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Boom, F.A.; Brun, P.P.H. le; Buehringer, S.; Sirks, M.; Touw, D.J.

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Microbiological controls of aseptic handling in Dutch hospital pharmacies: Results, limits, and methods for assessing.

Frits A. Boom [a,*], Paul.P.H. Le Brun [b], Stefan Bühringer [c], Madeleine Sirks [d], Daan J. Touw [e]

[a] Department of Clinical Pharmacy, Zaans Medical Center, Koningin Julianaplein 58, Zaandam 1502 DV, the Netherlands
[b] Department of Clinical Pharmacy and Toxicology, Leiden University Medical Center, the Netherlands
[c] Biomedical data Sciences, Medical Statistics and Bioinformatics, Leiden University Medical Center, the Netherlands
[d] Laboratory of Dutch Pharmacists, Royal Dutch Pharmacists Association KNMP, The Hague, the Netherlands
[e] Department of Clinical Pharmacy and Pharmacology, University Medical Center Groningen, University of Groningen, the Netherlands

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ABSTRACT

Introduction: 'Aseptic handling is the procedure to enable sterile products to be made ready to administer using closed systems' (EU Resolution CM/Res(2016)2). Microbiological controls are an important part of the overall assurance of process and product quality of aseptic handling. They consist of the End-of-Session Broth Test (ESBT) using Tryptone Soya Broth, Microbiological Monitoring (MM) using Ø 55- and 90-mm agar plates and a periodical Operator Broth Transfer Validation Test (OBTVT) using Tryptone Soya Broth. This study describes the results of these controls over a 7-year period, involving between 44 and 49 pharmacies (mostly hospital pharmacies). All pharmacies use a web-based programme for processing, evaluation and assessing microbiological controls ('Microbio'). Aggregated results in Microbio are used for benchmarking and feedback information.
Objective: The objective of this study is to analyse the results of the 7-year period and to develop methods for assessing of, and determining realistic limits for, microbiological controls during aseptic handling. As secondary objective the role of Microbio is highlighted.
Results and discussion: Results of ESBT are expressed as Contamination Rate (CR), which is the percentage of units filled in a process simulation that are positive for microbial growth after incubation. Compared with the first 3 years of the study, the results in the last 4 years were significantly better: mean CRs are 0.20 and 0.11, respectively (p-value <0.01). For assessing CRs of ESBT, the approach 'the more frequent samples with growth, the stronger the corrective actions' was adopted. Levels of investigative and corrective actions, based on the 95% Upper Confidence Limit, are suggested. Microbiological Monitoring (MM) during aseptic handling into a laminar airflow cabinet or safety cabinet consists of settle plates, glove prints, contact plates of the worktop and surface bioburden determination of disinfected ampoules and vials. The results are expressed as the Contamination Recovery Rate (CRR), which is the rate at which MM samples contain any level of contamination. During the study period, the results of glove prints and contact plates improved substantially. The most probable explanation of this finding is improved disinfection procedures of the gloved hands, the worktop inside LAF/SC and the ampoules and vials. Results of settle plates did not change. There were too few results available to evaluate the surface bioburden of disinfected ampoules and vials. Benchmarking and feedback information from Microbio may have contributed to the improved ESBT and MM results. Results of the Operator Broth Transfer Validation Test (OBTVT) are expressed as Contamination Rate (CR). The target is zero samples with growth (CR = 0). The overall CR result over the study period is 0.50%. This is worse than ESBT (overall CR is 0.14%). This is probably due to the high number of critical steps in OBTVT compared to ESBT.
Conclusion: Results of microbiological controls improved during the study period. Realistic limits as well as methods for assessing ESBT and MM results are given and discussed.

* Corresponding author.
E-mail address: Boom.f@zaansmc.nl (F.A. Boom).

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1. Introduction

'Aseptic handling is the procedure to enable sterile products to be made ready to administer using closed systems' (Resolution CM/Res, 2016). The starting materials are sterile and must be kept so during this process (Boom and Beaney, 2015). Aseptic handling itself is performed in a laminar airflow cabinet (LAF), a safety cabinet (SC) or in an isolator (I). The background area is the room where the LAF, SC or I are housed.

Microbiological controls are an important part of the overall quality assurance of process and product of aseptic handling. They consist of Aseptic Process Simulation (APS; also known as media fill), Microbiological Monitoring (MM), and the Operator Broth Transfer Validation Test (OBTVT) (Boom and Beaney, 2015). In aseptic processing, which is comparable to aseptic handing, a sterility test is normally carried out as a release test. However, because of the limited number of samples in a batch (often only one product), a sterility test with a representative number of samples is not possible in aseptic handling. Instead, a daily APS of one broth preparation can be considered as a useful alternative (Beaney, 2016; Boom et al., 2012; Crauste-Manciet et al., 2020). This test is called 'End-of-Session Broth Test' or 'On-going Simulation Test'. In this article we will use the term 'End-of-Session Broth Test' (ESBT). Because of the short time between preparation and administration (between hours and days), results of ESBT are generally not available at the time of administration. Therefore, these results have to be considered as process controls.

ESBT consists of a broth preparation which comprises all critical steps (see definition in Appendix A) that occur during aseptic handling (Boom en Beaney, 2015). For assessing the results, a Contamination Rate (CR) of $\leq$ 1% (95% UCL) is recommended (LNA, 2010). This rate is defined as the percentage of units filled in a process simulation that are positive for microbial growth after incubation (TR 22, 2011). However, taking zero samples with growth as the target and considering samples with growth as a coincidence seems to be more likely. This approach has been applied in EU GMP Annex 1 and means: the more frequent samples with growth, the stronger the corrective actions (EU GMP Annex 1, 2009). If the levels for samples with growth mentioned in Annex 1 are adjusted to aseptic handling, this approach can also be applied to this process (Boom et al., 2012).

MM of aseptic handling inside a LAF/SC consists of air sampling, glove prints, worktop prints and surface bioburden determination of disinfected ampoules and vials (LNA, 2019a). The Contamination Recovery Rate (CRR) is recommended for the assessment of the results (Boom et al., 2020a). This rate is defined as the percentage of samples that show any microbial recovery, irrespective of the number of cfu (USP 35, 2012). A CRR of less than 10% is recommended for MM results inside a LAF/SC (Z3. Aseptic Handling, 2022). Background information on the CRR is given in a previous article (Boom et al., 2020a).

OBTVT consists of a repeated number of aseptic key techniques and must be completed without growth in any sample (UK Pharmaceutical Aseptic Services Committee 2006; LNA, 2012). The test should be used for initial- and requalification of each operator (Beaney, 2016; Z3. Aseptic Handling, 2022).

ESBT, MM and OBTVT were introduced in Dutch hospital pharmacies in the late nineties. A few years later, standard procedures for these tests were developed by the Royal Dutch Pharmacists Association, division Laboratory of Dutch Pharmacists (LNA). Afterwards, the idea of a web-based programme for processing, evaluation (trending) and assessing microbiological controls arose. This programme was named Microbio. The objective of this study is to analyse the results of microbiological controls over a 7-year period and to develop methods for assessing of, and determining realistic limits for, microbiological controls during aseptic handling. As secondary objective the role of Microbio is highlighted.

This article starts with a brief description of Microbio. Subsequently, the results of ESBT, MM and OBTVT are evaluated and the support of Microbio is explained. Finally, the limits for ESBT and MM are discussed.

There is only limited experience with isolators in the Netherlands. Therefore, this study is restricted to microbiological controls for aseptic handling carried out in a LAF or SC.

2. Microbio

Microbio was developed by the KNMP in 2007 to store results of microbiological controls. The web-based application is a standardised tool which fully supports the processing of microbiological control data and enables the preparation of tables, diagrams and histograms for evaluation and assessment. Validation is carried out by several internal procedures and extensive testing. Microbio has been improved several times over the years. An advisory board of hospital pharmacists supports Microbio. After subscribing to Microbio, participants will receive access.

Results of ESBT, MM and OBTVT must be entered in Microbio. Parameters, such as preparation date, LAF or SC used, name of the primary operator (see definition in Appendix A) must be filled in, in addition to each result. Data are entered in a spreadsheet-like interface. Functionalities such as copy-and-paste are available, and unique sample identification numbers are generated automatically. To make data permanent, a verification step must be performed.

After entering data, results of a specific LAF/SC during a specific period can be evaluated and assessed by generating tables and diagrams. Examples are given in the Results section. For further analyses, such as comparing results of individual operators, data can be exported to Excel spreadsheets.

Each participant has the opportunity to benchmark their results online against the results of all participants. Examples of this option are also given in the Results section. Periodically, each participant will receive a newsletter with aggregated results of all participants as well as feedback from their own results from the previous year. Information about reducing the risk of non-sterility and improving the results of microbiological controls are permanent issues discussed in the newsletters. The objective of benchmarking and feedback information is reducing the risk of microbiological contamination and, with this, improving the results of microbiological controls.

In 2020 Microbio had 44 participants, two of which were community pharmacies and the other 42 were hospital pharmacies. Some of the 44 participants are authorised to produce for other pharmacies. The 42 hospital pharmacies comprise around 60% of all hospital pharmacies in the Netherlands with facilities for aseptic handling.

3. Materials and methods

Microbiological controls were performed according to the procedures of the Royal Dutch Pharmacists Association (LNA, 2010, 2012, 2019a, 2019b). These procedures are comparable to procedures described elsewhere (Beaney, 2016; Crauste-Manciet et al., 2020). The results of the controls were entered in Microbio and evaluated and assessed as mentioned above (see Section 2, Microbio). Aggregated results over a 7-year period were used for further analyses.

Definitions of terms, which are less common, are given in Appendix A.

3.1. End-of-Session broth test (ESBT)

ESBT was executed with Tryptone Soya Broth as a growth medium and should comprise all critical steps that occur during typical aseptic handling, such as withdrawing a solution from a vial or ampoule, dissolving a powder in a vial and adding a solution to an infusion bag or vial. The final products were incubated for 14 days at 30 °C and examined for either growth or no-growth. The recommended frequency is at least one ESBT per working day. For more information see Boom and Beaney (2015), LNA (2010). Note: 14 days incubation time at 30 °C is established and proved in the Netherlands as comparable to a dual temperature incubation regime (7 days 20–25 °C and 7 days 30–35 °C)

(LNA 2010, 2012).

3.2. Microbiological monitoring (MM)

The results used in this study were restricted to MM results inside a LAF/SC. They consisted of:

- Passive air sampling using Ø 90 mm agar plates (settle plate);
- Glove print of 5 fingers of one hand of the primary operator using Ø 90 mm agar plates;
- Surface print of the work zone (see definition in Appendix A) on the worktop using Ø 55 mm contact plates (contact plate).

The frequency of settle plates, glove prints and contact plates were one sample per MM at every working day.

The microbiological growth medium used in all cases was Tryptone Soya Agar. For more information about the MM procedures, see Boom et al. (2020a), LNA (2019a, 2019b).

3.3. Operator broth transfer validation test (OBTVT)

Two tests have been developed for the Dutch OBTVT procedure: LNA 1 and LNA 2 (LNA, 2012).

LNA 1: Transfer a microbiological growth medium (Tryptone Soya Broth, TSB) from one vial to another.

(a) Take a 3 ml syringe with needle, withdraw 3 ml from a 10 ml vial with TSB and add this to an empty vial. Repeat this 2 times (in total: 3 empty vials are filled with 3 ml TSB each).
(b) Take a new syringe with needle and repeat a) 9 times.

The final products are 30 vials with 3 ml TSB.

LNA 2: Transfer 4-fold strength TSB into an infusion bag.

(a) Remove 10 ml from a 100 ml sodium chloride 0,9% bag.
(b) Take a 3 ml syringe with needle, withdraw 1 ml from a 10 ml vial with 4 strength TSB and add this to the sodium chloride 0,9% bag. Repeat this 9 times.
(c) Repeat (b) 2 times with a new syringe and needle and another 10 ml 4 strength TSB vial.

The final product is one bag containing 120 ml single-strength TSB.

The final products (30 vials or one infusion bag) should be incubated for 14 days at 30 °C and assessed as either growth or no-growth. See Section 3.1. for an explanation of the used incubation time and temperature.

The test should be used for initial qualification as well as for annual re-qualification of each operator (Z3. Aseptic Handling, 2022).

3.4. Statistics

CRs of ESBT and CRRs of MM were compared using chi-square tests with Yates' continuity correction (Agresti, 2007). For calculating of *p*-values, an online calculator was used (GraphPad QuickCalcs, 2022).

The 95% Upper Confidence Limit (95% UCL) for the CRs of ESBT was calculated using the Pearson-Clopper procedure which results in exact, i.e., non-approximated, intervals similar to Fisher's test results in exact *p*-values in similar situations (Clopper and Pearson, 1934). This is necessary as CRs can be close to zero which can result in inaccurate confidence intervals if the standard, asymptotic approximation is being used. The R programming environment was used for the computation of Pearson-Clopper bounds (R-function *binom.test*) (R Core Team, 2018).

4. Results

Some participants did not use all capabilities of Microbio. Therefore, the number of participants of ESBT, MM and OBTVT differs from 44 and varies over time in the tables of the Results section.

4.1. End-of-Session Broth Test (ESBT)

Table 1 gives the ESBT results of all Microbio participants over a 7-year period (2014 up to and including 2020). Compared with the first 3 years of the study, the results in the last 4 years were significantly better. Interventions started in 2015, see Section 5.3.1. Therefore, the first 3 years and the last 4 years were compared.

In Table 2, ESBT results over a 4-year period (2017, 2018, 2019 and 2020) of 37 participants are compared. Participants were ranked firstly on CR values, and secondly on numbers of samples. The columns 'participant', 'n' and 'CR' were generated by Microbio. The columns 'growth' and '95% UCL' were calculated afterwards. The number of samples of 7 participants were too low to allow calculation of reliable CR values (< 300 samples). 11 participants had a CR of 0%. The maximum CR was 0,70%.

To assist with the assessment of ESBT results, a table with 95% UCLs for different sample sizes and different numbers of samples with growth was calculated. The resulting values are given in Table 3 and can conveniently be used as an aid without having to resort to statistical software. This will be explained further in Section 5.2.4.

Table 4 summarises ESBT results over a 4-year period (2017 up to and including 2020) for participants 17, 28 and 32 of Table 2. Data are collected by exporting ESBT results to Excel. Data before 2017 (grey shaded, participant 32) are necessary for assessment of the results. This will be explained further in Section 5.2.4.

4.2. Microbiological monitoring (MM)

As mentioned in the introduction, MM results are given as CRR. Table 5 gives the results over a 7-year period (2014 up to and including 2020) for non-hazardous products, antineoplastics and radiopharmaceuticals. Table 6 is an elaboration of the results of glove prints and contact plates (non-hazardous and hazardous products only; there are too few results of radiopharmaceuticals for further analyses).

During the 7-year period, the results of settle plates do not differ substantially. The results of glove prints and contact plates improved considerably (see Table 5). Compared to 2014 and 2015, all CRRs of glove prints and contact plates from 2016 up to and including 2020, except some contact plates of hazardous products, are significantly lower (see Table 6).

Microbio has online facilities for evaluation and assessing MM results. After selecting a specific LAF or SC and a specific period, two sets

Table 1
Results ESBT over a 7-year period.

year	number of participants	number of samples	samples with growth	CR (%)	*p*-value
2014	28	12,006	22	0.18	–
2015	25	12,128	24	0.20	–
2016	28	12,638	29	0.23	–
total 2014 to 2016	–	36,772	75	0.20	–
2017	29	15,976	14	0.09	–
2018	32	19,227	25	0.13	–
2019	33	18,164	26	0.14	–
2020	30	25,390	23	0.09	–
total 2017 to 2020	–	78,757	88	0.11	0.0091 *
total 2014 to 2020	–	115,529	163	0.14	–

CR = contamination rate.
* p-value comparison mean CR for period 2014 to 2016 with mean CR for period 2017 to 2020 including Bonferroni correction for size possible comparisons.

Table 2
Comparison of ESBT results of 37 participants over a 4-year period.

Participant	*n*	CR	growth	95% UCL	Participant	*n*	CR	growth	95% UCL
1	4819	0.00%	0	0.08%	20	1328	0.08%	1	0.42%
2	3672	0.00%	0	0.10%	21	2201	0.09%	2	0.33%
3	3397	0.00%	0	0.11%	22	1132	0.09%	1	0.49%
4	2958	0.00%	0	0.12%	23	1090	0.09%	1	0.51%
5	2530	0.00%	0	0.15%	24	2025	0.10%	2	0.36%
6	2454	0.00%	0	0.15%	25	2792	0.11%	3	0.31%
7	2148	0.00%	0	0.17%	26	879	0.11%	1	0.63%
8	1750	0.00%	0	0.21%	27	3642	0.19%	7	0.40%
9	1730	0.00%	0	0.21%	28	1967	0.20%	4	0.52%
10	1373	0.00%	0	0.27%	29	3402	0.24%	8	0.46%
11	1038	0.00%	0	0.35%	30	12,561	0.25%	31	0.35%
12	181	X	0	X	31	2493	0.28%	7	0.58%
13	39	X	0	X	32	694	0.29%	2	1.04%
14	5	X	0	X	33	986	0.30%	3	0.89%
15	3	X	0	X	34	860	0.70%	6	1.51%
16	2902	0.03%	1	0.19%	35	19	X	1	X
17	1782	0.06%	1	0.31%	36	4	X	1	X
18	1750	0.06%	1	0.32%	37	16	X	5	X
19	5976	0.07%	4	0.17%					

CR = contamination rate; growth = number of samples with growth; *n* = number of samples; UCL = Upper Confidence Limit; *X* = to little data to calculate a reliable CR.

Table 3
Exact 95% UCL for given sample sizes and given number of samples with growth.

sample size	growth	CR (%)	95% UCL	sample size	growth	CR (%)	95% UCL
300	0	0	1.22%	800	1	0.13	0.69%
300	1	0.33	1.84%	800	2	0.25	0.90%
400	0	0	0.92%	800	3	0.38	1.09%
400	1	0.25	1.38%	800	4	0.50	1.28%
400	2	0.50	1.79%	800	5	0.63	1.45%
500	0	0.00	0.74%	800	6	0.75	1.63%
500	1	0.20	1.11%	900	1	0.11	0.62%
500	2	0.40	1.44%	900	2	0.22	0.80%
500	3	0.60	1.74%	900	3	0.33	0.97%
600	0	0.00	0.61%	900	4	0.44	1.13%
600	1	0.16	0.93%	900	5	0.55	1.29%
600	2	0.33	1.20%	900	6	0.66	1.45%
600	3	0.50	1.45%	900	7	0.77	1.60%
600	4	0,67	1.70%	1000	0	0.00	0.37%
700	1	0.14	0.79%	1000	1	0.10	0.56%
700	2	0.29	1.03%	1000	2	0.20	0.72%
700	3	0.43	1.25%	1000	3	0.30	0.87%
700	4	0.57	1.46%	1000	4	0.40	1.02%
700	5	0.71	1.66%	1000	5	0.50	1.16%
				1000	6	0.60	1.30%
				1000	7	0.70	1.44%
				1000	8	0.80	1.57%

CR = contamination rate; growth = number of samples with growth; UCL = Upper Confidence Limit.

of figures with the results of settle plates, glove prints and contact plates can be generated. One set is called 'Sample history' and shows the number of cfu of samples with growth and a 'rolling mean cfu_{100}' diagram. The other is called 'Percentage contaminated' and shows a 'rolling CRR_{100}' diagram. Both diagrams can be created by calculating the mean cfu or CRR from the previous 100 samples for each sampling point. When new results are added, both values are recalculated, and the diagrams are updated. For more information, in particular the choice of 100 samples for the rolling diagrams, see reference (Boom et al., 2020a).

Fig. 1 shows the rolling mean cfu_{100} and the rolling CRR_{100} of glove prints from a particular LAF. The reference value (see definition in Appendix A) used for the blue line in Fig. 1b can be determined from the MM results of the last year, or a longer period if insufficient data are available (at least 250 samples) (Boom et al., 2020a).

4.3. Operator broth transfer validation test (OBTVT)

Table 7 gives the results of OBTVT for all participants over the 7-year period (2014 up to and including 2020). Most participants used LNA 2 (final product one bag containing 120 ml TSB). Tests from the category 'other' are often a combination of LNA 1 and 2.

5. Discussion

5.1. Microbio

When Microbio was introduced (2007), microbiological controls of aseptic handling were already operational in most Dutch hospital pharmacies. However, systematic collecting and regular evaluation of the results was not common practice at that time. Besides, possibilities for benchmarking were thought to be a stimulant to improve the results. The rapid increase in the number of participants in the first years (up to 49 in 2012), showed that Microbio met a need (Postma et al., 2012). Some fluctuations in the number of participants resulted in a slight decrease in the following years (44 participants in 2020). Hospital pharmacies that do not use Microbio, often use an Excel application, developed in-house.

Entering data into Microbio is comparable to using a spreadsheet application such as Excel (2015). Automatically generating unique sample numbers, as well as a verification step, makes Microbio a robust application. Evaluation (trending) and assessing results can be carried out online within seconds and does not require specific training. Comparing a pharmacy's own results to results of all participants (benchmarking) is another online option. The yearly newsletters (see Section 2) are public, making them available to non-Microbio users, as well as to organisations interested in the quality of aseptic handling (e.g., the healthcare inspectorate).

All participants use the standardised LNA procedures for microbiological controls. All data are collected and stored in the same way. Therefore, the chance of methodological differences between participants is small. This is of particular interest if results are compared. Additionally, aggregated results of Microbio give a reliable image of microbiological controls during aseptic handling in the Netherlands.

5.2. End-of-Session Broth Test (ESBT)

5.2.1. Results over a 7-year period

The better ESBT results in the second part of the study (2017 to 2020, see Table 1) could be expected, because the MM results of glove prints

Table 4
ESBT results of 3 participants over a 4-year period.

Participant 17			Participant 28			Participant 32		
sam-ple no	period	result	sam-ple no	period	result	sam-ple no	period	result
1 to 56	01/01/2017 – 16/02/2017	no growth	1 to 363	01/01/2017 - 13/07/2017	no growth	- *88*	08/06/2016: 4 years retrospectively after the latest sample with growth*	
57	17/02/2017: 1000 samples retrospectively, after the latest sample with growth*		**364**	**14/07/2017**	**growth**	1 to 288	01/01/2017 - 07/08/2018	no growth
58 to 1056	17/02/2017 – 02/04/2019	no growth	365 to 653	15/07/2017 - 28/02/2018	no growth	**289**	**08/08/2018**	**growth**
1057	**03/04/2019**	**growth**	*654*	29/02/2018: 1000 samples retrospectively, after the latest sample with growth*		290 to 603	09/08/2018 - 07/06/2020	no growth
1058 to 1782	05/04/2019 - 31/12/2020	no growth	655 to 734	30/02/2018 - 02/05/2018	no growth	**604**	**08/06/2020**	**growth**
			735	**03/05/2018**	**growth**	605 to 694	09/06/2020 - 31/12/2020	no growth
			736 to 1384	04/05/2018 - 24/09/2019	no growth			
			1385	**25/09/2019**	**growth**			
			1386 - 1653	26/09/2019 - 03/04/2020	no growth			
			1654	**04/04/2020**	**growth**			
			1655 - 1967	05/04/2020 - 31/12/2020	no growth			

* Explanation in Section 5.2.4.

Table 5
Mean MM results over a 7-year period in all LAF/SC units with ≥ 100 samples a year of each sampling method.

	non-hazardous products													
	2014		2015		2016		2017		2018		2019		2020	
sampling method	n	mean CRR	n	mean CRR	n	mean CRR	n	mean CRR	n	mean CRR	n	mean CRR	n	mean CRR
settle plate	51	2.19%	54	2.37%	50	2.35%	43	2.61%	49	2.47%	55	2.55%	66	4.37%
glove print	49	20.84%	53	22.09%	51	11.62%	45	8.86%	48	7.98%	58	11.66%	56	9.16%
contact plate	26	7.00%	30	6.72%	27	4.48%	27	3.29%	33	3.32%	44	2.97%	51	2.88%
	hazardous products													
	2014		2015		2016		2017		2018		2019		2020	
sampling method	n	mean CRR	n	mean CRR	n	mean CRR	n	mean CRR	n	mean CRR	n	mean CRR	n	mean CRR
settle plate	35	2.20%	36	2.64%	34	2.52%	32	2,23%	32	2.64%	31	2.11%	37	1.78%
glove print	35	17.96%	36	17.25%	34	13.05%	30	10.67%	31	11.42%	30	10.26%	29	8.97%
contact plate	12	7.61%	15	7.13%	12	5.94%	14	4.86%	15	5.45%	19	4.82%	24	6.27%
	radiopharmaceuticals													
	2014		2015		2016		2017		2018		2019		2020	
sampling method	n	mean CRR	n	mean CRR	n	mean CRR	n	mean CRR	n	mean CRR	n	mean CRR	n	mean CRR
settle plate	2	3.47%	2	2.00%	4	3.55%	3	2.04%	3	1.77%	3	1.62%	3	2.71%
glove print	3	12.50%	3	9.13%	5	14.01%	4	9.90%	4	14.95%	4	10.97%	5	8.28%
contact plate	2	4.21%	1	2.63%	3	2.22%	2	0.67%	3	1.12%	3	2.36%	4	3.21%

CRR = contamination recovery rate; n = number of LAF/SC units of which results were used in the given year.

improved after 2015 (see Table 6). The following explanation can be given for this assumption. The risk of contamination, caused by any accidental touch of a critical spot, is smaller if the CRR of the gloved hands is lower.

The improved MM results are among other things due to the feedback provided by the Microbio advisory board to the participants (see Section 5.3.1).

5.2.2. Results and the probability of non-sterility

Table 2 shows that all participants except 32 and 34 had a CR below the recommended 1% (95% UCL) (LNA, 2010). However, the following comments are relevant. Firstly, during aseptic handling the preparation time is sometimes longer, and the number of critical steps is regularly greater compared to ESBT. Therefore, ESBT is not always a worst-case simulation. Secondly, it is possible that operators are more careful when carrying out an ESBT, compared to 'normal' aseptic handling. To avoid part of this criticism, more critical steps could be included in an ESBT. The number of these steps can be determined by a method described by Ris et al. (see Appendix B) (Ris et al., 2010). Considering aseptic handling in different hospital pharmacies, an ESBT with > 30 critical steps will come close to a worst-case simulation.

Even if the number of critical steps is increased, it remains important to realise that not all aspects of the way of working can be controlled by ESBT (Boom et al., 2021c; TR 44, 2008). In other words, good ESBT results in case of aseptic handling does not automatically mean a low probability of non-sterility; it is merely an indication of acceptable technique. Therefore, additional controls are necessary to objectify this probability. In previous articles we advised a yearly audit off all operators as an important additional control (Boom et al., 2021b, 2021c).

5.2.3. Limits of ESBT results

Zero samples with growth is the target. However, an occasional sample with growth is inevitable (a coincidence). Therefore, as mentioned in the introduction, the approach 'the more frequent samples with growth, the stronger the corrective actions' is suggested. To develop this idea further, levels of Investigation and Corrective Actions (ICA level) should be determined. If these levels are expressed as 95% UCLs, both mean CR and the number of samples are taken into consideration.

In Table 8, 4 ICA levels are defined. The first one is the level of a coincidence. This level is fixed at a 95% UCL of 0.6%, which means only one sample with growth out of 1000 samples (see Table 3). Because of the coincidence, investigation and corrective actions are limited at this level. The upper limits of the other 3 ICA levels are determined by taking CRs in daily practice into consideration (Table 1). The investigations and corrective actions mentioned in Table 8 are derived from the articles about Risk Assessment and Risk Control during aseptic handling (Boom et al., 2022, 2021b).

It is common practice to always identify micro-organisms in samples with growth in ESBT. Therefore, this was added at ICA level 1. However,

Table 6
elaborated results (Table 4) of glove prints and contact plates of non-hazardous and hazardous products.

	non-hazardous products							hazardous products						
glove print	n	% LAF/SC	mean CRR (%)	pos	neg	p_1	p_2	n	% LAF/SC	mean CRR (%)	pos	neg	p_1	p_2
2014	49	53.1	20.84	3206	12,179	x	0.0065	35	45.7	17.96	1797	8204	x	0.1774
2015	53	52.8	22.09	3746	13,212	0.0065	x	36	47.2	17.25	1980	9494	0.1774	x
2016	51	58.8	11.62	1869	14,210	< 0.0001	< 0.0001	34	55.9	13.05	1407	9378	< 0.0001	< 0.0001
2017	45	62.2	8.86	1267	13,036	< 0.0001	< 0.0001	30	53.3	10.67	1018	8526	< 0.0001	< 0.0001
2018	48	77.1	7.98	1242	14,320	< 0.0001	< 0.0001	31	45.2	11.42	1179	9147	< 0.0001	< 0.0001
2019	58	62.1	11.66	2083	15,785	< 0.0001	< 0.0001	30	53.3	10.26	1046	9154	< 0.0001	< 0.0001
2020	56	60.7	9.16	1596	15,824	< 0.0001	< 0.0001	29	69.0	8.97	835	8478	< 0.0001	< 0.0001
contact plate	n	% LAF/SC	mean CRR (%)	pos	neg	p_1	p_2	n	% LAF/SC	mean CRR (%)	pos	neg	p_1	p_2
2014	26	69.2	7.00	495	6573	x	0.4982	12	66.7	7.61	243	2949	x	0.4829
2015	30	73.3	6.72	587	8152	0.4982	x	15	73.3	7.13	273	3548	0.4829	x
2016	27	92.6	4.48	274	5844	< 0.0001	< 0.0001	12	66.7	5.94	188	2970	0.0099	0.0516
2017	27	96.3	3.29	213	6260	< 0.0001	< 0.0001	14	92.9	4.86	180	3524	< 0.0001	< 0.0001
2018	33	97.0	3.32	261	7596	< 0.0001	< 0.0001	15	86.7	5.45	210	3639	0.0003	0.0027
2019	44	100	2.97	319	10,410	< 0.0001	< 0.0001	19	84.2	4.82	226	4464	< 0.0001	< 0.0001
2020	51	98.0	2.88	415	13,983	< 0.0001	< 0.0001	24	83.3	6.27	390	5832	0.0154	0.0937

% LAF/SC = percentage of LAF/SC according to the MM limit of less than 10%; CRR = contamination recovery rate; *n* = number of LAF/SC units of which results were used; neg = number of samples without growth; pos = number of samples with one or more cfu; $p_{1=}$p-value mean CRR 2014 compared to mean CRR of 2015, 2016, 2017, 2018, 2019 and 2020, respectively; $p_{2=}$p-value mean CRR 2015 compared to mean CRR of 2014, 2016, 2017, 2018, 2019 and 2020, respectively.

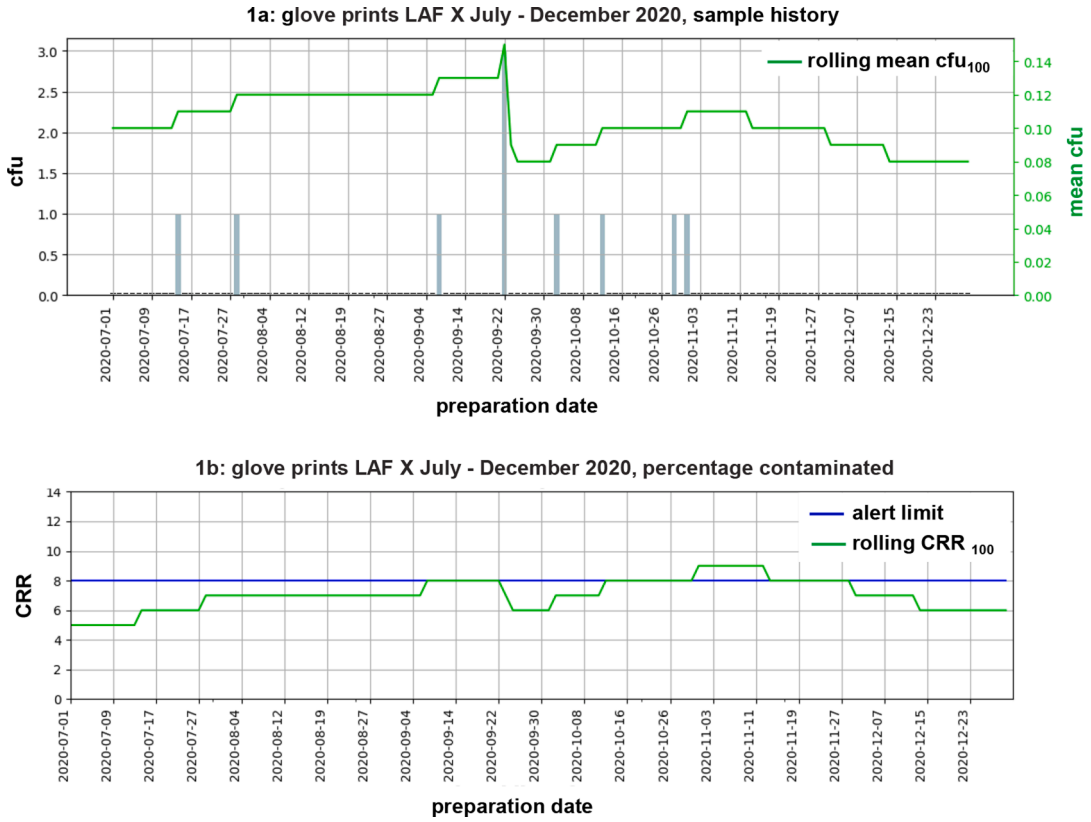

Fig. 1. Sample history (1a) and percentage contaminated (1b). Fig. 1a: each bar is the number of cfu in a sample with growth; the value for the Y-axis of the green line (rolling mean cfu_{100}) is given on the right side. Fig. 1b: the alert limit (blue line) is the reference value (see definition in Appendix A) plus 2%). For further explanation, see text Section 5.3.2.

Table 7
Results of OBTVT over a 7-year period.

year	parti-cipants	LNA 1		LNA 2		other		all OBTVT		
		n	pos	n	pos	n	pos	n	pos	%
2014	19	20	0	236	0	196	1	452	1	0.22
2015	19	23	0	323	4	114	0	460	4	0.87
2016	20	40	3	268	0	134	0	442	3	0.68
2017	25	30	0	381	4	183	0	594	4	0.67
2018	26	15	0	405	1	163	0	583	1	0.17
2019	28	45	0	472	2	80	0	597	2	0.34
2020	27	43	0	560	4	92	0	695	4	0.58
total 2014 to 2020	-	216	3	2645	15	962	1	3823	19	0.50

participants = number of participants for the OBTVT test; LNA 1 = vials as final product; LNA 2 = bags as final product; other = other OBTVT; *n* = number of tests; pos = number of tests with one or more samples with growth; % = percentage of tests with growth of all OBTVT in a particular year.

Table 8
4 levels of investigation and corrective actions for a sample with growth.

ICA level	95% UCL	investigations and corrective actions
1	≤ 0.6%	• Discuss the sample with growth with the operators. • Identification of micro-organism(s) in the sample with growth*.
2	> 0.6% en ≤ 1.0%	• Measures named at level 1. • Check the following SOPs have sufficient details and univocal text: (1) disinfection of materials with a non-sterile surface (2) glove handling (3) non-touch working. • Check results of microbiological monitoring, in particular glove prints (Boom et al., 2020a).
3	> 1.0% en ≤ 1.5%	• Measures named at level 2. • Audit of all operators (Boom et al., 2021b). • Surface bioburden determination of disinfected materials with a non-sterile surface (Boom et al., 2021a). • Audit of logistics and storage conditions of materials which are used in LAF/SC
4	> 1.5%.	• Measures named at level 3. • Audit by an external colleague and/or a technician, both experienced in aseptic handling.

* See text.

in the case of aseptic handling, the usefulness of identification is doubtful. Micro-organisms may inadvertently be introduced into a LAF/SC on the surface of disinfected ampoules and vials, even after thorough disinfection (Boom et al., 2021a, 2022). Gloves could be contaminated by these surfaces and microorganisms on gloves could contaminate the product by touch of critical spots. Because of the great number of ampoules and vials used during aseptic handling, matching of a micro-organism found in an ESTB sample with a micro-organism found on the surface of these items, seems to be impossible.

5.2.4. Assessing ESBT results

As mentioned in the introduction, a CR of ≤ 1% (95% UCL) is recommended, for assessing the results. According to the Dutch ESBT procedure, the minimum number of samples for calculating a reliable CR value is 300 (LNA, 2010). A maximum data collection period is not given for the calculation of this value. This is a shortcoming, as both operators and procedures will change over the years and with it, the probability of non-sterility. A maximum data collection period of 4 years is recommended. Additionally, it is important to use a fixed number of samples for assessing and comparing results. Because of the low CR values (see Table 1), the 300 samples mentioned above, is too small for calculating a reliable CR value. To minimise uncertainty about the true CR, a sample size of 1000 seems to be a reasonable compromise between accuracy and sensitivity to changes over time. For further explanation of assessing ESBT, the results of the 3 participants given in Table 4 were used. Considering participant 17 and 28: 1000 samples counted retrospectively, after the latest sample with growth, were reached on 17/02/2017 and 19/02/2018, respectively. The CRs and the corresponding 95% UCLs in these periods are 0.10 (one sample with growth) and 0.30 (3 samples with growth) and 0.56% and 0.87%, respectively (see Table 3). The corresponding ICA levels are 1 and 2, respectively (see Table 8).

Considering the results of participant 32 in Table 4: 1000 samples are not available within 4 years. In this case, all samples during 4 years, counted retrospectively after the latest sample with growth, were taken for the calculation of the CR and the 95% UCL. In the period 08/06/20 (final sample with growth) to 08/06/2016 (4-years backwards) results of 691 (88 + 603) samples are available (see Table 4). Two of these samples developed growth, yielding a CR of 0.29%. By using the Pearson-Clopper procedure (see Section 3.4) a 95% UCL of 1.04% can be calculated. The corresponding ICA level is 3. To avoid the complicated calculation of the UCLs by using the computation mentioned in Section 3.4, the following procedure can also be used: round down to the nearest hundred (e.g. 600 in case of 691; lower to avoid deriving an UCL that is too low) and determine the 95% UCL by using Table 3. Having 2 samples with growth, this limit is 1.20%. The corresponding ICA level by using this simple method will also be 3.

The influence of the sample size on the ICA level is reflected by comparing the results of participant 28 and 32. Despite small differences in CRs (0.30 versus 0.29, as mentioned above), the ICA levels differ (2 versus 3), resulting in substantially more investigations and corrective actions by participant 32 comparing to 28 (see Table 8).

5.3. Microbiological monitoring (MM)

As mentioned in the introduction, a CRR of less than 10% is recommended as a limit (Z3. Aseptic Handling, 2022).

5.3.1. Results over a 7-year period

The results of settle plates did not change during the study period (see Table 5). This is not surprising, because at the start of the study CRRs of settle plates were already very low. Investing in a more sensible determination method (active air sampling) is not advisable, because the value of viable air sampling inside a LAF/SC during aseptic handling is doubtful (Boom et al., 2020a).

In 2015 the Microbio advisory board started providing feedback related to high CRR values for glove prints. Advice to improve the disinfection procedure of materials with a non-sterile surface (ampoules and vials) and to increase the frequency of glove and worktop disinfection was given. This reduced the CRR values of glove prints and contact plates substantially and, except for some contact plates of hazardous products, significantly (see Table 6). These improved results may have contributed to the better ESBT results in the last 4 years of the study (see Section 5.2.1).

For many participants, it is still difficult to get the CRR of glove prints below the 10% limit. At the end of the study (2020), only 60.7% (34 out of 56 LAF/SC, non-hazardous products) and 69.0% (20 out of 29 SC, hazardous product) of LAF/SC meet this limit (see Table 6).

Due to the risk of antineoplastic spillage, the preparation of

hazardous products inside a SC will generally be carried out on a sterile pad. This pad protects the work zone, which is also the zone where the contact plate should be taken (see Section 3.2). Despite the expected lower recovery of a pad (soft surface) compared to the worktop surface (hard surface), performing the print in the work zone on the surface of the pad is recommended, because this zone is the most critical (the location with the highest risk of contaminating the product). Because of the risk of inadvertently transferring micro-organisms into a LAF/SC on disinfected materials with a non-sterile surface (ampoules and vials), MM of the outer surface of these materials was added to the Dutch MM procedures in 2019 (LNA 2019a, 2019b). Currently, however, it has not yet been adopted in most hospital pharmacies. The emphasis on this type of MM will inevitably increase when the new Annex 1 of the EU GMP comes into operation. Subsection 8.48 of this revised Annex recommends MM of the surface of materials, that are necessary for aseptic processing but cannot be sterilised (EU GMP Annex 1 revision, 2020). A method for MM of disinfected ampoules and vials is described in a previous article (Boom et al., 2020a).

5.3.2. Results of individual participants

The rolling mean cfu_{100} diagram (Fig. 1a) provides information on samples with growth. The rolling CRR_{100} diagram (Fig. 1b) is a practical tool for evaluating and assessing the results (Boom et al., 2020a). Generating these diagrams in Microbio is simple and will take only a few minutes. Therefore, it can be undertaken relatively frequently and without the help of staff with specific skills.

The rolling mean cfu_{100} (green line) in Fig. 1a is far below the limit of $<$ 1kve. This is not surprising, because the cfu limit is less critical compared to the CRR limit (Boom et al., 2020a).

In Fig. 1b, the green line (rolling CRR_{100}) is 12 days above the blue line (alert level). This period is too short to indicate a clear upward trend (Boom et al., 2020a). If this period becomes longer (for example a month, for practical reasons), intensified attention is necessary (exceeding the alert level). If the period continues above the blue line (for example two months, for practical reasons), action is necessary (exceeding the action level). In addition, a reference value above the 10% limit, must also be referred to as an exceeding of the action level (alert en action levels, see definitions in Appendix A).

In case of exceeding an alert level, it is advised that the operators are informed about this deviation. If the action level is exceeded, the following measures should be taken into consideration: checking the quality of the SOPs (sufficient details and unambiguous language) and an investigation into the working method within the LAF/SC (audit).

For pharmacies who do not use Microbio, an Excel template for making rolling CRR_{100} diagrams is available in a previous article (Boom et al., 2020a).

5.4. Operator broth transfer validation test (OBTVT)

Some participants registered the results of OBTVT in their own database. Therefore, the number of participants in Table 7 is lower compared to the number of participants for ESBT given in Table 1.

LNA 2 takes less time and less material compared to LNA 1. On the other hand, because there are 30 final products, LNA 1 gives more information about the probability of non-sterility compared to LNA 2 (1 final product). However, the target of OBTVT is zero samples with growth. Therefore, there is no preference for LNA 1 or 2 for the assessment of the results. In the case of growth, the operator involved must be retrained and must successfully carry out a new OBTVT (Beaney, 2016; LNA, 2012).

The risk of microbiological contamination increases as the preparation method becomes more complex (Crauste-Manciet et al., 2020). This is linked to the number of critical steps (Resolution CM/Res, 2016). This number is high for OBTVT, calculated according to the method of Ris et al. 130 and 123 of LNA 1 and 2, respectively (Ris et al., 2010). The number of critical steps in general applied ESBTs is much lower (15–30). This may explain the lower overall percentage contaminated in ESBT compared to OBVTV: 0.14 and 0.50, respectively (see Tables 1 and 7).

The number of key techniques in LNA 1 and 2 is low comparing to the OBTVT procedure used in the UK (UK Pharmaceutical Aseptic Services Committee, 2006). The latter consists of 6 different preparations which in total require 80 critical steps. To have more key techniques in the LNA procedure an improvement of LNA 2 could consist of transferring the TSB from the final bag into syringes, removing the needles, capping the syringes, and incubating the syringes.

The UK procedure notes that the OBTVT, in itself, does not conclusively prove that an operator can prepare aseptic dosage units accurately, precisely, and safely. Additionally, operators must be assessed on aseptic techniques (UK Pharmaceutical Aseptic Services Committee, 2006). This is in line with the remark about the limitations of ESBT and the advice for an annual audit of each operator (see Section 5.2.2).

6. Conclusion

Results of microbiological controls improved during the study period. Benchmarking in and feedback information from Microbio may have contributed to this. The aggregated results from the pharmacies participating in Microbio made it possible to establish science based as well as realistic limits for ESBT and MM. Methods for assessing ESBT and MM are developed and the application of these methods in daily practice are described.

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CRediT authorship contribution statement

Frits A. Boom: Investigation, Resources, Project administration, Conceptualization, Methodology, Writing – review & editing, Visualization. **Paul.P.H. Le Brun:** Conceptualization, Methodology, Validation, Writing – review & editing. **Stefan Bühringer:** Formal analysis, Writing – review & editing. **Madeleine Sirks:** Resources, Data curation, Writing – review & editing. **Daan J. Touw:** Conceptualization, Supervision, Writing – review & editing.

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Supplementary materials

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