IMPROVED TIME TO DETECTION OF SLOW-GROWERS MICROORGANISMS WITH AN AUTOMATED GROWTH-BASED METHOD

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INTRODUCTION

Automated Growth-based methods are used for rapid detection of microbial contamination in pharmaceutical products. Historically, regulators have classified such methods as "alternative" to the compendial U. S. Pharmacopoeia (USP) chapter <71> / European Pharmacopoeia (EP) chapter 2.6.1 (1, 2), requiring extensive validation prior to implementation. In recent years, the regulatory environment has been evolving to be more inclusive of rapid and alternative methods for designated products thanks to chapters such as the EP 2.6.27, USP draft <72> and USP <1071> (3-5).

EP 2.6.27 and USP <72> (3, 4) specifically support the use of technology of the BACT/ALERT® 3D DUAL-T solution as "compendial" with some restrictions. For short shelf-life products such as Cell And Gene Therapies (CAGT), every hour gained on release testing is crucial to quickly deliver therapies to patients in need. The BACT/ALERT® 3D DUAL-T system answers this need by providing automated, non-destructive, growth-based detection of most relevant microorganisms in less than 72 hours. Despite this, the current practice is to release the product after 7 days of incubation, due to the risk of contamination with "slow grower" microorganisms such as molds or *Cutibacterium acnes*. To improve the detection of such microorganisms, different culture media, growth factors, and incubation conditions were evaluated.

PURPOSE

To present data showcasing how optimization of BACT/ALERT® 3D DUAL-T incubation conditions can lead to notable improvements in Time-to-Detection (TTD).

MATERIALS AND METHOD

BACT/ALERT® 3D DUAL-T

- Growth/Respiration based method for detection of aerobic and anaerobic bacteria and fungi using a combination of aerobic & anaerobic industry bottles as required by regulatory bodies.
- Determines the presence of microorganisms based on dissolved CO_2 production monitoring using a colorimetric sensor and reflected light measurement.
- Continuous, automated, objective reading for early detection alerts.

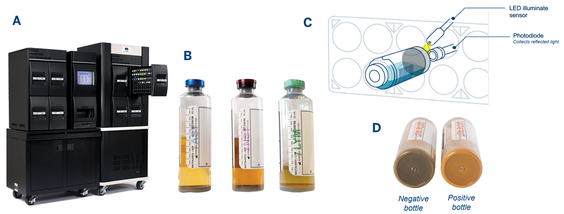

Figure 1. A/ BACT/ALERT® 3D DUAL-T system; B/ Industry culture media bottle; C/ Detection principle; D/ Colorimetric sensor indicative of positive result.

Study outline

- 11 microbial strains (aerobic and anaerobic bacteria, and fungi) were inoculated into BACT/ALERT® 3D DUAL-T Industry standard bottles (iAST, iNST or iLYM) or supplemented bottles in absence of product (Table 1).
- When applicable, supplementation consisted of addition of growth factor solutions (7-10) prepared in-house (Table 2) or commercially available ready-to-use supplement (BD BBL™ IsoVitaleX™) promoting growth of fastidious organisms.
- A minimum of 3 bottles were seeded at low concentration ≤ 10 CFU per bottle, with inoculum prepared from a fresh culture or a BIOBALL® and incubated at either 22.5°C, 32.5°C or 36°C.
- Inoculum levels were confirmed through enumeration on culture media plate.
- Un-inoculated bottles without/with supplementation were included as negative controls.
- Mean Time-To-Detection (MTTD) data for each microorganism was compared between the various conditions tested.

Table 1: List of microorganisms and incubation conditions evaluated.

Microorganism	Collection strain	Format	Initial Incubation conditions	Optimized Incubation conditions
Aspergillus brasiliensis	NCPF 2275	BIOBALL	iAST 22.5°C	iLYM 22.5°C
Bacillus spizizenii	NCTC 10400	BIOBALL	iAST 22.5°C	iAST 32.5°C
Candida albicans	NCPF 3179	BIOBALL		
Micrococcus luteus	Isolate	Fresh culture		
Pseudomonas paraaeruginosa	NCTC 12924	BIOBALL		
Streptococcus pyogenes	ATCC 19615	Fresh culture		
Bacteroides fragilis	ATCC 25285	Fresh culture	iNST 32.5°C	iNST 36°C or iNST 36°C + commercial supplement
Clostridium sporogenes	NCTC 12935	BIOBALL		
Cutibacterium acnes	DSM 1897	BIOBALL		
Phocaeicola vulgatus (formerly *Bacteroides vulgatus*)	ATCC 8482	Fresh culture		
Staphylococcus aureus	NCTC 10788	BIOBALL		

Table 2: List of growth factors evaluated against commercially available supplement.

Growth factors	Reference	Concentration of stock solution
Bovine Serum Albumin (BSA) Solution 30%	Sigma-Aldrich A9576	10 % (v/v)
Iron (III) nitrate nonahydrate	Sigma-Aldrich F8508	0,025 g/L
L-Cysteine hydrochloride mononhydrate	Sigma-Aldrich C7880	50 g/L
L-Cystine	Sigma-Aldrich C8755	1 g/L
L-Glutathione Reduced	Sigma-Aldrich G6013	50 g/L
N-Acetyl-L-cysteine	Sigma-Aldrich A9165	50 g/L
Nicotinamide-adénine-dinucléotide (NAD) hydrate	Sigma-Aldrich N7004	0,25 g/L
Tween 80	Sigma-Aldrich W291706	20% (v/v)

RESULTS

- All negative controls were confirmed negative after 7 days by the system & visual inspection (data not shown).
- All inoculum were ≤ 10 CFU/bottle with the exception of *B. fragilis* & *P. vulgatus* respectively at 21 & 12 CFU/bottle and *C. acnes* in iNST + L-Cysteine 50 g/L at 12 CFU/bottle.
- All aerobic microorganisms were recovered within 7 days at 22.5°C in iAST bottle (Figure 2). Increasing temperature to 32.5°C helped reduce MTTD of all aerobic bacteria tested, specifically *M. luteus* isolate.
- iLYM media, a fungal-specific aerobic bottle, improved recovery of *A. brasiliensis* at 22.5°C in less than 3 days (Figure 2).
- Anaerobic microorganisms were all recovered in iNST within 6 days at 32.5°C and within 5 days at 36°C; *C. acnes* had the longest observed MTTD (Figure 3).
- The addition of a commercial supplement reduced the MTTD on certain anaerobic microorganisms; in particular, the slow-growing organism *C. acnes*. The MTTD was reduced by approximately 1 day at 32.5°C and nearly 2 days at 36°C in absence of product.
- Supplementation with additional growth factors resulted in both delays and a range of MTTD improvements (Figure 4). The growth factors, Iron nitrate (0.25 g/L), NAD (0.25 g/L), BSA (10% v/v) and Tween 80 (20% v/v), had either limited effect on *C. acnes* growth or showed a detrimental effect. Bottle supplemented with sulfur-containing amino acids had observed faster MTTD of *C. acnes* (0.5 to 1 day gain) and were comparable at certain temperatures to the improvement observed with the commercial supplement.

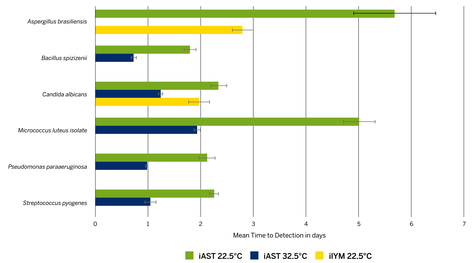

Figure 2: Mean time to detection in days of aerobic microorganisms using aerobic bottle iAST 22.5°C, iAST 32.5°C & fungal-specific aerobic bottle iLYM 22.5°C (n = 10).

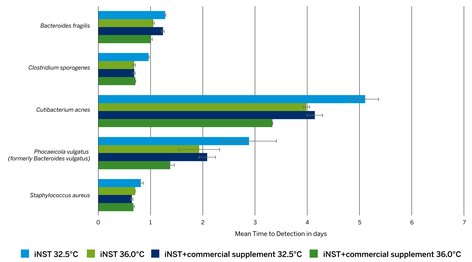

Figure 3: Mean time to detection in days of anaerobic microorganisms using iNST 32.5°C, iNST 36°C, iNST + commercial supplement at 32.5°C and iNST + commercial supplement at 36°C (n = 3 to 5 except for C. sporogenes, C. acnes & S. aureus *in iNST 32.5°C where n = 10).*

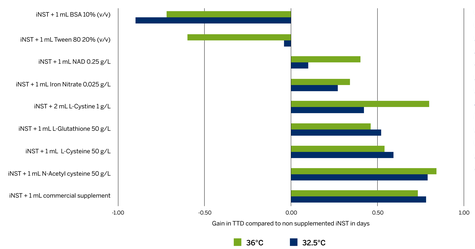

Figure 4: Gain in MTTD on C. acnes *recovery when using supplementation compared to non-supplemented iNST media at both 32.5 & 36°C (n = 3 to 5). Positive numbers indicate reduction in MTTD while negative numbers indicate a delay in MTTD.*

DISCUSSION

Sterility testing of CAGT products is described as compendial method in EP 2.6.27 (1) with an incubation time requirement of 7 days using both an aerobic & anaerobic media. Our data show that improvements in faster MTTD are possible and that the MTTD could be further optimized with a '3-bottle set'.

As previously described by Daane & Jeffrey (6), MTTD of molds can be optimized using a fungal-specific aerobic bottle (iLYM). Incubation at low temperature is critical to recover other environmental isolates including *Aspergillus* spp. or *Penicillium* spp. (6).

Increasing the incubation temperature from 22.5°C to 32.5°C for aerobic media improve that TTD of aerobic strains. Similarly, anaerobic bacteria will exhibit faster MTTD at 36°C.

Additionally, slow growing anaerobic organisms can be further optimized using growth factors (7-10) as Kumaran & Ramirez-Arcos (10) reported a gain in *C. acnes* MTTD of 1.2 days at 32°C in presence of platelets using commercial supplement. Similarly, we observed a gain of approximately 1 day at 32.5°C and nearly 2 days at 36°C with *C. acnes* in absence of product.

From the list of growth factors evaluated, sulfur-containing amino acids contributed positively to faster growth of *C. acnes* (9) and were comparable to the commercial supplement in certain conditions. However, more replicates should be performed to establish statistical significance.

Hence, a "3-bottle" set combination, iLYM 22.5°C - iAST 32.5°C - iNST 36°C, could help CAGT to further optimize their sterility testing method with an estimated gain in MTTD of about 1.5 days without supplementation or approximately 2 days with the addition of supplement in anaerobic media.

CONCLUSION

Automated growth-based BACT/ALERT® 3D DUAL-T system remains the method of choice for rapid sterility testing of CAGT products. Although, it is described as compendial method in EP 2.6.27 (1) with a 7 days incubation time, our data show that faster TTD is possible and could be further optimized. In particular, slow-growing anaerobic organisms can be improved through incubation at 36°C and/or supplementation of anaerobic media, leading to respectively 1 day and more than 1.5 days gain in MTTD in the absence of the product. Hence, an optimal '3-bottle' set (iLYM 22.5°C - iAST 32.5°C – iNST 36°C) and possible addition of supplement should be evaluated as part of the feasibility of an alternative method validation.

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