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European Community Reference Laboratory
for monitoring bacteriological and viral
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Report on 2nd FRNA bacteriophage ring trial, 2003

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CRL ring trial reference : RT6 (FRNA bacteriophage, 2003)

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Introduction

The draft regulations concerning Microbiological Criteria for Foodstuffs EN (SANCO/4198/2001 Rev. 9) include a proposal for the validation of commercial depuration process using the removal of FRNA bacteriophage. FRNA bacteriophage has also been proposed as a 'viral indicator' for other applications in shellfish sanitation. In view of the possible use of FRNA bacteriophage as a sanitary indicator it was agreed at the first workshops of NRLs to undertake specialist training and ring trial evaluation of this method. The methodology for enumeration of FRNA bacteriophage (ISO 10705-1) is well standardised and robust. In autumn 2002 the CRL organised and hosted FRNA training events for NRL staff. Training comprised both practical and theoretical work and delegates were provided with CRL standard operating procedures (SOP). In early 2003 the CRL organised a pilot FRNA bacteriophage ring trial which showed generally good laboratory performance of NRLs. At the second workshop of NRLs it was agreed to conduct a second FRNA bacteriophage ring trial to consolidate progress. A second FRNA ring trial took place during September, October and November of 2003. Three ampoules of MS2 were distributed to the participants and the results are reported below.

Preparation of samples and distribution

Freeze-dried glass ampoules of a suspension of the prototype FRNA bacteriophage MS2 were prepared by collaborating partners in the UK Health Protection Agency (HPA). 2 ampoules of MS2 were reconstituted in 0.1% peptone water and the titre of FRNA bacteriophage determined using a serial dilution series as described in appendix 1. FRNA bacteriophage were assayed as described in CRL SOP2 (Enumeration of male-specific RNA bacteriophage in molluscan bivalve shellfish; available on www.crlcefas.org). The results are given in table1.

Dilution factor	Replicate 1	Replicate 2
10^{-9}	142	64
10^{-10}	115	15
10^{-11}	6	7

Table 1: Concentration levels of MS2 in MS2 ampoules used for ring trial

Three glass ampoules were placed in a centrifuge tube with a sufficient amount of tissue to reduce the chances of the ampoules breaking during transportation of the samples to the participant's laboratory. Each package contained the relevant documentation describing how the ampoules should be opened, re-hydrated and the reporting of the results. Samples were distributed at ambient temperature on the 1st September 2003 and were refrigerated on receipt by the laboratories. Participants were requested to analyse 1 ampoule during each of the weeks beginning 22nd September, 20th October and 17th November 2003. Prior to analysis each ampoule was re-hydrated and serial diluted as described in appendix 1. On the basis of the results given in table 1 participants were asked to prepare dilution series from each distributed ampoule and to analysis 10 replicates of each of a 10^{-7}, 10^{-8} and 10^{-9} dilution (see appendix 1).

Participation in FRNA ring trial

All NRLs were contacted to see whether they wished to take part in the FRNA ring trail. 13 NRLs agreed to take part but only 11 NRLs returned results. Table 3 shows the NRLs participation in the ring trial distribution.

Country	Participation in ring trial	Ring trial material sent to these laboratories	Results returned to the CRL
Austria	No	No	No
Belgium/ Luxembourg	Yes	Yes	Yes
Denmark	Yes	Yes	Yes
Finland	Yes	Yes	Yes
France	Yes	Yes	Yes
Germany	Yes	Yes	Yes
Greece	Yes	Yes	No
Ireland	Yes	Yes	Yes
Italy	Yes	Yes	No
Netherlands	Yes	Yes	Yes
Norway	Yes	Yes	Yes
Portugal	Yes	Yes	Yes
Spain	Yes	Yes	Yes
Sweden	No	No	No
United Kingdom	Yes	Yes	Yes

Table 3: Ring Trial Summary FRNA

Analysis of results

On 4 occasions over the duration of the ring trial period the CRL reconstituted ampoules, performed serial dilutions, and analysed the 10^{-9} dilution for a total of 35 reference analysis. The results from these samples are shown in tables 4 and figures 1, 2, and 3.

The participants' results were assessed by comparison with those returned by other participants and with the reference results. To avoid interference from extreme values (outliers) robust estimators were used to assess performance. For each distribution a median value at the 10^{-9} dilution was established from the results reported by participants. Reference values were excluded from this calculation. Following this upper and lower limits around the median were calculated using the statistical estimator 'median absolute deviation' (MAD)(Staudte, R.G. & Sheather, S.J. 1990). A 'warning' limit was calculated at ±2 MAD and an 'action' limit at ±3 MAD in relation to the median.

The MAD is defined as: for data Yi i=1,...,n with M = median (Yi)
MAD = 1.4826 * median { abs(Yi - M)) } where abs is the absolute value function.
(Note : the 1.4826 value is used so that MAD matches standard deviation for normally distributed data.)

The median and the upper and lower MAD limits for each distribution are shown in table 4.

Log_{10} FRNA	Median	-2MAD	-3MAD	+2MAD	+3MAD
Reference results	1.415	1.188	1.075	1.641	1.755
Participants results vial 1	1.531	0.848	0.506	2.215	2.556
Participants results vial 2	1.574	1.006	0.722	2.142	2.426
Participants results vial 3	1.597	1.102	0.854	2.091	2.339

Table 4: FRNA median and performance criteria (±2 MAD warning and ±3 MAD action limits above and below the median value).

Participants results

Not all participants reported values for all 10 replicates of FRNA bacteriophage as requested. The FRNA results reported, and the reference results for FRNA bacteriophage for the three distributions, are shown in table 5, 6 and 7 and plotted in figures 1, 2 and 3.

Results for vial 1

ID No.	Vial No.	Dil. factor	1	2	3	4	5	6	7	8	9	10
NRL 001	1	-9	36	35	40	45	41	36	42	30	36	34
NRL004	1	-9	62	62	56	52	50	50	46	45	44	50
NRL005	1	-9	29	21	13	26	18	20	22	19	21	13
NRL007	1	-9	19	25	20							
NRL008	1	-9	38	30	44	35	35	46	40	24	23	26
NRL009	1	-9	56	54	56	50	49	66	54	63	47	47
NRL011	1	-9	0	0	0	0	0	0	0	0	0	0
NRL012	1	-9	>300	>300	>300	>300	>300	>300	>300	>300	>300	>300
NRL013	1	-9	0	0	0							
NRL014	1	-9	25	17	24							
NRL015	1	-9	12	17	10	10	18	12	16	13	12	16

Table 5. Results received from NRL participants for vial 1, September 2003.

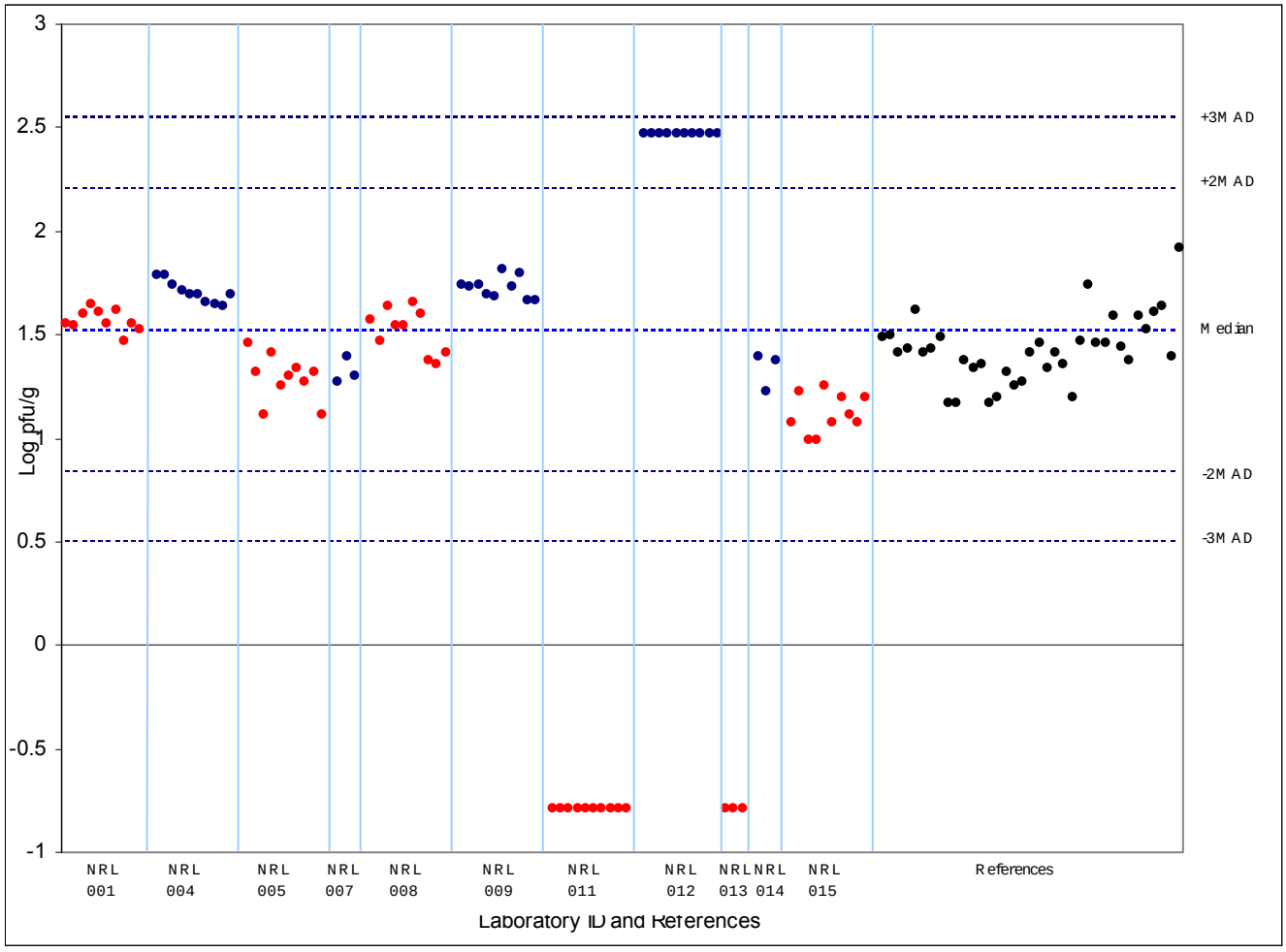

Fig 1. Results for FRNA ring trial distribution vial 1 (September 2003)

Results for vial 2

ID No.	Vial No.	Dil. factor	1	2	3	4	5	6	7	8	9	10
NRL 001	2	-9	66	51	51	49	48	61	66	70	55	58
NRL004	2	-9	34	31	34	35	37	34	28	25	33	37
NRL005	2	-9	40	62	36	33	42	45	39	46	37	64
NRL007	2	-9	59	50	61							
NRL008	2	-9	29	31	33	35	40	38	40	30	30	34
NRL009	2	-9	52	47	48	55	48	46	55	68	63	58
NRL011	2	-9	0	0	0	0	0	0	0	0	0	0
NRL012	2	-9	99	124	113	120	120	109	121	109	125	115
NRL014	2	-9	16	11	15							
NRL015	2	-9	24	19	14	15	19	14	22	23	16	14

Table 6. Results received from NRL participants for vial 2, October 2003.

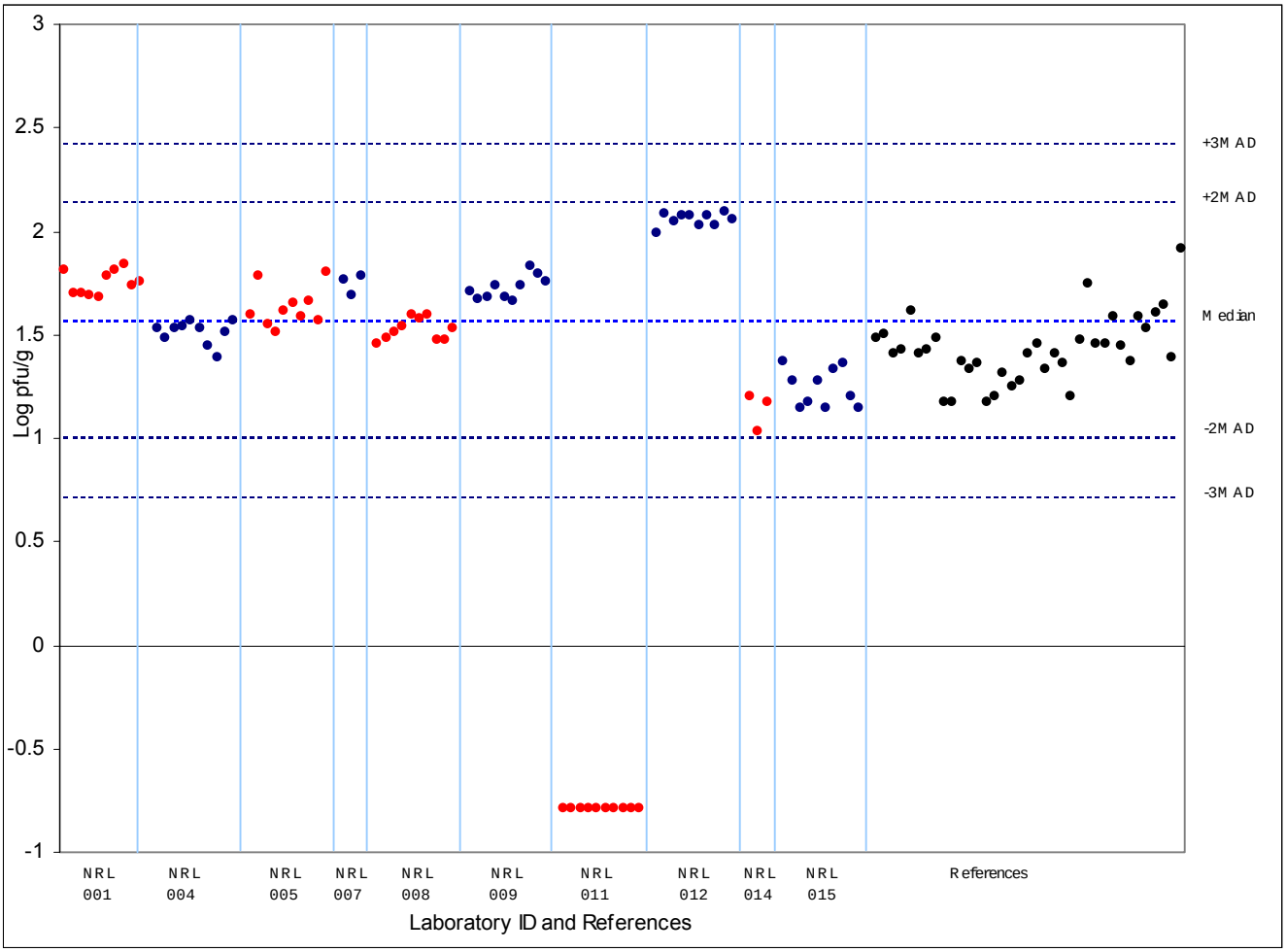

Fig 2. Results for FRNA ring trial distribution vial 2 (October 2003)

Results for vial 3

ID No.	Vial No.	Dil. factor	1	2	3	4	5	6	7	8	9	10
NRL 001	3	-9	40	42	39	45	38	64	48	42	38	50
NRL004	3	-9	39	40	48	58	56	45	51	51	52	40
NRL005	3	-9	33	45	46	56	40	36	22	47	68	52
NRL007	3	-9	0	0	0							
NRL008	3	-9	33	34	31	32	31	20	31	34	22	29
NRL009	3	-9	65	62	60	54	64	54	64	69	49	65
NRL012	3	-9	45	58	62	63	52	52	48	51	65	59
NRL013	3	-9	12	12	10	19	23	18	20	12	13	11
NRL014	3	-9	1	1	0							
NRL015	3	-9	0	0	0	0	0	0	0	0	0	0

Table 7. Results received from NRL participants for vial 3, November 2003.

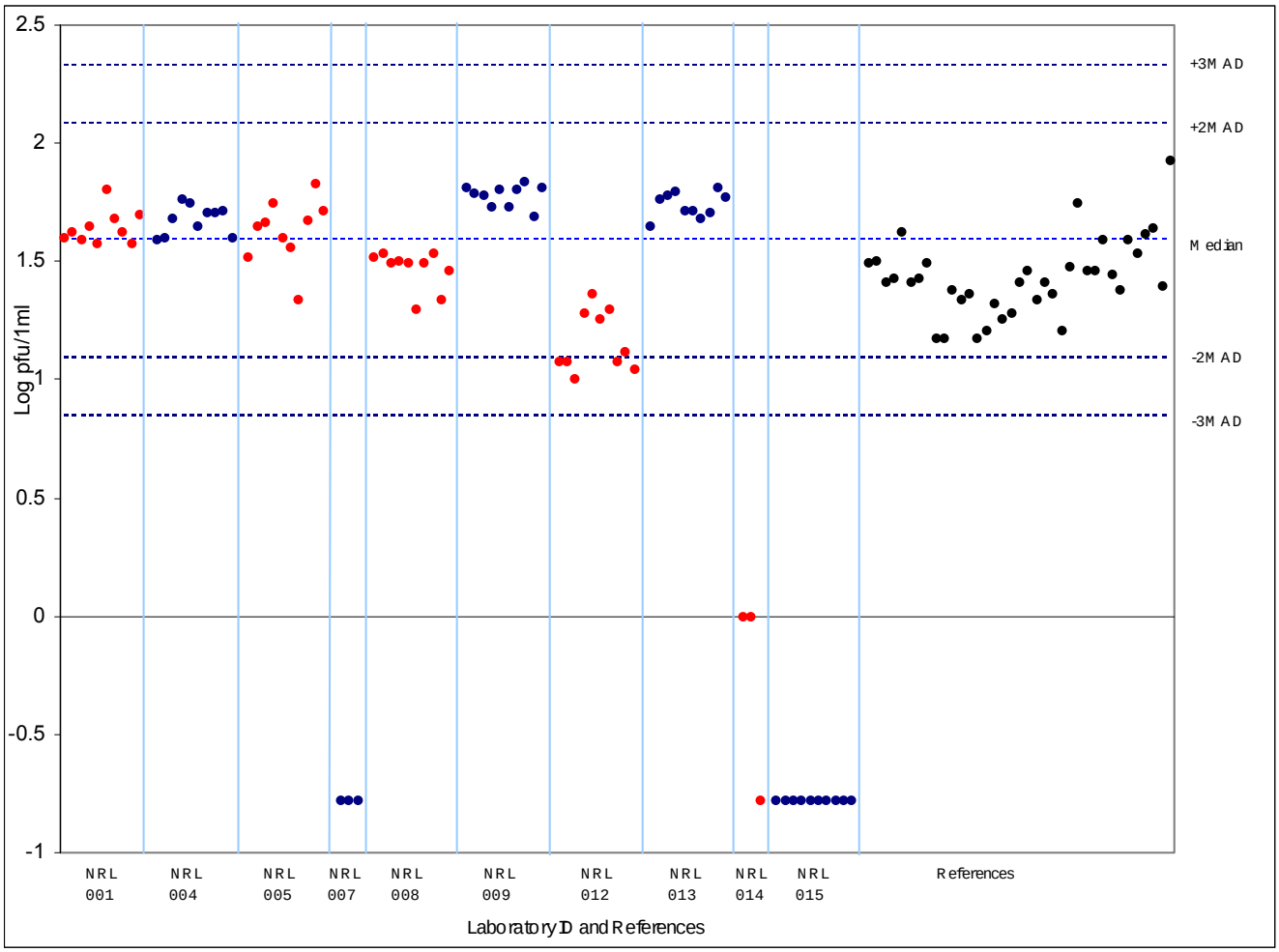

Fig 3. Results for FRNA ring trial distribution vial 3 (November 2003)

Comments

- Laboratory performance

Vial 1 (September)

8 out of 11 participants reported all results -conforming with the expected result. 1 participant reported all results higher than the upper warning limit (+2 MAD). 2 participants reported all results below the lower action limit (-3MAD).

Vial 2 (October)

9 out of 10 participants reported all results conforming with the expected result. 1 participant reported all results below the lower action limit (-3MAD).

Vial 3 (November)

6 out of 10 participants reported all results conforming with the expected result. 1 participant reported 5 of 10 results falling below the lower warning limit (-2 MAD). 3 participants reported all results below the lower action limit (-3 MAD).

It would be advisable for laboratories reporting results outside the warning and action limits to review their procedures.

Summary

- It is clear from the ring trial results that NRLs can run the FRNA bacteriophage assay reproducibly and consistently. When laboratories obtain results within the expected range they generally group closely both with other participants and with the reference results generated by the CRL. The existence of standard methods and the training exercises previously performed will have helped to achieve this good performance.

- However, the ring trials have also demonstrated that, on occasion, NRL results fall well outside of those expected. Generally such results are characterised by being very low, or completely negative. This is strongly indicative of a problem with the bacteriophage host and has been previously documented. Proper attention to the

internal control procedures laid out in ISO 10705-1 should overcome this problem. Mostly this problem occurs when the assay is not being run routinely in the laboratory.

- On one occasion an NRL reported results much higher than expected. This may have been a contamination occurrence or an error with the dilution series.

Appendix

Methodology for opening, preparation and dilution of FRNA bacteriophage ampoules

Opening of ampoules (wear protective gloves throughout the procedure.)

1. Care should be taken in opening the ampoule as the contents are in a vacuum.

2. Make a file mark on the ampoule near the middle of the cotton wool plug and either use a diamond pen to cut the glass or apply a red-hot glass rod to crack the glass.

3. Allow time for air, filtered by the plug, to seep into the ampoule and then gently remove the pointed top part. (If the pointed top part is snapped off suddenly the plug will be drawn to one end and may release fine particles of dried organisms into the air).

4. The plug may be impregnated with dried culture and should be regarded as dangerous to handle and removed with forceps.

Preparation of Samples

5. Flame the open end of the tube and rehydrate+ the contents of the ampoule in 1±0.1 ml of 0.1% peptone water.

6. Mix the contents carefully to avoid frothing or creating aerosols and leave the contents to rehydrate for a few minutes.

Dilution of Sample: serial dilute the sample as follows

7. Make the dilutions using 30ml sterile universals or equivalent; fill the first universal with 4.5ml of 0.1% peptone water. Fill eight further universals with 18.0ml of 0.1% peptone water. Label the universals consecutively 10^{-1} through to 10^{-9}. ***NB*** **All measurements must be exact.**

8. Transfer 500µl from the ampoule, using a 100µl pipette or equivalent, into 4.5ml of 0.1% peptone water (tube labelled 10^{-1}), vortex (or shake) for 30±10 sec to mix the sample. Then using a 2.0ml pipette or equivalent, transfer 2.0ml of the 10^{-1} sample and add to the next universal containing 18.0ml of 0.1% peptone water (10^{-2}), mix well and repeat down the series until all dilutions to 10^{-9} have been carried out.

9. Keep the dilutions out of direct sunlight, to avoid any risk of damaging the viral particles in the suspensions.

10. The last three of the nine universals (10^{-7}, 10^{-8} and 10^{-9}) contain the dilutions to be assayed. Ten replicate 1ml aliquots should be assayed for each dilution.

11. Parallel positive (MS2 NCO12487) and negative (0.1% peptone water) controls must be used throughout in triplicate.