Evaluation of growth based rapid microbiological methods for sterility testing of vaccines and other biological products

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A B S T R A C T

Most biological products, including vaccines, administered by the parenteral route are required to be tested for sterility at the final container and also at various stages during manufacture. The sterility testing method described in the Code of Federal Regulations (21 CFR 610.12) and the United States Pharmacopeia (USP, Chapter <71>) is based on the observation of turbidity in liquid culture media due to growth of potential contaminants. We evaluated rapid microbiological methods (RMM) based on detection of growth 1) by adenosine triphosphate (ATP) bioluminescence technology (Rapid Milliflex® Detection System [RMDS]), and 2) by CO2 monitoring technologies (Bact/Alert and the BACTEC systems), as alternate sterility methods. Microorganisms representing Gram negative, Gram positive, aerobic, anaerobic, spore forming, slow growing bacteria, yeast, and fungi were prepared in aliquots of Fluid A or a biological matrix (including inactivated influenza vaccines) to contain approximately 0.1, 1, 10 and 100 colony forming units (CFU) in an inoculum of 10 ml. These preparations were inoculated to the specific media required for the various methods: 1) fluid thiglycollate medium (FTM) and tryptic soy broth (TSB) of the compendial sterility method (both membrane filtration and direct inoculation); 2) tryptic soy agar (TSA), Sabouraud dextrose agar (SDA) and Schaedler blood agar (SBA) of the RMDS; 3) IAST and INST media of the Bact/Alert system and 4) Standard 10 Aerobic/F and Standard Anaerobic/F media of the BACTEC system. RMDS was significantly more sensitive in detecting various microorganisms at 0.1 CFU than the compendial methods (p < 0.05), whereas the compendial membrane filtration method was significantly more sensitive than the BACTEC and Bact/Alert methods (p < 0.05). RMDS detected all microorganisms significantly faster than the compendial method (p < 0.05). Bact/Alert and BACTEC methods detected most microorganisms significantly faster than the compendial method (p < 0.05), but took almost the same time to detect the slow growing microorganism P. acnes, compared to the compendial method. RMDS using SBA detected all test microorganisms in the presence of a matrix containing preservative 0.01% thimerosal, whereas the Bact/Alert and BACTEC systems did not consistently detect all the test microorganisms in the presence of 0.01% thimerosal. RMDS was compatible with inactivated influenza vaccines and aluminum phosphate or aluminum hydroxide adjuvants at up to 8 mg/ml without any interference in bioluminescence. RMDS was shown to be acceptable as an alternate sterility method taking 5 days as compared to the 14 days required of the compendial method. Isolation of microorganisms from the RMDS was accomplished by recollection of membranes with fresh SBA medium and microbial identification was confirmed using the MicroSEQ Identification System. Bact/Alert and BACTEC systems may be applicable as alternate methods to the compendial direct inoculation sterility method for products that do not contain preservatives or anti-microbial agents.

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1. Introduction

The current sterility test described in regulatory requirements [1] and Pharmacopoeia [2] has been used for many decades with only a few changes [3]. Major changes in sterility testing have included the use of anaerobic media, introduction of the membrane filtration (MF) method, and the use of isolators [3]. Though the current sterility testing method is well defined and has a good track record in ensuring sterility of biological products, there has been much discussion about its limitations, particularly with regard to the growth supporting properties of the media used in the test [4,5]. The current sterility test uses two types of liquid media, tryptic soy broth (TSB) and fluid thioglycollate medium (FTM), with incubation at 20–25°C and at 30–35°C, respectively, to detect fungal, yeast, aerobic and anaerobic bacteria [1,2]. Media incubation is required for 14 days to allow detection of slow growing microorganisms that may be in a dormant stage or injured (stressed) due to exposure to extreme environmental conditions during manufacturing (e.g. cleaning and disinfecting agents, ultraviolet light, and preservatives). Due to the shorter shelf life of certain biological products and the need to make products available rapidly during emergencies, such as pandemic outbreaks and bioterrorism attacks, an alternate sterility method that can provide faster results is highly desirable. Further, availability of a rapid sterility method would facilitate faster manufacturing decisions leading to more timely implementation of corrective and preventive actions, thereby improving product quality while reducing manufacturing time and cost.

Developments and advances in rapid microbiological methods (RMM) in the isolation and detection of microorganisms [6–10] have led to an evaluation of growth-based RMMs for sterility testing of biological products [11–16]. Appropriate evaluation and validation of RMMs is necessary to ensure suitability of these methods for their intended purpose [17–19]. Growth based RMMs rely upon the measurement of biochemical or physiological parameters that reflect growth on solid or in liquid medium, for examples, detection of adenosine triphosphate (ATP) by bioluminescence [6,11,20] and detection of CO2 production by colorimetric methods or by change in head space pressure [12,13,21–23]. The Rapid Milliflex® Detection System (RMDs) (Millipore), based on ATP bioluminescence technology, was developed for the rapid enumeration of microbial contaminants in filterable samples [6,20] and has been recently evaluated for sterility testing [15,16]. The BACTEC FX and the BacT/Alert systems, based on detection of CO2 generated due to growth of contaminants, were designed for the automated rapid detection of microorganisms in clinical specimens, [23–27]. In 2004, Genzyme received FDA approval to use the BacT/Alert system for sterility testing of the short shelf life cellular therapy product, Carticel, in order to better manage its release and use [13]. The use of BacT/Alert system has also been approved by FDA for the screening of platelets for bacterial contamination [28]. Scan RDI system based on solid-phase laser scanning cytometry (a RMM that is not based on growth) has been used to enumerate viable microorganisms in filterable products, including routine microbiological testing of pharmaceutical grade water [19,29,30]. The use of this system was also approved by FDA for sterility testing of water [19].

We evaluated three commercially available growth based RMMs, the RMDs, the BacT/Alert 3D and the BACTEC FX, for their suitability for sterility testing of biological products.

2. Materials and methods

2.1. Rapid microbiological methods (RMM)

The RMDs was from Millipore, USA and the BACTEC FX and the BacT/Alert systems, were from Becton Dickinson, USA and BioMerieux, France respectively. Media bottles used in the BACTEC and the BacT/Alert systems contain a sensor which responds to the concentration of CO2 produced by the metabolism of microorganisms. The sensor is monitored by the instruments every ten minutes for an increase in fluorescence (BACTEC system) and for color change (BacT/Alert system).

2.2. Strains

Microorganisms, representing Gram negative, Gram positive, aerobic, anaerobic, spore forming, yeast and fungi were used in the present study. These microorganisms included slow growers and those with fastidious nutritional requirements. Most of these microorganisms were from American Type Culture Collection (ATCC) and a few were environmental isolates (Table 1). This list covered microorganisms included in 21 CFR 610.12 [1] and USP [2] used for evaluating growth promotion qualities of test media used in sterility testing.

Stocks of these microorganisms were prepared in FTM and TSB, as appropriate. Microorganisms in logarithmic growth phase were harvested by centrifugation; cells were suspended in 10% skimmed milk, distributed in aliquots and stored at −80°C. Viable counts of these stocks were determined by standard plate count method on tryptic soy agar (TSA) in multiple tests (at least 3 tests) to determine average CFU used for preparation of different concentrations to evaluate the RMMs.

Table 1

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Strain ID</th>
<th>Anaerobic/aerobic</th>
<th>Gram Stain</th>
<th>CFR/USP</th>
<th>Incubation temperature°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptococcus pyogenes</td>
<td>ATCC 5174</td>
<td>aerobic</td>
<td>Gram-positive cocci</td>
<td>–</td>
<td>30–35</td>
</tr>
<tr>
<td>Micrococcus luteus/Kocuria rhizophila</td>
<td>ATCC 9341</td>
<td>aerobic</td>
<td>Gram-positive cocci</td>
<td>CFR</td>
<td>30–35</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>ATCC 12600</td>
<td>aerobic</td>
<td>Gram-positive cocci</td>
<td>USP</td>
<td>30–35</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>ATCC 9027</td>
<td>aerobic</td>
<td>Gram-negative rods</td>
<td>USP</td>
<td>30–35</td>
</tr>
<tr>
<td>Acinetobacter lwoffii</td>
<td>ATCC 17925</td>
<td>aerobic</td>
<td>Gram-negative rods</td>
<td>–</td>
<td>30–35</td>
</tr>
<tr>
<td>Clostridium sporogenes</td>
<td>ATCC 11437</td>
<td>anaerobic</td>
<td>Gram-positive rods</td>
<td>CFR/USP</td>
<td>30–35</td>
</tr>
<tr>
<td>Bacteroides vulgatus</td>
<td>ATCC 8482</td>
<td>anaerobic</td>
<td>Gram-negative rods</td>
<td>CFR</td>
<td>30–35</td>
</tr>
<tr>
<td>Candida parapsilosis</td>
<td>ATCC 22019</td>
<td>aerobic</td>
<td>Yeast</td>
<td>–</td>
<td>20–25</td>
</tr>
<tr>
<td>Candida albicans</td>
<td>ATCC 10231</td>
<td>aerobic</td>
<td>Yeast</td>
<td>CFR/USP</td>
<td>20–25</td>
</tr>
<tr>
<td>Aspergillus fumigatus</td>
<td>ATCC 1022</td>
<td>aerobic</td>
<td>Fungi</td>
<td>–</td>
<td>20–25</td>
</tr>
<tr>
<td>Aspergillus niger/Aspergillus brasiliensis</td>
<td>ATCC 16404</td>
<td>aerobic</td>
<td>Fungi</td>
<td>USP</td>
<td>20–25</td>
</tr>
<tr>
<td>Streptomyces halstedii</td>
<td>ATCC 10897</td>
<td>aerobic</td>
<td>Filamentous bacteria</td>
<td>–</td>
<td>30–35</td>
</tr>
<tr>
<td>Propionibacterium acnes</td>
<td>Environmental isolates</td>
<td>anaerobic</td>
<td>Gram-positive rods</td>
<td>CFR/USP</td>
<td>30–35</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>Environmental isolates</td>
<td>aerobic</td>
<td>Gram-positive rods</td>
<td>CFR/USP</td>
<td>30–35</td>
</tr>
</tbody>
</table>

* Temperature used to grow the microorganisms for preparation of stock culture.

* Both the old and currently accepted names for these microorganisms have been used throughout the manuscript.
2.3. Culture media and reagents

Sabouraud dextrose agar (SDA), TSA and 9–20 kGy gamma irradiated Schaedler blood agar (SBA) media filled cassettes for the RMDS were from Millipore, USA. FTM and TSB used for the compendial sterility test method and Fluid A were from commercial media suppliers. Standard 10 Aerobic/F and Standard Anaerobic/F media used for aerobic and anaerobic microorganisms, respectively, for the BACTEC FX system were from Becton Dickinson, USA and the iAST and iINST media used for aerobic and anaerobic microorganisms, respectively, for the BacT/Alert were from BioMerieux, France. Media used in the BACTEC and BacT/Alert systems are manufacturer’s propriety formulations developed for the isolation of microorganisms from clinical samples.

2.4. Matrices

For most of the studies, Fluid A (0.1% peptone) with and without 0.01% thimerosal (w/v) was used as a matrix for preparation of different concentrations of microorganisms. Inactivated influenza vaccine (both monovalent bulk and trivalent formulated bulk) with 0.01% thimerosal was also used as a matrix for evaluation of the alternate rapid sterility methods. Aluminum phosphate and aluminum hydroxide, each at 3–8 mg/ml, were evaluated to assess the compatibility of these adjuvants with the filtration procedure and ability to detect bioluminescence as used in the RMDS.

2.5. Preparation of dilutions of microorganisms

Serial 10-fold dilutions of stock cultures were prepared in Fluid A with and without 0.01% thimerosal or inactivated influenza vaccine containing 0.01% thimerosal to obtain microorganism concentrations at 0.1, 1, 10 and 100 CFU in 10 ml. These dilutions were based on viable count of stock cultures determined at the time of stock preparation. Back titrations of diluted microorganisms were performed in TSA to confirm viable counts used in the experiments. If count differences between stock culture and back titrations were more than two fold, results were expressed as CFU obtained from back titrations.

2.6. Compendial sterility testing

The sterility testing method was performed by both direct inoculation (DI) and MF with 10 ml inoculum for each TSB and FTM medium, as described in the CFR [1] and USP [2] for the bulk samples. A Steritest™ compact pump system from Millipore was used for the MF method. Media were observed daily for 14 days. If the culture medium was turbid by visual inspection, a sample was taken for contaminant identification by MicroSEQ ID as described in Section 2.10.

2.7. Rapid Milliflex Detection System (RMDS)

Initially, RMDS was evaluated as a hybrid method where the test was performed as per the compendial sterility method by inoculation of different concentrations of microorganisms (0.1–100 CFU per 10 ml) into TSB and FTM. This procedure met all requirements for the compendial sterility method; however, detection of growth was also evaluated using the RMDS in addition to a visual observation of turbidity. Media bottles not showing turbidity were tested for growth by the RMDS daily until growth was detected by RMDS. Detection of growth by RMDS was performed as per the manufacturer’s instructions and as previously described [15,16]. Briefly, a 1 ml sample from each inoculated media bottle was aseptically filtered through the 0.45 μm membrane using the Milliflex funnel, and followed by rinsing with 100 ml Fluid A. The membrane was then separated from the funnel base and dried aseptically in a laminar flow hood. After drying, ATP-releasing reagent and bioluminescence reagents were sprayed on the membrane using an RMDS-AutoSpray station. The processed membrane was analyzed in the RMDS detection tower. ATP from viable microorganisms reacts with the luciferase enzyme in the bioluminescence reagents to produce light. The photons generated by the ATP bioluminescence reaction were captured by a charged coupled device (CCD) camera, and the computer processed picture was analyzed. Results in CFU were displayed on the monitor by the integrated software. For the hybrid method, results were evaluated as qualitative (positive or negative), not quantitative as CFU.

In the standard RMDS method, multiple 10 ml matrix samples (Fluid A or inactivated influenza vaccine) inoculated with different concentrations of microorganisms (0.1–100 CFU per 10 ml) were evaluated. Each sample was aseptically filtered through 0.45 μm membrane, followed by filtration of 100 ml of fluid A, and the membrane was separated from the funnel base. Multiple membranes processed from each sample were incubated at 30–35 °C aerobically and anaerobically on SBA and TSA media separately; and at 20–25 °C aerobically on SBA and SDA. After incubation for 1–5 days, membranes were separated from solid media, dried and sprayed with ATP-releasing and bioluminescence reagents, as described above. For this method, quantitative CFU results reflected the number of microorganisms in the sample. Detection of 1 CFU was taken as a positive sterility test. If a microorganism showed visible colonies on the first day of incubation on solid media, the membrane was not processed for RMDS. Multiple sets of membranes were prepared with the same concentration of microorganism so that each set could be evaluated for growth by RMDS daily, from the first till the 5th day or until visible growth was detected.

Appropriate negative controls were included using matrices without microorganisms to evaluate background reaction in the RMDS.

Membranes showing positive growth by standard RMDS were placed on fresh media (same medium type that was used for initial detection of growth) to isolate the inoculated microorganisms. This was performed carefully to avoid air bubbles between the membrane and the medium; and the procedure was performed in a laminar flow hood to avoid any secondary contamination of the membranes. The incubation was performed under the same conditions as described above until visible growth of the microorganism was observed. Identification of visible growth was performed using the MicroSEQ ID System as described in Section 2.10.

Aluminum phosphate and aluminum hydroxide adjuvants at 3–8 mg/ml were also evaluated by standard RMDS for their compatibility with the filter, and for their potential impact on the bioluminescence background.

2.8. BACTEC and BacT/Alert systems

The proprietary media bottles used in BACTEC and BacT/Alert system contain 50 and 40 ml media, respectively. For these 2 systems, the only available option for sterility testing was the DI method, which was performed by inoculating 10 ml (in Fluid A or inactivated influenza vaccine matrix) containing different concentrations of microorganisms (0.1–100 CFU per 10 ml). Media bottles with bar codes and processed through the respective systems were incubated at 33 °C for 14 days. These systems monitor media bottles for growth every 10 min. The instrument gives an audible alarm when a positive growth is detected and records the time required to detect growth. Identification of microorganisms was performed by MicroSEQ ID System as described in Section 2.10.
Table 2
Sensitivity of rapid microbiological methods in comparison to the membrane filtration (MF) and direct inoculation (DI) methods with regard to observation of growth at various levels of inoculations. Data were evaluated statistically by the Chi square analysis.

<table>
<thead>
<tr>
<th>Methods</th>
<th>Number of positive experiments/total experiments performed (% positive experiments)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 CFU</td>
</tr>
<tr>
<td>Compendial Membrane Filtration Method</td>
<td>134/138 (97)</td>
</tr>
<tr>
<td>Direct Inoculation Method</td>
<td>53/56 (94)</td>
</tr>
<tr>
<td>Rapid Milliflex Detection System (TSA/SDA)</td>
<td>75/77 (97)</td>
</tr>
<tr>
<td>Rapid Milliflex Detection System (SDA)</td>
<td>40/40 (100)</td>
</tr>
<tr>
<td>BACTEC</td>
<td>74/77 (96)</td>
</tr>
<tr>
<td>BacT/Alert</td>
<td>89/95 (94)</td>
</tr>
<tr>
<td></td>
<td>1 CFU</td>
</tr>
<tr>
<td>Compendial Membrane Filtration Method</td>
<td>101/138 (73)</td>
</tr>
<tr>
<td>Direct Inoculation Method</td>
<td>36/56 (64)</td>
</tr>
<tr>
<td>Rapid Milliflex Detection System (TSA/SDA)</td>
<td>66/77 (86)</td>
</tr>
<tr>
<td>Rapid Milliflex Detection System (SDA)</td>
<td>39/40 (97)</td>
</tr>
<tr>
<td>BACTEC</td>
<td>38/77 (49)</td>
</tr>
<tr>
<td>BacT/Alert</td>
<td>47/97 (49)</td>
</tr>
<tr>
<td></td>
<td>0.1 CFU</td>
</tr>
<tr>
<td>Compendial Membrane Filtration Method</td>
<td>20/89 (22)</td>
</tr>
<tr>
<td>Direct Inoculation Method</td>
<td>10/50 (20)</td>
</tr>
<tr>
<td>Rapid Milliflex Detection System (TSA/SDA)</td>
<td>21/70 (30)</td>
</tr>
<tr>
<td>Rapid Milliflex Detection System (SDA)</td>
<td>29/33 (87)</td>
</tr>
<tr>
<td>BACTEC</td>
<td>12/57 (21)</td>
</tr>
<tr>
<td>BacT/Alert</td>
<td>10/56 (18)</td>
</tr>
</tbody>
</table>

* a Significantly more sensitive from Compendial membrane filtration method p < 0.05.
* b Significantly less sensitive from Compendial membrane filtration method p < 0.05.

2.9. Experimental design

For each microorganism, several experiments were performed by various methods. The compendial sterility method (DI and/or MF) was always performed in parallel as control. RMDS, BACTEC and BacT/Alert systems were evaluated simultaneously along with the compendial method. The same dilutions of microorganisms were used to evaluate multiple systems to minimize test to test variability.

2.10. Identification by MicroSEQ ID

The MicroSEQ ID 3130 four capillary Genetic Analyzer (Applied Biosystems, USA) based on 16s rRNA was used for identification of microorganisms from positive growth. DNA from the positive culture was extracted and amplified with a FAST MicroSEQ 16s rDNA PCR kit for bacteria, and a FAST MicroSEQ D2 LSU rDNA fungal PCR kit for fungi, and yeast using GeneAmp 9700 PCR System (Applied Biosystems, USA). Amplified PCR product was cleaned to remove excess primers and nucleotides using ExoSAP (USB, Affymetrix, USA). Sequencing cycle was performed using MicroSEQ 500 bacterial and fungal sequencing kit. After removal of excess dyes, the labeled amplicon was placed on a 3130 four capillary Genetic Analyzer (Applied Biosystems, USA). Sequence analyses were performed on MicroSEQ ID software to confirm species identification.

2.11. Statistical analysis

A Chi-Square test was performed to evaluate the sensitivity between the various methods by analyzing the number of positive growth cultures detected by the compendial method compared to those detected by the RMMS at each concentration of microorganisms. An Analysis of Variance (ANOVA) was performed to assess the compendial sterility test and the RMMS with regards to the difference in time required to detect growth after inoculating with 1 and 10 CFU preparations. All statistical analyses were performed using Minitab® Software. RMDS was statistically evaluated against MF method only, because MF method is considered superior to the DI method.

3. Results

Experiments with inoculations at 100 CFU showed growth for all systems evaluated in this study; therefore, data with 100 CFU inoculations are not presented. There were differences in the time required by various systems to detect growth at the 100 CFU inoculation level, particularly for slow growing microorganisms. Similar time differences were also reflected in the lower inoculum at 10, 1 or 0.1 CFU. These results are described below (Section 3.3).

3.1. Hybrid Rapid Milliflex Detection System

Most microorganisms, particularly at the 10 CFU inoculation level and higher, showed growth by the compendial method within 48 h and a few slow growing microorganisms showed growth in 96–240 h (data not shown). In a few cases, the RMDS detected growth a day earlier than the compendial method. Culture bottles showing growth within 24 h of the last negative RMDS test were not further tested by the RMDS. In such cases, the RMDS did not provide any advantage in detecting growth. In many instances the RMDS could not detect growth on a particular day when the culture bottles became visually turbid within 24 h. Slow growing microorganisms, B. vulgatus, P. acnes and C. albicans, particularly at inoculum of 1 CFU or lower, took 6–11 days for growth to be detected by turbidity (data not shown). In most of these instances, the RMDS did not detect growth earlier. Occasionally the hybrid RMDS presented technical problems with background noise, showing a positive signal in negative controls (media without microorganisms), which made it difficult to distinguish such false positive signals from low inoculation of microorganisms (0.1 and 1 CFU). Due to these observations and not being significantly faster than the compendial method, hybrid RMDS was not pursued further.

3.2. Limit of detection (sensitivity)

Table 2 shows the total number of cultures showing growth by various methods when inoculated with 10, 1 and 0.1 CFUs of the various microorganisms. Inoculations with the 10 CFU microorganisms showed growth for more than 90% of the cultures by all methods, where the RMDS using SBA medium had growth in 100% of the cultures and BacT/Alert and DI method had 94% of the cultures exhibiting growth. The RMDS using TSA/SDA and SBA media showed the highest rate of cultures with growth at both 1 and 0.1 CFU inoculation levels and showed significantly higher sensitivity than the MF and DI methods (p < 0.05). At the 0.1 CFU inoculation level, the RMDS with SBA medium detected most microorganisms, showing significantly higher sensitivity than the compendial method (p < 0.05) (Table 2). The compendial MF method had a significantly higher sensitivity than the BACTEC and BacT/Alert methods at 1 CFU inoculation (p < 0.01). At 0.1 CFU inoculation, the compendial MF and DI methods, BACTEC and BacT/Alert methods displayed similar sensitivity with approximately 20% of cultures showing growth.

3.3. Time required to detect growth

Table 3 and Figs. 1 and 2 compare the time required to detect the presence of the various microorganisms by the compendial method and the RMMS evaluated in this study. In general, time required to detect growth by a particular microorganism was shortest for the 10 CFU inoculum. Data obtained from the 0.1 CFU inoculations were not analyzed statistically for time required to detect growth.
Table 3  
Time required to detect growth by membrane filtration (MF), direct inoculation (DI) and rapid microbiological methods when evaluated at 10 and 1 CFU inoculations in fluid A. Data have been presented as mean ± standard deviation of time (h) required to detect growth in a number of experiments. Data were statistically analyzed by Analysis of Variance with regard to significant differences in time required to detect growth by various methods.

<table>
<thead>
<tr>
<th>Organisms</th>
<th>CFU in 10 ml Fluid A</th>
<th>Time required to detect growth (h)† (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Membrane filtration</td>
<td>Direct inoculation</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. aureus</td>
<td>10</td>
<td>32.7 ± 12.1</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>54.5 ± 30.5</td>
</tr>
<tr>
<td>S. pyogenes</td>
<td>10</td>
<td>24 ± 0</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>52.6 ± 28</td>
</tr>
<tr>
<td>S. halstedii</td>
<td>10</td>
<td>86.4 ± 40.1</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>96 ± 0</td>
</tr>
<tr>
<td>B. vulgatus</td>
<td>10</td>
<td>112 ± 27.7</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>148 ± 38.4</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>10</td>
<td>48 ± 24</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>62.4 ± 13.1</td>
</tr>
<tr>
<td>P. acnes</td>
<td>10</td>
<td>113.6 ± 26.8</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>141.6 ± 44.4</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>10</td>
<td>34.3 ± 12.8</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>65.1 ± 35.1</td>
</tr>
<tr>
<td>C. sporogenes</td>
<td>10</td>
<td>24 ± 0</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>33 ± 12.4</td>
</tr>
<tr>
<td>A. niger/A. brasiliensis</td>
<td>10</td>
<td>68.4 ± 16.2</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>102 ± 30.1</td>
</tr>
<tr>
<td>M. luteus/K. rhizophila</td>
<td>10</td>
<td>82.7 ± 55.1</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>62.4 ± 21.4</td>
</tr>
<tr>
<td>C. albicans</td>
<td>10</td>
<td>54.5 ± 18.6</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>78.0 ± 30.9</td>
</tr>
</tbody>
</table>

† Data from experiments with no growth at the end of the test were not included.
‡ Significantly faster in detection of growth than the membrane filtration method *p < 0.05;  
§ Significantly faster in detection of growth than the direct inoculation method *p < 0.05

because approximately 80% of experiments did not show growth at this level of inoculum (Table 2). The RMDS using solid media, TSA, SDA and SBA detected growth significantly faster (*p < 0.05) than the MF method for all the microorganisms. All microorganisms were detected by the RMDS within 5 days, whereas the compendial methods took up to 11 days to detect slow growing microorganisms, such as P. acnes and B. vulgatus (Figs. 1 and 2). BACTEC and BacT/Alert systems exhibited a significantly shorter time to detect growth at the 10 and 1 CFU inoculations (Table 3) when compared to the MF and DI methods for most microorganisms that showed growth within 72 h. There was no significant difference in time required to detect growth between the compendial method and BACTEC and BacT/Alert methods for slow growing microorganisms, such as P. acnes (*p > 0.05). Interestingly, BACTEC method was the fastest among all methods in detecting B. vulgatus with a mean detection time of approximately 25 h, whereas RMDS with SBA took 72–84 h to detect this microorganism. The maximum time duration to detect growth of all microorganisms by BACTEC and BacT/Alert methods was 6 days (Fig. 2).

Automatic notification of growth by generation of CO₂ was not recorded for the BACTEC and BacT/Alert systems for A. niger/A. brasiliensis even though fungal mycelial balls were visually observed in the media bottles.

### 3.4. Detection of microorganisms in matrix with preservative thimerosal

Table 4 and Figs. 3 and 4 show the time required to detect various microorganisms inoculated into a matrix containing 0.01% thimerosal. The MF method and the RMDS using SBA medium were the only methods that could detect all microorganisms at 10 and 1 CFU inoculations in the presence of 0.01% thimerosal. The RMDS with TSA/SDA media did not detect S. aureus, P. acnes and M. luteus/K. rhizophila at 10 and 1 CFU inoculations, and several more microorganisms were detected at 10 CFU but not detected at the 1 CFU inoculation in the presence of 0.01% thimerosal. The DI method also did not detect a number of microorganisms in the presence of 0.01% thimerosal at 10 CFU inoculation. The BACTEC detected S. aureus and anaerobic microorganisms, P. acnes, B. vulgatus and C. sporogenes in the presence of 0.01% thimerosal at both 1 and 10 CFU inoculations, whereas BacT/Alert detected S. aureus and C. sporogenes at 10 CFU inoculation. Microorganisms that could not be detected by the BACTEC and BacT/Alert methods, as shown in Table 4, were also not detected at 1000 CFU inoculation in the presence of 0.01% thimerosal (data not shown). The RMDS using SBA medium detected growth of inoculated microorganisms faster than the other methods in a matrix containing 0.01% thimerosal, with a statistically significant shorter time required to detect growth as compared to the MF method (*p < 0.05).

The RMDS was found to be compatible with inactivated influenza vaccine and aluminum phosphate or aluminum hydroxide adjuvants at up to 8 mg/ml and did not show any background interference with these matrices.

### 3.5. Isolation and identification of microorganisms

Inoculated microorganisms could not be isolated from the RMDS method when using TSA and SDA media (Fig. 5). However, the RMDS using SBA medium consistently allowed isolation of the inoculated microorganisms when the RMDS processed membranes were re-incubated with fresh SBA medium (Fig. 5). In all the
methods used in this study, identity of the inoculated microorganisms after recovery was confirmed by MicroSEQ ID.

4. Discussion

Most biological products cannot be terminally sterilized. These products are manufactured aseptically and tested for sterility at various stages of manufacture. Sterility testing along with aseptic process validation, environmental monitoring and use of sterilized equipment and reagents, provides assurance that biological products meet sterility requirements. Despite the limitation of the test in terms of demonstrating absolute product sterility [3,31–33], the current compendial method has served well in assuring sterility of biological products. With the shorter shelf life of certain biological products and an urgent need for quicker availability of products during times of emergency, such as pandemic influenza outbreaks and bioterrorism attacks, a sterility method that can provide faster results with the same degree of sterility assurance as the current compendial method is highly desirable.

This study shows that RMMs based on ATP bioluminescence technology and the detection of CO2 as a measure of growth could be used as rapid sterility methods for biological products. Recently the RMDS has been used in detection of microbial contaminants, including bacteria, yeast and fungi, for testing water, and the method had a sensitivity of detecting a single cell in the sample [11]. Gray et al. also found the RMDS with SBA medium a suitable method for sterility testing [15]. The use of BacT/Alert system has been approved for sterility testing of a cell therapy product [13]. The RMDS detected low concentrations of microorganisms in a significantly shorter period of time than the compendial method (Tables 2 and 3). The superiority of the RMDS can be attributed to the use of solid media in contrast to liquid media used in the compendial method. In general, solid media detected lower concentration of microorganisms in a shorter period compared to liquid media (At 0.1 CFU inoculations SBA and TSA/SDA had
approximately 87 and 30% positive cultures versus approximately 20% of positive cultures with each of FTM/TSB and the proprietary media used for the BACTEC and BacT/Alert systems (Table 2). This was also observed from experiments performed with the hybrid-RMDS (data not shown). Slow growing microorganisms and those exposed to matrix containing 0.01% thimerosal took a longer time to start growing in liquid media than on solid media. Growth promotion properties of FTM, a liquid medium used in the compendial sterility test, have been questioned for more than 30 years [4,5]. In 1973, Clausen reported that solid media, 5% blood agar and TSA have advantages over fluid media – dithionite-thioglycollate broth and soyabean caseine digest broth [4].

Theoretically, inoculations with the 0.1 CFU should show growth in approximately 10% of the cultures, when the same media are used for CFU titrations and for detection of growth. However, it has been reported earlier that membrane filtered microorganisms show higher counts than those by pour plate method [34,35]. We have additional explanations for obtaining consistent higher recovery at 0.1 CFU inoculations in our experiments. CFU values depend upon the media used for counting viable microorganisms. In the present study, TSA was used for determining the CFU concentration of stock cultures and for verification of the counts by the back titrations. Further, use of different dilutions for CFU titrations and for detection of growth may result in a discrepancy between expected positive cultures and those found. For example, CFU titrations are

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**Fig. 2.** Time required (h) to detect inoculated microorganisms by direct inoculation (DI) method (♦) and BACTEC FX system using Standard 10 Aerobic/F and Standard Anaerobic/F media for aerobic and anaerobic microorganisms, respectively (♦), and BacT/Alert system using iAST and INST media for aerobic and anaerobic microorganisms, respectively (●). Inoculations of various microorganisms at 1 CFU (A) and 0.1 CFU (B) were prepared in fluid A. Numbers adjacent to certain symbols represent number of experiments showing detection of a microorganism at same time point. Microorganisms not showing growth in 336 h (14 days) are shown as NG (No Growth) on right side of the graph. No symbol at NG column for a microorganism means all experiments showed positive growths at indicated time points.
Table 4
Time required to detect growth by membrane filtration (MF), direct inoculation (DI) and rapid microbiological methods when evaluated at 10 and 1 CFU inoculation in inactivated influenza vaccine and Fluid A, both with 0.01% thimerosal. Data have been presented as mean ± standard deviation of time (h) required to detect growth in a number of experiments. Data were statistically analyzed by Analysis of Variance with regard to significant differences in time required to detect growth by various methods.

<table>
<thead>
<tr>
<th>Organisms</th>
<th>CFU in 10 ml matrix</th>
<th>Time to detection growth (h)(^a) (mean ± SD)</th>
<th>CFR/USP method</th>
<th>Rapid microbiological methods</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Membrane filtration</td>
<td>Direct inoculation</td>
<td>Rapid Milliflex (TSA/SDA)</td>
<td>Rapid Milliflex (SBA)</td>
</tr>
<tr>
<td><strong>S. aureus</strong></td>
<td>10</td>
<td>48 ± 24.7</td>
<td>48 ± 0</td>
<td>NG</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>64 ± 27.7</td>
<td>48 ± 33.9</td>
<td>NG</td>
</tr>
<tr>
<td><strong>S. pyogenes</strong></td>
<td>10</td>
<td>28 ± 9.7</td>
<td>64 ± 13.8</td>
<td>24 ± 0</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>67.2 ± 31.2</td>
<td>176 ± 108.2</td>
<td>36 ± 16.9</td>
</tr>
<tr>
<td><strong>S. halstedii</strong></td>
<td>10</td>
<td>96 ± 0</td>
<td>NG</td>
<td>48 ± 0</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>96 ± 0</td>
<td>NG</td>
<td>64 ± 27.7</td>
</tr>
<tr>
<td><strong>B. vulgatus</strong></td>
<td>10</td>
<td>156 ± 16.9</td>
<td>NG</td>
<td>72 ± 0(^a)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>192 ± 0</td>
<td>NG</td>
<td>88 ± 27.7(^a)</td>
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<tr>
<td><strong>B. subtilis</strong></td>
<td>10</td>
<td>75 ± 5.20</td>
<td>24 ± 0</td>
<td>24 ± 0(^a)</td>
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<tr>
<td></td>
<td>1</td>
<td>116 ± 48.9</td>
<td>82.5 ± 22.9</td>
<td>24 ± 0(^a)</td>
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<tr>
<td><strong>P. aeruginosa</strong></td>
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<td>120 ± 25.2</td>
<td>NG</td>
<td>80 ± 13.8(^a)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>154.2 ± 33.0</td>
<td>NG</td>
<td>96 ± 13.1(^a)</td>
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<tr>
<td><strong>P. acnes</strong></td>
<td>10</td>
<td>45.3 ± 28.7</td>
<td>72 ± 33.9</td>
<td>28.8 ± 10.7</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>48 ± 12.8</td>
<td>192 ± 101.8</td>
<td>72 ± 0</td>
</tr>
<tr>
<td><strong>C. sporogenes</strong></td>
<td>10</td>
<td>36 ± 12.6</td>
<td>72 ± 0</td>
<td>48 ± 24</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>432 ± 9.7</td>
<td>72 ± 0</td>
<td>48 ± 3.9</td>
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<tr>
<td><strong>A. niger/A. brasiliensis</strong></td>
<td>10</td>
<td>144 ± 0</td>
<td>NG</td>
<td>144 ± 0</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>144 ± 0</td>
<td>NG</td>
<td>32 ± 13.8(^a)</td>
</tr>
<tr>
<td><strong>M. luteus/K. rhizophila</strong></td>
<td>10</td>
<td>68.5 ± 25.6</td>
<td>168 ± 0</td>
<td>NG</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>113.1 ± 30.8</td>
<td>NG</td>
<td>16 ± 0(^a)</td>
</tr>
<tr>
<td><strong>Calbicans</strong></td>
<td>10</td>
<td>96 ± 0</td>
<td>NG</td>
<td>48 ± 41.5(^a)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>96 ± 0</td>
<td>NG</td>
<td>40 ± 27.2(^a)</td>
</tr>
</tbody>
</table>

\(^a\) Data from experiments with no growth at the end of the test were not included.

\(^b\) Significantly faster in detection of growth than the membrane filtration method \(p < 0.05\).

\(^c\) Significantly faster in detection of growth than the direct inoculation method \(p < 0.05\); NG: No Growth.

Usually performed by inoculating 0.1 ml of the various dilutions containing approximately 300–1000 CFU/ml (pour plate method or spread method) to obtain 30–100 colonies on a media plate. In this study, dilution of 1000 CFU/ml was used for the back titration from the set of dilutions prepared for evaluation of growth characteristics of RMMs. In contrast, a dilution of 0.01 CFU/ml was used for evaluating growth characteristics of various RMMs at 0.1 CFU (in 10 ml) concentration. There is a possibility that microorganisms may form clumps at 1000 CFU/ml dilution compared to a 0.01 CFU/ml dilution. Microorganisms may tend to get dispersed when diluted to <1 CFU concentrations. Therefore, with different media and different dilutions of cultures used for CFU titrations and growth detection experiments, there is a possibility of having growth in more cultures than expected from theoretical counts. We further verified that the CFU counts were higher on SBA medium than those on TSA medium (unpublished data). This supports our observations of significantly higher detection at 0.1 CFU inoculation in SBA than in TSA and SDA. This may also be due to detection of injured or dormant microorganisms by SBA, as discussed below.

Among solid media evaluated in this study, SBA medium was found to be superior to TSA and SDA media in its ability to grow microorganisms in the presence of 0.01% thimerosal (a common preservative in vaccines) (Table 4), and also for its support of growth of those microorganisms that were exposed to ATP bioluminescence reagents during the RMDS procedure (Fig. 5). These findings are similar to those reported by Gray et al. [15,16]. Initially, we evaluated the RMDS using TSA and SDA media and were not able to isolate the inoculated microorganisms as the reagents used for ATP bioluminescence procedure ruptures the microbial cells to release ATP [15]. Isolation and identification of contaminants are important aspects of sterility testing and biological manufacturing operations. In order to maintain this feature, we evaluated SBA medium in the RMDS system despite concerns of sterility testing being performed on a medium containing blood, which cannot be claimed sterile. The SBA medium in this study was, therefore, gamma irradiated to assure its sterility and also to reduce bioluminescence background from the blood cells. Presence of blood, yeast extract and other supplements in the SBA medium has been considered important in growth of fastidious or injured microorganisms, particularly anaerobic microorganisms, as compared to TSA/SDA media [36].

An important aspect in sterility testing is evaluating the effect of product on the growth of potential contaminants, commonly referred as the bacteriostasis and fungistasis (B & F) testing or suitability testing [2]. In the USP suitability test for the MF method, the product is filtered through the membrane, followed by filtration of the challenge microorganisms in Fluid A with final rinsing of membrane with Fluid A. In this test, the microorganisms do not come in direct contact with the product. In contrast, all our experiments were performed by first mixing various concentrations of the microorganisms with the product, followed by filtration. Results from our study showed that the RMDS using TSA/SDA media detected growth of all inoculated microorganisms in the presence of 0.01% thimerosal when testing was performed by the USP suitability test (data not shown), whereas this procedure did not show growth for many microorganisms when these were mixed with a matrix containing 0.01% thimerosal (Fig. 2, Table 4). In contrast, RMDS with SBA medium detected growth of all microorganisms both from the USP suitability test (data not shown) and in the
experiments from this study (Fig. 2 and Table 4). Our experimental design mimics the situation in manufacturing operations where microorganisms are present as contaminants in the matrix. We are further evaluating the efficacy of the B & F evaluation as required by the USP method.

The BACTEC and BacT/Alert systems, which are similar to the DI method, use 33 °C incubation for detection of all microorganisms, including yeast and fungi, whereas the DI method requires incubation at 20–25 °C for detection of yeast and fungi. In this study, the BACTEC and BacT/Alert systems detected yeast and fungi representatives in a significantly shorter time than the DI method (Table 3). In previous studies, incubation temperature of 32 °C was found optimum for the growth of yeast and molds for both BACTEC and BacT/Alert systems [12,13]. The BACTEC and BacT/Alert methods showed significantly faster growth rate of microorganisms as compared to the MF and DI methods (Table 3), except for P. acnes where the time required to detect growth was almost the same for the BACTEC, BacT/Alert and the compendial methods. In an earlier study [12], P. acnes was not detected by the BacT/Alert system, and was detected by the BACTEC system in almost the same time as the compendial method. In another study [13], P. acnes took significantly longer time to show growth in the BacT/Alert system as compared to the MF method (p < 0.0001). In the presence of matrix containing 0.01% thimerosal, the BacT/Alert system did not detect most of the microorganisms, and the BACTEC was very efficient in detecting anaerobic microorganisms, B. vulgatus, P. acnes and C. sporogenes. In a previous study, M. luteus/K. rhizophila, a strict aerobe, showed inconsistent growth in both BACTEC and BacT/Alert systems, and no growth in the presence of antibiotics [12].

Based on these observations, it is evident that BACTEC and BacT/Alert systems could be used as alternate sterility methods to the DI method, particularly for products without preservatives or anti-microbial agents. Further modifications to these systems for use with MF and changes in amount of media may be helpful to
Fig. 4. Time required (h) to detect spiked microorganisms by direct inoculation (DI) method (♦) and BACTEC FX system using Standard 10 Aerobic/F and Standard Anaerobic/F media for aerobic and anaerobic microorganisms, respectively (▲). Inoculations of various microorganisms at 1 CFU (A) and 0.1 CFU (B) were prepared in a matrix containing 0.01% thimerosal and inactivated influenza vaccine with 0.01% thimerosal. Numbers adjacent to certain symbols represent number of experiments showing detection of a microorganism at same time point. Microorganisms not showing growth in 336 h (14 days) are shown as NG (No Growth) on right side of the graph. No symbol at NG column for a microorganism means all experiments showed positive growths at indicated time points.

expand the use of these systems for sterility testing purposes. Due to the absence of automatic growth signal based on CO₂ detection by the BACTEC and BacT/Alert systems for A. niger/A. brasiliensis, use of these systems for sterility method will require visual observation of media bottles on the last day of the test.

According to 21 CFR 610.9 [37], equivalent methods and processes in the manufacture of biological products can be used after demonstrating that these provide assurances of safety, purity, potency and effectiveness of biological products equal to or greater than the assurances provided by the current approved methods and processes. As per the compendial method, tests for growth promotion properties of media used in the sterility test and the B & F evaluation require ≤100 CFU inoculum of specified microorganisms [1,2]. A similar approach has been used to evaluate equivalency of an alternate sterility method by taking 10 to ≤100 CFU inoculum of specific microorganisms [12], probably due to a perception that the compendial sterility method is not very sensitive in detection of these microorganisms. Our study is one of the few studies that have systematically evaluated the sensitivity of the MF and DI methods and the results demonstrate that the compendial
sterility method detected all microorganisms used in this study with a similar degree of assurance as expected from the concentrations prepared for inoculation (Table 2). For example, approximately 100, 65 and 20% positive cultures at 10, 1 and 0.1 CFU inoculums, respectively. Therefore, we suggest that the future evaluation of alternate sterility methods should include inoculations with less than 1 CFU to demonstrate equivalency or better sensitivity of the alternate method. The USP also suggests use of as low as 0.05 CFU for the evaluation of RMMs [38].

In conclusion, the RMDs utilizing SBA medium appears to be a promising rapid alternate method to the compendial sterility method having the advantage of taking only 5 days for sterility testing of biological products, including inactivated influenza vaccines. This could accelerate the availability of biological products in a pandemic or during a bioterrorism attack. The RMDs can only be used for filterable products. The RMDs methodology used in this study consisted of 3 sets of inoculations to detect all possible contaminants (one each for aerobic and anaerobic incubations at 30–35 °C and an additional aerobic incubation for yeast and molds at 20–25 °C). This is consistent with the method used by Gray et al. [15], who found that incubation at 20–25 °C was necessary to detect all contaminants. Therefore, the RMDs procedure requires more sample compared to the MF method, where only 2 sets of inoculations are required (FTM at 30–35 °C for aerobic and anaerobic microorganisms and TSB at 20–25 °C for yeast and molds). For products without preservatives or anti-microbial ingredients that can only be tested by DI, the BACTEC and BacT/Alert systems may be suitable alternate methods. Suitability of all potential rapid sterility methods must be evaluated for each product using specific microorganisms, including those described in the compendia [1,2] and from those representing appropriate environmental isolates.

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References


Fig. 5. Detection and isolation of growth by Rapid Milliflex Detection System (RMDS) at 100 CFU (A, A1, B and B1) and 10 CFU (C, C1, D and D1) inoculations of P. acnes in the presence of inactivated influenza vaccine using SBA (top panels) and TSA (bottom panels) media. For isolation and identification of contaminant, membranes processed for detection of growth by RMDS were re-incuclated with respective media. Re-growth was observed after 4 days of incubation in SBA plates (A1 & C1) and no growth was observed in TSA plates after 14 days (B1 & D1). Identification of re-grown colonies was confirmed by MicroSEQ ID system.


