Comparison between NMKL methods and EN/ISO methods using PT-data

- required according to the EU Regulation 2073/2005[1]

According to the EU Regulation 2073/2005 on microbiological criteria for foodstuffs, EN/CEN methods or methods that provide equivalent results are to be used for microbiological testing.

As many laboratories are using NMKL methods, or would like to use NMKL methods, results from proficiency testing (PT) schemes arranged by the National Food Agency in Uppsala, Sweden, have been used in comparing the performance of NMKL and ISO methods. In the PT-schemes, other methods were also applied, however, only results obtained by the methods listed below were evaluated. The number of results/laboratories was sufficient for statistical evaluation of the methods of interest.

Thanks are given to the National Food Agency, Uppsala and to Christina Normark for facilitating the format of the PT-scheme reports, getting the laboratories to specify the methods and any modifications thereof, and for making the results available for NMKL.

The comparisons have been carried out on:

- *Listeria monocytogenes*: qualitative method: ISO 11290-1:2004 and NMKL 136, 2007
- *Listeria monocytogenes*: quantitative method: ISO 11290-2:2004 and NMKL 136, 2007
- *Salmonella spp.*: ISO 6579:2002/Corr 1:4 and NMKL 71, 1999
- *Enterobacteriaceae:* ISO 21528-2:2004 and NMKL 144:2005
- Aerobic microorganisms: ISO 4833:2003 and NMKL 86, 2006
- *Bacillus cereus:* ISO 7932:2006 and NMKL 67, 2003
- *E.coli:* ISO 16649-2: 2001 and NMKL 125, 2005
- Coagulase positive *Staphylococcus:* ISO 6888-2: 2004 and NMKL 66, 2003
- *E.coli O157*: ISO 16654:2001 and NMKL 164, 2005.

The results have been statistically evaluated, partly based on the draft ISO/DIS 16140 (doc N120, March 2012) and NordVal Protocol for validation of alternative microbiological methods (available at www.nmkl.org). The confidence interval of the probability of detection have been calculated according to Wilson score confidence intervals http://faculty.vassar.edu/lowry/prop1.html.

1) Commission Regulation (EC) No. 2073/2005 of 15 November 2005 on Microbiological Criteria for Foodstuffs

Listeria monocytogenes – comparison of results obtained by ISO 11290-1:2004 and NMKL 136, 2007

Listeria monocytogenes - Qualitative method

Results obtained by ISO 11290-1:2004 and by NMKL 136, 2007, in a PT- Scheme January 2012, were compared. The laboratories analysed three sample solutions (A, B and C). 33 laboratories had applied the ISO method and 20 laboratories had used the NMKL method. Information about the test solutions are given in Annex 1, table 2. The obtained results are given in table 1.

Table 1: Qualitative results for Listera monocytogenes obtained by ISO 11290-1:2004 and NMKL 136, 2007

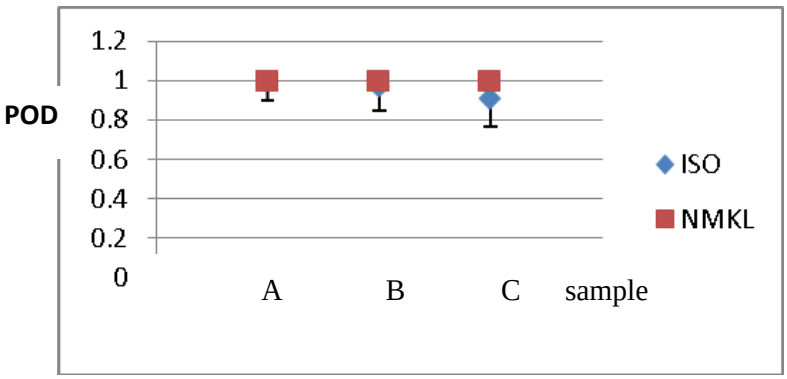
Method	**ISO 11290-1:2004**			**NMKL 136, 2007**		
No of labs	33			20		
Sample	A	B	C	A	B	C
No of pos	33	32	30	20	20	20
No of neg	0	1	2	0	0	0
POD*	1	0.97	0.91	1	1	1
Lower limit**	0.90	0.85	0.76	0.84	0.84	0.84
Upper limit**	1	0.99	0.97	1	1	1

*POD = probability of detection = number of positives/number of samples (laboratories)
** Lower and Upper limits of the 95% confidence interval for a proportion, according to E.B Wilson

All the (20x3) 60 results were positive with the NMKL method. Three samples out of (33x3) 99 samples were false negative with the ISO method. According to the draft ISO/DIS 16140 (doc N120), the acceptance limit (AL) for 60 test samples is 3, and 5 for 99 samples. This means that for 60 samples, 3 negative deviations (false negatives) are acceptable while when analysing 99 samples, 5 negative deviations are acceptable. Both methods obtained acceptable results, and hence for these samples the methods performs equivalent.

The figure below shows a plot of the probability of detecting positive samples analysing the three sample solutions using ISO method and NMKL method, respectively. The confidence interval included, is the range between lower and upper limits of the results obtained by the ISO method. As the results obtained by the NMKL methods falls within the confidence level of the ISO method, it illustrates that there are no significant differences between the results obtained by the two methods in the PT-scheme.

Figure 1: A plot of the probability of detection (POD) for the NMKL and ISO method, including the confidence limit of the ISO method.



***Listeria monocytogenes* - Quantitative method**

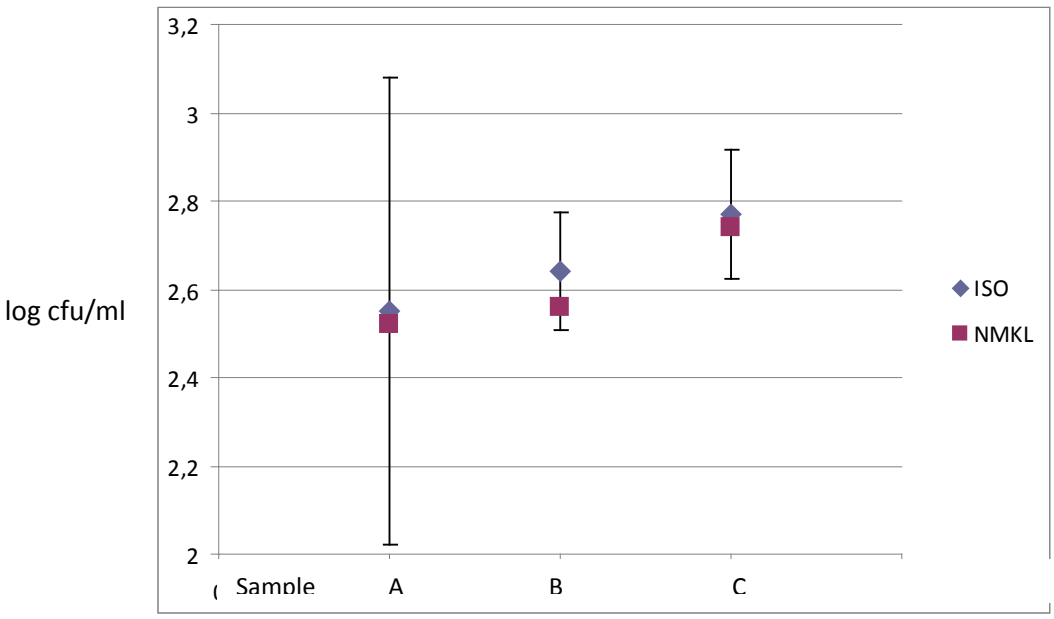
The results obtained in a PT- Scheme January 2012 by ISO 11290-2:2004 and NMKL 136, 2007, were compared. 34 laboratories had used the ISO method and 21 laboratories had used the NMKL method. The tests were carried out on samples given in Annex 1, table 2. The obtained results are given in table 2.

Table 2: Quantitative results for Listera monocytogenes obtained by ISO 11290-1:2004 and NMKL 136, 2007

Method	**ISO 11290-1:2004**			**NMKL 136, 2007**		
No of labs	34			21		
Sample	A	B	C	A	B	C
No of outliers	7	5	4	3	1	1
Mean (log cfu/ml)	2.77	2.55	2.64	2.75	2.53	2.56
SD (log cfu/ml)	0.53	0.13	0.15	0.10	0.16	0.16

By the ISO method, 16 of the (34x3) 102 results were outliers, while 5 of the (21x3) 63 results were outliers with the NMKL method. According to table 2, the mean values of the results obtained by the ISO and NMKL method are very close. The results obtained with the NMKL method falls within the confidence interval of the standard deviation of the ISO method. The standard deviation of sample A for the ISO method is not satisfactory. The mean values of the two methods are however overlapping. The results are also illustrated in the figure below.

Figure 2: Results (in log cfu/ml) *for Listera monocytogenes obtained by ISO 11290-1:2004 and NMKL 136for sample A, B and C*



The results show that there are no significant differences in the results obtained with the ISO and the NMKL method, except that the precision for sample A is not satisfactory for the ISO method.

Salmonella spp. - Comparison of results obtained by ISO 6579:2002/Corr 1:2004 and NMKL 71, 1999

Results obtained by ISO 6579 and NMKL 71, in a PT- Scheme January 2012, were compared. The laboratories analysed three sample solutions (A, B and C). 32 laboratories had applied the ISO method, and 39 laboratories had analysed according to the NMKL method. The tests were carried out on sample solutions given in Annex 1, table 2. The obtained results are given in table 3.

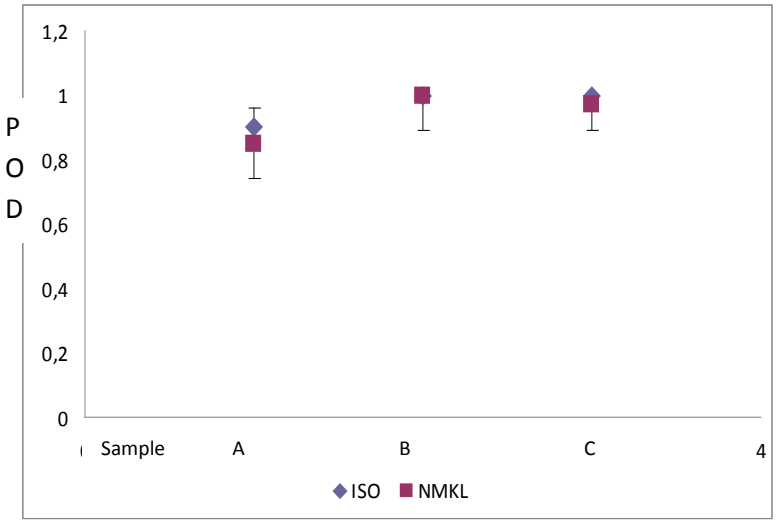
Table 3: Results for Salmonella spp. obtained by ISO 6579 and NMKL 71

Method	**ISO 6579**			**NMKL 71**		
No of labs	32			39		
Sample	A	B	C	A	B	C
No of pos	29	32	32	34	39	38
No of neg	3	0	0	5	0	1
POD*	0.9	1	1	0.85	1	0.97
Lower limit**	0.74	0.89	0.89	0.70	0.91	0.87
Upper limit**	0.96	1	1	0.94	1	1

*POD = probability of detection = number of positives/number of samples (laboratories)
** Lower and Upper limits of the 95% confidence interval for a proportion, according to E.B Wilson

Three out of (32x3) 96 samples were negative with the ISO method and 6 out of (39x3) 117 samples were negative with the NMKL method. According to the draft ISO/DIS 16140 (doc N120), the acceptance limit (AL) for 96 and 117 samples is 5 and 6, respectively. This means that for 96 samples, 5 negative deviations (false negatives) are acceptable while when analysing 117 samples, 6 negative deviations are acceptable. Both methods obtained acceptable results, and hence for these samples the methods perform equivalent. As the results obtained with the NMKL method fall within the confidence level of the probability of detection, POD, of the ISO method, the methods are providing equivalent results, see figure 3.

Figure 3: The probability of detection, including confidence interval of POD for sample A, B and C, by the ISO and the NMKL method for detection of Salmonella.



The figure shows that the results are overlapping. There are no significant differences in the results for the analysis of Salmonella using the ISO 6579: 2002 and the NMKL 71, 1999 method.

Enterobacteriaceae – Comparison of the results obtained by ISO 21528-2:2004 and NMKL 144:2005

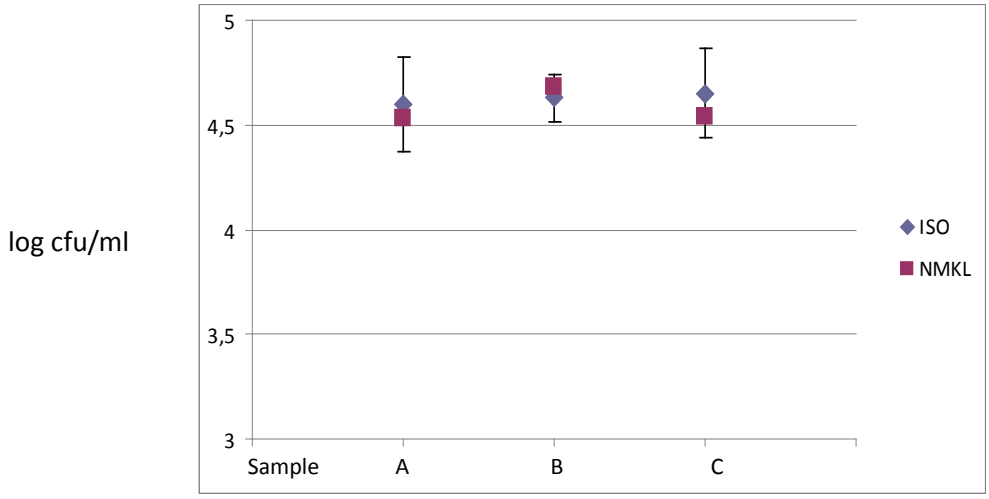
Results obtained by ISO 21528-2:2004 and NMKL 144, 2005 in a PT- Scheme January 2012 were compared. The laboratories analysed three sample solutions (A, B and C). 19 laboratories had applied the ISO method and 71 laboratories had analysed according to the NMKL method. Of the (19x3) 57 samples analysed with the ISO method, 11 results were outliers. Of the (71x3) 213 samples analysed using the NMKL method, three results were outliers. The tests were carried out on samples given in Annex 1, table 2. The obtained results are given in table 4.

Table 4: Results of Enterobacteriaceae obtained by ISO 21528-2 and NMKL 144

Method	**ISO 21528-2:2004**			**NMKL 144:2005**		
No of labs	19			71		
Sample	A	B	C	A	B	C
No of outliers	5	4	2	1	2	0
Mean (log cfu/ml)	4.63	4.60	4.65	4.68	4.53	4.54
SD (log cfu/ml)	0.23	0.11	0.21	0.13	0.19	0.22

The results obtained are close/overlapping and the variation of the results, when the outliers are omitted, is satisfactory. This is also illustrated graphically, in the figure below.

Figure 2: Results (in log cfu/ml) of Enterobacteriaceae obtained by ISO 21528-2 and NMKL 144 for sample A, B and C



The results obtained by the NMKL method fall within the results obtained by the ISO method, and hence the methods provide equivalent results.

Aerobic microorganisms – Comparison of the results obtained by ISO 4833:2003 and NMKL 86, 2006

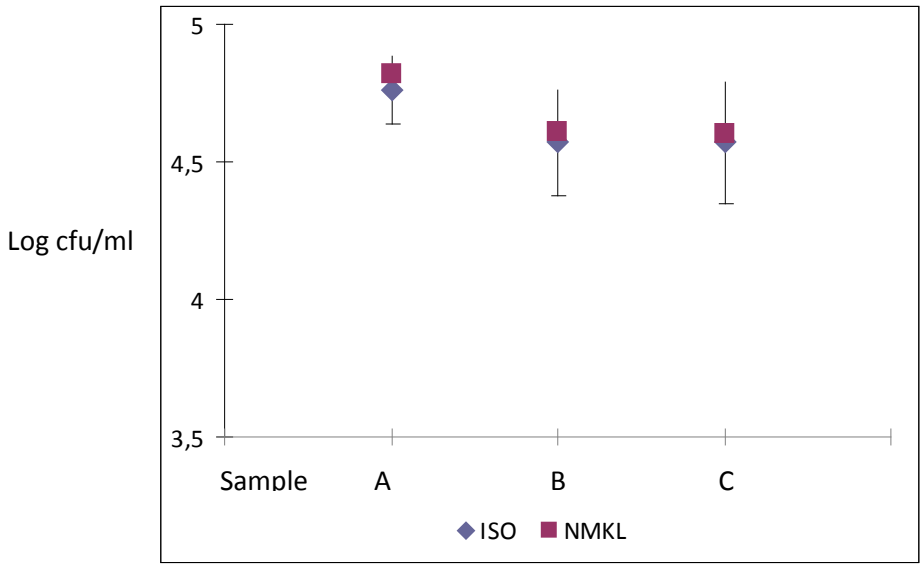
Results obtained by ISO 4833:2003 and NMKL 86, 2006 in a PT- Scheme January 2012 were compared. The laboratories analysed three sample solutions (A, B and C). 40 laboratories had applied the ISO method and 55 laboratories had analysed according to the NMKL method. Of the (40x3) 120 samples analysed with the ISO method, six results were outliers. Of the (55x3) 165 samples analysed with the NMKL method, six samples were outliers. The tests were carried out on samples given in Annex 1, table 2. The obtained results are given in table 5.

Table 5: Results of aerobic microorganisms with ISO 4833:2003 and NMKL 86, 2006

Method	ISO 4833:2003			NMKL 86, 2006		
Number of labs:	40			55		
Number of outliers	2	2	2	3	1	2
Sample	A	B	C	A	B	C
Mean (log cfu/g)	4.76	4.57	4.57	4.82	4.61	4.60
SD (log cfu/g)	0.12	0.19	0.22	0.12	0.24	0.22

The results obtained are close/overlapping and the precisions of the results are satisfactory. This is also illustrated graphically, in the figure below.

Figure 4: Results (in log cfu/ml) of aerobic microorganisms obtained by ISO 4833 and NMKL 86 for sample A, B and C



The results obtained by the NMKL method fall within the results obtained by the ISO method, and hence the methods provide equivalent results.

Bacillus cereus – Comparison of the results obtained by ISO 7932:2006 and NMKL 67, 2003

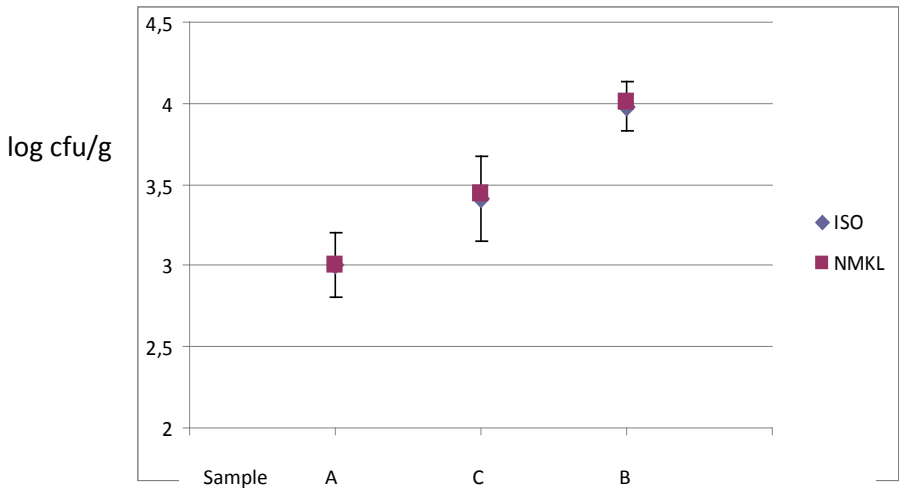
Results obtained by ISO 7932:2006 and NMKL 67, 2003, in a PT- Scheme October 2011, were compared. The laboratories analysed three sample solutions (A, B and C). 29 laboratories had applied the ISO method and 89 laboratories had analysed according to the NMKL method. Of the (29x3) 87 samples analysed with the ISO method, three results were outliers. Of the (89x3) 267 samples analysed with the NMKL method, nine samples were outliers. The tests were carried out on samples given in Annex 1, table 1. The obtained results are given in table 6.

Table 5: Results of Bacillus cereus with ISO 7932:2006 and NMKL 67, 2003

Method	**ISO 7932:2006**			**NMKL 67, 2003**		
Number of labs	29			89		
Number of outliers	2	1		3	4	2
Sample	A	C	B	A	C	B
Mean (log cfu/g)	3.00	3.98	3.41	3.00	4.02	3.44
SD (log cfu/g)	0.20	0.26	0.15	0.14	0.19	0.19

The results obtained are close /overlapping and the precisions of the results are satisfactory. This is also illustrated graphically, in the figure below.

Figure 5: Results (in log cfu/ml) of bacillus cereus obtained by ISO 7932 and NMKL 67 for sample solutions A, B and C



The results obtained by the NMKL method fall within the results obtained by the ISO method, and hence the methods provide equivalent results.

E.coli – Comparison of the results obtained by ISO 16649-2: 2001 and NMKL 125, 2005

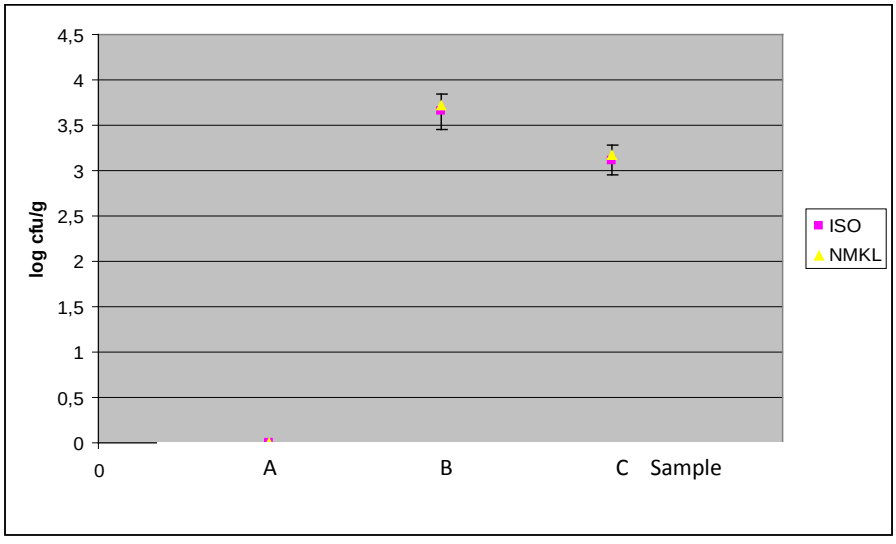
Results obtained by ISO 16649-2:2001 and NMKL 125, 2005, in a PT- Scheme October 2011, were compared. The laboratories analysed three sample solutions (A, B and C). 16 laboratories had applied the ISO method and 40 laboratories had analysed according to the NMKL method. Of the (16x3) 48 samples analysed with the ISO method, five results were outliers. Of the (40x3) 120 samples analysed with the NMKL method, nine samples were outliers. The test was carried out on samples given in Annex 1, table 1. The obtained results are given in table 7.

Table 7: Results of E.coliwith ISO 16649-2:2001 and NMKL 125, 2005

Method	**ISO 16649-2:2001**			**NMKL 125:2005**		
Number of labs	16			40		
Sample	A	B	C	A	B	C
Number of outliers	0	3	2	3	3	3
Mean (log cfu/g)	0	3.60	3.09	0	3.72	3.17
SD (log cfu/g)		0.15	0.16		0.12	0.12

The results obtained are close /overlapping and the variation of the results is satisfactory. This is also illustrated graphically, in the figure below.

Figure 6: Results (in log cfu/ml) of E.coliobtained by ISO 16649-2:2001 and NMKL 125, 2005for the solutions A, B and C



The results obtained by the NMKL method fall within the results obtained by the ISO method, and hence the methods provide equivalent results.

Coagulase positive Staphylococcus – Comparison of the results obtained by ISO 6888-2: 2004 and NMKL 66, 2003

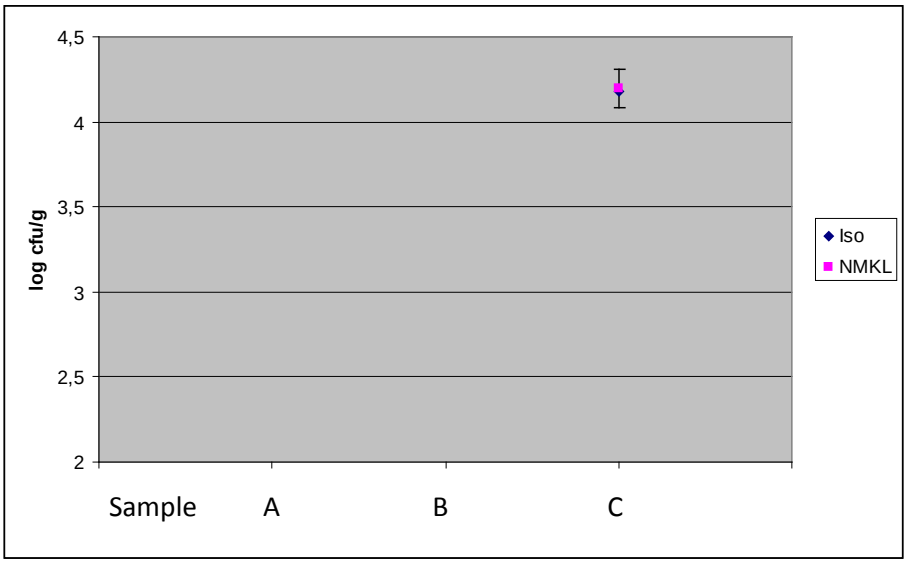
Results obtained by ISO 6888-2:2004 and NMKL 66, 2003 in a PT- Scheme October 2011 were compared. The laboratories analysed three sample solutions (A, B and C). 28 laboratories had applied the ISO method and 65 laboratories had analysed according to the NMKL method. The median of solution A and B was zero for both methods. A few laboratories had however obtained positive results for these samples. The results of the solution C are given in table 8. The content of the test solutions are given in Annex 1, table 1.

Table 8: Results of Coagulase Positive Staphylococcus ISO 6888-2:2004 and NMKL 66, 2003

Method	**ISO 6888-2:2004**	**NMKL 66:2003**
Number of labs	28	65
Sample	C	C
Number of outliers	4	2
Mean (log cfu/g)	4.20	4.21
SD (log cfu/g)	0.82	0.11

The results obtained are close /overlapping and the variation of the results is satisfactory. This is also illustrated graphically, in the figure below.

Figure 6: Results (in log cfu/ml) of ECoagulase Positive Staphylococcus ISO 6888-2:2004 and NMKL 66, 2003 for the solution C



The results obtained by the NMKL method fall within the results obtained by the ISO method, and hence the methods provide equivalent results.

E.coli O157 – Comparison of the results obtained by ISO 16654: 2001 and NMKL 164, 2005

Results obtained by ISO 16654:2001 and NMKL 164, 2005 in a PT- Scheme January 2012 were compared. The laboratories analysed three sample solutions (A, B and C). 10 laboratories had applied the ISO method and nine laboratories had analysed according to the NMKL method. The test was carried out on solutions given in Annex 1, table 2. The obtained results are given in table 9.

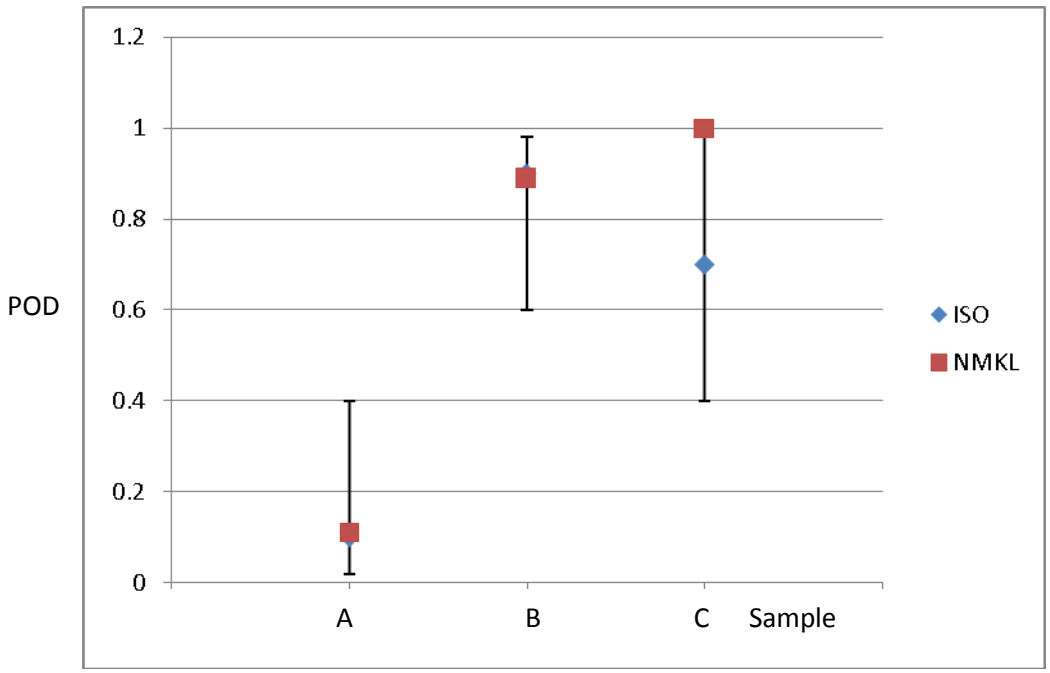
Table 9: Results for E.coli O157 obtained by ISO 16654 and NMKL 164

Method	**ISO 16654**			**NMKL 164**		
No of labs	10			9		
Solutions	A	B	C	A	B	C
No of pos	1	9	7	1	8	9
No of neg	9	1	3	8	1	0
POD*	0.10	0.90	0.70	0.11	0.89	1
Lower limit**	0.02	0.60	0.40	0.02	0.57	0.70
Upper limit**	0.40	0.98	0.89	0.44	0.98	1

*POD = probability of detection = number of positives/number of samples (laboratories)
** Lower and Upper limits of the 95% confidence interval for a proportion, according to E.B Wilson

The results obtained positive for sample A, are false positives. It is illustrated that it is a small probability of obtaining false positives. The probability of detection, including confidence limits, is illustrated in the figure below. As the results obtained with the NMKL method fall within the confidence level of the probability of detection, POD, of the ISO method, the methods are providing equivalent results.

Figure 3: Probability of detection for E.coli O157, including confidence interval of POD for the samples A, B and C by the ISO and the NMKL method



Annex 1: Test material used in the PT-schemes:

Each laboratory analysed three freeze dried microorganisms solutions, A, B and C. The test material was made and freeze dried in portions of 0.5 ml in vials according to Peterz och Steneryd (1). Before plating the content in each vial should be dissolved in 254 ml sterile dilution solution. The content of the test solutions are given in tables below.

Table 1.*Mikroorganisms in respective test solutions, October 2011*

Solution	Mikroorganisms	Strainlabelled
A	*Citrobacterfreundii*	SLV-424
	Enterococcus faecalis	SLV-051
	Bacillus thurigensis	SLV-478
	Klebsiellapneumoniae	SLV-564
B	*Proteus vulgaris*	SLV-476
	Bacillus cereus	SLV-556
	Escherichia coli	SLV-523
C	*Micrococcus sp.*	SLV-055
	Staphylococcus aureus	SLV-280
	Bacillus cereus group	SLV-518
	Escherichia coli	SLV-524

Table 2.*Mikroorganisms in the respective solutions of January 2012*

Solution	Mikroorganisms	Strainlabelled
A	*Escherichiacoli*	SLV-165
	Campylobactercoli	SLV-271
	Listeria monocytogenes	SLV-361
	Salmonella agona	SLV-318
B/C	*Klebsiella pneumoniae*	SLV-537
	Campylobacter jejuni	SLV-540
	Listeria monocytogenes	SLV-444
	Listeria innocua	SLV-312
	Salmonella bovismorbificans	SLV-443
	Escherichia coli O157	SLV-515

Quality control of the test samples

Homogenised test samples with identical volume in each vial are the assumption that every freeze dried samples from a test sample are comparable. Quality control of the test samples were carried out according to the National Food Administration Protocol (2). The results are given in table 3 (PT in October 2011) and table 4 (PT in January 2012), respectively.

The standard deviation in the different test solutions varied between 0.03 and 0.13 log. The requirement for homogeneity is that the standard deviation for 10 test samples should be less than 0.15 log and that the difference of the highest and the lowest value should not be more than 0.5 log.

In qualitative analyses, the microorganisms are to be detected in all samples. The content of Salmonella and *E. coli* O157 were determined in parallel solutions without background flora.

Table 3: The mean value *(m) and standard deviation (s) of analyses, in log_{10}cfu (colony forming units) per ml samples, obtained in the quality control of 10 vials per solution.*

Analysis and method	A		B		C	
	m	s	m	s	m	s
Aerobic microorganisms, 30 ˚C NMKL 86	4,6	0,11	4,6	0,08	4,5	0,03
Aerobic microorganisms, 20 ˚C NMKL86	4,5	0,13	4,6	0,05	–	–
Unknown microorganisms ISO 13559/IDF 153	4,6	0,11	4,5	0,07	4,5	0,03
Enterobacteriaceae NMKL144	4,3	0,13	3,6	0,05	3,5	0,08
Escherichia coli NMKL 125	–	–	3,8	0,04	3,3	0,03
Termotolerant coliform bacteria NMKL 125	2,9	0,03	3,8	0,04	3,3	0,03
Coliform bacteria, 30 ˚C NMKL 44	4,3	0,11	3,6	0,05	3,2	0,05
Coliform bacteria, 30 ˚C NMKL 44	4,3	0,12	3,6	0,08	–	–
Presumptive*Bacilluscereus* NMKL 67	3,2	0,06	4,2	0,12	3,4	0,08
Coagulaspositive staphylococcus NMKL 66	–	–	–	–	4,3	0,04
Enterococcus NMKL 98	3,5	0,03	–	–	–	–

– Organism missing or the analysis not conducted

Table 4: The mean value *(m) and standard deviation (s) of analyses, in log_{10}cfu (colony forming units) per ml samples, obtained in the quality control of 10 vials per solution.*

Analysis and method	A		B/C	
	m	s	m	s
Aerobic microorganisms, 30 ˚C NMKL86	4,7	0,07	4,4	0,08
Enterobacteriaceae NMKL 144	4,7	0,07	4,7	0,09
Termotolerant campylobacter, kvant, NMKL119	1,4	0,08	2,8	0,14
Termotolerant campylobacter, kvanl, NMKL119	pos	–	pos	–
Listeria monocytogenes, kvant, NMKL-metod nr. 136	2,8	0,03	2,7	0,04
Listeria monocytogenes, kval, NMKL-metod nr. 136	pos	–	pos	–

Salmonella NMKL-metod nr. 71	0,8*	0,05*	1,0*	0,04*
Escherichia coli O157 NMKL-metod nr. 164	–	–	1,5*	0,03*

* The value is based on parallell solutions

– Organism missing or the analysis not conducted