Title: Validation and Comparability of the BACTEC method and BD FX40 Device for Sterility Testing of CAR-T Drug Products: A Comprehensive Study

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Abstract:

The sterility testing of autologous cell therapies, such as CAR-T drug products, is critical to ensuring patient safety and product quality. In this study, we aimed to validate a rapid microbial method for sterility, using the BD BACTEC FX40 device as an efficient and reliable method allowing rapid sterility testing of frozen CAR-T drug products in just 5 days. We also sought to demonstrate the method's comparability to the compendial sterility method, 14-day sterility method by direct inoculation (Ph.Eur. Chapter 2.6.1 and USP <71>).

Introduction:

Sterility testing is an essential and critical step in the batch release process for CAR-T (Chimeric Antigen Receptor T-cell) therapies. CAR-T therapies involve genetically engineering a patient's own T cells to target and destroy cancer cells. These therapies are often used to treat patients with severe forms of cancer, particularly hematological malignancies. Given the highly specialized nature of CAR-T treatments and the immunocompromised state of the patients receiving them, ensuring the sterility of these therapies is of utmost importance. Unlike typical modalities, cell and gene Therapies are living final products. However, distinguishing between desired living cells (e.g., T-cells) and undesired components (e.g., bacteria, fungi) is crucial. This is especially vital for autologous CAR-T cells therapies as each small batch must be tested due to limited volume, and rapid testing is essential to ensure timely delivery for patient treatment (Source: https://www.pharmtech.com/view/contamination-control-for-cell-and-gene-therapies-needs-new-analytics). The standard reference used by the industry is the compendial sterility test, involving direct inoculation of micro-organisms into the medium (Ph.Eur. Chapter 2.6.1 and USP <71>). This method takes 14 days and is recognized by the industry for its adherence to regulatory standards, and reliability.

We have developed and qualified a rapid sterility test using the BACTEC (Bacterial Automated Detection and Enumeration) device, for early detection of potential contamination. We assessed the comparability of the BACTEC method with the compendial sterility test using three batches of a CAR-T product, intentionally contaminated with nine different microorganisms. The BACTEC method offers an automated and rapid microbiological solution that aligns with the adoption of efficient microbiological methods for advanced therapies (Regulatory Policy - ATMPs - Rapid Microbiology and Rapid Microbiological Methods (rapidmicromethods.com))

Methods:

Nine microbial reference strains (aerobic and anaerobic bacteria, and fungi, namely *Clostridium sporogenes, Cutibacterium acnes, Staphylococcus aureus, Streptococcus pyogenes,*

Brachybacterium nesterenkovii, *Pseudomonas aeruginosa, Bacillus subtilis, Candida albicans, Aspergillus brasiliensis*) were inoculated into the BACTEC vials (Bottle Plastic Bactec Std Aerobic/F 50Pk or Bottle Plastic Bactec Lytic/10 Anaer/F) in various concentrations (100, 50, and 10 CFU/vial), together with FOS supplement. Concentration 100 CFU was tested on three batches of frozen CAR-T drug product. Concentrations of 50 CFU and 10 CFU were tested in triplicates of each microorganism on one batch of frozen CAR-T drug product. Positive controls of microorganisms and negative control of selected media, FOS supplement, and CAR-T drug products were prepared. Correct concentration of inoculated microorganism was confirmed by plating on agar plates. All BACTEC vials were cultivated in the BACTEC FX40 at temperature 35 ± 1,5°C up to 14 days or until detection of microbial growth (positive status in the device).

Testing by the compendial method (Ph. Eu. 2.6.1) was carried out in parallel. Briefly, the sample was inoculated in Trypticase Soy Broth and in Clear Fluid Thioglycolate Medium and cultured 14 days at 20-25°C or 30-35°C respectively. Turbidity was evaluated every workday of the test.

For the comparability study, results in 100 CFU inoculated samples were compared between the BACTEC rapid sterility methods and the compendial method. For the method validation sensitivity and specificity comparison between the two methods was performed, using bacterial and fungal contaminants at 10 CFU and 50 CFU/vial concentrations. Specificity was verified through samples spiked with 50 CFU of microorganisms (positive) and not spiked samples (negative).

Three in-house isolates (aerobic bacteria) were also tested in concentration 100 CFU/vial without CAR-T product.

Results:

Method Optimization

We optimized the culture conditions, including the addition of FOS supplement, and the technique of vial inoculation into the BACTEC vials, particularly for anaerobic strains (e.g., *Clostridium sporogenes*). It was observed that anaerobic strains are sensitive for manipulation. It is necessary to prevent air in the tube when the samples are inoculated. The technique of inoculation was optimized.

Comparison of the rapid sterility test and compendial test

In the comparability experiment the samples of three batches of CAR-T drug product were inoculated by 100 CFU of microorganisms. Both methods were tested in parallel. All tested microbial strains (9 reference strains) showed positive results within four days in BACTEC FX40, in a comparable time frame to the compendial method. All types of negative control were without growth after 14 days. 3 in-house isolates from the clean room environment were tested without CAR-T product. See results in the table 1 and 2.

Table 1: Comparability of the BACTEC rapid sterility method and compendial method in the suitability test. Time to detection is recorded for BATEC FX40.

Tested sample	Microorganism 100 CFU	BACTEC FX40	Direct inoculation
CAR-T-1	*Clostridium sporogenes*	18 h 15 min	1 day
CAR-T-2		15 h 13min	1 day
CAR-T-3		15 h 3 min	1 day
Positive control		14 h 3 min	1 day
CAR-T-1	*Cutibacterium acnes*	3 days 17 h 37 min	5 days *
CAR-T-2		3 days 3 h 8 min	2 days
CAR-T-3		2 days 20 h 37 min	5 days *
Positive control		3 days 2 h 6 min	5 days *
CAR-T-1	*Staphylococcus aureus*	14 h 34 min	3 days *
CAR-T-2		14 h 5 min	3 days *
CAR-T-3		14 h 8 min	3 days *
Positive control		14 h 39 min	4 days
CAR-T-1	*Streptococcus pyogenes*	13 h 35 min	3 days *
CAR-T-2		13 h 16 min	3 days *
CAR-T-3		13 h 40 min	3 days *
Positive control		12 h	3 days *
CAR-T-1	*Brachybacterium nesterenkovii*	1 day 8 h 38 min	3 days *
CAR-T-2		1 day 8 h 39 min	3 days *
CAR-T-3		2 days 23 h 40 min	3 days *
Positive conrol		1 day 8 h 40 min	3 days *
CAR-T-1	*Pseudomonas aeruginosa*	15 h 6 min	3 days *
CAR-T-2		15 h 3 min	3 days *
CAR-T-3		15 h 4 min	3 days *

Tested sample	Microorganism 100 CFU	BACTEC FX40	Direct inoculation
Positive control		15 h 37 min	3 days *
CAR-T-1	*Bacillus subtilis*	10 h 49 min	2 days
CAR-T-2		12 h 7 min	1 day
CAR-T-3		11 h 48 min	1 day
Positive control		11 h 54 min	1 day
CAR-T-1	*Candida albicans*	1 day 2 h 8 min	2 days
CAR-T-2		1 day 3 h 46 min	1 day
CAR-T-3		23 h 35 min	1 day
Positive control		1 day 4 h 27 min	3 days
CAR-T-1	*Aspergillus brasiliensis*	1 day 11 h 5 min	3 days
CAR-T-2		2 days 10 h 50 min	2 days
CAR-T-3		1 day 20 h 21 min	2 days
Positive control		1 day 14 h 35 min	3 days

* The compendial samples were evaluated only on working days. The positivity could have been detected from one/two days earlier.

Table 2: Comparability of the BACTEC rapid sterility method and compendial method in three in-house isolates. Time to detection is recorded for BATEC FX40.

Microorganism 100 CFU	BACTEC FX40	Direct inoculation
Kocuria rhizophila	1 day 4 h	1 day
Moraxella osloensis	2 days 12 h	3 days
Bacillus cereus	8 h	1 day

Sensitivity experiment

Samples were inoculated with 10 or 50 CFU, in triplicates for all conditions. At 50 CFU all tested conditions were tested positive in both methods (data now shown). BACTEC FX40 confirmed

sensitivity as low as 10 CFU for all tested microorganisms which was even superior to the compendial methods where no growth was observed in the case of *Pseudomonas aeruginosa*. See the results in the table 3.

Table 3: Assessment of the method sensitivity. 10 CFU inoculation in triplicate in one batch of CAR-T product. Time to detection is recorded for BACTEC FX40.

Tested sample	Microorganism 10 CFU	BACTEC FX40	Direct inoculation
CAR-T -1 I	*Clostridium sporogenes*	16 h 8 min	1 day
CAR-T -1 II		17 h 36 min	1 day
CAR-T -1 III		16 h 4 min	1 day
Positive control		15 h 33 min	1 day
CAR-T -1 I	*Cutibacterium acnes*	3 days 20 h 11 min	6 days *
CAR-T -1 II		3 days 12 h 6 min	6 days *
CAR-T -1 III		3 days 23 h 8 min	6 days *
Positive control		3 days 15 h 41 min	6 days *
CAR-T -1 I	*Staphylococcus aureus*	16 h 4 min	2 days
CAR-T -1 II		16 h 5 min	2 days
CAR-T -1 III		15 h 38 min	2 days
Positive control		15 h 7 min	no growth
CAR-T -1 I	*Streptococcus pyogenes*	10 h 19 min	3 days *
CAR-T -1 II		10 h 25 min	3 days *
CAR-T -1 III		10 h 39 min	3 days *
Positive control		12 h 57 min	3 days *
CAR-T -1 I	*Brachybacterium nesterenkovii*	1 day 11 h 34 min	3 days *
CAR-T -1 II		1 day 10 h 4 min	3 days *
CAR-T -1 III		1 day 11 h 38 min	3 days *
Positive control		1 day 9 h 40 min	3 days *

Tested sample	Microorganism 10 CFU	BACTEC FX40	Direct inoculation
CAR-T -1 I	*Pseudomonas aeruginosa*	15 h 24 min	no growth
CAR-T -1 II		17 h 34 min	no growth
CAR-T -1 III		18 h 36 min	no growth
Positive control		17 h 38 min	no growth
CAR-T -1 I	*Bacillus subtilis*	13 h 3 min	2 days
CAR-T -1 II		12 h 53 min	2 days
CAR-T -1 III		12 h 36 min	2 days
Positive control		12 h 18 min	2 days
CAR-T -1 I	*Candida albicans*	1 day 1 h 8 min	2 days
CAR-T -1 II		1 day 9 h 36 min	2 days
CAR-T -1 III		1 day 7 h 31 min	2 days
Positive control		1 day 7 h 42 min	2 days
CAR-T -1 I	*Aspergillus brasiliensis*	3 days 5 h 43 min	3 days
CAR-T -1 II		3 days 11 h 27 min	3 days
CAR-T -1 III		3 days 20 h 6 min	3 days
Positive control		2 days 23 h 10 min	3 days

* The compendial samples were evaluated only on working days. The positivity could have been detected from one/two days earlier.

The study confirmed that the rapid sterility test using the BD BACTEC FX40 device effectively detected bacteria or fungi inoculated at 10 CFU, while the compendial sterility test did not always yield positive results at this concentration. The time to positivity for all microbial strains was within four days, indicating the rapidity and efficiency of the BACTEC method. The method's specificity was also confirmed, as it successfully detected negative samples.

Discussion:

The rapid sterility test using the BD BACTEC FX40 device demonstrated high sensitivity in detecting low levels of bacterial and fungal contamination in CAR-T drug products, even when the compendial method failed to do so. Moreover, all microorganisms detected by the BACTEC device

were successfully grown in subsequent subcultures, validating the reliability of the method. The study confirmed the fulfillment of all comparability and validation criteria set by the Pharmacopoeia.

Nevertheless, we encourage the testing of other cell therapy-specific environmental bacterial or fungal species. Strains from the environment that require lower culture temperature can be challenging to grow which can be overcome by the parallel testing with SDA plates as suggested by *England et al.* J Clin Microbiol. 2019 (https://pubmed.ncbi.nlm.nih.gov/30541938/). To ensure maximum safety, a conservative approach of combining BACTEC testing with the compendial method is recommended for conditional product release after five days of BACTEC testing, followed by the compendial sterility test (after 14 days) for the final release.

Rapid sterility testing is required to minimize the risk of contamination while accelerating products to patients. Several companies and organizations have been involved in developing sterility testing methods specifically tailored for CAR-T. In 2020, NIST launched the Rapid Microbial Testing Methods (RMTM) Consortium (https://www.nist.gov/publications/report-nist-workshop-launch-rapid-microbial-testing-methods-consortium). Input from the presenters, panelists, and attendees emphasized the need for the community to work together to efficiently overcome challenges that limit the use of RMTMs.
In the meantime, patients remain in critical need, so industry needs to evaluate and promote methods that ensure quick deployment of safe therapies. In summary, the rapid sterility test using BACTEC device can be explored within the context of the rapid microbiological methods landscape for ATMPs. The method's ability to address regulatory compliance, improve efficiency, mitigate risks, and integrate innovative technology makes it a pertinent topic within the realm of advanced therapies and quality control.

Conclusion:

The rapid sterility test using the BD BACTEC FX40 device proved to be a robust and sensitive method for sterility testing of frozen CAR-T drug products. Its rapid detection and comparability to the compendial sterility test make it an attractive option for routine testing.