

Food Microbiology

January 2017

Jonas Ilbäck



Edition

Version 1 (2017-04-26)

Editor in chief

Hans Lindmark, head of Biology department, National Food Agency

Responsible for the scheme

Jonas Ilbäck, microbiologist, Biology department, National Food Agency

PT January 2017 is registered as no. 2016/03619 at the National Food Agency.

Proficiency Testing **Microbiology – Food**

January 2017



Accred. no. 1457
Proficiency testing
ISO/IEC 17043

Quantitative analyses

- Aerobic microorganisms, 30 °C
- Enterobacteriaceae
- Thermotolerant *Campylobacter*
- *Listeria monocytogenes*

Qualitative analyses

- Thermotolerant *Campylobacter*
- *Listeria monocytogenes*
- *Salmonella*
- *Escherichia coli* O157
- Pathogenic *Vibrio* spp.
- *Yersinia enterocolitica*

Abbreviations

Media

ALOA	Agar <i>Listeria</i> according to Ottaviani and Agosti
APW 2%	Alcaline peptone water, 2 % NaCl
BPW	Buffered Peptone Water
BA	Blood Agar
BS	Bromthymol blue Saccharose agar
CIN	Cefsulodin Irgasan Novobiocin agar
CT-SMAC	Cefixime-tellurite-sorbitol-MacConkey-agar
ITC	Irgasan Ticarcillin potassium Chlorate broth
mCCDA	Modified Charcoal Cephoperazone Deoxycholate Agar
MKTTn	Muller-Kauffmann tetrathionate/novobiocin broth
MPCA	Milk Plate Count Agar
MRB	Modified Rappaport Broth
MSRV	Modified Semi-solid Rappaport-Vassiliadis enrichment media
mTSB	Modified Tryptone Soya Broth
PCA	Plate Count Agar
PSB	Peptone Sorbitol Bile salts broth
RVS	Rappaport-Vassiliadis Soy peptone broth
SP	Salt Polymyxin broth
SSDC	<i>Salmonella/Shigella</i> Sodium Deoxycholate Calcium chloride agar
TCBS	Thiosulphate Citrate Bile salts Sucrose agar
TSA	Tryptic Soya Agar
TSBY	Tryptone Soya Broth with Yeast extract
XLD	Xylose Lysine Deoxycholate agar
VRBG	Violet Red Bile Glucose agar

Organisations

AFNOR	French National Standardization Association
AOAC	AOAC INTERNATIONAL
ISO	International Organization for Standardization
NMKL	Nordic Committee for Food Analyses
SLV/NFA	Livsmedelsverket/National Food Agency, Sweden

Contents

General information on results evaluation.....	4
Results of the PT round January 2017.....	5
- General outcome	5
- Aerobic microorganisms, 30 °C.....	6
- Enterobacteriaceae	7
- Thermotolerant <i>Campylobacter</i>	9
- <i>Listeria monocytogenes</i>	11
- <i>Salmonella</i>	13
- <i>Escherichia coli</i> O157	14
- Pathogenic <i>Vibrio spp.</i>	15
- <i>Yersinia enterocolitica</i>	16
Outcome of the results of individual laboratory – assessment	18
- Box plot	19
Test material and quality control	24
- Test material	24
- Quality control of the mixtures	25
References	26
Annex 1: Results obtained by the participants	
Annex 2: z-scores of all participants	

General information on results evaluation

Statistical evaluation of the results

Highly deviating values that did not belong to a strictly normal distribution were identified as statistical outliers (Grubbs' test modified by Kelly (1)). In some cases, subjective adjustments were made to set limits, based on knowledge of the mixture's contents. Outliers and false results were not included in the calculations of means and standard deviations. Results reported as "> value" were excluded from the evaluation. Results reported as "< value" were interpreted as being zero (negative result). All reported results are presented in Annex 1.

According to EN ISO/IEC 17043, for which the proficiency testing programme is accredited, it is mandatory for the participating laboratories to report method information for all their analyses. Method information is sometimes difficult to interpret, since many laboratories report a medium that is not included in the standard method that they refer to. Results from laboratories that report contradictory data on methods/media have either been excluded from the method analysis, or been added to the group of "Others", together with results from methods and media that are only used by 1-2 laboratories.

Mean values and standard deviations are normally provided for the different analyses. When the total number of reported results for an analysis is fewer than 20, the median is provided instead of the mean value. For method groups with fewer than 5 results, only the number of false results and outliers are provided.

Uncertainty of measurement for the assigned values

The uncertainty of measurement for an assigned value is calculated as the standard deviation divided by the square root of the number of correct results ("standard error"). The assigned value of evaluated parameters is the mean value of the participants results.

Table and figure legends

Tables

N	number of laboratories that performed the analysis
n	number of laboratories with satisfactory result
m	mean value in \log_{10} cfu/ml (false results and outliers excluded)
s	standard deviation
F	number of false positive or false negative results
<	number of low outliers
>	number of high outliers
	global results for the analysis
	values discussed in the text

Figures

Histograms of the analytical results for each mixture are presented. The mean value of the analysis results is indicated in each histogram.

	values within the interval of acceptance (Annex 1)
	outliers
	false negative results
*	values outside of the x-axis scale

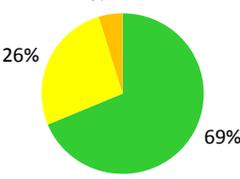
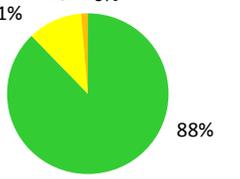
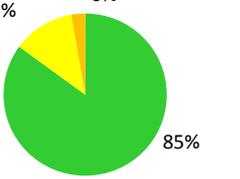
Results of the PT round January 2017

General outcome

Samples were sent to 153 laboratories, 33 in Sweden, 102 in other European countries, and 18 outside Europe. Of the 147 laboratories that reported results, 64 (44 %) provided at least one result that received an annotation. In the previous round with similar analyses (January 2016), the proportion was 41 %.

Individual results for each analysis in the PT round are listed in Annex 1 and are also available on the website after logging in: www2.slv.se/absint.

Table 1 Microorganisms in each mixture and % of deviating results (N: number of reported results, F%: false positive or false negative, X%: outliers).

		Mixture A				Mixture B				Mixture C			
% participants with 0 annotation 1 annotation 2 annotations >2 annotations													
Organisms		<i>Aeromonas hydrophila</i> <i>Campylobacter coli</i> <i>Escherichia coli O157</i> <i>Listeria monocytogenes</i>				<i>Bacillus cereus</i> <i>Micrococcus</i> sp. <i>Salmonella</i> Enteritidis <i>Vibrio cholerae</i> <i>Yersinia enterocolitica</i>				<i>Campylobacter jejuni</i> <i>Proteus mirabilis</i> <i>Salmonella</i> Enteritidis <i>Vibrio parahaemolyticus</i>			
Analysis		Target	N	F%	X%	Target	N	F%	X%	Target	N	F%	X%
Aerob. microorg. 30 °C		<i>A. hydrophila</i>	129	1	1	<i>Micrococcus</i> sp.	126	0	10	<i>P. mirabilis</i>	127	0	4
Enterobacteriaceae		<i>E. coli</i> O157 (<i>A. hydrophila</i>)	105	30	0	<i>Y. enterocolitica</i> <i>S. Enteritidis</i>	104	0	4	<i>P. mirabilis</i>	104	2	9
Thermotol. Camp.	Quant.	<i>C. coli</i>	11	27	0	-	11	0	0	<i>C. jejuni</i>	11	0	0
	Qual.		24	4	-		23	4	-		23	0	-
<i>L. monocytogenes</i>	Quant.	<i>L. monocytogenes</i>	61	2	13	-	62	0	0	-	62	2	0
	Qual.		95	1	-		93	0	-		93	0	-
<i>Salmonella</i>		-	117	3	-	<i>S. Enteritidis</i>	117	1	-	<i>S. Enteritidis</i>	117	6	-
<i>E. coli</i> O157		<i>E. coli</i> O157	25	0	-	-	24	0	-	-	24	0	-
Path. <i>Vibrio</i> spp.		-	20	10	-	<i>V. cholerae</i>	19	11	-	<i>V. parahaemolyticus</i>	19	11	-
<i>Y. enterocolitica</i>		-	13	0	-	<i>Y. enterocolitica</i>	12	0	-	-	12	0	-

- : no target organism or no value
 (microorganism): false positive before confirmation

Aerobic microorganisms, 30 °C

Mixture A

A strain of *Aeromonas hydrophila* was present in the highest concentration, and thus most colonies were from this species. The analyses were without problem for the majority of the 129 laboratories, but the results had a rather wide distribution, with a relatively large number of results lower than the main peak. One false negative result was also reported. Statistically, only one low outlier was identified among the low results. Participants should however be aware that the same mixture has been used also in a previous PT round (January 2016). In that earlier PT round, the results were distributed around a distinct peak, and results lower than \log_{10} 3.5 were considered outside the accepted interval.

Mixture B

A strain of *Micrococcus* sp. was present in the highest concentration, and thus most colonies were from this species. As a whole, the results were distributed around a distinct peak, but 10 low and 2 high outliers were reported.

Mixture C

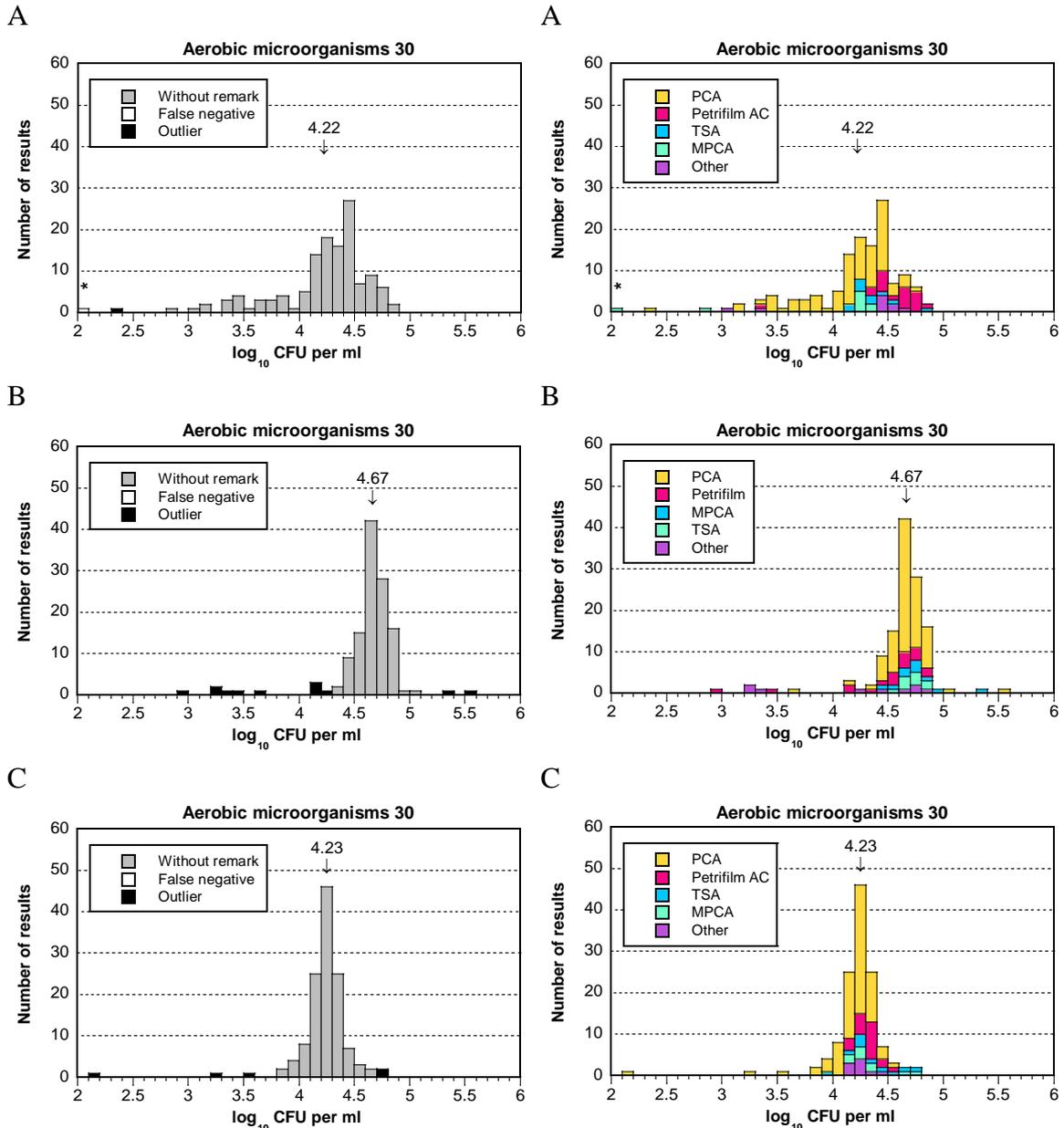
A strain of *Proteus mirabilis* was present in the highest concentration, and thus most colonies were from this species. The analysis was without problem for the laboratories, and the results were distributed around a distinct peak. Three low and 2 high outliers were reported.

General remarks

The results were for the most part without problem for the laboratories, with a relatively small number of outliers for mixture B, and occasional outliers for mixtures A and C. The wider range of results reported for mixture A does not have an obvious explanation. Results lower than the main peak are mainly associated with the use of PCA, and the methods NMKL 86 (2006 and 2013) and ISO 4833 (2003 and 2013). These methods are highly similar, and all stipulate incubation for 72 h at 30 °C. In contrast, the incubation time and temperature for 3M™ Petrifilm™ Aerobic Count (Petrifilm AC) varies depending on the method, and is for example 48 h at 35 °C according to AOAC® 990.12 and 72 h at 30 °C according to AFNOR 3M 01/1-09/89. Despite this, the results from Petrifilm AC are more clustered compared to those from PCA. Possibly the surface spreading technique used with this substrate is more gentle to the bacteria compared to the pour plate method used in NMKL 86:2013 and ISO 4833-1:2013. A milder treatment of the bacteria could also help explain the higher results for Petrifilm AC, compared to other media, in mixture A.

Results from analysis of aerobic microorganisms at 30 °C.

Media	N	Mixture A						Mixture B						Mixture C					
		n	m	s	F	<	>	n	m	s	F	<	>	n	m	s	F	<	>
All results	129	127	4.22	0.40	1	1	0	114	4.67	0.12	0	10	2	122	4.23	0.14	0	3	2
PCA	81	80	4.15	0.36	0	1	0	77	4.66	0.12	0	2	1	76	4.20	0.13	0	3	0
Petrifilm AC	20	20	4.53	0.31	0	0	0	14	4.64	0.13	0	4	0	20	4.31	0.10	0	0	0
TSA	10	10	4.36	0.23	0	0	0	9	4.70	0.14	0	0	1	9	4.31	0.21	0	0	1
MPCA	9	8	4.09	0.51	1	0	0	9	4.70	0.09	0	0	0	8	4.29	0.15	0	0	1
Other	9	9	4.20	0.58	0	0	0	5	4.67	0.13	0	4	0	9	4.24	0.10	0	0	0



Enterobacteriaceae

Mixture A

No target organism for the analysis of Enterobacteriaceae was present in the recommended dilutions. Laboratories that analysed the undiluted sample may however have detected *E. coli* O157, which is positive for the analysis of Enterobacteriaceae, and was present at a low concentration (\log_{10} 0.75 cells/ml in the undiluted sample). Results corresponding to the concentration of *E. coli* O157 were therefore judged as correct. However, 31 of the 105 reporting laboratories had clear false positive results with concentrations considerably higher than that of *E. coli* O157. Most of the false results corresponded to the concentration of *Aeromonas hydrophila*. The included strain of *A.*

hydrophila grows on violet red bile glucose agar (VRBG) with colonies that can be interpreted as belonging to Enterobacteriaceae. They should however be distinguished from Enterobacteriaceae in subsequent confirmation, since colonies of *A. hydrophila*, in contrast to Enterobacteriaceae, are oxidase positive. Identification of *A. hydrophila* as Enterobacteriaceae may be a consequence of not confirming or with having problems in the confirmation.

Mixture B

Strains of *Yersinia enterocolitica* and *Salmonella* Enteritidis were target organisms for the analysis, which in general was without problem for the laboratories. The results were distributed well, and 4 high outliers were reported.

Mixture C

A strain of *Proteus mirabilis* was target organism for the analysis. The analysis was without problem for the majority of laboratories, however low outliers were reported by 9 laboratories. Two laboratories reported false negative results.

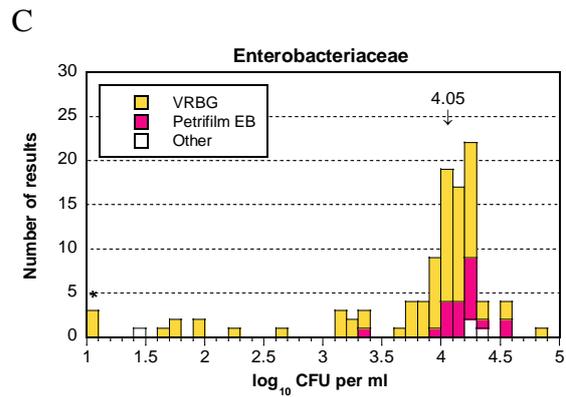
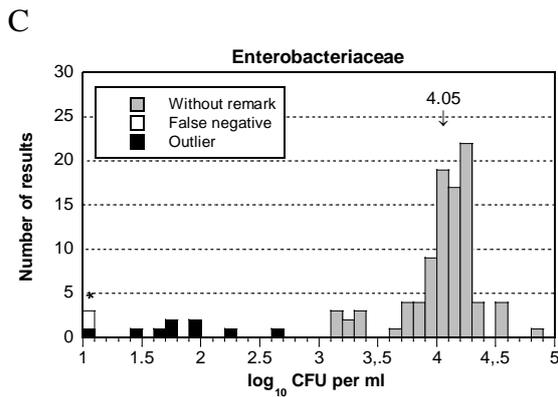
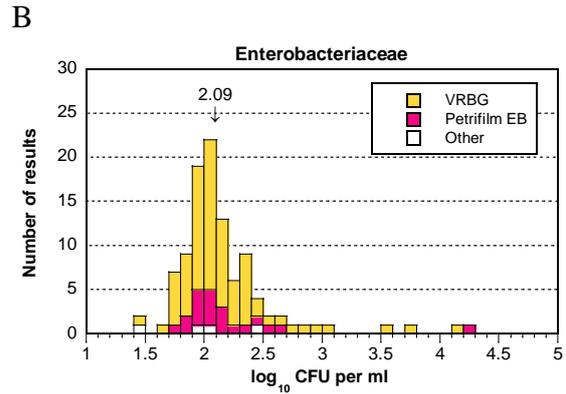
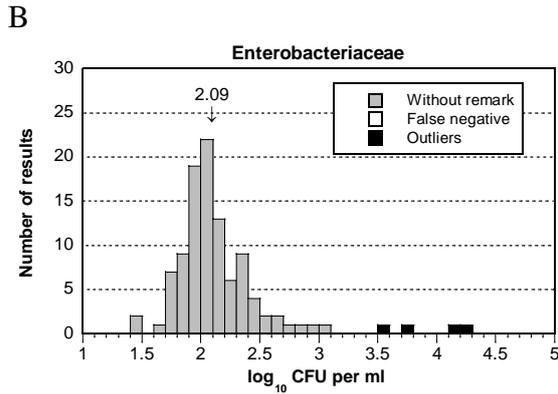
General remarks

The analyses of mixtures B and C were as a whole without problem for the laboratories. As in previous proficiency testing rounds, the majority of the laboratories reported the use of either NMKL 144:2005 or ISO 21528-2:2004. Consequently, most laboratories (77 %) reported VRBG as medium. Among the remaining laboratories 20 % used 3M™ Petrifilm™ Enterobacteriaceae (Petrifilm EB) and 3 % used other media. Regardless of method and media, equivalent results were reported for both mixture B and C.

For mixture C, 9 low outliers were reported. All but one of these results were reported by laboratories that used VRBG and followed NMKL 144:2005 or ISO 21528-2:2004. Enterobacteriaceae are oxidase negative and Gram-negative bacteria. They ferment glucose, with the production of acid by-products. On VRBG they form pink/red colonies, with or without a bile precipitation zone. None of the 9 outliers in mixture C were reported by users of Petrifilm EB, and it is possible that the colour indicator in Petrifilm EB assists in detecting acid by-products of glucose fermentation. In NMKL 144:2005, presumptive colonies from VRBG are confirmed by a negative oxidase test. In ISO 21528-2:2004, presumptive colonies are confirmed both with an oxidase test and with a glucose fermentation test. Low outliers were however reported both by laboratories that performed confirmation tests and those that did not. So far, no plausible explanation for the low outliers and false negative results has been identified. At the National Food Agency, the colonies growing on VRBG were without doubt identified as belonging to Enterobacteriaceae.

Results of Enterobacteriaceae analysis

Metod	N	Mixture A						Mixture B						Mixture C					
		n	m	s	F	<	>	n	m	s	F	<	>	n	m	s	F	<	>
All results	105	74	-	-	31	0	0	100	2.09	0.28	0	0	4	93	4.05	0.32	2	9	0
VRBG	81	64	-	-	17	0	0	77	2.09	0.29	0	0	3	70	4.00	0.33	2	8	0
Petrifilm EB	20	8	-	-	12	0	0	19	2.10	0.23	0	0	1	20	4.16	0.25	0	0	0
Other	4	2	-	-	2	0	0	4	-	-	0	0	0	3	-	-	0	1	0



Thermotolerant *Campylobacter*

Mixture A

A strain of *Campylobacter coli* was target organism. Of the 11 laboratories that performed the quantitative analysis, 3 reported false negative results. In contrast, only 1 of the 24 laboratories in the qualitative analysis reported a false negative result.

Mixture B

No target organism for this analysis was present in mixture B. All laboratories reported correct negative results for the quantitative analysis, whereas 1 false positive result was reported for the qualitative analysis.

Mixture C

A strain of *Campylobacter jejuni* was target organism. The analysis was without problem for the laboratories, and all reported results in both the quantitative and qualitative analysis were without remark.

General remarks

Eleven laboratories performed the quantitative analyses, which makes it difficult to evaluate the results statistically. Of the participants, 6 followed NMKL 119:2007 and 5 followed ISO/TS 10272-2:2006. False results were reported by users of both methods. All laboratories except one reported the use of modified charcoal cephaloperazone deoxycholate agar (mCCDA).

The results in the quantitative analysis had a relatively large distribution, something that has been observed in several earlier PT rounds. *Campylobacter* spp., are sensitive to

mechanical stress and to dehydration. Differences in the results might therefore be a consequence of a harsh surface spreading. At the National Food Agency, *Campylobacter* spp., are carefully spread onto the plates and the final drying of the bacterial suspension is done by leaving the lids of the plates slightly open.

Similar to the quantitative analysis, NMKL 119:2007 and ISO 10272-1:2006 were the most widely used methods for the qualitative analysis, and the majority of laboratories used mCCDA. Five laboratories used other methods (*e.g.* PCR-based methods and VIDAS), or other methods in combination with the NMKL and ISO methods. Somewhat more laboratories reported results for the qualitative analysis, but the results are still difficult to evaluate statistically.

The number of laboratories that performed a confirmation test was high; 92 % of the 24 laboratories in the qualitative analysis reported some type of confirmation. *Campylobacter* spp. are Gram-negative, oxidase positive and catalase positive bacteria. They can also be confirmed by their appearance; spiral-shaped rods that display a characteristic darting/rotating movement. Further, *C. jejuni*, *C. coli* and *C. lari* can be separated by differences in their hydrolysis of hippurate and indoxyl acetate and their sensitivity/resistance to nalidixic acid and cephalothin.

Results of thermotolerant Campylobacter quantitative analysis

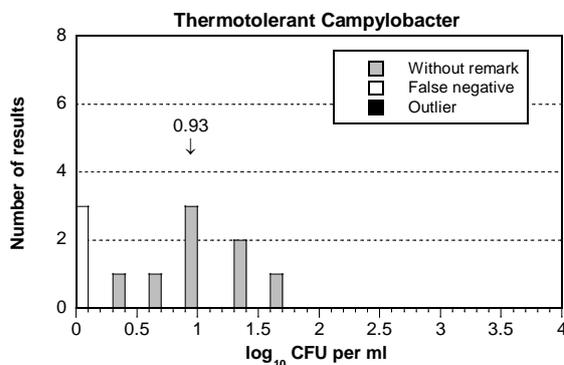
Method	N	Mixture A						Mixture B						Mixture C					
		n	Med*	s	F	<	>	n	m	s	F	<	>	n	Med*	s	F	<	>
All results	11	8	0.93	-	3	0	0	11	-	-	0	-	-	11	2.10	-	0	0	0
NMKL 119	6	4	0.75	-	2	0	0	6	-	-	0	-	-	6	2.09	-	0	0	0
ISO 10272-2	5	4	1.32	-	1	0	0	5	-	-	0	-	-	5	2.10	-	0	0	0

* Med = median

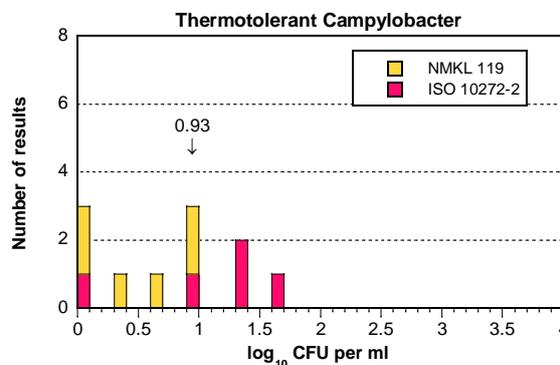
Results of thermotolerant Campylobacter qualitative analysis

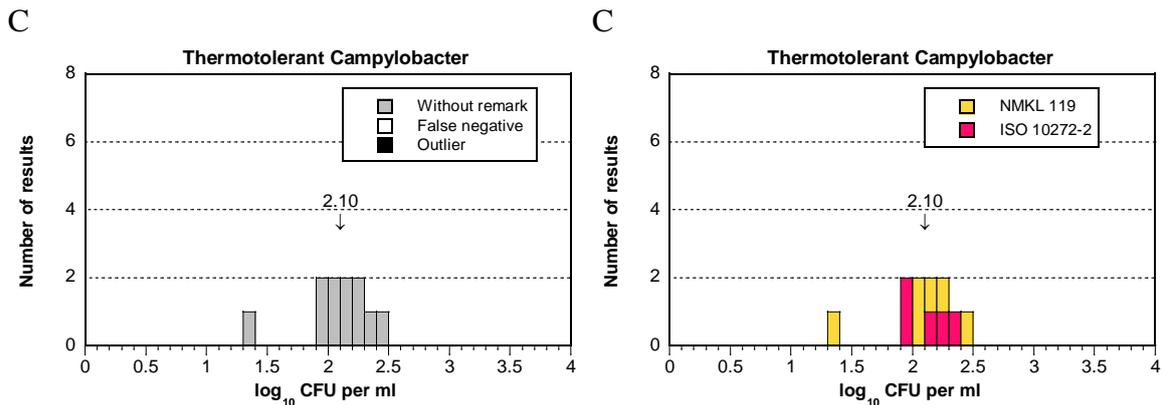
Method	N	Mixture A			Mixture B			Mixture C		
		n	+/-	F	n	+/-	F	n	+/-	F
All results	24	23	Pos	1	23	Neg	1	23	Pos	0
NMKL 119	13	13	Pos	0	13	Neg	0	13	Pos	0
ISO 10272-1	6	5	Pos	1	5	Neg	0	5	Pos	0
Other	5	5	Pos	0	4	Neg	1	5	Pos	0

A



A





Listeria monocytogenes

Mixture A

A strain of *Listeria monocytogenes* was target organism for the analysis. The quantitative analysis was without problem for the majority of the 62 laboratories; however 7 laboratories reported results that were all clearly lower than the main peak. Such low values for *L. monocytogenes* have been seen in previous PT rounds (January 2016), where they have statistically been determined as outliers. In the current PT round, the 7 values below log₁₀ 2.0 constituted a minor peak, clearly separated from the main peak. In these situations, the statistical test used is insensitive to discern these values as outliers. Considered individually, they were however easily determined as outliers. Taken together, values below log₁₀ 2.0 were therefore considered to be statistically unlikely, and were regarded as low outliers. The low outliers could not be attributed to the use of a specific method or media. In addition to the low outliers, one high outlier and one false negative result were reported. In the qualitative analysis, one of 95 laboratories reported a false negative result.

Mixture B

No target organism for this analysis was present in mixture B. The analyses were without problem for the laboratories, and all reported results in both the quantitative and qualitative analysis were without remarks.

Mixture C

No target organism for this analysis was present in mixture C. All laboratories that performed the qualitative analysis reported correct negative results. For the quantitative analysis, one laboratory reported a false positive result. The false positive result was possibly incorrectly reported, as it would have been unreasonably high even if *Listeria* sp. had been present in the mixture.

General remarks

With the exception of the low outliers in mixture A, the analyses were without problem for the laboratories. Regardless of method and media, equivalent results were reported.

ISO 11290-1 and ISO 11290-2 were the most used methods for the qualitative and quantitative analyses, respectively. The qualitative method (ISO 11290-1) is based on primary enrichment in half Fraser broth, followed by secondary enrichment in Fraser

broth. Aliquots from both half Fraser and Fraser are plated onto selective agar for *Listeria* according to Ottaviani and Agosti (ALOA) and also onto a second selective medium chosen by the individual laboratory. Typical colonies of *L. monocytogenes* are green-blue on ALOA due to β -glucosidase activity, and surrounded by an opaque halo due to hydrolysis of inositol in the media. The halo is sometimes weak or may not be present at all. Confirmation of *Listeria* spp. is by a positive catalase test and a positive Gram staining result. Confirmation of *L. monocytogenes* is by β -haemolysis on blood agar (BA), carbohydrate utilization (fermentation of rhamnose but not xylose) and increased and decreased β -haemolysis in the presence of *Staphylococcus aureus* and *Rhodococcus equi* respectively (CAMP test). In the quantitative method (ISO 11290-2), an initial suspension of the sample is made in buffered peptone water (BPW) or in half Fraser broth, and aliquots from this are transferred to ALOA. Confirmation is carried out essentially as in the qualitative method. The qualitative and quantitative methods utilized in NMKL 136 – the second most used method for the quantitative analysis in this PT round – are similar to the ISO methods. At the National Food Agency, colonies of *L. monocytogenes* present in mixture A were on ALOA blue-green and surrounded by a distinct opaque zone. In subsequent confirmation, the strain displayed β -haemolysis on BA, and fermented rhamnose but not xylose.

New versions of both ISO 11290-1 (detection method) and ISO 11290-2 (enumeration method) are scheduled for publication in 2017. In the revised methods, identification of *Listeria* spp. is included as a mandatory step. The incubation time in Fraser broth will also be shortened from 48 h to 24 h, and confirmation with catalase test and CAMP test will be optional. Gram staining will be optional if the isolation agar allows distinction between pathogenic and non-pathogenic *Listeria* spp.

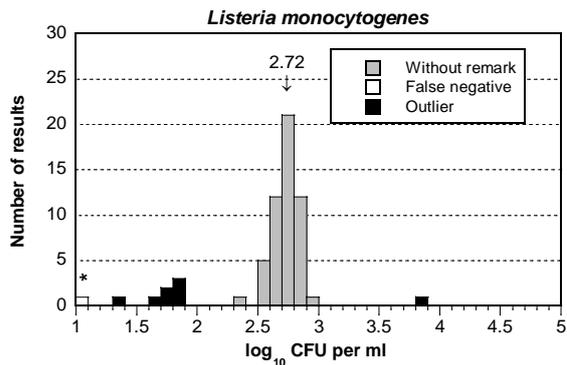
Results of *L. monocytogenes* quantitative analysis

Method	N	Mixture A						Mixture B						Mixture C					
		n	m	s	F	<	>	n	m	s	F	<	>	n	m	s	F	<	>
All results	62	52	2.72	0,11	1	7	1	62	-	-	0	-	-	61	-	-	1	-	-
ISO 11290-2	25	22	2.73	0,13	0	2	1	25	-	-	0	-	-	24	-	-	1	-	-
NMKL 136	17	14	2.74	0,10	0	3	0	17	-	-	0	-	-	17	-	-	0	-	-
RAPID' L.mono	14	13	2.69	0,10	0	1	0	14	-	-	0	-	-	14	-	-	0	-	-
Other	6	3	-	-	1	1	0	6	-	-	0	-	-	6	-	-	0	-	-

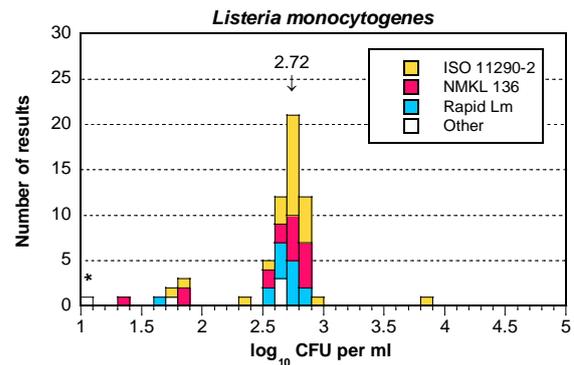
Results of *L. monocytogenes* qualitative analysis

Method	N	Mixture A			Mixture B			Mixture C		
		n	+/-	F	n	+/-	F	n	+/-	F
All results	95	95	Pos	1	93	Neg	0	93	Neg	0
ISO 11290-1	28	28	Pos	0	27	Neg	0	27	Neg	0
RAPID' L.mono	18	18	Pos	0	18	Neg	0	18	Neg	0
NMKL 136	16	16	Pos	0	16	Neg	0	16	Neg	0
VIDAS®	15	15	Pos	0	15	Neg	0	15	Neg	0
PCR	7	7	Pos	0	7	Neg	0	7	Neg	0
Other	11	10	Pos	1	10	Neg	0	10	Neg	0

A



A



Salmonella

Mixture A

No target organism for this analysis was present in mixture A. Of the 117 laboratories that performed the analysis, 3 reported false positive results.

Mixture B

A strain of *Salmonella* Enteritidis was target organism for the analysis. One laboratory reported a false negative result.

Mixture C

The same strain of *Salmonella* Enteritidis as in mixture B was target organism for the analysis in mixture C. Seven laboratories reported false negative results.

General remarks

The analyses were generally without problem, and no differences in results could be attributed to the use of a specific method or medium.

The same strain of *S. Enteritidis* was present in both mixture B and C, and in a similar concentration (\log_{10} 2.06 and 2.02 for mixtures B and C, respectively). Despite this, whereas only 1 laboratory reported a false negative result for mixture B, 7 laboratories reported false negative results for mixture C. No obvious explanation to this discrepancy could be found. All 7 laboratories also reported the use of a confirmation test. At the National Food Agency, the strain of *S. Enteritidis* was easily identified and displayed typical colony morphology on XLD and Brilliance™ in both mixtures.

NMKL 71:1999 was the most used method, followed by ISO 6579:2002, VIDAS, and various PCR-based methods. Nineteen laboratories used rare methods (used by 3 laboratories or less), that in the table below are included in the group “Other method”, together with laboratories that stated the use of more than one method, and laboratories that used older versions of the NMKL and ISO methods.

NMKL 71:1999 and ISO 6579:2002 are very similar. Both are based on pre-enrichment in buffered peptone water (BPW), followed by selective enrichment in Rappaport-Vassiliadis soy peptone broth (RVS) and subsequent plating onto selective xylose lysine deoxycholate agar (XLD) and a second selective agar medium chosen by the individual laboratory. ISO 6579:2002 differs from NMKL 71:1999 in that it also includes selective enrichment in Muller-Kauffmann tetrathionate/novobiocin broth (MKTn). Confirmation is by biochemical (e.g. mannitol, urea) and serological (*Salmonella* poly O- and poly H-antiserum agglutination) verifications. On XLD,

typical *Salmonella* colonies are transparent red and have a black center. As a complementary media to XLD, most laboratories in this PT round used a chromogenic media containing the substrates magenta-caprylate and X-glucoside, that detect caprylate esterase and β -glucosidase activity, respectively. On these media (*e.g.* Brilliance™ and COMPASS) colonies of *Salmonella* typically have a magenta/purple colour (caprylate esterase + and β -glucosidase –) that distinguish them from other microorganisms that may grow on the media.

A new ISO method for *Salmonella* is now available; ISO 6579-1:2017. Major changes compared to ISO 6579:2002 include the use of modified semi-solid Rappaport-Vassiliadis enrichment media (MSRV). This is suitable for detection of motile *Salmonella*, and is allowed for all sample types. For confirmation, colonies can be tested directly from the selective media if they are well separated on the plate. In the confirmation step, detection of β -galactosidase and indole test are optional, and a positive result for both O- and H-antigen agglutination is a requirement for a strain to be considered as *Salmonella*.

NMKL 187:2007 was used by 6 laboratories. This method is intended for the detection of motile *Salmonella*, and therefore differs from NMKL 71:1999 in that the selective enrichment broth (RVS) is substituted by MSR.V. The method was recently revised, and the new NMKL 187:2016 includes clarifications on the complementary selective agar medium and the MSR.V composition, as well as additional paragraphs for faecal samples and materials from primary animal production.

Results of *Salmonella* qualitative analysis

Method	N	Mixture A			Mixture B			Mixture C		
		n	+/-	F	n	+/-	F	n	+/-	F
All results	117	114	Neg	3	116	Pos	1	110	Pos	7
NMKL 71:1999	41	40	Neg	1	41	Pos	0	37	Pos	4
ISO 6579:2002	21	21	Neg	0	21	Pos	0	21	Pos	0
VIDAS	15	14	Neg	1	15	Pos	0	15	Pos	0
PCR method	15	15	Neg	0	15	Pos	0	15	Pos	0
NMKL 187:2007	6	6	Neg	0	6	Pos	0	6	Pos	0
Other method	19	18	Neg	1	18	Pos	1	16	Pos	3

Escherichia coli O157

Mixture A

A strain of *E. coli* O157 was target organism for the analysis in mixture A. All 25 laboratories that performed the analysis correctly reported positive results.

Mixture B

No target organism for this analysis was present in mixture B. All laboratories that performed the analysis correctly reported negative results.

Mixture C

No target organism for this analysis was present in mixture C. All laboratories that performed the analysis correctly reported negative results.

General remarks

Regardless of method and media, all laboratories performing the analysis reported correct results. The majority of laboratories used methods based on isolation of *E. coli* O157 on cefixime tellurite sorbitol MacConkey agar (CT-SMAC). For pre-enrichment, modified tryptone soya broth (mTSB) was the most common medium. The strain of *E. coli* O157 present in mixture A is sorbitol negative, and formed transparent colonies with a dark center on CT-SMAC at the National Food Agency. It should be noted that one laboratory reported the use of ISO 7251:2005, which is a Most Probable Number Method (MPN) for the detection of presumptive *E. coli*, and not suited for the identification of *E. coli* O157.

Results of *E. coli* O157 qualitative analysis

Method	N	Mixture A			Mixture B			Mixture C		
		n	+/-	F	n	+/-	F	n	+/-	F
All results	25	25	Pos	0	24	Neg	0	24	Neg	0
ISO 16654:2001	7	7	Pos	0	7	Neg	0	7	Neg	0
NMKL 164:2005	3	3	Pos	0	3	Neg	0	3	Neg	0
EB-SM-5036	4	4	Pos	0	4	Neg	0	4	Neg	0
VIDAS	3	3	Pos	0	3	Neg	0	3	Neg	0
PCR method	3	3	Pos	0	3	Neg	0	3	Neg	0
Other	5	5	Pos	0	4	Neg	0	4	Neg	0

Pathogenic *Vibrio* spp.

Mixture A

No target organism for this analysis was present in mixture A. Of the 20 laboratories that performed the analysis, 2 reported a false positive result.

Mixture B

A strain of *Vibrio cholerae* was target organism for the analysis. Two false negative results were reported.

Mixture C

A strain of *Vibrio parahaemolyticus* was target organism for the analysis. Two false negative results were reported.

General remarks

Only 20 laboratories performed the analysis, and most used similar methods and media. It is therefore difficult to evaluate differences between methods and media.

The majority of laboratories followed either NMKL 156:1997 or ISO/TS 21872-1:2007. ISO/TS 21872-1:2007 is based on enrichment in alkaline peptone water with 2 % NaCl (APW 2 %), followed by plating onto selective thiosulphate citrate bile salts sucrose (TCBS) agar plates. The procedure in NMKL 156:1997 is similar, but in addition to APW 2% also includes enrichment in salt polymyxin (SP) broth.

At the National Food Agency, *V. cholerae* in mixture B formed typical yellow colonies on TCBS, regardless of whether enrichment was carried out in APW 2% or in SP. Likewise, the strain of *V. parahaemolyticus* in mixture C formed typical blue-green

colonies on TCBS. Upon confirmation, both strains were oxidase positive and sensitive to vibriostaticum O129.

At the National Food Agency, *P. mirabilis* formed atypical small light green colonies on TCBS in the analysis of mixture C. These were oxidase negative, and could thus be distinguished from *V. parahaemolyticus* present on the same plate.

Results of pathogenic Vibrio spp. qualitative analysis

Method	N	Mixture A			Mixture B			Mixture C		
		n	+/-	F	n	+/-	F	n	+/-	F
All results	20	18	Neg	2	17	Pos	2	17	Pos	2
NMKL 156:1997	11	10	Neg	1	9	Pos	2	9	Pos	2
ISO/TS 21872-1:2007	7	7	Neg	0	6	Pos	0	6	Pos	0
Other	2	1	Neg	1	2	Pos	0	2	Pos	0

Yersinia enterocolitica

Mixture A

No target organism for this analysis was present in mixture A. All 13 laboratories that performed the analysis correctly reported negative results.

Mixture B

A strain of *Yersinia enterocolitica* was target organism for the analysis. All laboratories that performed the analysis correctly reported positive results.

Mixture C

No target organism for this analysis was present in mixture C. All laboratories that performed the analysis correctly reported negative results.

General remarks

The analyses were without problem for the laboratories, and no false results were reported. Most laboratories followed NMKL 117:1996 or ISO 10273:2003. The method in ISO 10273:2003 is based on parallel enrichment in semi-selective peptone sorbitol bile salts broth (PSB) and irgasan ticarcillin potassium chlorate broth (ITC), followed by isolation on cefsulodin irgasan novobiocin agar (CIN) and *Salmonella/Shigella* sodium deoxycholate calcium chloride agar (SSDC) respectively. NMKL 117:1996 differs somewhat, and is based on pre- and cold-enrichment in PSB, as well as selective enrichment in modified Rappaport broth (MRB). Following enrichment, colonies are isolated on CIN, but SSDC may also be used. Presumptive colonies are subcultured on bromthymol blue saccharose agar (BS) and saccharose positive colonies (yellow) are selected for confirmation.

Colonies of *Y. enterocolitica* have a typical appearance on CIN, with a red “bull’s eye” center and an outer transparent zone. At the National Food Agency, the strain of *Y. enterocolitica* in mixture B formed typical colonies on both CIN and on BS. The colonies were oxidase negative upon confirmation.

Two laboratories used in-house methods that were based on PCR. One laboratory followed NMKL 163:2013, which is based on growth in semi-selective PSB or in non-selective tryptone soya broth with yeast extract (TSBY), followed by DNA extraction

and real-time PCR detection of the chromosomal virulence-associated *ail* gene in *Y. enterocolitica*. Subculturing from the enrichment media onto CIN plates is optional. The method is suitable when high contamination levels are suspected, and use of NMKL 117:1996 or ISO 10273:2003 is recommended for samples with low suspected levels of *Y. enterocolitica*.

A revised version of ISO 10273 is scheduled for publication during early 2017. In the revised method, characteristic *Y. enterocolitica* colonies isolated on CIN can be confirmed either with the traditional biochemical confirmation steps, or with real-time PCR detection of the *ail* gene as in NMKL 163:2013.

Results of Y. enterocolitica qualitative analysis

Method	N	Mixture A			Mixture B			Mixture C		
		n	+/-	F	n	+/-	F	n	+/-	F
All results	13	13	Neg	0	12	Pos	0	12	Neg	0
ISO 10273:2003	6	6	Neg	0	5	Pos	0	5	Neg	0
NMKL 117:1996	3	3	Neg	0	3	Pos	0	3	Neg	0
NMKL 163:2013	1	1	Neg	0	1	Pos	0	1	Neg	0
Other	3	3	Neg	0	3	Pos	0	3	Neg	0

Outcome of the results of individual laboratory - assessment

The reported results of all participating laboratories are listed in Annex 1, together with the minimum and maximum accepted values for each analysis. Results that received a remark (false results and outliers) are highlighted in yellow, with bold font.

When laboratories appear to have mistakenly analysed the wrong mixture, the corresponding results are written in italics.

Z-scores (see below) for individual analyses are shown in Annex 2 and can be used as a tool by laboratories when following up on the results.

The laboratories are not grouped or ranked based on their results. The performance of a laboratory as a whole can only be evaluated from the number of false results and outliers that are listed in Annex 1 and below the box plots.

Information on the results processing and recommendations for follow-up work are given in the Scheme Protocol (2). Samples for follow-up can be ordered, free of charge via our website: www.livsmedelsverket.se/en/PT-extra.

Z-scores, box plots and deviating results

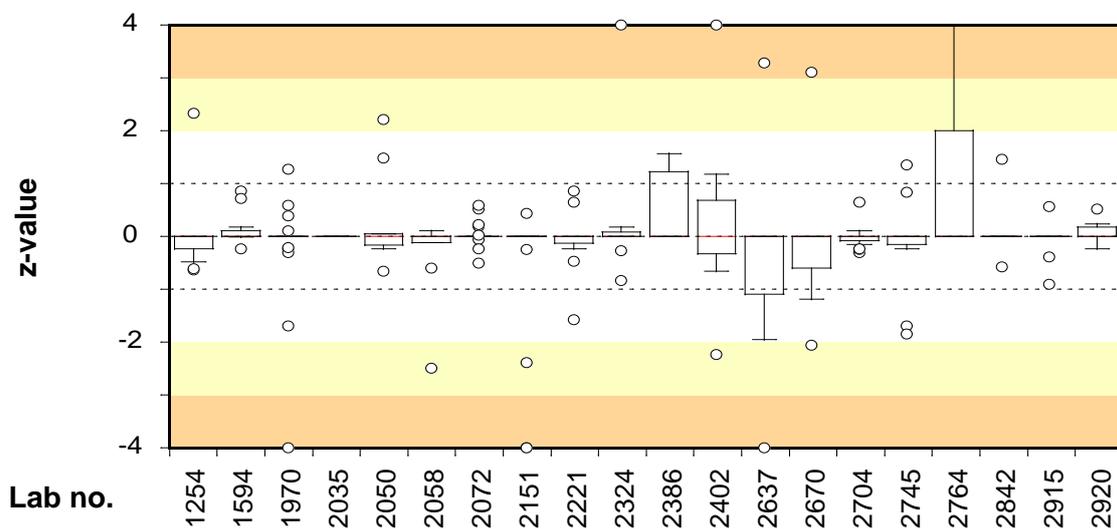
In order to allow comparison of the results from different analyses and mixtures, all results are transformed into standard values (z-scores). For quantitative analyses, a z-score is either positive or negative, depending on whether the individual result is higher or lower than the mean value calculated from all laboratory results for each analysis.

The box plots are based on the z-scores listed in Annex 2, and give a comprehensive view of the achievement of each laboratory. A small box, centred around zero, indicates the results of that individual laboratory, with false results excluded, are close to the general mean values calculated for all laboratory results. The range of z-scores is indicated by the size of the box and, for most laboratories, by lines and/or circles above and beneath the box. For each laboratory, the number of false results and outliers are also listed in the tables below the box plots.

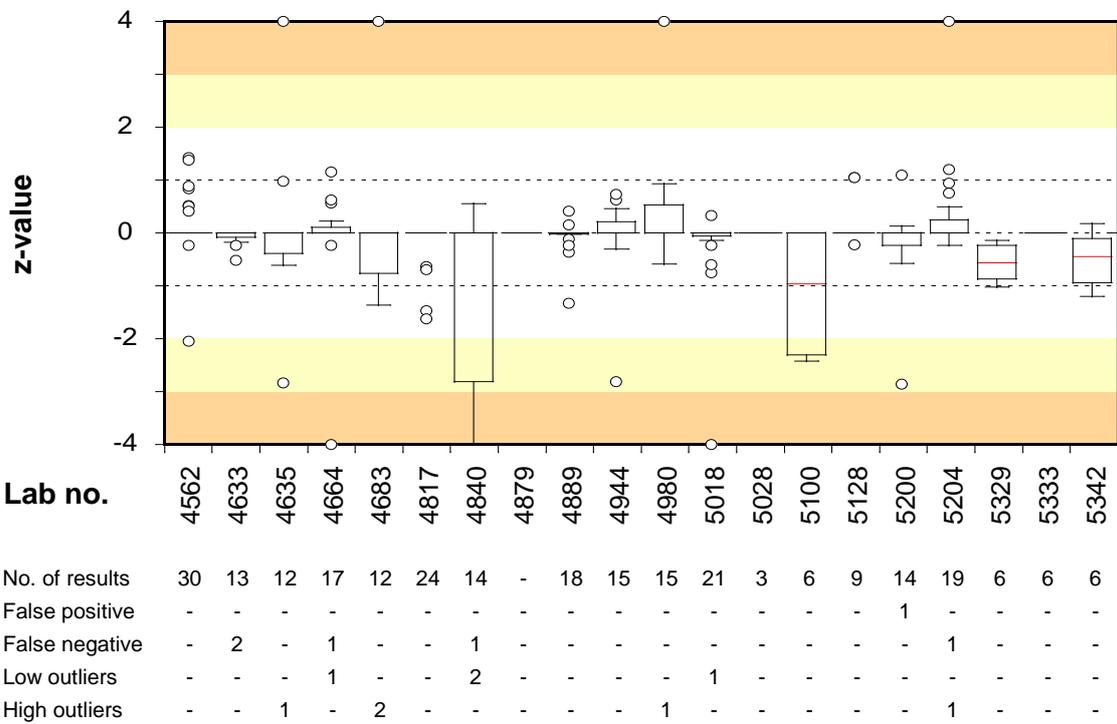
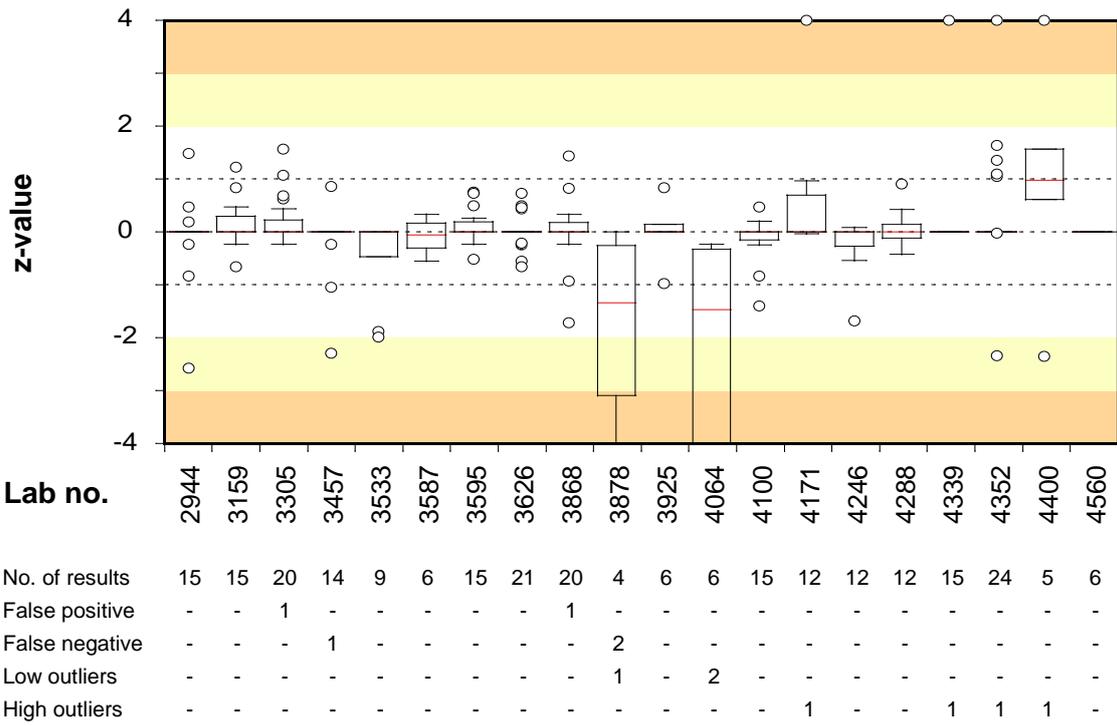
Box plots and numbers of deviating results for each laboratory

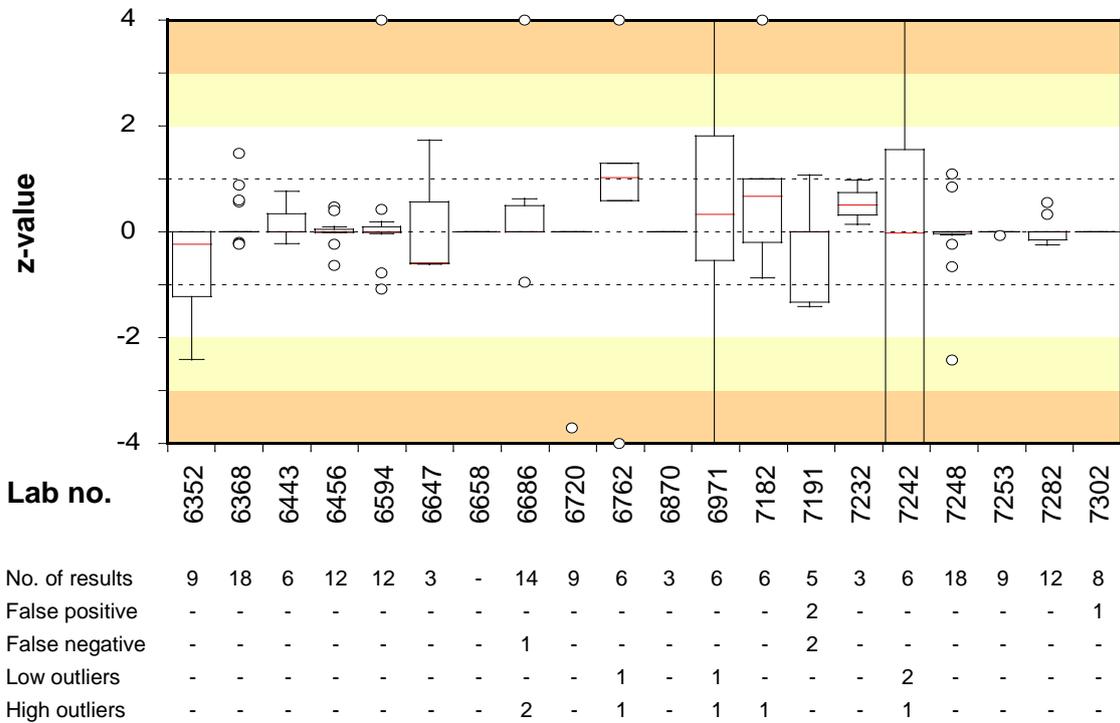
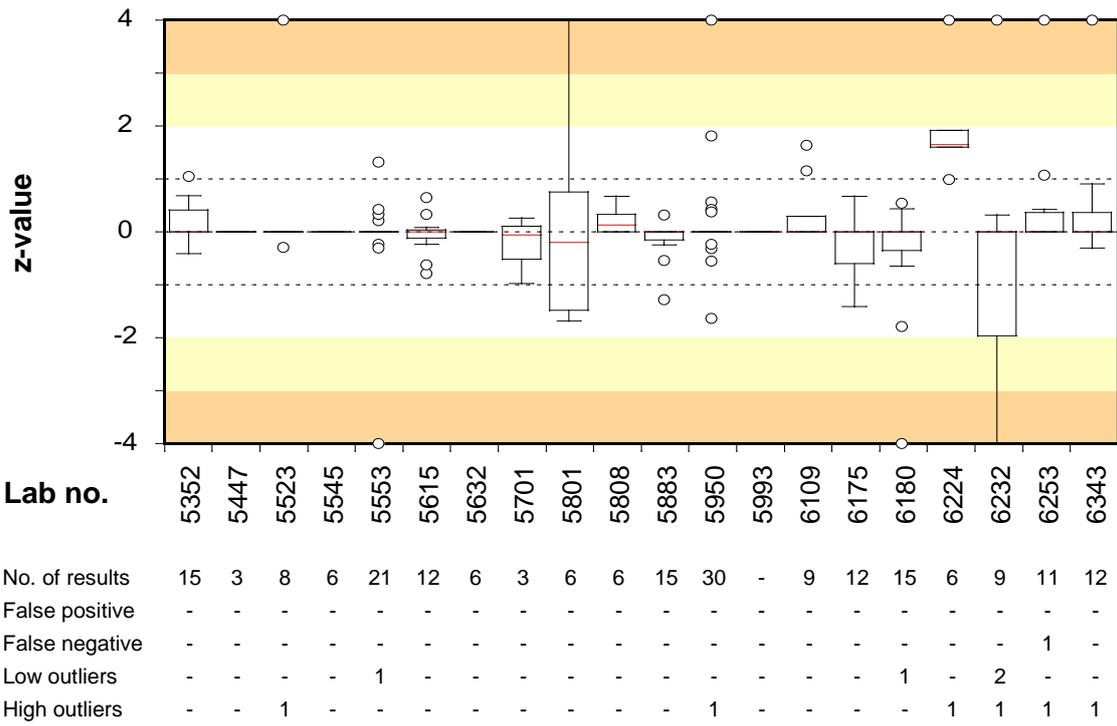
- Z-scores are calculated according to the formula: $z = (x-m)/s$, where x is the result of the individual laboratory, m is the mean of the results of all participating laboratories, and s is the standard deviation of the participating laboratories.
- Outliers are included in the figures after being calculated to z-scores in the same way as for other results.
- False results do not generate any z-scores, and are not included in "No. of results".
- Correct results for qualitative analyses and correct negative results for quantitative analyses without target organism generate a z-score of 0.
- The laboratory median value is illustrated by a horizontal red line in the box.
- The box includes 50 % of a laboratory's results (25 % of the results above the median and 25 % of the results below the median). The remaining 50 % are illustrated by lines and circles outside the box.
- A circle is for technical reasons shown in the plot when a value deviates to certain degree* from the other values. This does not by itself indicate that the value is an outlier.
- z-scores $>+4$ and <-4 are positioned at $+4$ and -4 , respectively, in the plot.
- The background is divided by lines and shaded fields to simplify identifying the range in which the results are located.

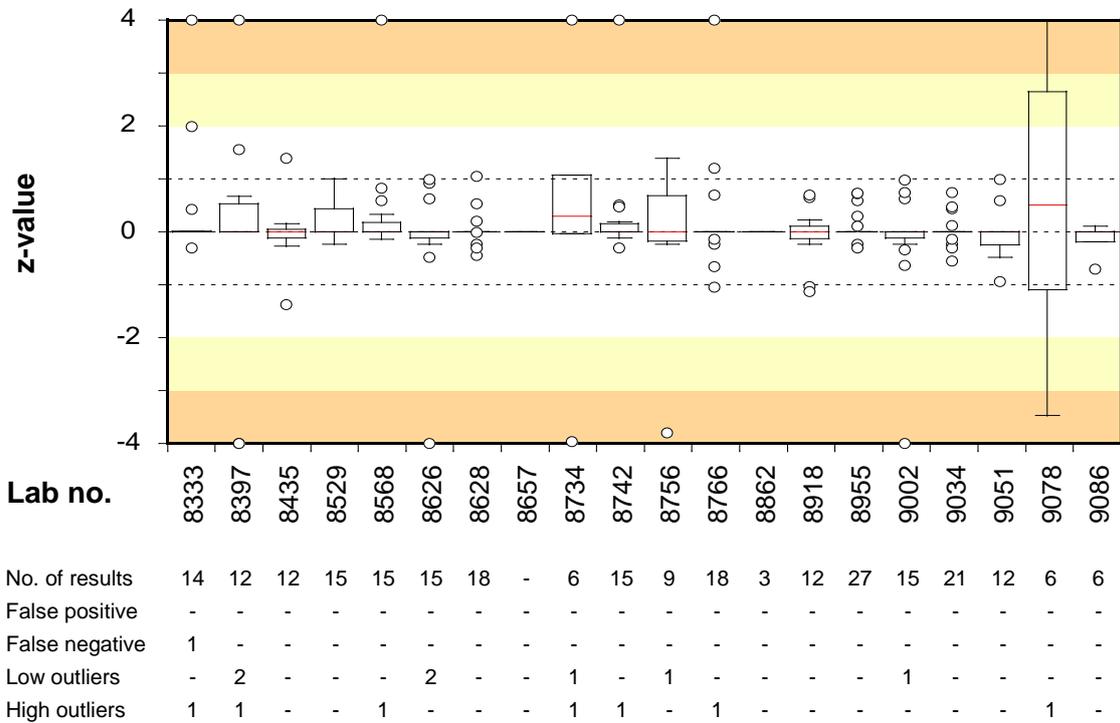
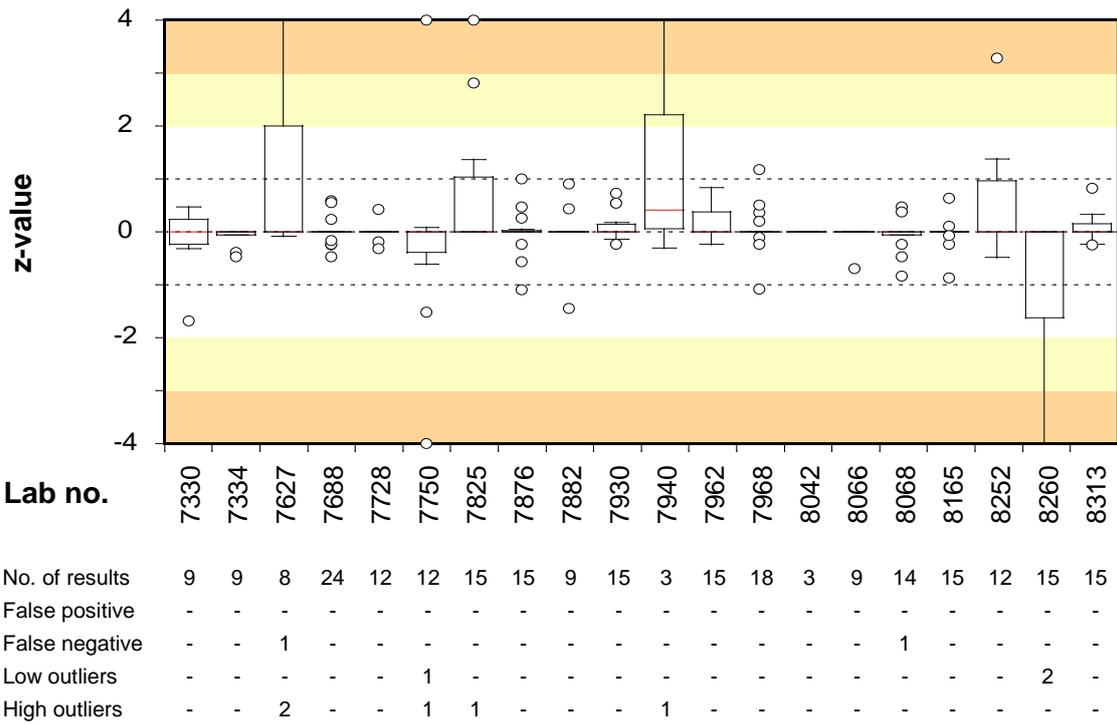
* $< [\text{lowest value in the box} - 1,5 \times (\text{highest value in the box} - \text{lowest value in the box})]$ or $> [\text{highest value in the box} + 1,5 \times (\text{highest value in the box} - \text{lowest value in the box})]$.

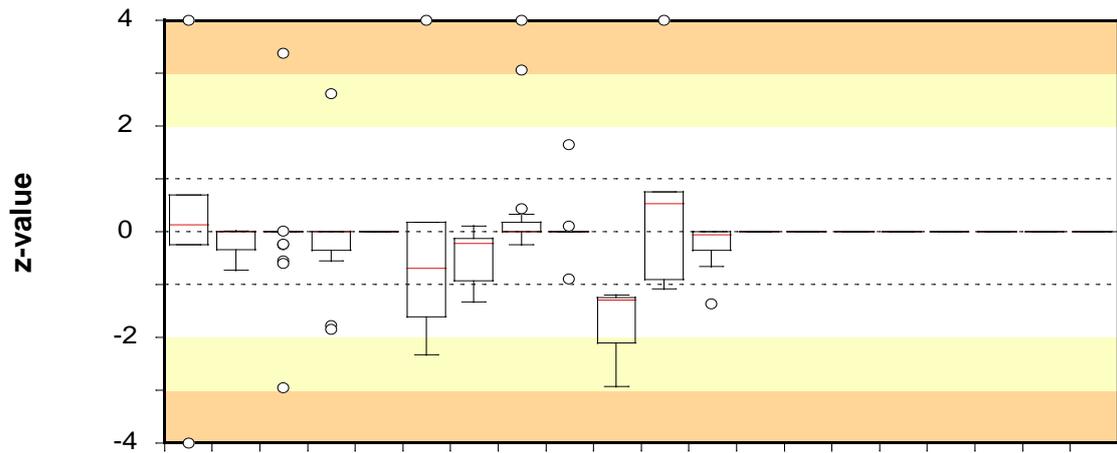


Lab no.	1254	1594	1970	2035	2050	2058	2072	2151	2221	2324	2386	2402	2637	2670	2704	2745	2764	2842	2915	2920
No. of results	15	12	23	3	9	9	22	17	15	12	9	8	15	8	15	15	15	18	11	9
False positive	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
False negative	-	-	1	-	-	-	2	1	-	-	-	-	-	1	-	-	-	-	-	-
Low outliers	-	-	1	-	-	-	-	2	-	-	-	-	1	-	-	-	-	-	-	-
High outliers	-	-	-	-	-	-	-	-	-	1	-	1	-	-	-	-	1	-	-	-









Lab no.	9217	9429	9436	9441	9453	9512	9555	9662	9716	9747	9890	9903	9950
No. of results	6	12	23	15	-	6	6	15	12	3	6	11	-
False positive	-	-	1	-	-	-	-	-	-	-	-	-	-
False negative	-	-	-	-	-	-	-	-	-	-	-	1	-
Low outliers	1	-	-	-	-	-	-	-	-	-	-	-	-
High outliers	1	-	-	-	-	1	-	1	-	-	1	-	-

Test material and quality control

Test material

Each laboratory received three manufactured freeze-dried microbial mixtures, designated A-C. The test material was freeze-dried in portions of 0.5 ml in vials, as described by Peterz and Steneryd (3). Before analysing the samples, the contents of each vial had to be dissolved in 254 ml of sterile diluent. The organisms present in the mixtures are listed in Table 2.

Table 2. *Microorganisms present in mixtures A-C.*

Mixture ¹	Microorganism	Strain	
		SLV no. ²	Reference ³
A	<i>Aeromonas hydrophila</i>	SLV-454	CCUG 30208
	<i>Campylobacter coli</i>	SLV-271	CCUG 45147
	<i>Escherichia coli</i> O157	SLV-479	SMI 81186
	<i>Listeria monocytogenes</i>	SLV-444	CCUG 69007
B	<i>Bacillus cereus</i>	SLV-516	CCUG 44740
	<i>Micrococcus sp</i>	SLV-055	CCUG 35073
	<i>Salmonella</i> Enteritidis	SLV-436	-
	<i>Vibrio cholerae</i>	SLV-530	CCUG 45388
	<i>Yersinia enterocolitica</i>	SLV-408	CCUG 45643
C	<i>Campylobacter jejuni</i>	SLV-540	Chicken, 2003
	<i>Proteus mirabilis</i>	SLV-374	CCUG 43605
	<i>Salmonella</i> Enteritidis	SLV-436	-
	<i>Vibrio parahaemolyticus</i>	SLV-529	CCUG 38981

¹ The links between the mixtures and the randomised sample numbers are shown in Annex 1.

² Internal strain identification no. at the National Food Agency

³ Origin or culture collection (CCUG: Culture Collection University of Gothenburg, Sweden ; ATCC: American Type Culture Collection, SMI: Public Health Agency of Sweden)

Quality control of the mixtures

It is essential to have aliquots of homogeneous mixture and equal volume in all vials in order to allow comparison of all freeze-dried samples from one mixture. Quality control is performed on 10 randomly chosen vials in conjunction with manufacturing of the mixtures or on 5 vials if an “old” mixture was used and the last quality control was performed more than 6 months ago. Homogeneity of a mixture is approved if, for each analysis, the values obtained for the test of reproducibility (T) and the test “Index of dispersion” between vials (I₂) do not simultaneously exceed 2.6 and 2.0, respectively. (For definitions of T and I₂, see references 4 and 5 respectively.)

Table 3. Concentration mean (*m*), *T* and *I*₂ values from the quality control of the mixtures; *m* is expressed in log₁₀ cfu (colony forming units) per ml of sample.

Analysis and method	A ¹			B ²			C ²		
	<i>m</i>	<i>T</i>	<i>I</i> ₂	<i>m</i>	<i>T</i>	<i>I</i> ₂	<i>m</i>	<i>T</i>	<i>I</i> ₂
Aerobic microorganisms 30 °C NMKL method no. 86	4.501	1.28	0.46	4.701	1.34	1.01	4.491	1.61	1.56
Enterobacteriaceae NMKL method no. 144	-	-	-	2.477	1.36	0.71	4.384	1.10	0.55
Thermotolerant campylobacter, quant. NMKL method no. 119	1.456	2.89	0.58	-	-	-	2.948	1.30	1.53
Thermotolerant campylobacter, qual. NMKL method no. 119	Pos	-	-	Neg	-	-	Pos	-	-
<i>Listeria monocytogenes</i> , quant. NMKL method no. 136	2.810	1.10	0.14	-	-	-	-	-	-
<i>Listeria monocytogenes</i> , qual. NMKL method no. 136	Pos	-	-	Neg	-	-	Neg	-	-
<i>Salmonella</i> NMKL method no. 71	Neg	-	-	2.062*	1.24	0.34	2.023*	3.50	0.70
<i>Escherichia coli</i> O157 NMKL method no. 164	0.752	1.00	0.00**	Neg	-	-	Neg	-	-
Pathogenic <i>Vibrio</i> spp. NMKL method no. 156	Neg	-	-	3.098*	1.72	9.17	2.485*	2.85	1.86
<i>Yersinia enterocolitica</i> NMKL method no. 117	Neg	-	-	2.402*	1.39	1.63	Neg	-	-

– No target organism and therefore no value

¹ n = 5 vials analysed in duplicate

² n = 10 vials analysed in duplicate

* Value based on results from analysis of parallel mixture

** Low value due to a small number of colonies on the plates

References

1. Kelly, K. 1990. Outlier detection in collaborative studies. *J. Assoc. Off. Anal. Chem.* 73:58-64.
2. Anonymous, 2015. Protocol, Microbiology. Drinking Water & Food, The National Food Agency, Sweden.
3. Peterz, M., Steneryd. A.C. 1993. Freeze-dried mixed cultures as reference samples in quantitative and qualitative microbiological examinations of food. *J. Appl. Bacteriol.* 74:143-148.
4. Mooijman, K.M., During, M. & Nagelkerke, N.J.D. 2003. MICROCRM: Preparation and control of batches of microbiological materials consisting of capsules. RIVM report 250935001/2003. RIVM, Bilthoven, Holland.
5. Heisterkamp, S.H., Hoekstra, J.A., van Strijp-Lockefeer, N.G.W.M., Havelaar, A.H., Mooijman, K.A., in't Veld, P.H., Notermans, S.H.W., Maier, E.A. ; Griepink, B. 1993. Statistical analysis of certification trials for microbiological reference materials. Luxembourg: Commission of the European Communities, Report EUR 15008 EN.

Internal and external control for microbiological analyses of food and drinking water

All analytical activities require work of a high standard that is accurately documented. For this purpose, most laboratories carry out some form of internal quality assurance, but their analytical work also has to be evaluated by an independent party. Such external quality control of laboratory competence is commonly required by accreditation bodies and can be done by taking part in proficiency testing (PT).

In a proficiency test, identical test material is analysed by a number of laboratories using their routine methods. The organiser evaluates the results and compiles them in a report.

The National Food Agency's PT program offers

- External and independent evaluation of laboratories analytical competence.
- Improved knowledge of analytical methods with respect to various types of organisms.
- Expert support.
- Tool for inspections regarding accreditation.
- Free extra material for follow-up analyses.

For more information visit our website: www2.slv.se/absint

The National Food Agency's reference material

As a complement to the proficiency testing, but without specific accreditation, the National Food Agency also manufactures a number of reference materials (RM) for internal quality control of food and drinking water microbiological analyses, including pathogens.

More information is available on our website: www.livsmedelsverket.se/en/RM-micro