Prevalence, risk factors and molecular epidemiology of \textit{Brachyspira pilosicoli} in humans on the island of Bali, Indonesia

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The purpose of this study was to investigate the prevalence and epidemiology of the anaerobic intestinal spirochaete \textit{Brachyspira pilosicoli} amongst Indonesians living in rural and urban settings on the island of Bali. Faecal samples ($n = 992$) were collected on two occasions, 4 months apart, from people living in four traditional farming villages, one peri-urban location and one urban area. Samples were cultured anaerobically on selective agar and intestinal spirochaete isolates were confirmed to be \textit{B. pilosicoli} by using a species-specific PCR. Forty-eight of the 121 isolates obtained were typed by using PFGE. A questionnaire was administered to participants and analysed in order to identify potential risk factors for colonization. Overall prevalence of carriage on the two visits was 11.8 and 12.6 %, respectively. Prevalence at different locations varied from 3.3 to 2.3 %, with the highest prevalence occurring in the peri-urban location. Considerable strain diversity was found, with the 48 isolates being divided into 44 PFGE types. There was no significant association between colonization and ownership of animals, contact with animals, farming, age or gender. On the first visit, colonization was significantly more common in people who used well water compared to those who used tap water. On the second visit, colonization was significantly more common in people with wet faeces than in those with normal faeces.

**INTRODUCTION**

The anaerobic intestinal spirochaete \textit{Brachyspira (Serpulina) pilosicoli} was initially described in the context of being the agent of a condition called porcine intestinal spirochaetosis (Taylor et al., 1980; Trott et al., 1996b). Pigs infected with \textit{B. pilosicoli} have mild colitis and diarrhoea. Subsequently, it emerged that the spirochaete also colonizes the large intestines of a variety of other species, including dogs (Duhamel et al., 1998), birds (McLaren et al., 1997) and human beings (Lee & Hampson, 1994; Trivett-Moore et al., 1998).

Previous studies of \textit{B. pilosicoli} in humans have shown that faecal carriage varies greatly, depending on the population group being investigated. One study found these spirochaetes in 32.6 % of faecal samples that were collected mainly from children in an Aboriginal community in the remote north-west of Western Australia (Lee & Hampson, 1992). Amongst Indians living on tea estates in India, 25.3 % were colonized by \textit{B. pilosicoli} (Munshi et al., 2004). In a study in Papua New Guinea (PNG), the overall prevalence of colonization was 22.8 % (Trott et al., 1997a). In contrast, colonization is generally uncommon in developed countries (Tompkins et al., 1986; Lee & Hampson, 1992; Brooke et al., 2001), apart from amongst individuals with HIV infection (Kästohrner et al., 1990) and homosexual males (Cooper et al., 1986; Trivett-Moore et al., 1998). In these latter groups, prevalence is more similar to that found in developing countries. In addition to the spirochaete's ability to colonize the large intestine, \textit{B. pilosicoli} has been isolated from the bloodstream of a small number of immunocompromised patients in France, the USA and Greece (Fournié-Amazouz et al., 1995; Trott et al., 1997b; Kanavaki et al., 2002).

Human beings can also be colonized by another intestinal spirochaete species, \textit{Brachyspira aalborgi}. This spirochaete has been associated with a range of intestinal symptoms that are similar to those of \textit{B. pilosicoli} infection, but it has extremely fastidious growth requirements and has rarely been isolated from faeces (Brooke et al., 2003; Calderaro et al., 2003).

The purpose of the present study was to investigate the

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occurrence and potential risk factors for intestinal colonization by *B. pilosicoli* in residents living in different environments on the island of Bali, Indonesia. This island and the different locations were chosen for study because it was suspected that there would be a high (but variable) prevalence of colonization, which would facilitate the identification of putative risk factors for colonization.

**METHODS**

**Ethics.** This study was undertaken with the approval of the Murdoch University Human Ethics Committee.

**Collection of samples.** Faecal samples were collected at two intervals, 4 months apart (August and December 1999), from individuals living in six locations in Bali. Four of these sites are traditional Balinese farming villages (Badung, Karang Suwung, Melinggih and Payangan Desa) that are located in the central region of Bali, approximately 40 km north of the capital, Denpasar. The fifth location, Sesetan, is a heavily populated, peri-urban area that is located close to Denpasar, whilst the sixth location is near the centre of Denpasar.

The villages of Badung, Melinggih and Payangan Desa were divided into four smaller subvillages. Each subvillage was divided into compounds, with up to four houses present in each compound. Three generations of a family were typically present within a compound. In these three villages, a systematic sampling regime was implemented, with every fourth compound being sampled. In the fourth village, Karang Suwung, every household was included in the study.

In the peri-urban region of Sesetan, the majority of dwellings (120) were not contained within discrete compounds, but were small, semi-detached houses. Sampling in Sesetan was based on street maps. Several households in each street were selected for sampling. Where nobody was present in a selected house, the neighbouring house was visited. This continued until an owner who agreed to participate in the study was found.

People sampled in Denpasar were staff and students who were associated with the Veterinary School, Udayana University and the Disease Investigation Centre (a regional veterinary diagnostic laboratory) and their family members. The majority of the people sampled in Denpasar had had a university education.

Households were visited up to three times at each collection time. On the first occasion, an adult family member was interviewed to collect information about the household, including the age of family members, their gender, primary occupation, ownership of animals, source of water and presence of clinical symptoms (diarrhoea, headache, constipation, muscle/joint pain, abdominal pain) and antibiotic administration in the month that immediately preceded sampling. Sample pots were left with the household for faecal collection. On the following morning, the household was revisited to collect faecal samples. If samples were not provided on this visit, the house was revisited on the following day.

At the second collection in December, the same households were visited, but not all individuals were available to be resampled. Consequently, additional samples were collected from other members of the family or from individuals in neighbouring households. In Denpasar, individuals from only 10 of the original 35 households were sampled.

Faecal samples were stored on ice for transport to the laboratory. Faecal consistency was categorized as normal (dry to slightly moist), wet (clay consistency) or watery (loose/watery).

**Culture.** Faeces were plated onto trypticase soy agar (BBL) that was supplemented with 5% defibrinated bovine blood, 400 μg spectinomycin/ml⁻¹, 25 μg vancomycin ml⁻¹ and 25 μg colistin ml⁻¹ (Sigma). Plates were incubated at 37 °C in anaerobic jars in an atmosphere of 94% H₂/6% CO₂ for 6–15 days. Spirochaete growth was indicated by low, flat, confluent growth, surrounded by areas of weak β-haemolysis. The presence of spirochaetes was confirmed by examining bacterial growth, resuspended in PBS, under a phase-contrast microscope.

Isolates were subcultured two or three times on agar to ensure purity, then a straight wire was used to transfer a discrete area of growth to Kunkle’s anaerobic broth medium (Kunkle et al., 1986). This was incubated at 37 °C on a rocking platform until mid-exponential phase was obtained, at a density of approximately 10⁸ cells ml⁻¹. Cells were harvested by centrifugation (10 000 g, 4 °C, 20 min) and washed with PBS. Aliquots of broth also were stored at –80 °C for later use in PFGE analysis.

**PCR.** A PCR that is specific for *B. pilosicoli*, developed previously in our laboratory (Mikosza et al., 1999, 2001a, 2001b), was used to confirm the identity of the spirochaetes. The reverse primer was 5’-CCCTTACAATATCCAGACT-3’. This reaction amplified a 439 bp segment of the *B. pilosicoli* 16s rRNA gene, equivalent to positions 204–676 of the 16s rRNA gene of *Escherichia coli*.

One positive control (*B. pilosicoli* P43/6/78T) and two negative controls (*B. aalborgi* 513A² and *Brachyspira hydysenteriae* B78) were included in each batch of PCR amplifications. One blank reaction also was used, to confirm that no contamination had occurred.

Amplification consisted of an initial denaturation step at 94 °C for 2 min, followed by 33 cycles of denaturation at 94 °C for 30 s, annealing at 46 °C for 45 s and extension at 72 °C for 30 s. After the last cycle, the product was incubated at 72 °C for 10 min. The amplification product was analysed by electrophoresis on 1·5% agarose gel in Tris/borate (TBE) buffer (0·09 M Tris, 0·09 M borate, 0·02 M EDTA, pH 8·0). The current for electrophoresis was set at 0·60 V for 50 min. Bands were stained by immersion in 0·5 μg ethidium bromide ml⁻¹ for 30 min and gels were viewed under UV light.

**PFGE.** Six reference strains of *B. pilosicoli* were included in the PFGE analysis. These were the porcine type strain (P43/6/78T), three strains from Aboriginal children (H21, 167 and Web; Lee & Hampson, 1992), one from an Omami child (31B; Barrett, 1990) and one from an Australian homosexual male (GAP 401; Trivett-Moore et al., 1998). These strains were obtained from the culture collection held at the Reference Centre for Intestinal Spirochaetes at Murdoch University.

Spirochaetes were recovered from –80 °C storage in Kunkle’s broth and grown anaerobically on blood agar at 37 °C for 5 days. The culture was harvested with a sterile cotton swab and resuspended in TE buffer (10 mM Tris, 0·1 M EDTA, pH 7·6) to a density that was equivalent to 10⁸ cells ml⁻¹. Cells were harvested with centrifugation (10 000 g, 4 °C, 20 min) and washed with PBS. Aliquots of broth also were stored at –80 °C for later use in PFGE analysis.

Plug preparation, DNA restriction and electrophoresis were carried out as described previously (Brooke et al., 2001). Plugs were prepared by mixing 50 μl lysostaphin and 100 μl 1·8% molten low-melting-point agarose in 0·5× TBE buffer (1·68 g Tris base l⁻¹, 5·5 g boric acid l⁻¹, 0·01 M EDTA, pH 8·0). The mixture was pipetted into sterile, pre-chilled moulds and allowed to set at 4 °C for 20 min. Plugs were placed in 500 μl lysis buffer (6 mM Tris/HC1, pH 7·6; 1 M NaCl; 100 mM EDTA, pH 7·6; 0·5% Brij; 0·2% sodium deoxycholate; 0·5% sodium lauryl sarcosine) and 12·5 μl lysozyme at 37 °C for 18 h; they were then incubated at 50 °C for 18 h in 500 μl TES (50 mM Tris, pH 7·4; 50 mM EDTA, pH 8·0; 1% sodium lauryl sarcosine) with 20 μl proteinase K (20 mg ml⁻¹). The plugs were washed six times in TE buffer, with a final wash for 30 min in 1× restriction enzyme (RE) buffer.

Digestion was with *MluI* (Biotechnology International) or *Smal* (Promega).
Smal digestion was only undertaken on isolates that had identical PFGE patterns with MluI. For MluI digestion, 50 U MluI, 3 μl BSA and 300 μl fresh RE buffer was added to the plugs and incubated at 37°C for 24 h. For Smal digestion, plugs were treated as for MluI and incubated at 25°C. Plugs were kept in TE buffer at 4°C.

Electrophoresis was run by using a contour-clamped, homogeneous electric field-DRIII system (Bio-Rad). Prior to electrophoresis, half of each plug was heated at 56°C for 8 min and loaded into the wells of an agarose gel (1% in 0.5× TBE buffer) and scaled with plug agarose. A ladder DNA size standard (Bio-Rad) was included for comparison. For MluI, electrophoresis was run at 200 V for 20 h, with pulse-time ramped at 1–40 s. For Smal-digested plugs, it was run for another 2 h, with a pulse-time of 45–60 s. The gel was stained by soaking in ethidium bromide solution and images were viewed under UV light by using a Bio-Rad Gel Doc 2000+ system and analysed by using Bio-Rad Molecular Analyst software. A dendrogram was created from the Dice clustering fusion strategy.

Statistical analysis. Data were entered into a spreadsheet (Excel; Microsoft) and analysed with the statistical package Statistix (version 7.0; Analytical Software). Data from the two visits were analysed independently. For continuous data, such as age, a one-way analysis of variance was used to test for significant differences between positive and negative individuals. For categorical data, a chi-square test for independence or Fisher’s exact test were used. Odds ratios and their 95% confidence intervals (CIs) were also calculated, to determine the importance of risk factors on positivity. Cohen’s k-statistic was calculated, to determine the degree of agreement between the first and second samplings. Prevalence and 95% CIs were calculated.

RESULTS

Prevalence

In total, 500 faecal samples were collected in August and 492 in December (Table 1). On the two visits, *B. pilosicoli* was cultured from 59 (prevalence, 11.8%; 95% CI, 9.0–14.6%) and 62 (prevalence, 12.6%; 95% CI, 9.7–15.5%) people, respectively. No isolates of *B. aalborgi* and *B. piloncilli* were obtained. Altogether, 375 samples were collected from the same individuals on both visits. Of these individuals, 74 (19.7%) were positive on one or other of the two visits, but only 18 (4.8%) of these were positive on both visits (Table 2). There was no significant difference in prevalence between visits ($\chi^2 = 0.15, df = 1, P = 0.7$). The level of agreement in results was low ($\kappa = 0.31$).

On both visits, prevalence between locations was significantly different (first visit: $\chi^2 = 13.4; df = 1, P < 0.05$; second visit: $\chi^2 = 24.7; df = 1, P < 0.001$) (Table 1).

On the first visit, prevalence was significantly higher in Sesetan (23.4%) than in all other places ($P < 0.05$ for all). People from Sesetan were 3.1 (95% CI, 1.6–5.9) times more likely to be positive than people from the four villages and 2.3 (95% CI, 1.0–5.1) times more likely to be positive than people from Denpasar (Table 1). On the second visit, prevalences in Denpasar (22.6%) and the village of Karang Suwang (21.4%) were both greater than on the first visit, and were similar to the prevalence in Sesetan (20.3%). The increase in prevalence in Denpasar was not significant, but the increase was significant at Karang Suwang ($\chi^2 = 4.9; P < 0.05$). Measurements of prevalence in Denpasar, Karang Suwang and Sesetan at the second visit were all significantly higher ($P < 0.05$) than in Melinggih (8.9%), Badung (6.8%) and Payangan Desa (3.3%).

Table 2. Detection of *B. pilosicoli* in 375 individuals who provided faecal samples on both visits

<table>
<thead>
<tr>
<th>Culture result for:</th>
<th>No. people sampled (%)</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>First visit</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>76 (2–83)</td>
<td>76.2–84.3</td>
</tr>
<tr>
<td>Positive</td>
<td>26 (5–8)</td>
<td>2.6–7.0</td>
</tr>
<tr>
<td>Second visit</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>27 (7–2)</td>
<td>4.6–9.8</td>
</tr>
<tr>
<td>Positive</td>
<td>30 (7–7)</td>
<td>5.0–10.4</td>
</tr>
</tbody>
</table>

Table 1. Prevalence of *B. pilosicoli* during visits in 1999

<table>
<thead>
<tr>
<th>Village</th>
<th>No. households present</th>
<th>Culture result for:</th>
<th>No. people sampled</th>
<th>September visit</th>
<th>December visit</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No. households sampled</td>
<td>No. people positive/ examined (%)</td>
<td>95% CI</td>
<td>No. households sampled</td>
</tr>
<tr>
<td>Badung</td>
<td>102</td>
<td>26</td>
<td>7/69 (10.1%)</td>
<td>3.0–17.3</td>
<td>27</td>
</tr>
<tr>
<td>Karang Suwung</td>
<td>50</td>
<td>30</td>
<td>7/77 (9.1%)</td>
<td>2.7–15.5</td>
<td>31</td>
</tr>
<tr>
<td>Melinggih</td>
<td>120</td>
<td>30</td>
<td>7/106 (6.6%)</td>
<td>1.9–11.3</td>
<td>30</td>
</tr>
<tr>
<td>Payangan Desa</td>
<td>118</td>
<td>31</td>
<td>9/79 (11.4%)</td>
<td>4.4–18.4</td>
<td>30</td>
</tr>
<tr>
<td>Sesetan</td>
<td>120</td>
<td>26</td>
<td>18/77 (23.4%)</td>
<td>13.9–32.8</td>
<td>30</td>
</tr>
<tr>
<td>Denpasar</td>
<td>NA</td>
<td>35</td>
<td>11/92 (12.0%)</td>
<td>5.3–18.6</td>
<td>10</td>
</tr>
<tr>
<td>Total</td>
<td>178</td>
<td>59/500 (11.8)</td>
<td>9.0–14.6</td>
<td></td>
<td>155</td>
</tr>
</tbody>
</table>

Within a column, superscripts with the same letter are significantly different ($P < 0.05$). NA, Not applicable.
Risk factors

On both visits, colonization was not affected significantly by age, gender, occupation, exposure to animals or having taken antibiotics.

On the first visit, significantly more positive people used well water than tap water ($\chi^2 = 5.05; df = 1, P = 0.0246$). On this visit, people who used well water were 1.9 (95% CI, 1.1–3.2) times more likely to have positive faecal samples than people who used tap water. On the second visit, the difference in colonization rates between people who used well water and those who used tap water was not significant.

Clinical signs

On neither visit was there any significant difference between culture-positive and culture-negative individuals in the occurrence of reported clinical symptoms of diarrhoea, headache, constipation, muscle and joint pain or abdominal pain in the month prior to sampling.

On the first visit, B. pilosicoli prevalence was highest in people with watery diarrhoea (22.7%). In contrast, B. pilosicoli was detected in only 6.7% of samples classified as watery on the second visit, but was isolated more frequently from people with wet-clay faeces (19.7%). However, there was no significant difference in detection of B. pilosicoli between the three different faecal consistencies (first visit: $\chi^2 = 4.3; df = 1, 2; P = 0.12$; second visit: $\chi^2 = 5.7; df = 1, 2; P = 0.06$) (Table 3).

On the second visit, people with faeces that were categorized as wet (wet-clay or watery) (15.8%) were 1.8 (95% CI, 1.0–3.1) times more likely to have spirochaetes detected in their faeces than people with normal faecal consistency (9.5%) ($\chi^2 = 4.44; df = 1; P < 0.05$).

PFGE

It was only possible to successfully revive 48 of 121 stored Balinese isolates for use in PFGE. Thirty-three isolates were from the first sampling and 15 from the second. Only two pairs of isolates were from two individuals who were culture-positive at both samplings. Digestion of DNA with Mlu I gave 7–11 DNA bands, with clear and reproducible patterns (Fig. 1). PFGE with Mlu I on these isolates and the control strains divided them into 50 PFGE types (Table 4; Fig. 2). The six control strains each had a unique PFGE pattern, whilst the 48 Balinese isolates were divided into 44 PFGE types. There was no clear pattern of genetic relationship between isolates by town, village or family of origin. The six reference strains were spread throughout the dendrogram (PFGE types 5, 10, 13, 27, 41 and 46; Fig. 2).

By using Mlu I, only three PFGE groups contained multiple isolates. PFGE type 24 contained two identical isolates: one from a 25-year-old female from Sesetan, which was obtained at the first visit, and the other from a 20-year-old male from Karang Suwung, obtained at the second visit. PFGE type 29 contained two isolates from a 9-year-old boy from Sesetan, which were obtained at an interval of 4 months, and one from a 60-year-old male who came from a different household in Sesetan, which was collected at the first visit. PFGE type 47 contained two identical isolates that were obtained at the second visit from two males, aged 14 and 60 years, who came from the same family and household in Karang Suwung. Digestion of all these isolates with Smal I did not differentiate them further.

Table 3. Association between faecal consistency and presence of B. pilosicoli

<table>
<thead>
<tr>
<th>Faecal consistency</th>
<th>First visit</th>
<th>Second visit</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. people</td>
<td>Positive, %</td>
</tr>
<tr>
<td></td>
<td>examined</td>
<td>(95% CI)</td>
</tr>
<tr>
<td>Normal</td>
<td>243</td>
<td>13.2 (8.9–17.4)</td>
</tr>
<tr>
<td>Wet-clay</td>
<td>235</td>
<td>9.4 (5.6–13.9)</td>
</tr>
<tr>
<td>Watery</td>
<td>22</td>
<td>22.7 (5.2–40.2)</td>
</tr>
</tbody>
</table>
In addition, there were several sets of isolates that differed by only one or two DNA bands after digestion with MluI, and hence appeared to be closely related. Related PFGE types 8 and 9 were obtained from members of different families in Melinggih. PFGE type 28 came from a 60-year-old male in Sesetan and the three isolates in related PFGE type 29 were also from Sesetan. An isolate in PFGE type 30 also differed slightly from those in PFGE type 29, but in this case the individual, a 13-year-old male, was from Payangan Desa. Isolates in PFGE types 31 and 32 were again similar, but they originated from individuals in different locations, Melinggih and Sesetan.

The second pair of isolates from the same individual obtained at the two visits that were available for PFGE typing originated from a 30-year-old female in Denpasar. These isolates were different from each other, having PFGE types 2 and 49.

DISCUSSION

The results of this study demonstrate that B. pilosicoli commonly colonizes individuals in Bali, with an overall prevalence of 11.8–12.6 %. This finding is consistent with previous evidence, suggesting that the spirochaete is commonly found in the faeces of people from developing countries (Barrett, 1990; Trott et al., 1997a; Brooke et al., 2001; Munshi et al., 2004). No isolates of B. aalborgi were obtained; this is not surprising, given the fastidious growth requirements of this species.

Prevalence of infection at the different sites examined varied considerably. Except in the case of the second visit to Karang Suwung, where prevalence was 21.4 %, prevalence in the four villages was generally low on both occasions (3.3–11.4 %). A similarly low prevalence was found in one of five villages in the Eastern Highlands of PNG (8.6 %), whilst prevalence in the other four villages studied was 22.6–30.1 % (Trott et al., 1997a). Amongst Indians on three tea estates in Assam, prevalence of colonization again varied, being 36.6, 18.9 and 8.2 % (Munshi et al., 2004). The relatively low prevalence in Balinese villages compared to rural villages in other developing countries may be associated with the Balinese villages having better sanitary and environmental conditions. Vi-
lages in Bali are generally well-organized and stable communities, where inhabitants are very aware of the importance of preserving a clean environment and water supply.

In contrast to the generally low prevalence in Balinese villages, prevalence in people from the peri-urban area of Sesetan (23.4 and 20.3% at the two visits) was significantly higher. Sesetan is crowded, with large numbers of transient residents from other parts of Indonesia, a large population of pigs that are fed on hotel food wastes, and a generally poor environment. Contamination of the water supply by spirillochaetes that originated from animals or humans may have contributed to the high prevalence of colonization. In this area, drinking water is often drawn from open shallow wells that are located adjacent to septic tanks and open drains. A possible role for water as a means of transmission of \( B. \) pilosicoli has been suggested previously (Trott et al., 1997a; Oxberry et al., 1998) and the use of well water was identified as a significant risk factor for \( B. \) pilosicoli colonization in a recent study in India (Munshi et al., 2004). Consistent with this, analysis of results from the questionnaire in the current study identified an increased risk of colonization amongst people who used well water compared to people who had access to tap water, at least at the first visit. The second visit in December coincided with the wet season and it is likely that polluted groundwater was diluted and/or washed away by the heavy rainfall.

The rate of colonization in people from Denpasar on the initial visit (12%) was similar to that of people from the four traditional villages, whilst on the second visit, prevalence was considerably higher (22.6%). The apparent increase in prevalence between visits may have been biased by the relatively small number of people who provided samples on the second visit. It would be expected that prevalence amongst relatively affluent and well-educated individuals in the city would be lower than that found in Sesetan, and more consistent with that found during the more extensive sampling at the first visit. In the study in PNG (Trott et al., 1997a), whilst there were generally high rates of colonization in villages, colonization was detected in only 9.3% of indigenous people living in an urban environment and in none of a group of expatriates living in this environment. Again, this low prevalence was attributed to the availability of relatively good sanitation and appropriate hygienic conditions in that particular urban environment.

The second visit to the village of Karang Suwang unexpectedly also gave a higher prevalence than the first visit (21.4% compared to 9.1%), with this difference being significant. There was no obvious explanation for this increase, especially as 95.1% of individuals in the study population were sampled on both visits. One possible scenario would be that the whole village was exposed to a new source of \( B. \) pilosicoli prior to the second sampling, perhaps through faecal contamination of the water supply. Isolates that were recovered at the second visit were equally as diverse, in terms of their PFGE pattern, as those that were recovered at the first visit; therefore, the increase in prevalence is unlikely to be due to widespread exposure to a particular epidemic strain of \( B. \) pilosicoli.

It was disappointing that analysis of results from the questionnaire failed to identify potential risk factors for colonization. As stated previously, the only factor that was significant was an increased prevalence amongst individuals who obtained their water from wells, rather than taps. Munshi et al. (2004), in their study in India, found the following to be significant factors for colonization by \( B. \) pilosicoli in logistic regression: other family members being colonized; water obtained from a well; water treatment; and not having visited a doctor in the last 12 months.

Analysis of the questionnaire results also failed to identify a significant link between colonization and disease. Although \( B. \) pilosicoli is a recognized enteric pathogen in animals and

Fig. 2. Dendrogram showing relationships between 50 \( B. \) pilosicoli isolates, as demonstrated by PFGE analysis.
despite the fact that human strains have been used experimentally to reproduce intestinal spirochaetosis and/or diarrhoea in animals (Trott et al., 1995, 1996a; Sacco et al., 1997), the precise role of the spirochaete as a potential human pathogen remains uncertain. Numerous studies have reported that people can carry intestinal spirochaetes without necessarily displaying clinical signs (Lee et al., 1971; Nielsen et al., 1983; Lee & Hampson, 1992; Trott et al., 1997a), whilst others have reported an association between the presence of intestinal spirochaetes (generally uncharacterized) and chronic diarrhoea and/or rectal bleeding (Gad et al., 1977; Douglas & Cruciani, 1981; Padmanabhan et al., 1996; Pehgin et al., 2000). In a study where a volunteer ingested approximately 2\times10^9 \text{B. pilosicoli} cells that were initially isolated from an Aboriginal child with diarrhoea, clinical symptoms of nausea, headache and abdominal bloating occurred 30 days after challenge (Oxberry et al., 1998).

The current study did identify the fact that individuals with watery or wet-clay faeces were more likely to have \text{B. pilosicoli} in their faeces than individuals with normal stools, at least at the second visit. Similarly, studies amongst Aboriginal children in Australia and villagers in PNG found that isolates were more common in individuals with watery stools than in those with normal stools (Lee & Hampson, 1992; Trott et al., 1997a). This finding supports the possibility that \text{B. pilosicoli} may be involved in causing long-standing diarrhoea in humans, although it does not provide direct causal evidence that this occurs.

PFGE analysis demonstrated that the isolates obtained were extremely heterogeneous. \text{B. pilosicoli} has a recombinant population structure (Trott et al., 1998), and the heterogeneity found in Bali is consistent with that found previously in other human populations (Trott et al., 1998; Brooke et al., 2001) and in other species, such as pigs (Atyeo et al., 1996). Unfortunately, many of the isolates could not be revived from storage; this limited the amount of information that could be deduced about strain dissemination and persistence. There was evidence for the presence of the same strain in two individuals in one family (PFGE type 47), in unrelated individuals in the same village (PFGE type 29) and in two individuals from different locations (PFGE type 24). The majority of isolates, however, were distinct from each other and the patterns obtained were clear and reproducible.

In total, 18 (4-8\%) people who were sampled 4 months apart were positive on both visits. This finding suggests that colonization in some people can last for at least 4 months, although it is not known whether these individuals were colonized continuously or expelled the bacteria and were subsequently reinfected. In an experimental study, a human volunteer remained colonized for 8 weeks after drinking a culture of \text{B. pilosicoli}, before the spirochaete was removed by specific antimicrobial treatment (Oxberry et al., 1998). In a study in PNG, 27 of 29 (93-1\%) individuals were colonized at two samplings that were undertaken 6 weeks apart (Trott et al., 1998). Furthermore, nine of 19 (47-4\%) culture-positive individuals were colonized by the same \text{B. pilosicoli} PFGE type at both samplings. Unfortunately, in the current study, only two pairs of samples from the same individuals, sampled 4 months apart, were available; one pair was identical and the other pair was different. Again, the first individual may have been infected continuously or may have become reinfected with the same strain. The second individual may have been reinfected with a different strain at the second visit or may even have been colonized by multiple strains, with different strains recovered at the two samplings.

In summary, this study has demonstrated that \text{B. pilosicoli} is endemic in Indonesians living in Bali, with many individual strains that circulate amongst the population. The highest prevalence of human colonization occurred in a crowded peri-urban area with a poor water supply.

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