Microbial integrity test for preservative-free multidose eyedroppers or nasal spray pumps

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ABSTRACT

Multidose packagings for preservative-free nasal and ophthalmic drugs are well established. A critical quality attribute for such products is maintenance of a sterile container content during the whole lifetime. Tests used so far rely on the repeated challenge of the orifice using *Pseudomonas aeruginosa*. The new test design includes 2 more germs, which brings the procedure closer to the preservative efficacy tests. A mixed suspension containing *P. aeruginosa*, *Staphylococcus aureus* and *Candida albicans* was used to test an eyedropper. The challenge consists of the repeated submersion of the dosing orifice during actuation. The challenge period is done at ambient temperature and lasts for 4 days, followed by 3–5 days incubation at 32 °C. This incubation period is sufficient to cause turbidity of the content in the case of microbial ingress. This procedure is a relevant worst-case simulation and allows an assessment of the protective barrier function of the dispenser. The use of well-known indicator germs and the comparable short test period reduce the complexity of the method. The results allow reliable evaluation of the efficacy of the antimicrobial protective measures.

ZUSAMMENFASSUNG

Ein mikrobiologischer Integritätstest für konservierungsmittelfreie Mehrdosen-Augentropfer und Nasensprays

Konservierungsmittelfreie Mehrdosenbehältnisse haben sich für Nasalia und Augentropfen am Markt etabliert. Kritisch ist die Aufrechterhaltung der Sterilität des Flascheninhalts über die gesamte Lebenszeit des Produkts. Bisherige Tests basieren auf der wiederholten Challenge der Dosieröffnung mit *Pseudomonas aeruginosa*. Diese Methode wurde um relevante Keime ergänzt und an Tests zur Prüfung auf ausreichende Konservierung von Arzneimitteln angenähert. Am Beispiel eines Augentropfers wird ein mikrobiologischer Challenge-Test beschrieben, für den nun neben *P. aeruginosa* auch *Staphylococcus aureus* und *Candida albicans* verwendet werden. Die Dosieröffnung des Tropfers wird wiederholt in die gemischte Keimsuspension eingetaucht und das System betätigt. Diese Phase dauert 4 Tage und wird bei Raumtemperatur ausgeführt, gefolgt von einer 3- bis 5-tägigen Inkubationsphase bei 32 °C. Dieser Test simuliert eine Anwendung unter widrigsten Bedingungen und ermöglicht somit eine objektive Bewertung der Verlässlichkeit konservierungsmittelfreier Dosiersysteme. Die Verwendung etablierter Testorganismen und die vergleichsweise kurze Dauer vereinfachen die Durchführung und Bewertung der Versuche.

KEY WORDS

- microbial integrity testing
- preservative-free multidose systems
- eyedropper
- microbial contamination

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1. Introduction

Preservative-free multi dose dispensers are now well established on the market, e.g., for nasal spray or eye drop products. The technology behind such container closures systems ensures that the container content remains sterile during storage within shelf life and the in use period which lasts often at least for 28 days. For historically used standard multidose devices, the sterility of the formulation had to be maintained by the added preservatives, in most cases benzalkonium chloride (BAC). The diverse preservative-free technologies which make the use of preservatives obsolete (e.g., tip seal or shut-off valve mechanisms, silver-containing applicators) have been

around for a few years, but there is still no standard test procedure available to prove the microbial barrier function under harsh or simulated worst-case conditions. In the 1990s, the so-called Wiedemann test was developed to demonstrate the microbial safety of Aeropump's 3K® nasal spray pump and the Comod® eye dropper systems, which both contain silver as an antimicrobial barrier. The challenge consists of repeated exposure of the dosing orifice to a bacterial suspension containing *Pseudomonas aeruginosa*, a silver susceptible germ. In 2004, a tip seal integrity test was developed which also used this particular motile Gram-negative germ but applying some more challenging handling conditions [1]. Up to now this tip seal integrity test is used for the microbial characterization of the wide range of preservative-free multidose systems within the Aptar corporate group. Recently, a new tip seal integrity challenge procedure was developed to align it with the preservative efficacy tests described in the USP <51> [2] and EP 5.1.3 [3] (Table 1).

The new tip seal integrity test version TSIT 2.0 is based on a long series of experiments and reflects the collected experience with a wide range of dispensing devices. It is intended to provide a reasonable and standardized challenge procedure for preservative-free multidose dispensers. The setup of such a test should enable its use during the development of a new delivery system filled with growth medium as well as for final pharmaceutical products, which may contain formulations with potentially bactericidal properties.

2. General considerations for microbial challenge tests

A well-known and still widely used microbial testing procedure is the immersion of sealed test containers into a bacterial suspension and subsequent sterility tests of the container content. Such a probabilistic test method can be considered more uncertain in the assay results as tests rely on a series of sequential and/or simultaneous events, each associated with random outcomes described by probability distributions. Thus, the findings are associated with uncertainties that necessitate large sample sizes and rigorous test-condition controls to obtain meaningful results. In addition, such microbial challenge tests are prone to produce false positive results [4].

For a container closure system which also serves as a metering and dosing device, it is even more demanding to select and adapt an appropriate microbial test method, as the different materials and geometry of parts and functions may generate some technical hurdles for the challenge procedure. With respect to cross contamination, the sterile recovery of the container content for sterility testing is more often than not extremely difficult. The generation of appropriate damaged (positive) control samples pose an additional challenge as pump or dropper systems need to remain functional to stay as close as possible to the test samples. As authorities are familiar with such microbial tests, the manufacturers are often faced with requests to perform such studies. Therefore, it is wise to develop a reasonable microbial integrity test design and to complement this with data on the properties of the formulation related to microbial growth. Such "growth promotion" evaluation should follow the tests described in USP <51> and EP 5.1.3 for preservative efficacy testing. In addition, some microbial challenge tests according to the described methods with the final product configuration will certainly satisfy the assessors at the competent authorities.

2.1 Considerations for a microbial tip seal challenge test design

As mentioned, a well-known procedure for nasal spray pumps and eyedropper is the so-called Wiedemann test [5]. This test was developed according to the needs of the system, which releases silver ions into the formulation. The acceptance criteria are a low microbial burden of the delivered dose and no contamination of the bottle content. For this test, a single organism was selected: *P. aeruginosa*. This is a Gram-negative rod measuring 0.5–0.8 µm by 1.5–3.0 µm. Almost all strains are motile by means of a single polar flagellum. *Pseudomonas* is one of the most vigorous, fast-swimming bacteria seen in pond water samples and has very simple nutritional requirements. It is tolerant to a wide variety of physical conditions, including temperature. *P. aeruginosa* can also cause devastating infections in the human eye and is one of the most common causes of bacterial keratitis [6]. So it is one of the most relevant and challenging candidates to prove proper tip seal function for multidose dispensers under experimental conditions. On the other hand, mixed suspensions with a wider range of germs are closer to reality. Such a challenge suspension should contain Gram-negative and -positive bacteria as well as yeasts, which can be found even on healthy skin. During normal handling, these germs located on skin and mucosa may reach the dosing orifice.

In the past years, Aptar engaged several studies with a mixed germ suspension. Initially, a mix consisting of *P. aeruginosa*, *Staphylococcus aureus*, *Candida albicans* and *Escherichia coli* was used for tip contamination studies of the Ophthalmic Squeeze Dispenser (OSD, Aptar Pharma's preservative-free multidose eye dropper) to evaluate the survival time of the indicator germs on the surface of the dropper and how they are transferred into the next delivered doses. From these studies, it was concluded that *"the abundance of microbes is highly reduced with an ongoing drying of the applied fluid. Mainly Gram-negative bacteria are sensitive against such drying effects (none detectable after 4–6 hours). Also, viable yeasts disappear after 24 hours. Only Gram-positive bacteria like S. aureus are detectable after 24 hours"* [Aptar data 2009]. These results were confirmed in tests of 3 different eye

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■ **Table 1**

Comparison of Wiedemann and the established Aptar TSIT with the newly developed TSIT 2.0 (Source: the authors based on [1] and [5]).

	Wiedemann test	established Aptar TSIT	TSIT 2.0
Rationale for test	The test was developed and optimized for Comod and 3K system (1993–94).	Aptar TSIT adapted ~2004 to APF nasal spray pump.	• request from authorities (e.g., BfArM) for broader challenge based on antimicrobial effectiveness testing • EP 5.1.3 • USP <51>, <771>
Test medium	physiological saline	growth medium	growth medium, tryptic soy broth (TSB)
Indicator germs	*Pseudomonas aeruginosa* (ATCC 9027) $>10^6$ CFU/ml	*Pseudomonas aeruginosa* (ATCC 9027) $>10^7$ CFU/ml	• *Pseudomonas aeruginosa*, ATCC 9027 • *Staphylococcus aureus*, ATCC 6538 • *Candida albicans*, ATCC 10231 at least 10^6 CFU/ml for each germ
Challenge procedure	tip submersed in challenge suspension, then removed and actuated, 8 times within 4 days	tip submersed in challenge suspension and actuated, 10 times within 5 days	tip submersed in challenge suspension and actuated, 10 times within 4 days
Incubation temperature	during challenge period ambient temperature, afterwards at 32 °C	whole test period at 32 °C	during challenge period ambient temperature (20–25 °C), afterwards at 32 °C
Parameters analyzed	analysis of spray and container content	analysis of spray and container content	only analysis of container content (other parameters may be included on request)

drop systems (one preserved with benzalkonium chloride) containing 2 % povidone artificial tears following a tip contamination using a mix suspension consisting of *P. aeruginosa*, *S. aureus* and *C. albicans* [Aptar data 2010].

Based on these results, further tip seal integrity tests were performed using a mixed suspension consisting of *P. aeruginosa* (Gram-negative bacterium), *S. aureus* (Gram-positive bacterium) and *C. albicans* (yeast). *Escherichia coli* (Gram-negative bacterium) was no longer included due to its similar survival kinetics in comparison to *P. aeruginosa*.

According to the USP and EP chapters on preservative efficacy testing, spores from *Aspergillus brasiliensis* should also be used. For the current test design, the spores were not included since the challenge potential of an immobile particle with 4–5 µm diameter was judged to be extremely low [7]. Its inclusion would have also led to a much higher complexity of the study design, as the speed of growth and optimum growth temperatures are significantly lower compared to the other indicator germs used.

Based on the preliminary experiments and the recommendations received from regulatory authorities, it was decided to follow an approach using a mixed suspension consisting of *P. aeruginosa*, *S. aureus* and *C. albicans* at a concentration of at least 10^6 CFU/ml for each indicator germ.

2.2 New tip seal integrity test design and method validation

For the old test procedure, a challenge period of 5 days was used, which was now reduced to 4 days. This change was made in order to ensure that the challenge suspension is in its optimal state (exponential growth phase) and that the challenge procedure can be executed within a 5-day working week. The test items are challenged by a 4-day contamination phase. On the first 3 days each test item is challenged 3 times daily with an interval of approx. 3–4 hours. On the fourth day, a single challenge is executed resulting in a total of 10 challenges per test item as with the old test design.

The test samples are handled and stored at room temperature (20–25 °C) during the challenge period as done for the Wiedemann procedure and as recommended for such challenge procedures [8]. Following the last challenge, the samples are incubated for another 3–5 days according to the EP and USP guidelines at 30–35 °C to provide optimum growth conditions for any indicator germs which might invade the system (fig. 1).

For the validation of the new TSIT 2.0, some more extensive studies were performed to generate data on the reliability and reproducibility of the new test method. These tests were also used to establish a justification of the minimum incubation period after the challenge procedure.

OSDs with standard OSD Röchling 10 ml polyethylene bottles were filled with growth medium (Tryptic Soy

Table 2

Visible contamination of container content after inoculation with a low number of germs in a Tryptic Soy Broth-filled OSD.

	P. aeruginosa	*S. aureus*	*C. albicans*	Mixed suspension
Inoculum CFU/OSD	25	8	10	30
Turbidity of container				
1 day after inoculation	5 of 5 +	5 of 5 +	none	5 of 5 +
2 days after inoculation	5 of 5 +	5 of 5 +	5 of 5 +	5 of 5 +
Delivered drop analysis	5 of 5 +	5 of 5 +	5 of 5 +	5 of 5 +
+ turbid medium or positive for growth				

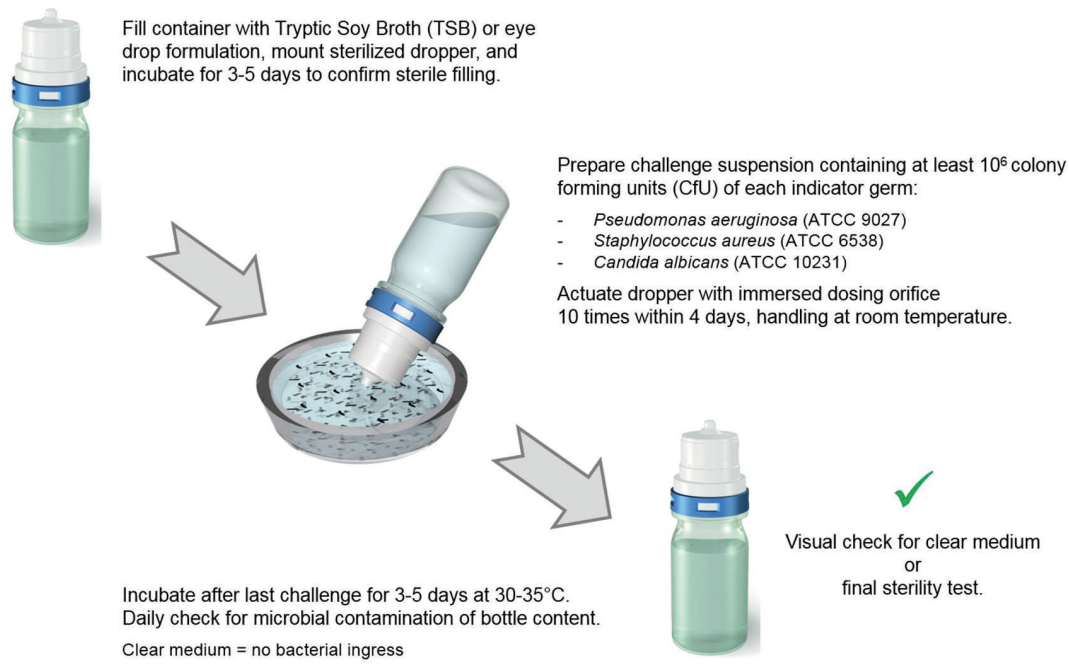

Figure 1: Principle of the TSIT 2.0 (Source: All figures were made by the authors.).

Broth, TSB) under sterile conditions. In order to simulate the challenge time of the TSIT, the filled containers were stored for 5 days at room temperature. Then the OSDs were opened under sterile conditions. 5 containers per microorganism species were inoculated with a low number of colony-forming units (CFU) of one of the respective test organisms and 5 containers were inoculated with a mixed suspension of all test microorganisms. One glass tube for each test organism suspension was inoculated for positive control. The inoculum for *P. aeruginosa*, *S. aureus*, *C. albicans* and the mixed suspension contained 25, 8, 10, and 30 CFU per OSD, respectively. Then the systems were reclosed and incubated at 30–35 °C for 2 days. At the first and second day after the inoculation, the potential microbial contamination of 1–2 delivered drops were tested using agar plates and the turbidity within the container was recorded (Table 2).

Each individual test organism including the mixed suspension of test organisms showed adequate microbial growth within the OSD already after 1 day (bacteria and mixed suspension) or 2 days (yeast) of incubation. On all agar plate subcultures derived from the delivered drops, the growth of the test microorganisms was confirmed in each of the inoculated OSD.

From this study it was concluded that a very low number (<100 CFU/container) is sufficient to cause visual turbidity of the OSD container content following at least 2 days of incubation.

In a second study, the bioburden within the dosage volume of an OSD after a single microbiological tip challenge was investigated. For the test, OSD filled with sterile TSB and a so-called "liner cap" (protection cap containing some porous material to speed up drying of remaining liquid) was used. After dipping the tip of the

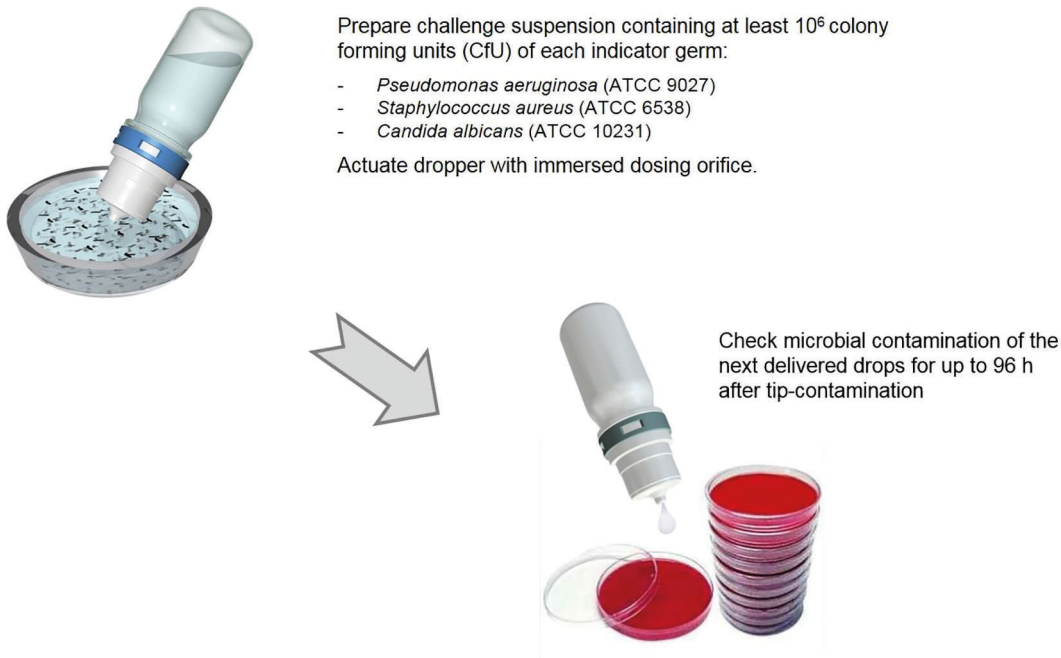

Figure 2: Principle of the next dose contamination study.

OSD into a challenge suspension containing approx. 4×10^6 CFU/ml of a mixed suspension of *P. aeruginosa*, *S. aureus* and *C. albicans*, the following drop doses were analyzed for microbial contamination for up to 4 days. At each time point, drops from 5 OSDs were analyzed by membrane filtration and/or plating methods (fig. 2).

Directly after the tip challenge approx. 5×10^3 CFU were found in each drop, rapidly decreasing within one day. On the next day after the challenge, a mean bioburden of 3.4 CFU was found in each drop. *S. aureus* showed the longest survival time of 3 days. 4 days after the challenge, no microorganisms could be detected within the dosed volumes.

This study indicates that a drop analysis following the challenge with a suspension containing *S. aureus* is not useful, as this particular indicator germ is able to survive at low numbers on the outer side of the dropper.

A complete study of the new tip seal integrity test in the designated design was done with additional sterility tests for the container content (membrane filtration test). For the test, the tips of the TSB media-filled OSD were repeatedly immersed into a challenge suspension. After the 4-day multiple challenge (a total of 10 challenges as described above), the test items were incubated at 30–35 °C for 5 days. Throughout the study, the medium within the container was visually analyzed for microbial growth. Additionally, after the incubation period a drop dose of each test item was transferred on agar medium for microbial growth evaluation. Then the OSD containers were opened and an aliquot of the contents was transferred on agar medium for additional growth evaluation.

For all 32 OSD samples, the container content remained sterile judged by a clear medium in the container and negative growth test on the agar plates. The positive controls with damaged dosing orifice had visual turbidity in the container at the end of the second challenge day (Table 3). The analyzed drops at the end of the incubation period were positive for microbial growth for all test samples and the positive controls. This was mainly *S. aureus* with partially delimited number of colonies (approx. >100 CFU) on the agar plates.

This finding confirms that an analysis of a delivered drop following the challenge procedure with *S. aureus* is not useful as some germs may survive outside the dropper without being able to contaminate the container content.

3. Summary

Although the updated USP <1207> chapter encourages the use of so-called deterministic methods (e.g., tracer liquid or gas tests or high voltage integrity testing), microbial challenge tests (probabilistic methods) remain in the test portfolio for multidose dispensers, which are designed to handle unpreserved formulations. Aptar Pharma has therefore developed a microbial tip seal challenge procedure further, which uses a well-established set of indicator germs. The considered indicator germs provide a robust and reliable challenge scenario, that is able to detect any relevant damages to the con-

Table 3

Results from a test run with the OSD.

Test day	Challenge total CFU/ml	Test samples	Positive controls	Negative controls
		32	3	3
1	4.3×10^6	–	–	–
2	6.8×10^6	–	3 of 3	–
3	5.1×10^6	–	3 of 3	–
4	8.6×10^6	–	3 of 3	–
9	–	–	3 of 3	–
Sterility test		–	not done	–
Dose analysis		32 of 32	3 of 3	–

(–) clear medium — number of samples positive for growth

tainer closure system. Using such a mixed microbial suspension is close to reality and not too complex for handling in the lab. Compared to the USP <51> and EP 5.1.3 chapters for preservative efficacy testing in preserved formulations, non-tip seal function-relevant germs were excluded.

The most relevant expected outcome for the tip seal integrity test is that the germs are not able to contaminate the remaining container content during dose delivery, as there will be no preservative inside to control microbial growth. Hence, the method provides the opportunity to perform sterility testing for the occurrence of indicator germs within the container if the test is performed with formulation instead of the growth medium. A useful complement to such a tip seal challenge is to test the particular formulation for some inherent antimicrobial properties according to USP <51> or EP 5.1.3 and to include the results into the risk analysis.

Less important is the microbial analysis of the delivered doses for microbial contamination. As demonstrated in different tests, Gram-negative as well as Gram-positive germs are markedly reduced when the surface of the closure system dries, but some robust germs like *S. aureus* may survive in low numbers for a few days. Therefore, a "next dose analysis" is of limited significance. Detecting the indicator germs in delivered doses following such a microbial challenge procedure only serves to confirm the quality of the recovery and analytical method. Furthermore, it should be considered that even preserved formulations are not able to eliminate potential microbial contaminations immediately either.

The described TSIT 2.0 procedure provides a significant challenge for preservative-free multidose devices as it uses a relevant challenge scenario. Combined with an analysis of the microbial properties of a formulation (growth promoting or even bacteriostatic) this method is well suited for the risk evaluation of new preservative-free multidose delivery systems.

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