DIAGNOSTIC MICROBIOLOGY

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 1×10^4 to 1×10^1 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 10^1 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. Spirochaetaemias 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 [2, 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 spirochaetes 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, arteriopathy, peritonitis or myeloma. Two had diarrhoea and four subsequently died. The spirochaetes were isolated

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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 Bioargos 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.

Spirochaetaemia 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 translocation 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 spirochaetaemia 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 P43/6/78^T (ATCC 51139^T). 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 10^4 , 10^3 and 10^2 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 1×10^4 , 1×10^3 , 1×10^2 and 1×10^1 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 spirochaete concentration 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 (BHIB) and thioglycollate broth bottles. These three strains were also tested separately in the two automated systems with BACTEC PLUS Anaerobic/F with and without Fastidious Organism Supplement (FOS), and BacT/Alert Anaerobic and FAN Anaerobic culture bottles. The other six strains, 28/94, RA87, H1, WesB, H121 and P43/6/78^T, 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

Table 1. Blood culture systems and media tested

Blood culture system	Media	Number of strains tested
Hémoline	Diphasic	3*
	Anaerobic	3
BBL Septi-Chek	TSB	3
	BHIB	3
	Thioglycollate broth	3
BACTEC 9240	PLUS Anaerobic/F with FOS	3
	PLUS Anaerobic/F	3 + 6†
BacT/Alert	FAN Anaerobic	3
	Anaerobic	3 + 6

TSB, trypticase soy broth; BHIB, 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 P43/6/78^T

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 10¹ and 10⁴ 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 P43/6/78^T 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 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

Systems and media	Lowest inoculum detected (minimum time to detectable growth in days)		
	strain 382/91	strain PE90	strain H171
Hémoline Diphasic	10 ¹ (2)	10 ³ (5)	10 ⁴ (7)
Hémoline Anaerobic	10 ² (2)	10 ² (2)	10 ⁴ (10)
BBL Septi-Chek TSB	10 ¹ (2)	10 ² (2)	10 ¹ (7)
BBL Septi-Chek BHIB	10 ¹ (2)	10 ² (5)	10 ² (7)
BBL Septi-Chek Thioglycollate Broth	10 ¹ (2)	10 ¹ (5)	—
BACTEC Plus Anaerobic/F	10 ¹ (5.6)*	10 ² (10.7)*	10 ³ (7.3)*
BACTEC Plus Anaerobic/F FOS§	10 ² (6.7)*	10 ² (9.1)*	10 ³ (7.3)*
BacT/Alert FAN Anaerobic	—	—	—
BacT/Alert Anaerobic†	10 ⁴ (7)	10 ³ (14.1)*	10 ³ (9.5)*

—, no growth detected.

Strains 382/91 and PE90 were from human blood, H171 was from human faeces.

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

†No bottles inoculated at 10¹ spirochaetes/ml of blood.

Table 3. Limits of detection and time to detection for *B. pilosicoli* strains 28/94, RA87, H1, WesB, H121 and P43/6/78^T tested in automated systems

Strain no.	Lowest inoculum detected (minimum time to detection in days)	
	BACTEC PLUS Anaerobic/F	BacT/Alert FAN Anaerobic [†]
28/94	10 ² (7.4)	—
RA87	10 ² (8.3)	10 ⁴ (14)
H1	10 ³ (14.9)	—
WesB	10 ¹ (5.7)	10 ³ (7)
H121	10 ¹ (8.5)	10 ⁴ (7)
P43/6/78 ^T	10 ¹ (6.9)	10 ¹ (7)

—, no growth detected.

*Detection by automated signal.

[†]Detection by subculture, not signal. Subcultures were only taken on days 7, 14, 21 and 28 and required incubation for a further 5–8 days before detection was achieved.

strain H1 took 16.6 days to give a signal at the highest inoculum. Spirochaetes 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 10¹ (strains WesB, H121 and P43/6/78^T) and 10³ (strain H1) spirochaetes/ml of blood in the original inoculum. Positive bottles contained between 4 × 10⁷ and 5 × 10⁸ 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^T at all concentrations, WesB at 1 × 10⁴ and 1 × 10³, and H121 and RA87 at 1 × 10⁴ spirochaetes/ml of blood. Time to first detectable growth varied between 7 days for strains WesB, H121 and P43/6/78^T and 14 days for strain RA87 (Table 3). Except for faecal isolate H121 at the 1 × 10⁴ 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 × 10¹ spirochaetes/ml of blood were detected in some cases (Tables 2 and 3). Although it is not known how many organisms are present in natural spirochaetaemias with *B. pilosicoli*, they have been isolated in patients with Hémoline media; in the present study these media had limits of detection of between 10¹ and 10⁴ 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, spirochaetaemias 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 polyethol 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 spirochaete *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 10³ or 10⁴ spirochaetes/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 10¹ spirochaetes/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* spirochaetemia 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 spirochaetemias 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.

Spirochaetemia 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 spirochaetemias associated with *B. pilosi-*

coli occur as a clinical complication in these high-risk groups of patients should be initiated.

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