Uluslararası Fen Araştırmalarında Yenilikçi Yaklaşımlar Dergisi
International Journal of Innovative Approaches in Science Research 2020, Vol. 5 (1), 01-99
https://doi.org/10.29329/ijiasr.2021.338.1
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Original article

Evaluation of BACTEC™ FX Blood Culture System for Rapid Sterility Testing of Human Medicinal Solutions for Infusion

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Abstract

Human medicinal products for infusion should be tested for sterility at various stages of manufacturing including the final step before marketing. Conventional sterility methods recommended in pharmacopeia need a 14 days incubation period in order to get reliable analytical results. However, rapid alternative methods based on automated detection of microbial growth have the advantage of taking only 5 days for sterility testing of medicinal products which can be very important especially during pandemics and emergency conditions. The aim of the present study is to evaluate the microbial detection potential and capacity of the BACTEC FX system for the rapid sterility testing of Paracerol, a 10 mg/mL paracetamol containing pediatric medicine for infusion, and to specify time required for the detection of a variety of microorganisms. Accordingly, the results showed that there were no significant differences between the BACTEC and conventional membrane filtration methods for detecting contamination. All positive/negative controls and all samples intentionally contaminated with microorganisms were determined correctly by using both methods. BACTEC FX system detected all microorganisms including slow growers significantly faster than the membrane filtration method ($p<0.05$). This system can be considered as a rapid alternative over conventional sterility methods for the release of human medicinal products for infusion to the markets especially under emergency conditions. However, for each particular products, validation steps should be executed according to European Pharmacopoeia by using a broad range of microorganisms.

Keywords: Rapid Sterility Test, BACTEC Method, Paracetamol, Membrane Filtration.

Received: 09 March 2021 * **Accepted:** 11 March 2021 * **DOI:** https://doi.org/10.29329/ijiasr.2021.338.1

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INTRODUCTION

The currently accepted and most widely used sterility tests described in Pharmacopoeia (Pharmacopeial Forum USP 33-NF 28, 2011) have included membrane filtration method and direct inoculum which need 14 days incubation period for the detection of slow-growing organisms (Bowman, 1969; Bugno et al., 2015; van Doorne et al., 1998) The development of rapid microbiological methods (RMMs) allows the implementation of more rapid decisions and preventive measures to assure quality control processes, as well as reduce the cost and time for the safe release of sterile products to the markets (Parveen et al., 2011). Although RMMs in pharmaceutical manufacturing gain acceptance with the availability of new generation fully automated systems, most companies still prefer to use manual and traditional methods. However, with the recent COVID-19 pandemic, pharma and biotech companies have realized the urgent need to respond to such public health situations faster than ever. Therefore, the transition from traditional techniques to automated RMMs in sterility testing for the rapid supply of the drugs and vaccines provides compelling benefits to manufacturers and patients during pandemic outbreaks and emergency conditions. Automated RMMs minimize human handling, increase speed and efficiency, reduce risk for contamination, and enable remote control (Menchinelli et al., 2019). Among today's automated systems, BACTEC FX (BACTEC; Becton Dickinson, Sparks, MD, USA) designed for assessing the microbial growth in clinical specimens particularly in blood samples, is based on the detection of carbon dioxide generated from microorganisms by using fluorescent technology (Fuller & Davis, 1997; Reisner & Woods, 1999; Schroeter, Wilkemeyer, Schiller, & Pruss, 2012; Somily et al., 2018). Briefly, microorganisms utilize nutrients in the cultured vials, and they release carbon dioxide reacting with a dye found in the sensor located at the bottom of culture bottles. This reaction modulates the amount of light absorbed by a fluorescent material in the sensor and finally, the level of fluorescence corresponding to the amount of carbon dioxide released by the organisms is measured by the photodetector of the instrument (Riedel et al., 2006).

The aims of the current study were to validate the microbial detection potential and capacity of the BACTEC FX system for the rapid sterility testing of the parenteral human medicinal products and to specify the time required for the detection of a variety of microorganisms including the bacteria that only generate limited amounts of carbon dioxide (e.g. Pseudomonas aeruginosa). We performed validation studies according to the European Pharmacopoeia (Ph.Eur.) monograph "Alternative Methods for Control of Microbiological Quality" by testing the following parameters: specificity, the limit of detection, robustness, and ruggedness (Jouette, 2007). A membrane filtration test was also performed in parallel as a control.

MATERIALS and METHODS

Materials

Insturument

The BACTEC FX system contains a sensor for detecting the concentration of CO_2 produced by the metabolism of microorganisms, which was from Becton Dickinson, USA. The instrument monitors the sensor every ten minutes for an increase in fluorescence change.

Culture media, reagents, and sample

Tryptic Soy Broth (TSB=BD BACTEC Plus Aerobic/F Culture Vials) and Fluid Thioglycollate Medium (FTM=BD BACTEC Plus Anaerobic/F Culture Vials) were used for aerobic and anaerobic microorganisms, respectively, for the BACTEC FX system. These media are commercial formulations from the manufacturer. To assess whether growth media were able to promote microbial development 10 mL of microorganisms were inoculated in TSB and FTM at 20-25 °C and 30-35 °C, respectively for 14 days. At the end of the incubation period, colonies were counted and evaluated for concentrations. Negative controls were used for all analyses. Paracerol containing 10 mg/mL Paracetamol solution for I.V. infusion was used as a matrix with and without different microorganisms listed in Table 1 in both BACTEC FX rapid sterility system and membrane filtration method.

Strains

In this study, yeast, fungi, Gram-negative and Gram-positive microorganisms from American Type Culture Collection (ATCC), and environmental isolates (Table 1) were used. These microorganisms included aerobic, anaerobic, spore-forming, slow growers, and those with fastidious nutritional requirements.

The effectiveness of the growth media to promote microbial development was tested as described in Ph.Eur. under subsection “2.6.13 Microbiological Examination of Non-Sterile Products: Test for Specified Microorganisms”. From stock strains, by using 0.9% isotonic sodium chloride solution dilutions were prepared and directly inoculated into an agar medium. While *Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Burkholderia cepecia complex, Paracoccus yeei* strains were inoculated into Tryptic soy agar (TSA) at 30-35°C for 2 days; *Candida albicans* and *Aspergillus brasiliensis* were inoculated into TSA at 20-25°C for 5 days. For *Clostridium sporogenes* microorganisms were incubated at 30-35°C for 2 days under anaerobic conditions. In the case of *Staphyloccous epidermidis*, microorganisms were inoculated at 30-35°C or 20-25 °C for 2 days under both aerobic and anaerobic conditions, respectively. Then, colonies were counted and concentrations were evaluated. Negative controls were used in all analyses. Growth media were incubated as described but without inocula.

Table 1. List of microorganisms used in the evaluation of BACTEC and membrane filtration method for sterility testing of Paracerol solution.

Microorganisms	Strain ID	Aerobic Anaerobic	Gram Stain	Incubation Temp. (°C)
Staphylococcus aureus	ATCC 6538	Aerobic	Gram-positive cocci	30-35
Pseudomonas aeruginosa	ATCC 9027	Aerobic	Gram-negative rods	30-35
Bacillus subtilis	ATCC 6633	Aerobic	Gram-positive rods	30-35
Candida albicans	ATCC 10231	Aerobic	Yeast	20-25
Aspergillus brasiliensis	ATCC 16404	Aerobic	Fungi	20-25
Clostridium sporogenes	ATCC 19404	Anaerobic	Gram-positive rods	30-35
Staphylococcus epidermidis	*EI	Anaerobic Aerobic	Gram-positive cocci	30-35 20-25
Paracoccus yeei	EI	Aerobic	Gram-negative coccobacilli	30-35
Burkholderia cepacia complex	EI	Aerobic	Gram-negative	30-35

*EI: Environmental Isolates

Method

Membrane Filtration Method

The aim of the membrane filtration test is to collect microorganisms on top of the membrane after filtering from 0.45 µm pore-sized cellulose nitrate filter. At the end of this process, membranes are either incubated with TSB to provide growth of aerobic organisms including yeast and fungi, or with FTM for anaerobic organisms. TSB and FTM were incubated at 20-25°C and 30-35°C, respectively for 14 days.

BACTEC Method

The proprietary media bottles used in BACTEC were labeled as BD BACTEC Plus Aerobic/F culture vial containing 30 mL medium and BD BACTEC Plus Anaerobic/F culture vial containing 25 mL medium. For the BACTEC system, the operational mode for the sterility testing is the direct inoculation method which was performed by inoculating 10 ml Fluid A or paracerol matrix with 1 ml of microorganism solution containing (10–100 CFU or ≤5 CFU). 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.

Experimental Design

Validation studies were performed according to the Ph.Eur. monograph “Alternative Methods for Control of Microbiological Quality” at three different phases by testing the following parameters: specificity, the limit of detection, robustness, and ruggedness as described below (Jouette, 2007).

Phase 1-Specificity of the method: The validation of the performance of the BACTEC FX system started with the experiments for testing "the specificity of the method" based on its ability to detect a range of microorganisms that may be present in the test sample. For each organism, 10 mL of Paracerol solution were intentionally contaminated with 1 mL of microbial suspension to finally obtain 10-100 CFU/mL microbial load, and then this suspension was subjected to rapid sterility test through an automated BACTEC FX system. The experiment was repeated for the product coming from three different batches. For each batch, the experiment was repeated six times. Each product from a different batch was also tested without organisms. The membrane filtration method was always performed in parallel as a control. Negative controls of the culture media were included to confirm their sterility. Positive controls of the inocula were utilized to assure their viability and as well as ability to grow.

Phase II-Limit of Detection: In this part of the validation procedure, 10 mL of Paracerol solution were intentionally contaminated with 1 mL of microbial suspension to finally obtain microbial load ≤5 CFU. The experiment was repeated for the product coming from three different batches. For each batch, the experiment was repeated four times. Each product from a different batch was also tested without organisms. The membrane filtration method was performed in parallel as a control. Negative controls of the culture media were included to confirm their sterility. Positive controls of the inocula were utilized to assure their viability and as well as ability to grow.

Phase III-Determination of the Robustness and Ruggedness of the Method: Being a validation parameter, robustness can be defined as a measure of a test's capacity to remain unaffected by the introduction of small intentional challenges to the method parameters. Herein, we performed two separate intentional variations: 1) Preincubation test: BACTEC AEROBIC/F and BACTEC ANAEROBIC/F media bottles were preincubated at 40 °C for 48 hours. And then 10 mL of Paracerol solution containing 1mL of microbial suspension to finally obtain 10-100 CFU/mL of microbial load inoculated to these bottles. The experiment was repeated for the product coming from six different batches. 2) Challange test: First Paracerol samples without microorganisms were incubated in the BACTEC FX system for 5 days at normal operating conditions and analyzed for contamination. Samples without contamination (negative samples) were inoculated with 1 mL of microbial suspensions to finally obtain 10-100 CFU/mL microbial load and incubated for another 5 days in the BACTEC FX system than results were evaluated. For each batch, the experiment was repeated four times. The experiment was repeated for the product coming from three different batches Each product from a different batch was also tested without organisms. Negative controls of the culture media were included to confirm their sterility. Positive controls of the inocula were utilized to assure their viability and as well as ability to grow.

The ruggedness of a qualitative microbiological method is the degree of precision of test results obtained by analysis of the same samples under a variety of normal test conditions, such as different analysts, instruments, reagent lots, and laboratories. Ruggedness is another validation parameter defined as the intrinsic resistance of the results of the microbiological method to the influences exerted by operational and environmental variables. In the current study, to obtain data for the ruggeddness of the method, two different analysts performed parallel studies by using two different strains *P. aeruginosa* and *S. aureus.*

Statistical Analysis

An Analysis of Variance (ANOVA) and unpaired t-test was performed to evaluate membrane filtration and BACTEC methods regarding the difference in time required to detect growth after inoculating with microorganisms at two different concentration ranges (10-100 CFU and ≤ 5 CFU). All statistical analyses were performed using GraphPad Prism (GraphPad Software 8.0.1). We considered differences with $p<0.05$ statistically significant.

RESULTS and DISCUSSION

Table 2 demonstrates the total number of cultures exhibiting microbial growth by BACTEC and membrane filtration methods with respect to relevant validation criteria. Accordingly, the level of agreement between the BACTEC and membrane filtration methodologies applied to detect microbial growth was 100% for relevant tested parameters i.e. specificity, the limit of detection, and ruggedness. In the experiments, all positive controls and all Paracerol samples intentionally contaminated with either 10-100 CFU or ≤ 5 CFU microbial loads demonstrated growth of inserted test microorganisms. All negative controls did not exhibit any microbial growth.

Table 2. Number of positive results in BACTEC method as compared to membrane filtration method according to tested parameters.

Parameters Tested	**BACTEC (*PE/TE)**	**Membrane Filtration (*PE/TE)**
Method Specificity (10 – 100 CFU)	180/180	180/180
Limit of Detection (≤ 5 CFU)	120/120	120/120
Robustness of the method (10 – 100 CFU)	60 /60	**NA
Ruggedness of the method (10 – 100 CFU)	12/12	12/12

*PE/TE= Number of positive experiments/total experiments performed, **NA: not applicable.

Table 3, Table 4, and Fig. 1 compare the time required to detect (TTD) the growth of the various organisms intentionally inserted into Paracerol samples by the BACTEC and membrane filtration

methods at different microbial load ranges. BACTEC method can effectively detect microbial contamination at either 10-100 or ≤ 5 CFU. All microorganisms including slow growers (*Pseudomonas aeroginosa* and *Candida albicans*) were detected by the BACTEC FX system less than 48 h at both concentration ranges. Consistent with our findings Somily et al., observed similar TTDs for clinical blood culture specimens (Somily et al., 2018). In an evaluation study, Parveen et al. showed that while the BACTEC FX system determined all microorganisms significantly faster than the compendial method in fluid A solution (0.1% peptone), it did not detect growth for many of the tested microorganisms when these were mixed with inactivated influenza vaccines containing preservative 0.01% thimerosal (Parveen et al., 2011). In the present study, Paracerol contains mannitol, cysteine hydrochloride monohydrate, disodium phosphate dihydrate, sodium hydroxide, and water as excipients. These compounds together with the active ingredient (paracetamol) seem to be compatible with the detection ability of BACTEC method. In the current study, the membrane filtration method took up to 3 days and 5 days to detect bacteria and fungus/yeast, respectively.

Table 3. The time required to detect the growth of the various organisms by the BACTEC and membrane filtration methods for the 10-100 CFU inoculum.

	Microorganisms	**Time required to detect growth (h) (Mean ± SEM)**		***p-value***
	10-100 CFU	**BACTEC FX**	**Membrane Filtration**	
Gram-positive bacteria	*Bacillus subtilis*	14.9 ± 0.2[a]	72.0 ± 0.0	*p < 0.0001*
	Clostridium sporogenes	29.2 ± 0.3[a]	72.2 ± 0.1	*p < 0.0001*
	Staphylococcus aureus	26.9 ± 0.5[a]	72.2 ± 0.1	*p < 0.0001*
	Staphylococcus epidermidis (anaerobic)	44.5 ± 1.9[a]	72.3 ± 0.1	*p < 0.0001*
	Staphylococcus epidermidis (aerobic)	42.1 ± 1.5[a]	72.4 ± 0.2	*p < 0.0001*
Gram-negative bacteria	*Burkholderia cepacia complex*	20.2 ± 0.2[a]	72.2 ± 0.1	*p < 0.0001*
	Pseudomonas aeruginosa	22.7 ± 0.4[a]	72.2 ± 0.1	*p < 0.0001*
	Paracoccus yeei	19.9 ± 0.3[a]	72.4 ± 0.2	*p < 0.0001*
Yeast	*Candida albicans*	26.7 ± 1.0[a]	120.4 ± 0.2	*p < 0.0001*
Fungus	*Aspergillus brasiliensis*	44.7 ± 1.0[a]	120.2 ± 0.1	*p < 0.0001*

[a] Significantly faster in the detection of growth than the membrane filtration method. [***]p < 0.0001

Table 5 compares the TTD values of two different intentional variations (preincubation and challenge tests) for BACTEC methodology with the aim of testing the robustness of the process. Herein,

we introduced two different intentional variations to test conditions as described in the materials and method section. When the effects of two different variations on the TTD values obtained by BACTEC were compared, there were no significant changes for most of the microorganisms except *S. epidermis* grown under aerobic conditions and fungus. The introduction of either preincubation or challenge variations to the system increased TTD values significantly from 45 h to 51 and 54 h, respectively for fungus. A similar pattern was also observed for *S.epidermis* (aerobic) in case of challenge variation only. However, for both microorganisms, TTD values were maintaned less than 72 hours.

Table 4. The time required to detect the growth of the various organisms by the BACTEC and membrane filtration method for the microbial load ≤5 CFU.

Types	Microorganisms	Time required to detect growth (h) (Mean ± SEM)		*p-value*
	≤5 CFU	**BACTEC FX**	**Membrane Filtration**	
Gram-positive bacteria	*Bacillus subtilis*	16.3 ± 0.1[a]	72.3 ± 0.2	p < 0.0001
	Clostridium sporogenes	29.4 ± 0.7[a]	72.6 ± 0.4	p < 0.0001
	Staphylococcus aureus	31.0 ± 0.3[a]	72.0 ± 0.0	p < 0.0001
	Staphylococcus epidermidis (anaerobic)	42.5 ± 0.5[a]	72.5 ± 0.2	p < 0.0001
	Staphylococcus epidermidis (aerobic)	37.8 ± 0.9[a]	72.8 ± 0.4	p < 0.0001
Gram-negative bacteria	*Burkholderia cepacia complex*	21.6 ± 0.2[a]	72.4 ± 0.2	p < 0.0001
	Pseudomonas aeruginosa	26.7 ± 0.3[a]	72.1 ± 0.8	p < 0.0001
	Paracoccus yeei	21.0 ± 0.2[a]	72.3 ± 0.2	p < 0.0001
Yeast	*Candida albicans*	23.5 ± 0.1[a]	120.3 ± 0.2	p < 0.0001
Fungus	*Aspergillus brasiliensis*	54.1 ± 1.0[a]	121.1 ± 0.5	p < 0.0001

[a] Significantly faster in the detection of growth than the membrane filtration method, $^{***}p < 0.0001$

Table 6 represents the ruggedness of both BACTEC and membrane filtration methods. Herein, this parameter was determined as a measure of reproducibility of test results from analyst to analyst by using two different microorganisms. There were no significant differences in TTD values obtained by two different analysts for the membrane filtration method in both tested microorganisms. In the detection of *S. aeruginosa* by the BACTEC method, a very slight difference (approximately 1 h) was observed between TTD values obtained by analysts.

Table 5. Robustness of the BACTEC FX system in case of two different intentional variations (preincubation, challenge) introduced separately to the method.

Types	Microorganisms	Time to detection (TTD, h) (Mean ± SEM)			Statistical significance *p-values*		
	10-100 CFU	**No Intervention**	**Preincubation**	**Challange**	**Preincubation vs Challange**	**Preincubation vs No intervention**	**Challenge vs No intervention**
Gram-positive bacteria	*Bacillus subtilis*	14.9 ± 0.2	16.0 ± 0.1	16.0 ± 0.1	ns	ns	ns
	Clostridium sporogenes	29.2 ± 0.3	27.3 ± 0.3	27.4 ± 0.2	ns	ns	ns
	Staphylococcus aureus	26.9 ± 0.5	26.4 ± 0.8	25.7 ± 1.4	ns	ns	ns
	Staphylococcus epidermidis (anaerobic)	44.5 ± 1.9	43.3 ± 0.9	44.4 ± 0.7	ns	ns	ns
	Staphylococcus epidermidis (aerobic)	42.1 ± 1.5	46.5 ± 5.7	58.9 ± 2.5	< 0.0001***	ns	< 0.0001***
Gram-negative bacteria	*Burkholderia cepacia complex*	20.2 ± 0.2	21.2 ± 0.6	22.5 ± 0.3	ns	ns	ns
	Pseudomonas aeruginosa	22.7 ± 0.4	25.4 ± 0.4	25.6 ± 0.7	ns	ns	ns
	Paracoccus yeei	19.9 ± 0.3	20.7 ± 0.4	21.4 ± 0.2	ns	ns	ns
Yeast	*Candida albicans*	26.7 ± 1.0	29.3 ± 0.6	29.8 ± 0.2	ns	ns	ns
Fungus	*Aspergillus brasiliensis*	44.7 ± 1.0[a]	51.4 ± 1.9	54.1 ± 2.9	ns	< 0.05*	< 0.0001***

Table 6. The ruggedness of the BACTEC method when the test was repeated by two different analysts.

Microorganisms	**Time to Detection (TTD, h) (Mean ± SEM)**					
10-100 CFU	**Membrane Filtration**			**BACTEC FX**		
	Analyst 1	**Analyst 2**	***p-value***	**Analyst 1**	**Analyst 2**	***p-value***
Pseudomonas aeruginosa	72.0 ±0.0	72.7 ± 0.7	ns	20.5 ± 0.2	19.2 ± 0.1	$p < 0.05$
Staphylococcus aureus	72.0 ±0.0	72.3 ± 0.3	ns	22.7 ±0.1	22.3 ± 0.2	ns

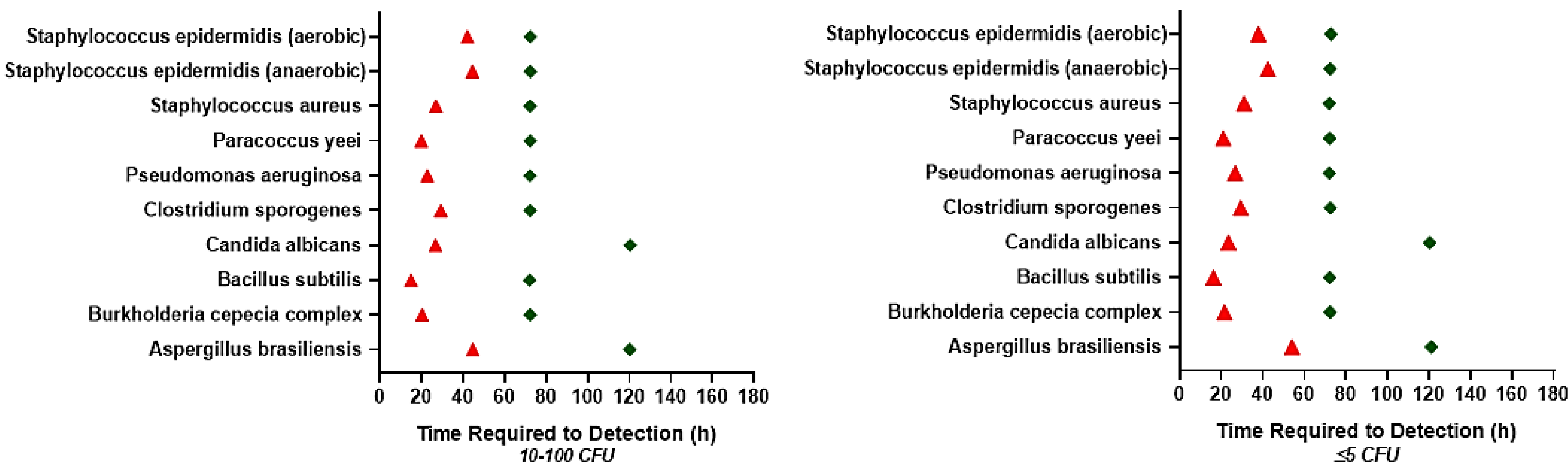

Figure 1. The time required to detect growth by membrane filtration method and Rapid Detection System Method, BACTEC, for both the 10-100 CFU and ≤5 CFU inoculum.

Our study is one of the few studies that have systematically evaluated the BACTEC FX automated system in medicinal products for infusion according to relevant validation criteria recommended by Ph. Eur (Jouette, 2007). Results demonstrated that the BACTEC method detected all organisms used in the current study with the same assurance as expected from the membrane filtration method but within a significantly shorter period of time. In conclusion, The BACTEC method seems to be a rapid alternative to the conventional sterility methods, therefore, having the great advantage of taking 5 days for sterility testing of medicinal products. This advantage may turn into a necessity in case of pandemic or emergency situations. However, the convenience of all potential RMMs should be evaluated for each medicinal product by using a wide range of specific microorganisms according to the relevant validation criteria described in compendia (Jacobs et al., 2017; Jouette, 2007; Totty et al., 2017).

Conflict of Interest

U.K. and E.K. have equity interests in and F.Y., E.Y. and M.K. are employed by Polifarma Pharmaceuticals, Industry and Trade Inc. No other potential conflicts of interest relevant to this article were reported.

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