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ORIGINAL ARTICLE

Evaluation of an Amplified ATP Bioluminescence Method for Rapid Sterility Testing of Large Volume Parenteral

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Abstract
The sterility test described in pharmacopeial compendia requires a 14-day incubation period to obtain a valid analytical result. Therefore, the use of alternative methods to evaluate the sterility of pharmaceuticals, such as the Celsis AKuScreen™ Advance™ system, is particularly interesting because it allows a reduced incubation period and higher efficiency. The present study was aimed to evaluate and compare the performance of Celsis AKuScreen™ Advance™ system with the pharmacopeial sterility test. There was no significant difference between the ability of detection of microbial contamination observed within pharmacopeial method and test method. The Celsis AKuScreen™ Advance™ system allowed a faster detection of the challenge microorganisms, which indicates that the system is a viable alternative for assessing the sterility of injectable products.

Keywords Celsis AKuScreen™ Advance™ · Performance · Rapid microbiological method · Sterility test

Introduction

A sterility test confirms whether a drug substance, drug product, or other pharmacopeial article is sterile as defined by the compendial method. The compendial sterility test requires an incubation period of 14 days to verify the sterility, i.e., the absence of visually discernable microbial growth in the samples evaluated [1].

The availability of a rapid sterility test, capable of detecting the presence of viable microorganisms more swiftly, facilitates rapid decision-making for the implementation of corrective and preventive actions to ensure product quality and reduces the time and costs to produce sterile products, due to the possibility of faster release of production. The development of rapid microbiological methods (RMM) for the isolation and detection of microorganisms has led to evaluate their suitability for use in sterility testing [2–9].

ATP bioluminescence is a generation of light by a biological process which uses the firefly luciferase enzymes to allow rapid and quantitative determination of ATP amount; the technology has gained acceptance in the quality control of food products and cosmetics [10–13], but transfer of new analytical technologies to the pharmaceutical industry has been a slow process. Clinical samples, food products, and cosmetics have microbiological quality requirements very different from pharmaceutical products and, initially, it was necessary to adapt methods so that they fulfill their new purposes. In addition, the regulatory agencies had also maintained caution regarding the approval of the new technologies due to the scarce information available regarding their adequacy. Now, it has been established that these new methods can be applied to improve efficiency with no compromise in terms of product

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safety, as long as they are validated and proven to be equivalent to the classical methods.

ATP analysis is based on the detection of adenosine triphosphate, which is present in all living organisms as a vital part of their energy metabolism and makes the method suitable for the detection of any type of viable cells that are utilizing energy in the form of ATP, including microorganisms. Detection is accomplished by using a bioluminescent enzyme-acceptor system (luciferase/luciferin). In the presence of ATP, the luciferase transfers energy from the ATP for the conversion of luciferin to oxyluciferin that then emits the energy as a photon of green light at 564 nm. The light emitted can be measured using photomultiplier or photodiode technology. Under controlled conditions, the amount of light produced is directly dependent on the amount of ATP provided to the enzyme-acceptor system [10, 14–18].

Although ATP bioluminescence is a very sensitive technique that provides a much more rapid detection system than waiting for visible colonies to grow on culture media, the technique is limited by the fact that organisms contain a finite amount of ATP, thus limiting the amount of ATP present for the bioluminescence reaction. However, biological cells that utilize ATP also contain adenylate kinase (AK), another component of the energy metabolism, which can be used to generate almost unlimited amounts of ATP from ADP and phosphate and thus increasing the sensitivity of an ATP bioluminescence assay since the ATP content is no longer limited to the amount present in the original cells but has been increased by the kinase reaction [7, 10, 14, 16, 18, 19].

The use of rapid microbiological methods for sterility testing relies on their ability to recover and detect microorganisms occasionally present in pharmaceuticals with performance equivalent to the compendial methods [1, 4, 18, 20, 21]. Pharmacopeial compendia [22] and alternative microbiological method validation guide [4] indicate which validation parameters should be evaluated according to the type of microbiological test; for qualitative tests such as the sterility test, various parameters like specificity and detection limit must be assessed.

Considering the setting of development of rapid microbiological methods, the intent of this study is to demonstrate the suitability of the technology employing adenylate kinase-enhanced ATP bioluminescence—the Celsis AKuScreen™ Advance™ (Celsis system)—in the sterility assessment of sterile pharmaceuticals, in comparison to the sterility test by membrane filtration, as defined in the pharmacopeial compendium.

Materials and Methods

Matrices

Experimental batches of 0.9% sodium chloride solution (M1), 5% dextrose solution (M2), injectable metronidazole (M3), and ringer solution with sodium lactate (M4) were contaminated by each challenge microorganisms at different concentrations.

Microorganisms

Following microorganism strains were used as indicated in the pharmacopeial compendia for evaluating the growth-promoting ability of culture media used in sterility testing, all of which were in the form of *BioBall*® Single Shots (BioMerieux, France) containing 30 CFU/unit: *Staphylococcus aureus* (NCTC 10788), *Bacillus subtilis* (NCTC 10400), *Pseudomonas aeruginosa* (NCTC 12924), *Clostridium sporogenes* (NCTC 12935), *Candida albicans* (NCPF 3179), and *Aspergillus brasiliensis* (NCPF 2275).

BioBall® Single Shot Control

Before evaluation of the media performance, eight units of each *BioBall*® Single Shot were tested to confirm the microbial concentration; four units were used on each of two different days. Each *BioBall*® Single Shot unit was dissolved in 1 mL of 0.9% sterile saline solution, provided by BioMerieux (Marcy-l'Étoile, France) and inoculated on tryptic soy agar (TSA) for *Staphylococcus aureus*, *Bacillus subtilis*, and *Pseudomonas aeruginosa*, with subsequent incubation at 32.5 ± 2.5 °C for 72 h; TSA for *Candida albicans*, incubated at 22.5 ± 2.5 °C for 5 days; Dichloran Rose Bengal Chloramphenicol (DRBC) agar for *Aspergillus brasiliensis*, incubated at 22.5 ± 2.5 °C for 5 days; or blood agar for *Clostridium sporogenes*, incubated at 32.5 ± 2.5 °C for 72 h under anaerobic conditions.

Plate counts were performed after incubation periods and all plates were inspected for purity. Gram stain was used to confirm the absence of contamination. This investigation was carried out for all the lots used in this study.

Preparation of Microbial Suspensions

For each challenge microorganism, nine *BioBall*® Single Shots were dissolved in 27 mL of 0.9% sterile saline solution (BioMerieux, France). From this suspension, 20 mL, 4 mL, and 800 μL aliquots were transferred to sterile flasks, and the final volume of each flask was adjusted to 100 mL with 0.9% sterile saline solution (BioMerieux, France) to obtain suspensions with concentrations approximately of 10 CFU/5 mL, 2 CFU/5 mL, and 0.4 CFU/5 mL, respectively, which were used to intentionally contaminate different matrices.

Additionally, 2 mL aliquot of the initial suspension was inoculated on TSA for *Staphylococcus aureus*, *Bacillus subtilis*, and *Pseudomonas aeruginosa*, with subsequent incubation at 32.5 ± 2.5 °C for 72 h; TSA for *Candida albicans* incubated

Table 1 Results of the test used to confirm the *BioBall*® Single Shots concentration compared with the microbial concentration described in the certificate of analysis

Microorganism	Lot	Average (CFU/*BioBall*®)	CV (%)	Predict average [$CI_{95\%}$] for 09 *BioBall*® (CFU)	CoA (CFU/*BioBall*®)
Staphylococcus aureus	1	28.2	8.9	253 [220: 293]	28.3
	2	28.4	9.3	256 [221: 296]	29.0
	3	29.4	8.1	265 [233: 301]	29.6
Pseudomonas aeruginosa	1	31.2	8.0	281 [247: 319]	31.0
	2	31.4	7.6	283 [251: 319]	31.5
	3	29.9	9.5	269 [233: 311]	31.3
Bacillus subtilis	1	29.7	8.9	267 [232: 307]	30.1
	2	29.4	7.1	265 [237: 297]	29.6
	3	29.9	8.6	269 [235: 309]	30.5
Clostridium sporogenes	1	29.9	8.2	269 [237: 306]	29.9
	2	30.2	8.3	272 [238: 310]	30.7
	3	30.2	9.1	271 [234: 314]	30.3
Candida albicans	1	28.7	9.2	258 [223: 299]	30.2
	2	29.4	7.1	265 [237: 297]	30.5
	3	29.2	7.6	263 [232: 298]	30.7
Aspergillus brasiliensis	1	29.4	9.8	265 [226: 309]	28.5
	2	29.9	9.8	269 [230: 315]	30.7
	3	29.4	7.1	265 [237: 297]	29.7

at 22.5 ± 2.5 °C for 5 days; DRBC agar for *Aspergillus brasiliensis* incubated at 22.5 ± 2.5 °C for 5 days; or blood agar for *Clostridium sporogenes* incubated at 32.5 ± 2.5 °C for 72 h under anaerobic conditions, in order to verify the microbial concentration. Plate counts were performed after incubation periods and all plates were inspected for purity and Gram stain was performed to confirm the absence of contamination.

Preparation of Samples

To evaluate the ability of the method to detect a representative range of microorganisms that may present in the test article, the compatibility of the method with different types of matrices and define the experimental condition related to pre-incubation period, 15 units of each product were artificially

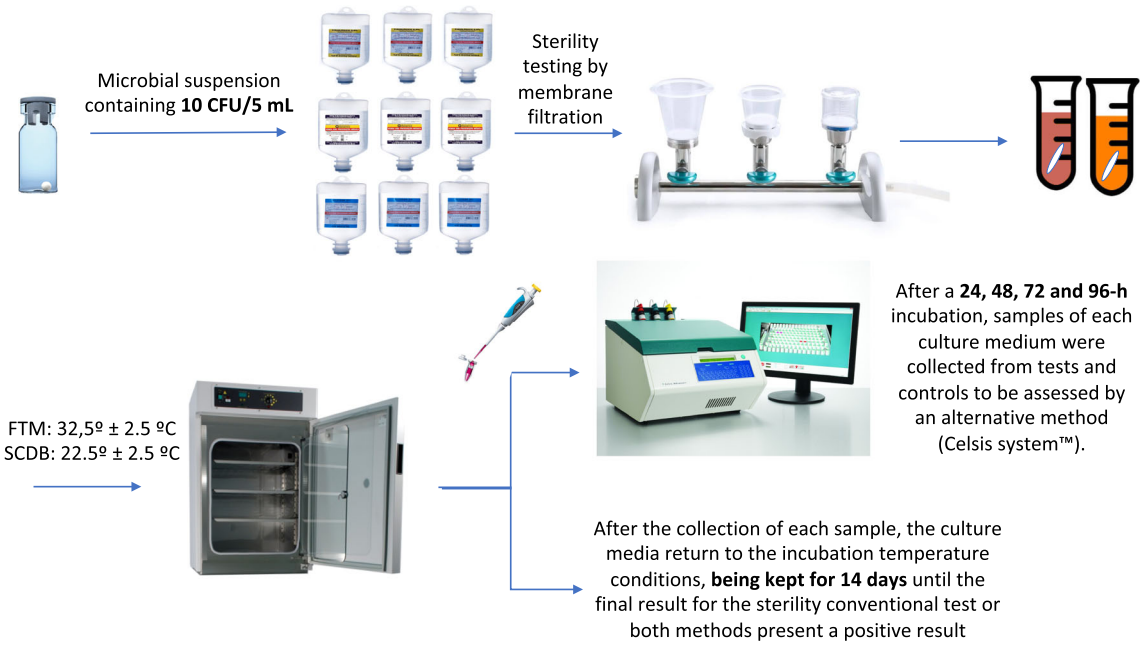

Fig. 1 Graphical representation of the first experiment

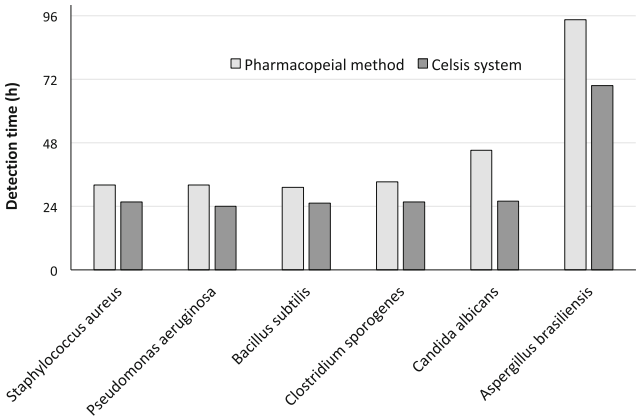

Fig. 2 Mean time required to detect microbial growth for each methodology employed

contaminated with a suspension containing 10 CFU/5 mL of each microorganism.

For verifying the lowest number of microorganisms in the sample that can be detected under the stated experimental conditions, 15 units of 0.9% sodium chloride solution were artificially contaminated with a suspension containing 10 CFU/5 mL of each compendial microorganisms, the same number of units were also contaminated with a suspension containing 2 CFU/5 mL and the same number of units were contaminated with a suspension containing 0.4 CFU/ 5 mL.

Sterility Test

The Celsis system was evaluated by comparing it to the pharmacopeial membrane filtration sterility testing method.

Each of the samples was filtered under aseptic conditions using a cellulose nitrate filter having nominal pore size not greater than 0.45 μm and about 50 mm in diameter. After filtration of samples, the membrane was washed three times by filtering 100 mL of Fluid A; then, the membrane was cut aseptically into two equal parts and one half was transferred to conventional culture media for the pharmacopeia sterility testing (FTM and SCDM), supplied by BioMerieux (France) in 100 mL flasks. The containers of FTM were incubated at 32.5 ± 2.5 °C and the containers of SCDM at 22.5 ± 2.5 °C for 14 days. Both types of culture media were evaluated daily to detect the presence of microbial growth.

Initially, to define the pre-enrichment incubation period in conventional culture media prior to evaluation on the Celsis system, 1 mL aliquot of each conventional culture media was aseptically collected to the Celsis system tubes, after 24, 48, 72, and 96 h of incubation, to be assessed by alternative method and was returned to incubator until complete 14 days of incubation. Considering that after 96-h incubation period, all microorganisms could be recuperate, in the second experiment, 1 mL aliquot of each culture media was collected to the Celsis system tubes to be assessed by alternative method and was returned to the incubator until complete 14 days of incubation.

A 50 μL aliquot was pipetted into a cuvette in duplicate and placed into the luminometer for testing. The luminometer automatically injected all three reagents: Lumin AMP (a purified source of ADP that is the substrate for adenylate kinase), Lumin EX (makes microbial cells permeable to AK and ATP), and Lumin ATE (final reagent responsible for the light reaction when ATP is present). The RLU reading was recorded within 25 s of reagent injection. The first two samples of each run were a sterile, negative broth calibrator. The cutoff values to determine negative and positive results were derived from the RLU results of these first two cuvettes, so the broth calibrator was the same lot as the broth used to suspend the samples. Celsis system information management software automatically averaged the results of the two cuvettes and then multiplied this value by two. Any result less than two times the broth RLU was considered negative. Any result over two times the broth RLU was considered a positive result. If a positive result was detected, the sample in question was re-assaying to confirm the Celsis system result.

Negative controls consisting of filtrations of each of the matrices that had not been intentionally contaminated were included in all the assays. Negative controls of the culture media were also used to confirm the sterility of these reagents.

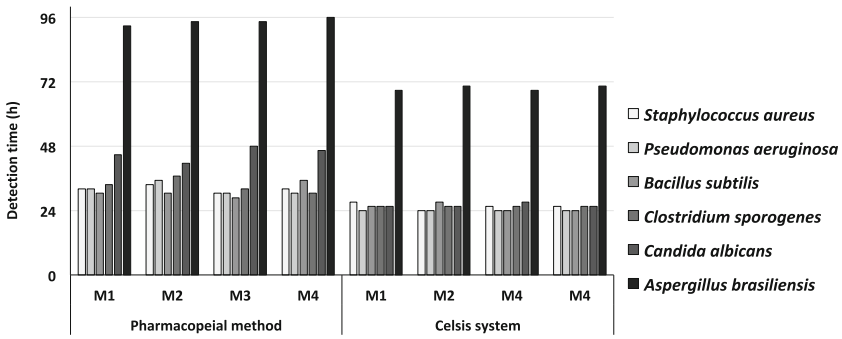

Fig. 3 Mean time required to detect microbial growth for each methodology employed considering the types of sample matrices used

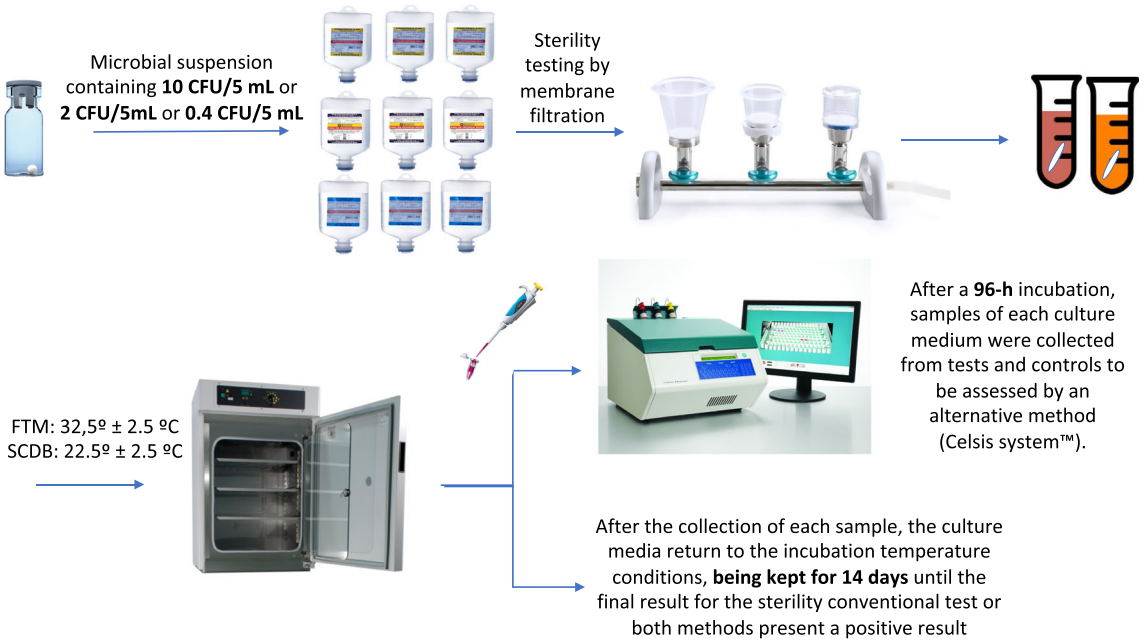

Fig. 4 Graphical representation of the experiment

Positive controls of the inoculum were used to confirm their viability and ability to grow in the culture media.

All of the flasks containing culture media that had microbial growth were subjected to Gram staining and subcultured to identify the microorganism and confirm the purity of the inoculum used.

Statistical Analysis

The chi square test was used to evaluate the effectiveness of the Celsis system. Analysis of variance (ANOVA) two-way was used to examine the differences in the time required to detect microbial growth. The p value of < 0.05 was considered statistically significant.

Results and Discussion

BioBall® Single Shot Control

Table 1 shows the data from the tests used to confirm the microbial concentration of each type of *BioBall*® Single Shots. All *BioBall*® Single Shots were found to be suitable for the present study considering the variability allowed in estimating microbial populations, which ranges from < 15% [1, 20].

Pre-Enrichment Incubation Period

Considering that the concentration of ATP per cell varies at different points in the growth curve and may be quite low as

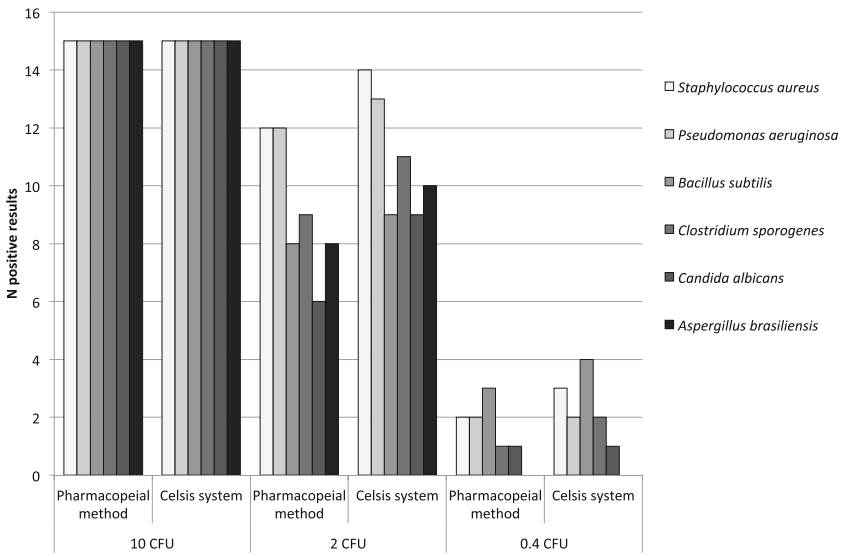

Fig. 5 Distribution of positive results obtained for each level of contamination according to the methodology employed

the microorganisms are not growing and dividing actively [16], the performance of the Celsis system as a rapid sterility test method began with an experiment designed to define the pre-enrichment incubation period in culture media per compendial sterility test prior to evaluation on the Celsis system (Fig. 1).

Each of the 360 trials at 10 CFU contamination level was assessed by Celsis system after 24, 48, 72, and 96 h of incubation and all tests evidenced microbial growth by the conventional method and by Celsis system until 96 h. Although there are no differences ($p > 0.05$) between the abilities of the methods to detect a 10 CFU contamination, the Celsis system detected microbial growth more rapidly than pharmacopeial methods (Fig. 2), independently of the types of sample matrices employed (Fig. 3).

Performance Evaluation

The ability of the method using Celsis system to detect a range of microorganisms that may be present in a sample [1, 4, 20] and the compatibility with different types of sample matrices were evaluated considering the results obtained in the prior experiment in a pre-incubation period of 96 h (Fig. 4).

All 360 trials performed to detect a 10 CFU contamination level by the conventional method and by Celsis system and demonstrated that there is no significant difference ($p > 0.05$) in the ability to detect the presence of the challenge microorganisms between the alternative and compendial methods. Also, the results evidenced that there was no interference of product matrix with the detection mechanism of Celsis system.

A total of 270 trials were performed using experimental batches of 0.9% sodium chloride solution (M1) to evaluate the ability to recover low inoculum concentrations of the microbial strains. The absolute number of contaminated samples detected using each method decreased as the microbial concentration decreased (Fig. 5) and the difference between abilities to detect microbial contamination was not significant ($p > 0.05$), thus indicating that the two methods have equivalent sensitivities.

Conclusion

Despite the limitations of sterility testing in demonstrating the absolute sterility of a product, the conventional method has adequately confirmed the sterility of products. However, a method that can generate results faster with same degree of sterility assurance as the conventional method is highly desirable. The results obtained in this study indicate that the technology employing adenylate kinase-enhanced ATP bioluminescence can be an alternative method for assessing the sterility of injectable products.

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