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Test of Keofitt Aseptic Sampling Bag with 3-way Valve

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Prepared for: Keofitt A/S

Preface

This report describes the tests performed in the assessment of the Keofitt aseptic sampling bag with 3-way valve with regard to saturated steam sterilisability. The study has been carried out under the authors' guidance. The report is a complete and accurate description of the methods applied and the data obtained.

DB Lab A/S is a contract laboratory approved by the Danish Medicines Agency according to GMP. We perform chemical as well as microbiological analyses for the pharmaceutical industry, biotech companies, the foodstuff industry and the chemical industry among others. Furthermore, we are experienced in development and optimisation of fermentation processes, downstream processing and purification of fermentation products in pilot scale.

Odense, October 14th 2011

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Table of contents

1. Abstract

Keofitt aseptic sampling bag with 3-way valve was tested with regard to saturated steam sterilizability. 3-way valves coated with *Bacillus subtilis* spores were treated with flushing saturated steam at selected time intervals (5, 10, 20, 30, 40 *and* 60 seconds) in order to find the shortest time interval where no growth was observed after media fill. No growth was observed after 30 seconds of heat treatment with flushing saturated steam. Furthermore, valves were heat treated in two different ways: A) with saturated steam at 2 bar absolute (121°C) for 1 minute using a steam trap and B) with flushing saturated steam at 1.7 bar absolute, respectively, both followed by media fill. Test requirements were not fulfilled for 3-way valves heat treated with saturated steam at 121°C for 1 minute whereas all test requirements were fulfilled for 3-way valves heat treated with flushing saturated steam.

2. Introduction

This report presents the study of an aseptic sampling bag with a 3-way valve with regard to steam sterilisability. This system is manufactured by Keofitt Sampling Bags ApS. The 3-way valve and the connected sampling bag were sterilized by gamma irradiation prior to this study. The 3-way valves were coated with *Bacillus subtilis* spores. When microorganisms of the genus *Bacillus* are in the dormant spore stage, they are resistant to heat, radiation, disinfectants and desiccation which results in species being troublesome contaminants. Spores of *Bacillus subtilis* typically have D values at 121°C (saturated steam) of approximately 0.5min.

3. Definitions and abbreviations

B. subtilis	*Bacillus subtilis* ATCC 6633
CFU	Colony Forming Unit
Cf	confer
LAF	Laminar Air Flow
Min	minute
ml	milli liter
µl	micro liter
3-way valve	Keofitt Aseptic Sampling Bag with 3-way valve
TSA	Tryptone Soya Agar
TSB	Tryptone Soya Broth
D value	Decimal reduction time

The D value is the time required, at a certain temperature (eg. 121°C), to reduce the amount of a specific microorganism by 90% of the initial value.

4. Materials and methods

4.1 Product to be examined

The samples to be examined were supplied by Keofitt Sampling Bags ApS.

Table 1. Samples used in this study.

Sample	Lot no	Internal number
Aseptic sampling bag 500ml with 3-way valve	45481-0000	15437,01/15501,01

The protocol "P-11-056 1st edition. Protocol for test of Keofitt 3-way valve" was followed. 50µl instead of 100µl spore suspension were loaded to the 3-way valves to reduce the drying time.

4.2 *Bacillus subtilis* spore suspension

Bacillus subtilis ATCC 6633 was spread plated onto TSA and incubated at 37°C. After 5 days of incubation spores were observed at 1000× magnification and cells were collected and transferred to 50ml 0.9% NaCl in 250ml flask with glass beads and mixed for 3min. 2ml of the spore suspension was heat treated at 80°C for 10min to kill vegetative cells. This spore suspension was diluted and used for growth promotion test of liquid growth medium, determination of limit of detection and for the test of the Keofitt aseptic sampling bag with 3-way valve.

4.3 Growth promotion test and determination of limit of detection in TSB

Decreasing known numbers of *B. subtilis* spores were added in duplicate to test tubes containing TSB in order to determine the lowest number of spores that would result in visible growth in the TSB. The test tubes were incubated at 37°C and monitored on a daily basis in order to determine the minimum number of days required to obtain visible growth.

4.4 Determination of limit of detection in 3-way valve

Decreasing known numbers of spores were added in triplicate to 3-way valves and dried out in the LAF bench. When the 3-way valves were dried, 20ml TSB was added through the valve into the sampling bag and then incubated at 37°C and monitored on a daily basis in order to determine the minimum number of spores and the minimum number of days required to obtain visible growth in the sampling bag.

4.5 Test of aseptic sampling bag with 3-way valve

4.5.1 Test of steam flushing of 3-way valves at selected exposure times

The purpose of this first test was to determine the shortest steam flushing time required to kill or remove all spores.

The test consisted of 18 3-way valves. 12 3-way valves were spiked with $\geq 10^6$ *Bacillus* spores and exposed to flushing steam at selected exposure times in order to find the minimum required time of exposure of flushing steam to prevent growth of *Bacillus* spores. 1L flasks with 100ml of TSB were connected to the exhaust tubes on the 3-way valves for the detection of any live *Bacillus* spores.

Six control 3-way valves were included: Two positive control 3-way valves (spiked with *Bacillus* spores, no heat treatment), two negative controls (not spiked with *Bacillus* spores and no heat treatment) and two negative steam controls (not spiked with *Bacillus* spores. Heat treated for 1 minute).

The steam was generated from an AWA 30 autoclave (a photo of the test setup can be viewed in Appendix A). Aseptic techniques were applied to all handling steps with the 3-way valves in order to minimize the risk of contamination.

50µl of spore suspension containing approximately ≥ 10^6 spores were added to the inner surfaces of 14 3-way valves (12 valves for test and two valves for positive controls). The valves were dried overnight at room temperature under LAF.

The 3-way valves were connected to the steam generator tube at the inlet valve and exhaust tubes were connected to 1L flasks.

Two steam negative control 3-way valves were treated with flushing steam for 1 minute and spiked 3-way valves were tested in duplicate at the following exposure times: Approximately 5 seconds, 10 seconds, 20 seconds, 30 seconds 40 seconds and 1 min, respectively. The steam from the exhaust tubes were collected in sterile 1L flasks containning 100ml TSB and incubated at 37°C up to 5 days. Visually observed growth was examined by microscopy. After heat treatment the 3-way valves were transferred to a LAF bench and handled according to Keofitt instruction, cf. Appendix B.

After handling according to Keofitt instruction, 20ml of sterile TSB was poured through all the 3-way valves and collected in the 500ml sampling bags. The 3-way valves and bags were subsequently incubated at 37°C for 3-5 days. After incubation, the sampling bags were visually inspected for growth.

4.5.2 Test of heat treatment with saturated steam of 3-way valves

The purpose of this second test was to confirm the repeatability of the 60 seconds heat treatment at two different conditions: A) Steam trap connected to the exhaust and B) free flowing steam to the drain.
The test consisted of 18 3-way valves. Six 3-way valves were spiked with ≥ 10^6 *Bacillus* spores and exposed to saturated steam at 121°C for 1 minute. 1 bar of overpressure was reached by the connection of a steam trap to the exhaust tube on the 3-way valve. Furthermore, six 3-way valves were spiked with ≥ 10^6 *Bacillus* spores and exposed to flushing steam for 1 minute. Six control 3-way valves were included: Two positive control 3-way valves (spiked with *Bacillus* spores, no heat treatment), two negative controls (not spiked with *Bacillus* spores and no heat treatment) and two negative steam controls (not spiked with *Bacillus* spores. Heat treated for 1 minute).

After heat treatment the 3-way valves were transferred to a LAF bench and handled according to Keofitt instruction, cf. Appendix B.

After handling according to Keofitt instruction, 20ml of sterile TSB was poured through all the 3-way valves and collected in the 500ml sampling bags. The 3-way valves and bags were subsequently incubated at 37°C for 3-5 days. After incubation, the sampling bags were visually inspected for growth.

4.6 Technical assistance

The experiments were performed by Allan Nielsen, Jesper Pedersen and Marianne Hansen September - October 2011.

5. Results & Discussion

5.1 Growth promotion test and limit of detection in TSB

Bacillus subtilis spores ranging from 3×10^6 spores to 3 spores in 10-fold dilutions were added in duplicate to test tubes with 10ml TSB and incubated at 37°C. After 24h of incubation visible growth were observed in all tubes. No growth was observed in the negative controls. Thus, addition of as low as 3 spores resulted in visible growth after 24h of incubation.

5.2 Limit of detection in 3-way valve

Bacillus subtilis spores ranging from 3×10^6 spores to 3 spores in 10-fold dilutions were added in triplicate (A, B, and C) to 3-way valves, dried overnight, TSB added and incubated at 37°C. After 2 days of incubation visible growth was observed from 3-way valves listed with "+"in table 2. No growth was observed in negative controls. Only two out of three 3-way valves spiked with 3000, 300 and 30 spores, respectively, resulted in growth in the sampling bags. No growth was observed from 3-way valves spiked with approximately 3 spores. Some variation in the results must be expected when working with living organisms.

The limit of detection of *B. subtilis* spores in the 3-way valves is expected to be approximately 30 spores after minimum two days of incubation at 37°C.

Table 2. Detection limit of *B. subtilis* spores in 3-way valves

Spores added	Visually observed growth		
	A	B	C
3×10^6	+	+	+
3×10^5	+	+	+
3×10^4	+	+	+
3×10^3	+	+	÷
3×10^2	+	+	÷
30	+	+	÷
3	÷	÷	÷

+ Growth, ÷ No growth.

If no growth is observed in sampling bags with 3-way valves, the result will be reported as < approximately 30 spores.

5.3 Test of steam flushing of 3-way valves at selected exposure times

The number of spores added to each 3-way valve were determined to 3×10^6 spores. The 3-way valves were tested in duplicate (A and B) at the following exposure times: Approximately 5 seconds, 10 seconds, 20 seconds, 30 seconds 40 seconds and 1 min, respectively. The steam from the exhaust tubes were collected in sterile 1L flasks containing 100ml TSB and incubated at 37°C. After 1 day of incubation growth was observed in all flasks. The growth was examined by microscopy and consisted of motile rods (approximately 1µm ×4-8µm). Spores were also observed.

All sample bags containing TSB were visually inspected after 1 day and 2 days of incubation at 37°C. Growth was observed from one 3-way valve flushed for 5 seconds and from two 3-way valves flushed for 10 seconds and 20 seconds, respectively. No growth was observed from 3-way valves flushed for 30 seconds, 40 seconds and 1 minute, respectively, cf. table 3.

Surprisingly, there was no growth from sample B after exposure of flushing steam for 5 seconds. This result is not in line with results obtained after 10 and 20 seconds of exposure of flushing steam where growth was observed in both duplicate samples. One possible explanation is that no or very few spores were attached to the inner surface of the first part of the valve when the spores were loaded. If the majority of the spores then ended up in the valve rod cavity they could have been pushed away by the valve rod action and thus not exposed to TSB when the sampling bag was filled through the 3-way valve.

Table 3. Results from steam flushing of 3-way valves at selected exposure times.

Time of steam exposure	3-way valve		Flasks exposed to exhaust steam	
	A	B	A	B
5 seconds	+	÷	+	+
10 seconds	+	+	+	+
20 seconds	+	+	+	+
30 seconds	÷	÷	+	+
40 seconds	÷	÷	+	+
60 seconds	÷	÷	+	+
Neg. control	÷	÷	÷	÷

÷ ~ < 30 spores (limit of detection)

In the control samples only growth from the spiked 3-way valves were observed. No growth from negative control 3-way valves or negative steam control 3-way valves was observed, indicating that the 3-way valves were sterile prior to the test and the steam used and experimental setup was appropriate for the test, cf. table 4.

Table 4. Results from visual inspection of control sampling bags with 3-way valves after 2 and 5 days of incubation at 37°C. ÷ No visible bacterial growth. + Visible growth.

Test no.	Negative control	Negative steam control	Positive control
1	÷	÷	+
2	÷	÷	+

During the flushing of steam the pressure in the 3-way valve was approximately 1.7 bar absolute. The results indicate that flushing of steam through the 3-way valve for 30 seconds is sufficient to remove 3×10^6 spores of *B. subtilis*. The majority of the spores are removed from the 3-way valve and not killed. With a D value (121°C) of *B. subtilis* of approximately 0.5 min the number of living cells after exposure of steam at approximately 0.7 bar overpressure for 30 seconds should be more than 3×10^5 spores. Living spores were detected in the exhaust steam (cf. table 3) and thus support that the spores are washed out of the 3-valve and not killed. However, the

flushing of steam was efficient since no spores were detected after 30 seconds, 40 seconds and 60 seconds, respectively. These results also indicate that spores of *Bacillus subtilis* were not tightly attached to the interior of the 3-way valve due to the smooth and confined area that was flushed with steam.

5.4 Test of heat treatment with saturated steam of 3-way valves

The number of spores added to each 3-way valve were determined to 3×10^6 spores. Six 3-way valves exposed to saturated steam at 121°C for 1 minute all failed the test, since growth was observed in all sampling bags. The six 3-way valves exposed to flushing steam for 1 minute all passed the test, since no growth was observed in any of the six sampling bags, cf. table 5. Even though no steam trap was used in the latter test a counter pressure of approximately 0.7 bar, due to the small opening in the 3-way valve, was observed during the flushing of steam.

Table 5. Results from visual inspection of test sampling bags with 3-way valves after 2 and 5 days of incubation at 37°*C*.

Test no.	Steam trap connected	Approximate overpressure, bar	Result
1	Yes	1	Growth
2	Yes	1	Growth
3	Yes	1	Growth
4	Yes	1	Growth
5	Yes	1	Growth
6	Yes	1	Growth
7	No	0.7	No growth
8	No	0.7	No growth
9	No	0.7	No growth
10	No	0.7	No growth
11	No	0.7	No growth
12	No	0.7	No growth

As discussed in pt. 5.3, 1 minute of exposure of saturated steam at 121°C is not sufficient to kill all spores (3×10^6 spores). The speed of the flow of steam was slowed down when the steam trap was connected and thus prevented the spores from being washed out of the 3-way valve. When no steam trap was connected, the spores were all washed out of the 3-way valve and thus no growth was observed in the sampling bags.

6. Conclusion

The performed tests show that under the conditions described, the Keofitt aseptic sampling bag with a 3-way valve can be considered as steam sterilisable by flushing of saturated steam for 1 minute. Tests have revealed that after approximately 30 seconds of flushing with saturated steam no growth of the added spores (3×10^6) could be detected. Flushing of saturated steam for less than 30 seconds resulted in growth from the 3-way valves. Growth was also observed from flasks connected to the exhaust tubes of the 3-way valves during flushing of steam. These observations strongly indicate that the spores are not all killed in the 3-way valve during heat treatment, but efficiently washed out.

7. Documentation

This report as well as the analytical results are filed at DB Lab A/S under "validations" in fire cabinet.

8. References

Protocol P-11-056 1st edition. "Protocol for test of Keofitt 3-way valve".
Bergey's Manual of Systematic Bacteriology, 2nd edition, Vol. Three, The Firmicutes, 2009, Springer

9. Appendix

Appendix A: Photos of test setup at DB Lab A/S
Appendix B: Keofitt aseptic sampling bag handling instructions.

DB Lab

Appendix A

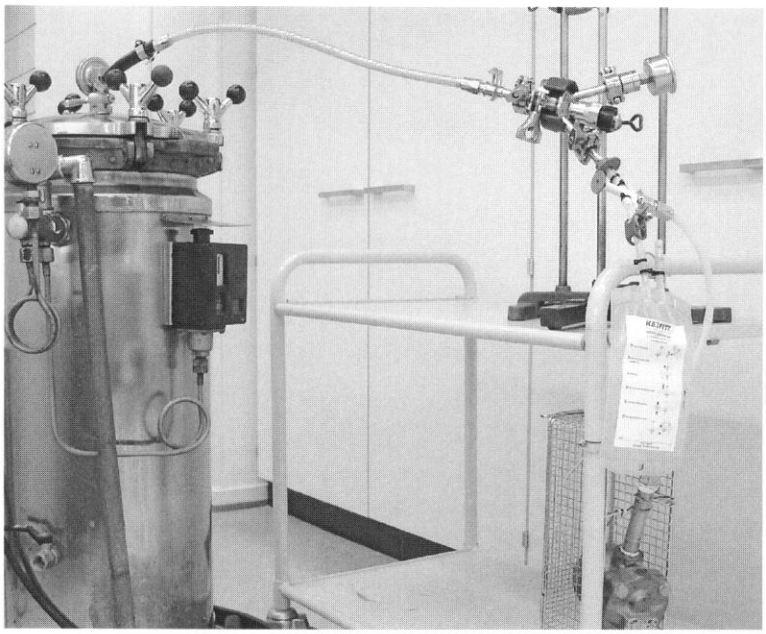


Photo 1.
Test set up with 3-way valve connected to steam generator at the inlet and steam trap at the exhaust.

Photo 2.
Test set up with 3-way valve connected to steam generator at the inlet and a sterile flask with TSB at the exhaust.

KEOFITT®

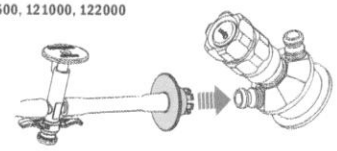
SAMPLING DISPOSABLES™

ASEPTIC SAMPLING BAG

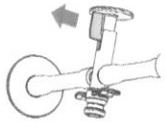
⚠ USE PROTECTION GOGGLES AND GLOVES

Item no. 120500, 121000, 122000

1. ATTACH TO VALVE OUTLET

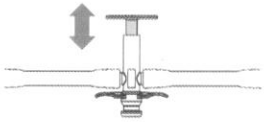


2. STEAM OR FLUSH STERILISATION (WARNING HOT)

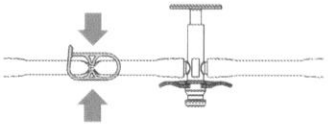


3. REMOVE SEAL

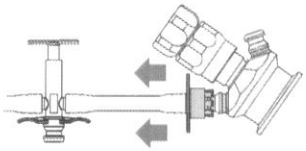
4. PUSH VALVE TO FILL BAG AND PULL TO STOP



5. PUSH PINCH CLAMP TO SEAL BAG



6. PULL QUICK COUPLING TO RELEASE



NOTE:

PATENT PENDING

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