Comparison of three bacterial detection methods under routine conditions


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Background and Objectives Since 2004, bacterial screening of platelets has been required in the USA and is also done on a voluntary basis in many European countries. The German Red Cross blood donor services conducted a prospective multicentre study in order to investigate the prevalence of bacterially contaminated pool platelet concentrates and apheresis platelet concentrates. This substudy compares three different bacterial detection systems.

Study Design and Methods Platelet concentrates were tested in parallel with BacT/ALERT, Scansystem™ and Pall eBDS (n = 6307) in pool platelets. Apheresis platelets were tested in parallel with BacT/ALERT and Pall eBDS (n = 4730). All initially positive results were evaluated by a standardized procedure including evaluation by a microbiology reference laboratory.

Results One in 6307 pool platelets were confirmed positive by BacT/ALERT, whereas Pall eBDS and Scansystem failed to detect these samples. Only three samples were initially reactive with Pall eBDS without proof of any bacteria strains. The rate of false-positive results was substantially higher for BacT/ALERT (0.25%, 28 in 11 037 tested samples) than for eBDS (0.03%, 3 in 11 037 tested samples) or Scansystem (0.0%, 0 in 6307 tested samples). Three of 4730 apheresis platelets were confirmed positive by BacT/ALERT. These were negative with Pall eBDS.

Conclusion Sensitivity was best for BacT/ALERT, whereas specificity was enhanced for Pall eBDS and Scansystem. Scansystem required specially trained staff, whereas BacT/ALERT and Pall eBDS were easy, quick, user-friendly and objective methods.

Key words: BacT/ALERT, bacterial detection, Pall eBDS, routine conditions, Scansystem.

Introduction

Releasing safe blood products is a high priority task for blood donor services. Because of the AIDS scandal in the mid-1990s, the attention of the transfusion community was drawn to the prevention of transfusion-transmitted virus infections [1–3]. Implementation of antibody screening and nucleic acid amplification technique (NAT) significantly reduced the risk of acquiring a transfusion-related virus infection [4]. NAT was voluntarily integrated in blood-donor screening at the German Red Cross (GRC) since 1996 subsequently for hepatitis C virus (HCV), human immunodeficiency virus (HIV) and hepatitis B virus (HBV), and later in Germany it became mandatory for HCV and HIV in 1999 and 2004, respectively [5–9]. Based on
data from the GRC NAT study, the remaining risk for a transfusion-associated virus infection is estimated at 0.22, 0.37 and 1.51 per 10^6 donations for HIV, HCV and HBV, respectively [10].

It has been known since the early 20th century that microorganism infections can be transmitted by blood transfusion. Borden [11] published on the topic of transfusion-related deaths caused by massive bacterial contamination, and the bacterial contamination of blood products remains a persistent problem in modern transfusion medicine. In Germany, sterility control is done only for quality assurance, and random samples are thus tested after standard operating procedures. Two factors, room temperature storage and the biological composition of platelet concentrates and their media, make platelet concentrates close-to-ideal culture media for a wide variety of Gram-positive and Gram-negative organisms.

In this study, we evaluated three commercially available bacterial detection methods under routine conditions. Two culture methods (BacT/ALERT® and Pall eBDS) and one rapid bacterial detection system (Scansystem®) were compared. Diagnostic sensitivity, manageability and efficiency of the systems were evaluated.

Material and methods

Study design and platelet concentrates

The GERMS Group (German Evaluation of Regular Monitoring Study Group) [12] conducted a multicentre study to evaluate commercially available bacterial detection systems. Three methods (BacT/ALERT, Scansystem and Pall eBDS) were compared under routine screening conditions at four sites: the GRC Institute in Muenster and Breitscheid, and the BRC (Bavarian Red Cross) in Munich and Nuremberg. Based on a statistical calculation, at least 4500 samples were needed for each method in order to compare all three systems. A total of 6307 pool platelet concentrates (PPC) were tested with BacT/ALERT and Pall eBDS. Pool platelets were produced immediately after sampling and were analysed within 70 min. All samples without a positive reaction in each of the three tests were considered negative. Samples with a reactive signal in one of the three tests, but no microbiological confirmation of the bacterial strain, were labelled as initially reactive. Samples with both a reactive signal and microbiological confirmation were regarded as initially positive, and samples in which the initial positive signal could be repeated either with a second sample of the satellite bag, a sample of the original platelet bag, or a sample of a related erythrocyte bag in the case of a PPC were denoted confirmed positive.

All initial reactive samples were forwarded to microbiological reference laboratories (Departments of Microbiology, Universities of Bochum and Frankfurt, Germany) where Gram stains, subcultivation on solid medium and bacterial identification were performed as previously described [12].

Donor arm disinfection

Donor arm disinfection is also standardized at the GRC with a two-step procedure: donor skin is sterilized twice with a swab (single use) and disinfection solution listed in GSHM (German Society for Hygiene and Microbiology; e.g. Octeniderm®, Schülke & Mayr, Norderstedt, Germany, containing 30% 1-propanol, 45% 2-propanol and 0.1% Octenidindihydromochlorid). Bacterial diversion systems (diversion of the first 30 ml blood) were used for whole blood donations as well as for apheresis platelets.

Production of PPCs

Pool platelet concentrates were prepared according to the procedures routinely used at the GRC Institutes in Muenster and Breitscheid. Briefly summarized, whole blood units were centrifuged and separated into plasma, red cell concentrate anduffy coat (70 ± 10 ml) using an automated device (Compomat, Fresenius Hemocare, Bad Homburg, Germany). Fouruffy coats were pooled by sterile docking and mixed with 200 ml plasma. Following soft spin centrifugation at 548 g for 10 min, the platelet-rich plasma was transferred to a storage container using the Compomat and then filtered. After preparation, the PPCs contained at least 2 × 10¹¹ platelets per bag.

Production of APCs

Apheresis platelet concentrates were produced by different automatic continuous-flow or discontinuous-flow separators. All APCs from Munich (n = 2970) were produced by Fresenius COM.TEC (Fresenius, Bad Homburg, Germany), whereas in Nuremberg 918 APCs were produced by Fresenius COM.TEC and 842 were produced by Haemonetics MCS plus (Haemonetics, Munich, Germany). In total, 151 products were collected into a single bag, and all other APCs were split into two bags.
After preparation, APCs contained at least $2 \times 10^{11}$ platelets per bag, 280 ml of plasma, $< 1 \times 10^6$ leucocytes and $< 1 \times 10^9$ erythrocytes.

**BactT/ALERT**

BactT/ALERT, a culture method for bacterial detection, has been used for quality assurance at the GRC institutes since 1997. In the present study, BacT/ALERT Classic 240 (bioMérieux, Marcy, l’Etoile, France) was used at each institute. All samples were tested for aerobic and anaerobic bacteria. Bacterial detection with BacT/ALERT is based on the evolution of carbon dioxide by proliferating bacteria [13]. A carbon-dioxide-sensitive liquid emulsion sensor at the bottom of the culture bottle changes colour, which is detected through alteration of light reflected on the sensor [14]. In all assays, 7–5 ml of sample volume was inoculated into each test bottle (aerobic and anaerobic) and tested up to 7 days. Prior inoculation the bottle tops were cleaned with alcohol. The signals were continuously recorded with software (Bact/Link E.20, bioMérieux). Results were manually read every 12 h. BacT/ALERT sensitivity was 1 CFU/ml according to the manufacturer. Bottles were inoculated under laminar air conditions. The whole procedure took 5–10 min per sample. The process is completely barcode controlled. Signals were automatically analysed. Growth curves could be plotted for each sample.

**Scansystem**

Scansystem is a rapid bacterial detection system and has been described in detail by Jacobs et al. [15] and Schmidt et al. [16]. Aliquots of three platelet concentrates were pooled. Briefly summarized, bacteria were stained with PicoGreen (Hemosystem, Marseilles, France) and analysed within 70 min. Three millilitre of three platelet concentrates were each inoculated in a plastic chamber. Luer locks were then broken and all three samples were mixed. Three millilitre of this mixture (dilution 1 : 3) were incubated with 1 ml of BLS1 (polyethyleneimine, 60 mg/l; monoclonal antibody CD9 GB1, 30 mg/l; 1 : 2000 dilution of PicoGreen nucleic acid-binding dye) and placed on a flatbed agitator for 40 min to aggregate platelets. After filtering through a 5-μm pore size filter, bacterial cell membranes were permeabilized and stained with BLS2 (ethylenediaminetetraacetate acid, 1-86 g/l; nisin, 8 mg/l; N-octyl-β-D-glucopyranoside, 2-5 g/l; chlorhexidine diacetate, 150 mg/l, all in distilled water). After this step, all fluid was filtered onto a 0-4-μm pore size black membrane, scanned with an argon laser (488 nm excitation light) and analysed under an epifluorescence microscope. At least 50 microscopic fields were visually analysed. If more than 10 bacteria-specific fluorescence spots were detected, the ratio between detected bacteria and analysed fields was $\geq 0.2$. These samples were consequently assumed positive. In the case of a positive pool result, individual tests of the three platelet concentrates were conducted. The entire process was barcode controlled with the exception of the final steps (scanning the black membrane with an argon laser and microscopic analysis). Staining, scanning and analysis took 2-5 h for 12 pool samples. One technician was therefore able to analyse 36 pool samples in 8 h (108 samples per 8 h). To differentiate negative samples from weak positive samples, specially trained staff was required.

**Pall eBDS**

Pall eBDS is another culture method and was described in detail by Ortolano et al. [17], Holme et al. [18] and McDonald et al. [19]. In brief, 3 ml of the sample volume were inoculated into a sample pouch. Afterwards, the pouch was incubated at 35 °C for 24 h. Next, pouches were removed from the incubator and kept at room temperature for at least 2 min. Bacterial detection with the Pall eBDS system is based on the measurement of reduced oxygen levels in contaminated platelet components caused by microbial oxygen consumption during proliferation. The probe for oxygen detection was inserted into the pouch and the air in the pouch was automatically analysed by the oxygen analyser and displayed on a monitor. Oxygen content less than or equal to 9.5% was deemed positive. The entire process was barcode controlled. Results were analysed independently of the examiner. Environment cross-contamination by the technician was inhibited because it was a closed system. The total preparation time was 2 min per sample (for inoculation, scanning pouches and incubation in the heater), and another 2 min per sample for oxygen analysis. One technician was therefore able to handle 120 samples per day. No specially trained staff was needed.

**Results**

All three bacterial detection methods (BacT/ALERT, Scansystem and Pall eBDS) were used according to the manufacturers’ instructions. Table 1 summarizes the specification of each method. All methods were Communauté Europeenne (CE) marked. The inoculated sample volume differed between each method; BacT/ALERT required an inoculated volume between 4 and 10 ml per bottle (in the present study, the inoculated volume was standardized to 7-5 ml), whereas Pall eBDS and Scansystem analysed only 3 ml per sample. The performance of BacT/ALERT and Pall eBDS demonstrates an easy and quick handling. In contrast, specially trained staff was needed for Scansystem. The maximum sample handling per technician and day was comparable for all three methods (Table 1).

As shown in Table 2, 25 PPCs were initially reactive by BacT/ALERT (six only in the aerobic bottle, 18 only in the anaerobic
Table 1: Specification of bacterial detection systems according to manufacturers’ instructions

<table>
<thead>
<tr>
<th>Category</th>
<th>BacT/ALERT</th>
<th>Pall eBDS</th>
<th>Scansystem</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detection system</td>
<td>Single sample</td>
<td>Single sample</td>
<td>Mini pool (three samples)</td>
</tr>
<tr>
<td>Inoculated volume</td>
<td>4–10 ml per bottle</td>
<td>2–3 ml per pouch</td>
<td>3 ml per sample</td>
</tr>
<tr>
<td>Test performance</td>
<td>Continuous culture at 35 °C for 7 days</td>
<td>Incubation of the inoculated pouch at 35 °C for 24 h</td>
<td>3 ml of a 9-ml mixture is analysed</td>
</tr>
<tr>
<td></td>
<td>– pH sensitive disk in the bottom of the bottle</td>
<td>– O₂ measurement</td>
<td>– 40 min incubation with BLS1</td>
</tr>
<tr>
<td></td>
<td>– Color changes from grey into yellow for positive samples</td>
<td>– Samples with an O₂ concentration less than 9.5% are positive</td>
<td>– filtering</td>
</tr>
<tr>
<td></td>
<td></td>
<td>– scanning with argon laser</td>
<td>– filtering on black membrane</td>
</tr>
<tr>
<td></td>
<td></td>
<td>– microscopic analysis</td>
<td>– positive samples have a ratio between detected bacteria/analysed fields ≥ 0.2</td>
</tr>
<tr>
<td>Testing time after sample collection</td>
<td>up to 7 days</td>
<td>24 h–25 h</td>
<td>1–2 h</td>
</tr>
<tr>
<td>Maximum test number per technician per day</td>
<td>96 samples</td>
<td>120 samples</td>
<td>108 samples</td>
</tr>
<tr>
<td>Barcode-controlled process</td>
<td>Entire process is barcode controlled</td>
<td>Entire process is barcode controlled</td>
<td>Entire process with the exception of the last two steps (scanning black membrane and microscopic analysis) is barcode controlled</td>
</tr>
<tr>
<td>Analysis</td>
<td>Automatically; qualitative results</td>
<td>Automatically; qualitative results</td>
<td>Manually; qualitative results</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>1 CFU/ml</td>
<td>1 CFU/ml</td>
<td>1000 CFU/ml</td>
</tr>
<tr>
<td>Staff training</td>
<td>No specially trained staff necessary</td>
<td>No specially trained staff necessary</td>
<td>Specially trained staff needed</td>
</tr>
</tbody>
</table>

BacT/ALERT and Pall eBDS are culture methods, whereas Scansystem is a rapid detection method. The bacterial test systems differed from one another in regard to the categories listed here.

Table 2: Comparison of BacT/ALERT, Scansystem and Pall eBDS

<table>
<thead>
<tr>
<th>Product</th>
<th>Total</th>
<th>Aerobic bottle</th>
<th>Anaerobic bottle</th>
<th>Total</th>
<th>Aerobic bottle</th>
<th>Anaerobic bottle</th>
<th>Total</th>
<th>Aerobic bottle</th>
<th>Anaerobic bottle</th>
<th>Total</th>
<th>Aerobic bottle</th>
<th>Anaerobic bottle</th>
<th>Total</th>
<th>Aerobic bottle</th>
<th>Anaerobic bottle</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pool PC</td>
<td>6307</td>
<td>7</td>
<td>19</td>
<td>6</td>
<td>6</td>
<td>8</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pool PC</td>
<td>6307</td>
<td>7</td>
<td>19</td>
<td>13</td>
<td>29</td>
<td>6</td>
<td>17</td>
<td>0</td>
<td>3</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Apheresis PC</td>
<td>4730</td>
<td>10</td>
<td>29</td>
<td>39</td>
<td>6</td>
<td>17</td>
<td>0</td>
<td>3</td>
<td>3</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>11037</td>
<td>64</td>
<td>36</td>
<td>23</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Pool platelet concentrates (PCs) were tested in parallel with BacT/ALERT, Scansystem and Pall eBDS. Apheresis PCs were tested with BacT/ALERT and Pall eBDS. All reactive samples were confirmed by microbiological tests. Initially reactive samples were only positive in the screening test. A bacterial strain was proved in initial positive samples and confirmed positive samples, which were also positive in a second volume.

\(^{a}\)Staphylococcus saccharolyticus.

\(^{b}\)Staphylococcus hominis and Staphylococcus epidermidis.

\(^{c}\)Propionibacterium acnes.

One APC contaminated with K. pneumoniae was negative in BacT/ALERT and Pall eBDS.

NT, not tested.
Table 3 Bacterial strains detected by BacT/ALERT

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Initial positive</th>
<th>Confirmed positive</th>
<th>Mean time to positive signal (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus circulans</td>
<td>1</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>Bacillus endophyticus</td>
<td>1</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>Bacillus pumilus</td>
<td>1</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Bacillus spp.</td>
<td>2</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>1</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>Corynebacterium coyleae</td>
<td>1</td>
<td>44</td>
<td></td>
</tr>
<tr>
<td>Micrococcus luteus</td>
<td>4</td>
<td>76</td>
<td></td>
</tr>
<tr>
<td>Propionibacterium acnes</td>
<td>20</td>
<td>3</td>
<td>112-3*</td>
</tr>
<tr>
<td>S. aureus</td>
<td>1</td>
<td>22-1</td>
<td></td>
</tr>
<tr>
<td>S. capitis</td>
<td>1</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>S. epidermidis</td>
<td>2</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>S. lugdunensis</td>
<td>1</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>S. saccharolyticus</td>
<td>1</td>
<td>55-2*</td>
<td></td>
</tr>
<tr>
<td>Uncultured soil bacterium clone</td>
<td>1</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>37</td>
<td>4</td>
<td>80-42</td>
</tr>
</tbody>
</table>

Fourteen different bacterial strains were detected by BacT/ALERT. Most of the strains were anaerobic or facultative anaerobic bacteria. Mean time points between inoculation and identification were 36.2 ± 17.3 h and 98.0 h ± 28.6 h (P < 0.01) for initial positive and confirmed positive results. *Confirmed positive samples.

bottle, and one PC was reactive in both bottles). Bacterial strains were proofed in 13 out of 25 samples (five only in the aerobic bottle, seven only in the anaerobic bottle, and one was positive in both bottles). Only one PPC sample was confirmed positive in both bottles (Staphylococcus hominis and Staphylococcus epidermidis). None of these samples were reactive or positive in Scansystem as well as in Pall eBDS. Only three PPCs were initially reactive with Pall eBDS. For APCs 39 samples were initially reactive by BacT/ALERT, 23 were initial positive and only three were confirmed positive. None of these three samples were reactive in Pall eBDS. An additional sample, which was negative in BacT/ALERT, was reactive in Pall eBDS. Microbiology confirmative analysis found two bacteria (Staphylococcus hominis and Staphylococcus epidermidis) in this sample. However, one APC that was contaminated with Klebsiella pneumoniae was undetected by both BacT/ALERT and Pall eBDS. Sensitivity was calculated by the number of initial positive samples (samples with confirmation of a bacterial strain) divided by the number of all positive samples. Due to the fact that the real number of positive samples was unknown (screening of unknown samples related to microbiology contaminations), relative sensitivities between all three methods were calculated. For APCs BacT/ALERT detected 13 initial positive samples (13/13; sensitivity 100%), which were failed in total by Scansystem (0/13; sensitivity 0%) and Pall eBDS (0/13; sensitivity 0%). For APCs BacT/ALERT found 23 initial positive samples and failed in one sample that were reactive by Pall eBDS. For all products sensitivity was best for BacT/ALERT (36/37 samples; 97.3%) followed by Pall eBDS (1/37 samples; 2.7%) and Scansystem (0/13 samples; 0%).

The mean time of incubation until a positive signal was obtained with BacT/ALERT differed from 20 h for Bacillus pumilus to 112.3 h for Propionibacterium acnes (Table 3) with a mean time of 36.2 h and 98.0 h for initial reactive and confirmed positive results, respectively. In 12 of the 6307 (0.19%) of PPCs and 16 of the 4730 (0.34%) of APCs, no bacteria were identified in the BacT/ALERT bottle. Neither the percentage of initially reactive samples nor the percentage of confirmed reactive samples was significantly different between PPCs and APCs (P = 0.12 for initially reactive samples; P = 0.3 for confirmed reactive samples). In comparison, both Pall eBDS and Scansystem also showed significantly less initially reactive PPCs than did BacT/ALERT (P < 0.002). Bacteria of all samples that were confirmed positive were detected in the anaerobic BacT/ALERT bottle after 55.2 h, 122.4 h, 123.4 h and 129.2 h for S. (Peptococcus) saccharolyticus and three times for P. acnes, respectively. The sample that was contaminated with S. (Peptococcus) saccharolyticus was also confirmed positive in the aerobic bottle.

Discussion

Three commercially available bacterial detection methods were compared with regard to their sensitivity, manageability and efficiency. The evaluated methods can be subdivided into culture methods (BacT/ALERT and Pall eBDS) and rapid detection methods (Scansystem). Clinical sensitivity was best for BacT/ALERT, which confirmed four samples positive, while Scansystem and Pall eBDS failed to detect 1/1 and 4/4 of the Bac/ALERT-positive samples, respectively. The negative results of the Pall eBDS system can be partially attributed to the fact that this technology focuses on the detection of aerobic bacteria. In this regard, Pall eBDS has a disadvantage compared to BacT/ALERT and Scansystem. Nevertheless, Scansystem failed to detect the confirmed positive PPC. This case may be attributed to the moderate analytical sensitivity of Scansystem, which is indicated by the manufacturer as 1000 CFU/ml, compared to 1 CFU/ml for BacT/ALERT and Pall eBDS. The long incubation time of 55.2 h for this confirmed positive sample in the BacT/ALERT suggests that the initial bacterial number in the PPC was below Scansystem’s detection limit. In the meantime, sensitivity of Scansystem was enhanced by development of an optimized Scansystem and by implementation of a quenching dye to reduce non-specific background fluorescence [20]. Additionally, and potentially more importantly, rapid detection systems like fluorescence-activated cell sorter (FACS) [21,22] or NAT [23] offer the opportunity for late
sample collection. Late sampling allows bacterial growth in contaminated blood products prior to testing and to reduce the risk for sampling errors. Therefore, late sampling potentially enhances the overall sensitivity of these techniques.

Mean time points between inoculation and identification with BacT/ALERT were significantly different between initial reactive samples (mean time points 36·2 h ± 17·3 h) and confirmed positive samples (mean time points 98·0 h ± 28·6 h; \( P < 0·01 \)). More than two-thirds of all platelets of our blood donor service, however, were released within 72 h (data not shown). These data therefore indicate the disadvantage of culture methods, where reactive results may occur after the contaminated product has already been transfused. Similar findings were also published by te Boekhorst et al. [24], Larson et al. [25] and Munksgaard et al. [26].

In this study BacT/ALERT and Pall eBDS failed to detect one bacterially contaminated APC. The APC was split into two bags and both concentrates were transfused at the end of product shelf live. The products caused severe transfusion adverse reactions in the recipients immediately after transfusion. One recipient died 10 days later because of multiple adverse reactions in the recipients immediately after transfusion. The products caused severe transfusion reactions in the recipients immediately after transfusion.

To summarize, no commercially available bacterial detection system currently appears to be perfect. The high sensitivity of BacT/ALERT is convincing as a good screening test, but testing takes up to 7 days, whereas the shelf life of platelets is limited to 5 days. Pall eBDS also has high sensitivity, but it only detects aerobic bacteria (most of the detected bacteria were anaerobic bacteria strains). Scansystem detects both aerobic and anaerobic bacteria and the testing procedure takes only 70 min. But sensitivity is lower than that of the other culture methods. A late sample collection might also enhance its overall sensitivity. Other rapid detection methods like FACS [21,22] or NAT [23] also represent alternatives to culture methods and should be tested in field trial studies.

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