

The same PCR methodology was used in this study as in our other studies, and consequently these results can be compared. Previously, prevalence rates for *B. aalborgi* of ~6 % were found amongst four different population groups in Australia (Brooke *et al.*, 2006) and in villagers in Assam, India (Munshi *et al.*, 2004). Consequently, the prevalence for *B. aalborgi* in Bali at all locations was unexpectedly high. Furthermore, the prevalence of *B. aalborgi* in Bali was greater than the prevalence of *B. pilosicoli*, and again this was the reverse of previous findings in other countries where comparable surveys have been conducted using faeces from unselected individuals from the local populations (Brooke *et al.*, 2006; Munshi *et al.*, 2004). The reasons for the differences in prevalence rates in Bali are not immediately obvious, but presumably are a result of specific local conditions. These could include factors associated with ethnicity, the local diet and the colonic microbiota, or differences in other aspects of the biology of the spirochaete strains that were detected.

Of the 118 individuals who were PCR-positive for *B. aalborgi* at the first sampling, 75 (63.3 %) were also positive at the second sampling 4 months later. These individuals may have

been reinfected during this interval and/or they may have been persistently infected. As only 29 individuals (6.2 % of the total population) were newly positive in December, the mean duration of colonization would need to have been at least 3 months to maintain the overall high prevalence rates at the different locations (15.6–41.5 %; mean positivity 22.6 %). Furthermore, the disparity at Sesetan between the number of new infections in December (7.7 %) and the high prevalence (~40 %) would suggest that colonization was more prolonged at this location and/or that this large subset of individuals was being regularly reinfected. Again, these possibilities are likely to be due to factors associated with the poor socio-economic environment in Sesetan.

Further work is required to determine why the apparent prevalence of *B. aalborgi* is so high in Bali compared with the situation in other communities. In particular, it would be useful to apply molecular typing methods to help determine the duration of infection with specific strains and to obtain full 16S rRNA gene sequences to determine whether the 'lineages' or strains of *B. aalborgi* that are distributed in Bali are different from those present in other countries and regions.

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