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Application Note

Detection of low-level bacterial contamination on stainless steel using ICR Swabs and Contact Plates

Manufacturing drugs in isolators is a clearly observable trend in the pharmaceutical industry that has raised the necessity to detect microorganisms on isolator surfaces, many of which are made of stainless steel.

Contact plates and swabs are commonly used for surface monitoring in the aseptic manufacturing of pharmaceuticals. Both methods are recommended by the current European and US GMP guidance.

The published recovery rates for swabs and contact plates vary due to differences in the chosen methods, surfaces and test microorganisms. This variability is also reported in USP chapter <1116>. The methods used should be able to detect low levels of microorganisms, especially in grade A cleanrooms or isolators.

This study was designed to prove the suitability of ICR Swabs **(Ref. No. 146529)** and lockable TSA w. LTHThio contact - ICR+ **(Ref. No. 146783)** plates, to detect low numbers of different bacterial test strains from stainless steel coupons. The ICR Swab is designed for presence/absence tests on dry and hard-to-access surfaces, whereas ICR contact plates are suitable for the enumeration of microorganisms on flat, dry surfaces in cleanrooms and isolators. As a control, the "agar overlay" method was chosen to determine the number of surviving microorganisms.

Material & Methods

Stainless steel coupons (5 cm x 5 cm plates) were cleaned and autoclaved. One surface of each was then inoculated with ten 10 µL drops of one of two different dilutions (high and low inoculation levels) made from the microbial suspensions of four bacteria strains (in the case of *Bacillus subtilis*, its endospores). A homogeneous dispersion was obtained on the surfaces. **Table 1** lists the chosen microorganisms and the CFU count in the total volume (100 µL) of each dilution used to inoculate the test surfaces, as determined by the spread plate method on 90 mm TSA plates. A higher inoculum level was used for Gram-negative bacteria because they were expected to be more sensitive towards the subsequently applied drying procedure of the inoculated surfaces.

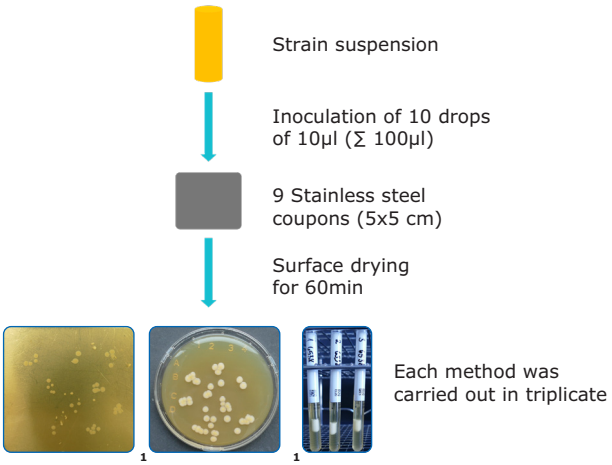
Table 1. Selected Test Stains and CFU contained in 100 µL of the respective dilutions

Test strain	High inoculation levels	Low inoculation levels
Staphylococcus aureus **ATCC® 6538**	93	24
Bacillus subtilis **ATCC® 6633 (spores)**	26	6
Escherichia coli **ATCC® 8739**	650	130
Pseudomonas aeruginosa **ATCC® 9027**	228	32

Each steel plate was dried for 60 min under a laminar flow hood without air stream. The detection of the microorganisms from the dried surfaces was performed in parallel using 3 different methods and each test was performed in triplicate. The procedures are described as follows and shown in **Figure 1.**

1. **Agar overlay:** As the control method the "agar overlay" method was chosen. Assuming that a high number of microorganisms would be killed by the drying process, this test method was used to determine how many can survive on the steel surface. The molten TSA agar was poured onto the inoculated and subsequently dried steel surface. All plates were incubated for up to seven days at 30-35 ˚C. The colonies were counted every day.
2. **Contact plate method:** The dried stainless steel surface was sampled using a TSA w. LTHThio contact - ICR+ plate, which was pressed on the test surface for 10 seconds with a standardized pressure of 500 g. All plates were incubated for up to seven days at 30-35 ˚C. The colonies were counted every day.
3. **Swab method:** The dried surface was carefully sampled with the premoistened swab tip of an ICR Swab in longitudinal and cross directions. The broth medium from the reservoir was then squeezed onto the swab tip, and the swab incubated at 30-35 ˚C for up to seven days to take into account a prolonged lag phase due to desiccation. The tubes were inspected for turbidity every day.

Figure 1. Test procedure for the detection of microbes on the stainless-steel coupons by the agar overlay method, TSA w. LTHThio contact- ICR+ plates and ICR Swabs



Incubation for up to 7 days at 30-35 ˚C and daily evaluation (CFU on overlay and ICR contact plates, turbidity of Swab media)

1 = e.g. *S. aureus* (high inoculum) after 48 h incubation

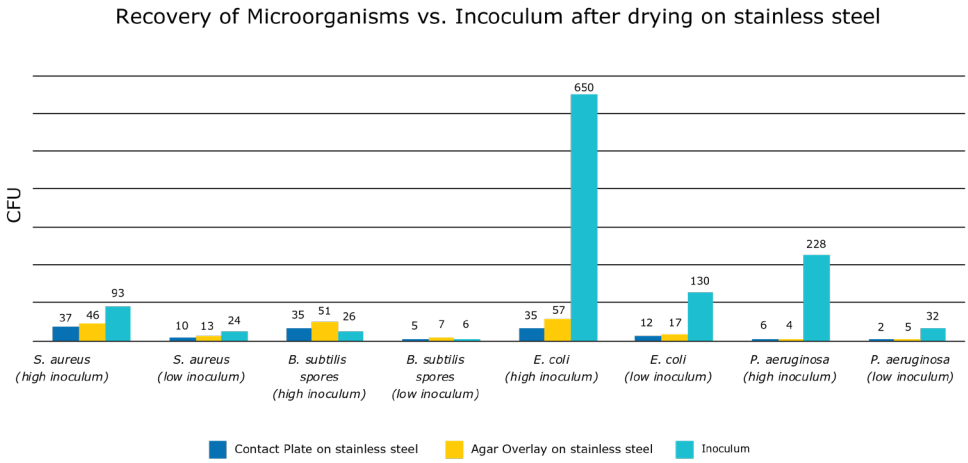
Results and Discussion

1. "Agar overlay" method vs. ICR Contact Plates

The detected CFUs for the test strains when using the agar overlay and ICR Contact Plate methods are indicated in **Figure 2**. **Table 2** shows the approximate recovery rates in percent. The listed percentage ranges cover the results of both the low and the high dilutions and were calculated relative to the respective inoculum counts. All positive results were achieved within an incubation period of 3 days. Prolonging incubation to up to 7 days did not lead to higher recoveries.

The recoveries of the surviving microorganisms varied depending on the bacterial strain. The Agar Overlay method recovered higher CFUs than the ICR contact plate in most cases.

Figure 2. Survival in CFU of test strains on stainless steel after a drying period of 60 minutes (averages of 3 replicates)

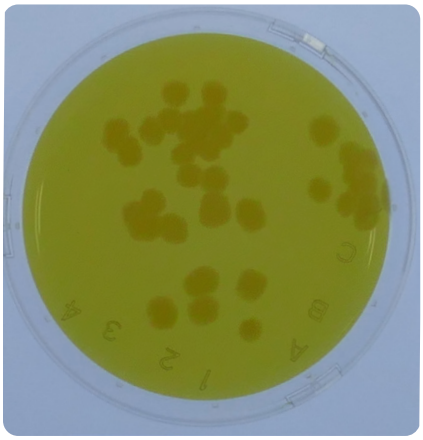


Note: For *Bacillus subtilis* spores with the agar overlay method, only one out of the three replicates of each inoculation level was countable, with the others showing coalesced colonies. For *P. aeruginosa* with the agar overlay method, the CFU was calculated based on only two replicates, with the third being negative at both the high and low inoculation levels.

Table 2: Approximate Recovery Rates using ICR Contact Plates after 60 min of dehydration on the stainless-steel coupon and with the "Agar Overlay" method. For *Bacillus subtilis* spores with the agar overlay method, only one out of the three replicates of each inoculation level were countable, with the others showing coalesced colonies. For *P. aeruginosa* with the agar overlay method, the CFU was calculated based on only two replicates, with the third being negative at both the high and low inoculation levels

Method	*S aureus*	*B.subtilis (spores)*	*E.coli*	*P. aeruginosa*
Agar Overlay	50-54 %	116 - 200 %	8-13 %	2-16 %
ICR Contact Plate	41 %	83-134 %	<10 %	<6 %

Figure 3 Growth from *Bacillus subtilis* spores that were recovered from stainless steel plates when using the overlay method and contact plates (same inoculation level)



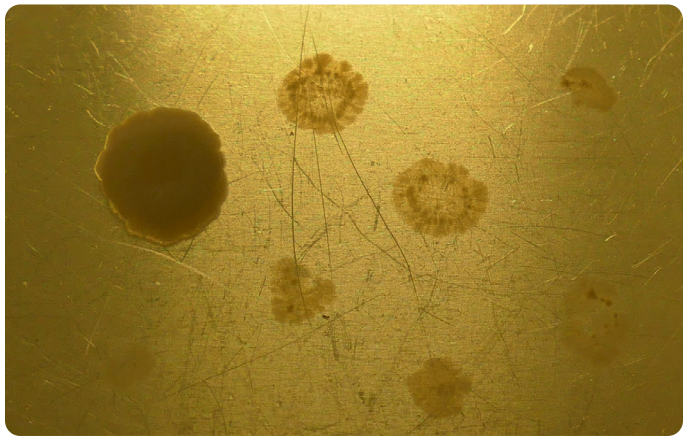


Figure 3. (left) illustrates the bacterial growth performance from *Bacillus subtilis spores* that were recovered from stainless steel plates with the overlay method (using TSA agar). The photo suggests that *Bacillus subtilis spores* tended to cluster in the original drop after drying and were not separated by applying the melted agar to form individual colonies. For both inoculation levels, colonies were observed on all three replicates, but countable only on one, because the colonies were coalesced on the other two replicates. The contact plate method **(right)** provided a better distribution of the colonies, and these were therefore countable. The high recoveries with all three ICR contact plate replicates (83-135 %) clearly showed that the spores of *B. subtilis* were the most resistant towards dehydration, followed by *S. aureus* with 41 %. The Gram-negative bacteria *E. coli* and *P. aeruginosa* proved much more sensitive towards the dehydration of the test surface and resulted in recovery rates of less than 10 % with the ICR contact plates. Overall, the overlay method resulted in slightly higher recoveries.

2. Comparison of detection rates when using ICR Swabs, the overlay method and ICR Contact Plates

Table 3 shows the colony counts of each replicate for contact plates and the agar overlay method as well as the number of positive results with the ICR swab. The results demonstrate the suitability of ICR swabs to detect even low numbers of microorganisms on stainless steel surfaces. Particularly the result that *P. aeruginosa* yielded positive ICR Swab results at an inoculation of ≤ 3 CFU suggest the suitability of ICR swabs to detect low level bacterial contaminations.

Table 3. Recoveries in CFU from stainless steel plates using contact plates, the agar overlay method and the number of positive results with ICR swabs

Test Strain	Inoculated CFU to Surface	CFU ICR Contact Plate 1	CFU ICR Contact Plate 2	CFU ICR Contact Plate 3	CFU Agar Overlay Plate 1	CFU Agar Overlay Plate 2	CFU Agar Overlay Plate 3	Positive ICR Swabs
S. aureus	24	9	10	12	14	15	10	3 of 3
	93	42	38	31	40	58	41	3 of 3
***B. subtilis* (spores)**	6	3	6	7	positive*	positive*	7	3 of 3
	26	24	39	43	51	positive*	positive*	3 of 3
E. coli	130	10	13	13	21	16	15	3 of 3
	650	36	29	41	61	56	55	3 of 3
P. aeruginosa	32	1	3	1	8	6	0	3 of 3
	228	3	9	7	12	0	1	3 of 3

*positive: growth visible, but no individual colonies countable

Conclusion

In summary, this study showed that both ICR Swabs and ICR contact plates are suitable to detect low numbers of Gram-positive and Gram-negative microorganisms from stainless steel surfaces.

Despite the low bacterial count of some samples, the ICR Swab results were always positive and therefore comparable to the contact plates.

Surprisingly, the overlay method did not prove to be the best method to detect *Bacillus subtilis* spores.

Possible explanations are that the stainless-steel surface could prevent the homogeneous spreading of the spores on the surface or that the spores could have a tendency to build aggregates during pouring of the liquid media onto the slide.

The calculated approximate recovery rates after 60 minutes of dehydration showed the survival rates of Gram-negative bacteria like *Escherichia coli* and *Pseudomonas aeruginosa* to be lower than those of Gram-positive strains. This was observed with both the contact plates and the overlay method.

Literature and further readings

1. FDA Guidance for Industry (2004): Sterile Drug Products Produced by Aseptic Processing – Current Good Manufacturing Practice.
2. United States Pharmacopoeia 40 NF 35: <1116> Microbiological Control and Monitoring of Aseptic Processing Environments.
3. EU GMP Guide (2008): Volume 4 EU Guidelines to Good Manufacturing Practice Medicinal Products for Human and Veterinary Use - Annex 1 Manufacture of Sterile Medicinal Products (corrected version).
4. Application Note - Detection of Low Bacterial Contamination from Neoprene® Gloves with ICR Swabs.

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09/2020