Evaluation of blood culture systems for detection of the intestinal spirochaete *Brachyspira (Serpulina) pilosicoli* in human blood

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The anaerobic intestinal spirochaete *Brachyspira (Serpulina) pilosicoli* has been isolated from the bloodstream of French patients by manual blood culture systems. The purpose of this study was to determine whether the automated and manual blood culture systems used in Australia are suitable for growth and detection of this organism. Strains of *B. pilosicoli* were added to human blood to give concentrations ranging from $1 \times 10^4$ to $1 \times 10^5$ spirochaetes/ml and 10-ml volumes were inoculated into the media. Three strains of *B. pilosicoli* grew slowly in all manual Hémoline and BBL Septi-Chek formulations tested. Subcultures taken between 2 and 10 days after inoculation yielded growth only after incubation for a further 5–8 days. Growth and automated detection were achieved in the BACTEC system with Anaerobic/F medium with or without Fastidious Organism Supplement. Minimum time to signal for nine strains varied between 5.6 and 14.9 days, with a minimum concentration of $10^5$ spirochaetes/ml of blood being detected. None of nine strains gave a positive signal in the Bact/Alert system when FAN Anaerobic culture bottles were used; however, four strains were detected by subculture taken at 7 or 14 days after inoculation. When Anaerobic medium was used in the Bact/Alert system, two of three strains gave a signal and the other strain grew and was detected by subculture. Spirochaetemias caused by *B. pilosicoli* may be unrecognised because detection time by the signal or subculture exceeds 5 days.

Introduction

*Brachyspira* (previously *Serpulina* *pilosicoli*) is an anaerobic spirochaete that colonises the large intestine of man and many animal species [1–3]. In pigs, large numbers of these spirochaetes are found attached by one end of their cell to the colonic mucosa, and this colonisation is associated with colitis and diarrhoea [3, 4]. In man the end-on attachment of spirochaetes is referred to as intestinal spirochaetosis (IS) [1, 5]. Colonisation of the large intestine by *B. pilosicoli* apparently occurs quite commonly (>30% prevalence) in man in developing countries [3, 6], in Australian Aborigines [7], in homosexual males [5] and in patients with human immunodeficiency virus (HIV) infection [8]. Clinically, colonisation has been linked to various intestinal disorders, including chronic diarrhoea and rectal bleeding [1, 9, 10]. A volunteer who drank cultures of a human isolate of *B. pilosicoli* became colonised and developed abdominal discomfort and headaches after a prolonged incubation period (>1 month) [11]. Invasive colitis and hepatitis associated with intestinal spirochaetae have also been recorded [12, 13], although in some cases *B. aalborgi* rather than *B. pilosicoli* may have been involved [14, 15].

The potential pathogenicity of *B. pilosicoli* was emphasised recently by its isolation from the bloodstream of seven patients in France and the USA [16, 17]. The patient in the USA had HIV infection and was receiving chemotherapy for Kaposi’s sarcoma. The six French patients had various unrelated clinical problems, including stroke, alcoholic shock, arterio-pathy, peritonitis or myeloma. Two had diarrhoea and four subsequently died. The spirochaetes were isolated
with a manual blood culture system and specialised media. Primary isolation of five isolates was achieved in Hémoline blood culture bottles (bioMérieux, Marcy L’Etoile, France) and the other in Bioargs Sanofi
diagnostic anaerobic medium (Institut Pasteur, Paris,
France) [16]. The isolate from the USA grew in the
ESP automated blood culture system (Difco, Detroit,
MI, USA), but failed to produce a signal [17]. Changes
in the medium were noticed by an observant operator,
who then subcultured from the bottle.

Spirochaetemia associated with B. pilosicoli has not
been reported in Australia despite the existence of
groups of individuals known to have a high rate of
intestinal carriage and who have other debilitating
conditions which might predispose to bacterial translo-
cation across the intestinal wall. These groups include
Aboriginal people living in remote communities, recent
migrants from developing countries, homosexual males
and patients with HIV infection. Automated blood
culture systems are now used routinely in most large
hospital diagnostic laboratories in Australia, and it was
hypothesised that these systems may fail to detect
spirochaetemia caused by B. pilosicoli. The main
automated blood culture systems currently used are the
BACTEC (Becton Dickinson Diagnostic Instrument
Systems, Sparks, MD, USA) and BacT/Alert (Organon
Teknika Corporation, Durham, NC, USA) systems;
therefore, these were investigated. Both are fully
automated, non-radiometric and use continuous, non-
invasive monitoring of CO2 to detect microbial growth
[18]. The BACTEC system uses a fluorescence sensor
located in the medium [19]. The BacT/Alert system uses a colorimetric method of detection; when growth of
the micro-organism produces CO2, the colour of the
sensor changes from green to yellow [20].

Two manual systems were also investigated. Two types
of manual Hémoline blood culture bottles were
evaluated, as they were used to isolate B. pilosicoli
from the blood of some of the original French patients.
Manual BBL Septi-Chek blood culture bottles (Becton
Dickinson Microbiology Systems, Cockeysville,
MD, USA) with three different types of media were also
tested, as these are often used to culture blood obtained
from Australian Aboriginal patients in remote rural
communities.

Materials and methods

B. pilosicoli strains

The nine strains of B. pilosicoli studied were obtained
from the Reference Centre for Intestinal Spirochaetes
at Murdoch University. They comprised five strains
from blood (French strains 382/91, PE90, 28/94 and
RA87, and the USA strain H1), three from human
faeces (H171, WesB and H121) and the porcine type
strain P43/6/784 (ATCC 511399). The frozen (−70°C)
strains were thawed and inoculated into a pre-reduced
anaerobic broth medium as described by Kunkle et al.
[21] and checked for purity by phase contrast
microscopy and culture on Columbia agar base
containing horse blood 5% (BA). Plates were incubated
in an anaerobic chamber (Don Whitley Scientific,
Shipley, Yorkshire) in an atmosphere of N2 80%, H2
10%, CO2 10% at 37°C for 5 days. The spirochaetes
grew as a thin haze with weak, incomplete haemolysis.

Preparation of inocula

The isolates were grown to log phase in the pre-
reduced anaerobic broth, diluted in phosphate-buffered
saline (PBS), pH 7.2, and counted with a Helber
counting chamber, as B. pilosicoli does not form
discrete countable colonies. Each suspension was
adjusted to 1 × 10^5 organisms/ml in PBS, then serial
10-fold dilutions were made in PBS to obtain
concentrations of 10^9, 10^7 and 10^5 organisms/ml. A
1-ml volume of each suspension was added to 9 ml of
human blood (Australian Red Cross) to give final
concentrations of 1 × 10^5, 1 × 10^3, 1 × 10^1 and 1 ×
10^3 organisms/ml of blood. Blood comprised outdated
RBCs from which plasma had been removed and the
cells reconstituted with 100 ml of Adsol (Baxter),
consisting of dextrose, NaCl, adenine and mannitol.
The 10-ml volumes were used to inoculate the
proprietary media. The viability of each strain was
determined immediately after inoculation by subculture
from the bottle with the highest spirochaeta concentra-
tion on to BA, followed by anaerobic incubation at
37°C for 5 days.

Blood culture systems and media

A summary of the blood culture systems and media
used, and number of strains examined in each is
presented in Table 1. Three strains of B. pilosicoli
(382/91 and PE90 from blood, H171 from faeces) were
tested in the manual Hémoline system, with both a
trypticase soy agar slope in trypticase soy broth
(diphasic) and a modified Wilkens Chalgren broth
(anaerobic), and in the manual BBL Septi-Chek system
with trypticase soy broth (TSB), modified brain heart
infusion broth (BHB) and thiglycollate broth bottles.
These three strains were also tested separately in the
two automated systems with BACTEC PLUS Anaero-
bic/F with and without Fastidious Organism Supple-
ment (FOS), and BacT/Alert Anaerobic and FAN
Anaerobic culture bottles. The other six strains, 28/49,
RA87, H1, WesB, H121 and P43/6/784, were tested at
the same time, but only in the BACTEC 9240
system with PLUS Anaerobic/F culture vials and in the
BacT/Alert system with FAN Anaerobic culture bottles.

Detection of spirochaete growth

All bottles were incubated for up to 28 days. For the
manual bottles, a 0.5-ml sample was taken on days 2,
5, 7, 10, 14, 18, 21, 24 and 28 and plated on to BA

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which was incubated anaerobically for up to 14 days. Where there was a suspicion of microbial growth at the time of sampling because of a colour change or build up of gas, a sample was also examined by phase contrast microscopy. For the manual bottles, the endpoint was defined as the day on which a sample was taken which subsequently yielded a growth of spirochaete on BA. Actual times between inoculation and confirmation of a positive result were longer than this because of the need for prolonged incubation of the plates.

For the automated systems, time to positivity was defined as the time of initial incubation until an automatic signal was detected or until a positive subculture was obtained. For bottles that did not give a signal, 0.5-ml samples were taken at 7, 14, 21 and 28 days and plated on to BA which was incubated anaerobically for up to 14 days. Care was taken not to disturb any gas present in the bottles when samples were removed. Where positive signals were obtained, the bottles were removed and a sample of broth was examined by phase contrast microscopy and plated on to BA. Spirochaete numbers in the broth were estimated with the counting chamber, after centrifugation at 150 g for 5 min to remove red blood cells.

Results

Growth of strains 382/91, PE90 and H171 in all systems

All three strains were viable when inoculated. Strain H171 was not detected in the manual BBL Septi-Chek system with thioglycollate broth, and none of the strains was detected by signal or subculture in the BacT/Alert system with FAN Anaerobic medium (Table 2). All three strains grew in the BacT/Alert Anaerobic medium, although strain 382/91 failed to produce a signal and was detected only by subculture. All the other systems and media supported growth of the spirochaete strains, but at various limits of detection and times to detection (Table 2). Strain 382/91 tended to be detected earlier and from a lower initial inoculum than the other two strains. Overall results were similar for a given strain for different manual systems or media with detection limits of between 10^1 and 10^4 spirochaetes/ml of blood in the original inoculum. Individual manual bottles sampled between 2 and 10 days were positive by subculture and the plates generally took 5–8 days to show growth. In most cases, spirochaetes were recovered by subculture before they were detected by phase contrast microscopy.

Growth of another six strains in automated systems

Strains 28/94, RA87, H1, WesB, H121 and P43/6/78T were all viable, and grew and produced signals in the BACTEC system with PLUS Anaerobic/F without FOS (Table 2). Time to first detection by signal in this system for the six strains ranged from 5.7 to 14.9 days (mean 7.5 days) (Table 3) although, unexpectedly,

<table>
<thead>
<tr>
<th>Blood culture system</th>
<th>Media</th>
<th>Number of strains tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hémoline</td>
<td>Diphasic</td>
<td>3^</td>
</tr>
<tr>
<td>Anaerobic</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>BBL Septi-Chek</td>
<td>TSIB</td>
<td>3</td>
</tr>
<tr>
<td>BHIIB</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>THIOGLYCOLATE BROTH</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>BACTEC 9240</td>
<td>PLUS Anaerobic/F with FOS</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>PLUS Anaerobic/F</td>
<td>3 + 6^</td>
</tr>
<tr>
<td>BacT/Alert FAN Anaerobic</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>BacT/Alert Anaerobic</td>
<td></td>
<td>3 + 6</td>
</tr>
</tbody>
</table>

TSB, tryptic soy broth; BHIIB, modified brain heart infusion broth; FOS, fastidious organism supplement.

^ The three strains tested throughout were 382/91, PE90 and H171.

The other six strains were 28/94, RA87, H1, WesB, H121 and P43/6/78T.

Table 2. Limits of detection and minimum time to detection at any inoculum concentration for B. pilosicoli strains 382/92, PE90 and H171 in manual and automated blood culture systems

<table>
<thead>
<tr>
<th>Systems and media</th>
<th>Strain 382/91</th>
<th>Strain PE90</th>
<th>Strain H171</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hémoline Diphasic</td>
<td>10^0(2)</td>
<td>10^0(5)</td>
<td>10^0(7)</td>
</tr>
<tr>
<td>Anaerobic</td>
<td>10^0(2)</td>
<td>10^0(2)</td>
<td>10^0(4)</td>
</tr>
<tr>
<td>BBL Septi-Chek TSIB</td>
<td>10^0(2)</td>
<td>10^0(2)</td>
<td>10^0(7)</td>
</tr>
<tr>
<td>BBL Septi-Chek BHIIB</td>
<td>10^0(2)</td>
<td>10^0(2)</td>
<td>10^0(7)</td>
</tr>
<tr>
<td>BBL Septi-Chek THIOGLYCOLATE BROTH</td>
<td>10^0(2)</td>
<td>10^0(5)</td>
<td>–</td>
</tr>
<tr>
<td>BACTEC Plus Anaerobic/F</td>
<td>10^0(5.6)^*</td>
<td>10^0(10.7)^*</td>
<td>10^0(7.3)*</td>
</tr>
<tr>
<td>BACTEC Plus Anaerobic/F FOS^</td>
<td>10^0(6.7)^*</td>
<td>10^0(9.1)^*</td>
<td>10^0(7.3)*</td>
</tr>
<tr>
<td>BacT/Alert FAN Anaerobic</td>
<td>10^0(7)</td>
<td>10^0(14.1)*</td>
<td>10^0(9.5)*</td>
</tr>
<tr>
<td>BacT/Alert Anaerobic</td>
<td></td>
<td>10^0(5)</td>
<td>10^0(9)</td>
</tr>
</tbody>
</table>

^ No growth detected.

Strains 382/91 and PE90 were from human blood; H171 was from human feaces.

^ Detected by automated signal; all other bottles in all systems detected by subculture only.

^ No bottles inoculated at 10^0 spirochaetes/ml of blood.
strain H1 took 16.6 days to give a signal at the highest inoculum. Sprochetaes could be seen in wet mount preparations at the time of signalling. Generally, bottles containing the highest inoculum of a given strain signalled first. Limits of detection for the six strains varied between 10^2 (strains WesB, H121 and P43/6/78) and 10^3 (strain H1) sprochetaes/ml of blood in the original inoculum. Positive bottles contained between 4 × 10^1 and 5 × 10^3 organisms/ml, confirming that the strains had proliferated. B. pilosicoli was subsequently detected by subculture from all bottles that signalled positive, in the subculture taken immediately before signalling. Growth from subculture took 2–8 days.

None of the bottles signalled positive in the BacT/Alert system with the FAN Anaerobic medium. However, four of the six strains were detected by subculture, P43/6/78 at all concentrations, WesB at 1 × 10^4 and 1 × 10^5, and H121 and RA87 at 1 × 10^6 sprochetaes/ml of blood. Time to first detectable growth varied between 7 days for strains WesB, H121 and P43/6/78 and 14 days for strain RA87 (Table 3). Except for faecal isolate H121 at the 1 × 10^6 concentration, viable organisms were observed by microscopy and recovered by subculture from the vials at successive sampling points. A considerable build up of gas at 21 and 28 days was noticed in these vials. The graph of CO₂ production over time produced by the BacT/Alert computer had a positive slope, indicating CO₂ production in the vials, and the bases of the bottles also became yellow, but nevertheless they did not generate a signal.

Discussion

This study demonstrated that blood culture systems and protocols in common use in Australian diagnostic laboratories are suboptimal for detection of B. pilosicoli.

Studies of automated and manual systems in a clinical setting have shown that the majority of organisms encountered in blood require <7 days for detection. The 5-day retention of blood culture samples is regarded as a working compromise between maximum detection and cost-effectiveness [19, 22–24]. The present study held samples for 28 days so as to evaluate the recovery times of the slow-growing B. pilosicoli. As few as 1 × 10^1 sprochetaes/ml of blood were detected in some cases (Tables 2 and 3). Although it is not known how many organisms are present in natural sprochetaemias with B. pilosicoli, they have been isolated in patients with Hémoline media; in the present study these media had limits of detection of between 10^3 and 10^8 organisms/ml of blood (Table 2). Overall, there was considerable variation in detection thresholds between the various strains tested, but the basis of this variability was not investigated. No consistent differences in growth were found between strains of faecal or blood origin.

Of the two automated systems tested, the BACTEC system performed best, with all B. pilosicoli strains growing and being detected by signal in the media. Even with the BACTEC system, the time required for detection considerably exceeded the 5-day retention normally used by clinical diagnostic laboratories. Consequently, sprochetaemias with B. pilosicoli would not usually be detected. Addition of FOS to the media did not improve detection time. Besides enhancing the growth of fastidious organisms, FOS has a neutralising effect on sodium polynol sulphonate (SPS), which is known to affect blood culture yield [25]. SPS is an anticoagulant agent that is also used to counteract the bactericidal activity of serum. The results suggest that B. pilosicoli is not susceptible to SPS at the concentration used in these media.

The BacT/Alert system performed poorly for the detection and recovery of B. pilosicoli. Strains 382/91, PE90 and H171 grew in the Anaerobic medium but not in the FAN Anaerobic medium. FAN Anaerobic medium contains Ecosorb, a proprietary material composed of activated carbon particles and Fuller’s earth which is thought to non-specifically bind inhibitory factors other than antimicrobial agents [23]. Whilst designed to increase organism recovery, Ecosorb may have bound necessary growth requirements for B. pilosicoli, such as cholesterol, which is essential for growth of the closely related intestinal sprochetae B. hyodysenteriae [26]. Even with the Anaerobic medium, one of three strains did not generate a signal and time to detection was longer than with the BACTEC system. B. pilosicoli was only recovered from bottles inoculated with 10^3 or 10^4 sprochetaes/ml of blood, which suggests that it was not optimal for growth of B. pilosicoli. Unfortunately, the other six strains were tested only in the FAN Anaerobic medium, and only one strain was detected in a bottle inoculated with 10^1 sprochetaes/ml of blood. It is possible that if the
Anaerobic medium had been tested more strains may have grown and may have been more rapidly detected at lower concentrations.

Detection algorithms also contributed to the poor performance of the BacT/Alert system. The system did not signal the presence of *B. pilosicoli* even when a build up of CO₂ had been generated and the organism had been recovered by subculture (4 of 9 strains; 8 of 36 bottles). It is unlikely that an algorithm would be changed to accommodate slow-growing organisms such as *B. pilosicoli*.

Although they are not widely used in Australia, growth in the two manual Hémoline preparations confirmed the utility of these media for isolation of *B. pilosicoli* from blood [16]. The manual Septi-Chek bottles gave comparable results. Therefore, either Hémoline or Septi-Chek could be used with some confidence to detect *B. pilosicoli* sprochaetamias in laboratories where automated systems are not available, provided that the vials were incubated for a longer time to accommodate the organism’s slow growth. The manual bottles required considerable time and labour to monitor and, in particular needed regular blind subculturing to detect the organism. While minimum time to positivity by subculture varied from 2 to 10 days, which was earlier than for the BACTEC system, the organisms were often not at a high enough concentration in the bottles to be visible by microscopy. Also, subcultures on plates needed to be incubated anaerobically for 5–8 days before growth was detected.

This study indicates that the BACTEC system is better than the BacT/Alert system for the detection of *B. pilosicoli* in seeded blood culture and, hence, is likely to be superior for the detection of sprochaetamias in patients. Unfortunately an 18-day incubation period with the BACTEC PLUS Anaerobic/F Culture Vials would be needed to detect the majority of clinical isolates. If the BacT/Alert system is used then BacT/Alert Anaerobic Culture Bottles should be considered and terminal subculturing performed. Both manual bottles tested required a minimum of an 18-day protocol with blind subculture every 3 days to enable early detection of viable strains. In addition, an aspirate from any positive bottle should be examined by phase contrast microscopy, as *B. pilosicoli* is slender and very difficult to detect by Gram’s stain. Subcultures on BA should be incubated anaerobically for up to 10 days before being discarded.

Sprochaetamias associated with *B. pilosicoli* should be considered in febrile patients from groups known to have a high rate of intestinal carriage with the organism. Appropriate media and conditions, including prolonged incubation times, are required to detect these organisms. Prospective studies to determine how frequently sprochaetamias associated with *B. pilosicoli* occur as a clinical complication in these high-risk groups of patients should be initiated.

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References