European Community Reference laboratory
for monitoring bacteriological and
contamination of bivalve molluscs

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Report on the PILOT *Vibrio parahaemolyticus* ring trial, 2007

CRL ring trial reference: RT 20 (*V. para* 2007)

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Introduction

Regulation (EC) No 882/2004 designates the Centre for Environment, Fisheries and Aquaculture Science at Weymouth U.K. as the Community Reference Laboratory (CRL) for monitoring the viral and bacteriological contamination of bivalve molluscs. Under Article 32 the laboratory is responsible for organising comparative testing by national reference laboratories.

At the 5th annual workshop of NRLs Weymouth 2006, it was established that the previous *Vibrio parahaemolyticus* ring trial had been beneficial and should be offered in 2006/07. The workshop agreed to continue with *V. parahaemolyticus* ring trial for detection, and determination of pathogenicity principles, and if possible enumeration, using the methods of the laboratories own choice. (Resolution 5).

Preparation of samples

Samples were comprised of laboratory constructed LENTICULES (1 - 2). LENTICULES were constructed following the methods of Codd *et al* (1998) with minor modifications. In brief, fully characterised *Vibrio parahaemolyticus* (CRL accession number V05/014) was grown at 30±2˚C on Haem agar for 24±2h after which, bacteria was suspended in solution to an optical density of ≥4.0 at 600nm. This was then added at a 1:5 ratio to modified lenticulating fluid. Strain V05/014 was both thermostable haemolysin direct (tdh) and thermostable haemolysin direct related (trh) positive as determined by PCR (Tada *et al* 1999) and nucleic acid hybridisation (McCarthy *et al* 1999; Nordstrom *et al* 2006). The lenticulating fluid/bacterial culture mix was aliquoted onto parafilm in 25µl volumes and placed in a desiccating chamber at 4±2˚C for 1 week. After 1 week LENTICULES were transferred to <-15˚C for 2 days prior to analysis using the CRL standard method. A second batch of LENTICULES was prepared using *V. alginolyticus* (CRL accession no. VA12160) prepared under the same conditions.

Note: Significant bacterial die off was evident in the preparation of LENTICULES carrying *tdh* and *trh* genes.

Expected results

Prior to the distribution three randomly selected LENTICULES were selected and enumerated using CRL standard methods. The expected results for each LENTICULE are given in Table 1.

Table 1. Expected results for RT 20

Sample	***V. parahaemolyticus***		
	Total *V. parahaemolyticus*	***tdh***	***trh***
RT 20-01	4.2 x 10^5-1.7 x 10^6/LENTICULE	+	+
RT 20-02	*V. alginolyticus*	-	-

Distribution

The *V. parahaemolyticus* ring trial 2006-07 was designated as RT 20. The LENTICULES were packaged according to IATA regulations, UN3373 as diagnostic specimens, division 6.2 under the packing instruction code 650. All participating laboratories received 2 sealed plastic tubes containing duplicate LENTICULES 1 - 2 within a single thermal control unit (CL-2/4) (Air Sea Containers Ltd., Birkenhead, U.K. CH42 1LE). Relevant transport documentation and import permits were included with the package whilst instruction sheets and result forms were sent to participants through email. The LENTICULES were distributed at ambient temperature by City Sprint dedicated courier services on 26th February 2007. On receipt, participants were requested to complete the analyses and return results by 16th March 2007.

Participation

Invitations inviting expressions of interest in the *V. parahaemolyticus* ring trial were sent to all designated MS NRLs, candidate countries laboratories, EFTA and selected third countries. Table 2 summarises the participation in the ring trial distribution (RT 20). Material was dispatched to nineteen laboratories. One laboratory requested multiple samples (n=6) for subsequent in country distribution. Addresses of participating laboratories are included as Annex I. All laboratories returned results for the trial (Table 2).

Table 2. Participation in the *Vibrio parahaemolyticus* ring trial distribution (RT 20)

Member State	Material dispatched	Results returned to CRL
Austria	Yes	Yes
Belgium & Luxembourg	No	-
Bulgaria	No	-
Cyprus	No	-
Czech Republic	No	-
Denmark	No	-
Estonia 1	Yes	Yes
Estonia 2	Yes	No
Finland	Yes	Yes
France	Yes	Yes
Germany	Yes	Yes
Greece	No	-
Hungary	No	-
Ireland	No	-
Italy	Yes	Yes
Latvia	No	-
Lithuania	Yes	Yes
Malta	No	-
Netherlands	Yes	Yes
Poland	Yes	Yes
Portugal	Yes	-
Romania	No	-
Slovakia	Yes	Yes
Slovenia	Yes	Yes
Spain	Yes	No[a]
Sweden	No	-
United Kingdom	Yes	Yes
Accession country		
Croatia 1	Yes	Yes
Croatia 2	Yes	Yes
Turkey	Yes	Yes
ETFA		
Norway	No	
Iceland	No	
Third country		
USA	Yes	Yes

[a] unable to carry out tests due to reported lack of controls

Confidentiality of results

To preserve anonymity of participants a confidential identification number was used to identify each laboratory.

Reference results

Reference analyses on *V. parahaemolyticus* LENTICULES stored at 4±2˚C were undertaken using the CRL standard methods. Fourteen randomly selected LENTICULES were enumerated using up to 10 parallel culture based analyses under repeatability conditions for total *V. parahaemolyticus* and *tdh*/*trh* positive colonies. Two LENTICULES failed to grow, whereas 2 yielded all replicates >300 cfu/100µl and were not enumerated on agar plates. The remaining CRL reference testing results from LENTICULES yielding replicate plates between 1-300 cfu/100µl are presented as boxplots in Appendix II.

Participants' results

Seventeen (89%) from 19 laboratories returned results to the CRL. Eleven (65%) used methods that enabled detection of *tdh* and *trh* and two (12%) used methods that enabled enumeration. Four laboratories failed to obtain growth of *V. parahaemolyticus* in LENTICULE 1 (the expected +ve) and 3 laboratories recorded no growth for LENTICULE 2 (the expected *non-parahaemolyticus*). All laboratories that reported growth from LENTICULE 1 correctly identified *V. parahaemolyticus*, all laboratories that analysed for *tdh* and *trh* correctly assigned LENTICULE 1 as positive for both putative pathogenicity determinants. All laboratories that recorded growth of LENTICULE 2 correctly reported *V. parahaemolyticus* as not detected, three laboratories correctly speciated the bacterium as *V. alginolyticus*, 1 laboratory incorrectly identified the contents of LENTICULE 2 as *V. metschnikovii.* Participants' results are shown in Tables 3 and 4. Results are provided for information only. No performance assessments were carried in this pilot ring trial due to high LENTICULE variability demonstrated following CRL reference testing.

Table 3. Participants' results for LENTICULE 1

Lab ID number	*V. parahaemolyticus*[a]	*tdh*	*trh*
6	Detected	+	+
7	Detected	+	+
9	Detected	+	+
10	No growth	-	-
14	Detected	nt	nt
17	No growth	-	-
19	Detected	+	+
23	Detected	nt	nt
26	No growth	-	-
27	nt	-	-
32	Detected ($4.6x10^5$ MPN/LENTICULE)	+	+
33	Detected	+	+
35	Detected	+	+
36	Detected	+	+
39	Detected	+	+
41	Detected	+	+
55	Detected (mean = $1.6x10^3$MPN/LENTICULE)	+	+
74	nr	-	-
90	No growth	-	-

nt-not tested
nr-not returned
[a] enumeration results in parentheses

Table 4. Participants' results for LENTICULE 2

Lab ID number	*V. parahaemolyticus*[a]	*tdh*	*trh*
6	Not detected	-	-
7	Not detected	-	-
9	*V. alginolyticus* detected	-	-
10	No growth	-	-
14	Not detected	-	-
17	No growth	-	-
19	Not detected	-	-
23	Not detected	-	-
26	Not detected	-	-
27	nt		
32	Not detected	-	-
33	*V. alginolyticus* detected	-	-
35	*V. metschnikovii* detected	-	-
36	No growth	-	-
39	Not detected	-	-
41	*V. alginolyticus* detected	-	-
55	No growth	-	-
74	nr	-	-
90	Not detected	-	-

nt-not tested
nr-not returned

Discussion

All laboratories that returned results and recorded bacterial growth correctly detected *V. parahaemolyticus* in LENTICULE 1, all laboratories that returned results and noted bacterial growth recorded absence in LENTICULE 2.

Of those laboratories correctly identifying *V. parahaemolyticus* and using methods that enabled detection of the pathogeniicity principle (*tdh* and *trh*) all recorded accurate designation of the strain as both *tdh* and *trh* positive.

Two laboratories that correctly identified *V. parahaemolyticus* in LENTICULE 1 provided enumerative data. One recorded *V. parahaemolyticus* cfu/LENTICULE within the expected range, whilst the order reported approximately $2\log_{10}$ lower than the anticipated levels.

Examination of standard deviations derived from replicated tests on individual LENTICULES demonstrated little within LENTICULE variability (data not shown).

However, analysis of variance on data generated by the CRL from reference testing (homogeneity and stability testing) demonstrated that between LENTICULE variability was high ($p<0.001$).

Individual laboratory performance assessments were not undertaken on participants' results due to the high level of LENTICULE to LENTICULE variability.

Further work on modified lenticulating fluid undertaken subsequent to ring trial (RT 20) has improved the integrity of LENTICULES and future distributions will utilise the improved formulations.

References

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Tada J, Ohashi T, Nishimura N, Shirasaki Y, Ozaki H, Fukushima S, Takano J, Nishibuchi M, and Takeda Y. 1992. Detection of the thermostable direct haemolysin gene (*tdh*) and the thermostable direct haemolysin-related haemolysin gene *trh*) of *V. parahaemolyticus* by polymerase chain reaction. Mol.Cell Probe. **6**:477-487.

McCarthy S.A, Depaola A, Cook D.W, Kaysner C.A, and Hill W.E. 1999. Evaluation of alkaline phosphatase- and digoxigenin-labelled probes for detection of the thermolabile hemolysis (tlh) gene of *Vibrio parahaemolyticus*. Letters in Applied Microbilogy **28**:66.

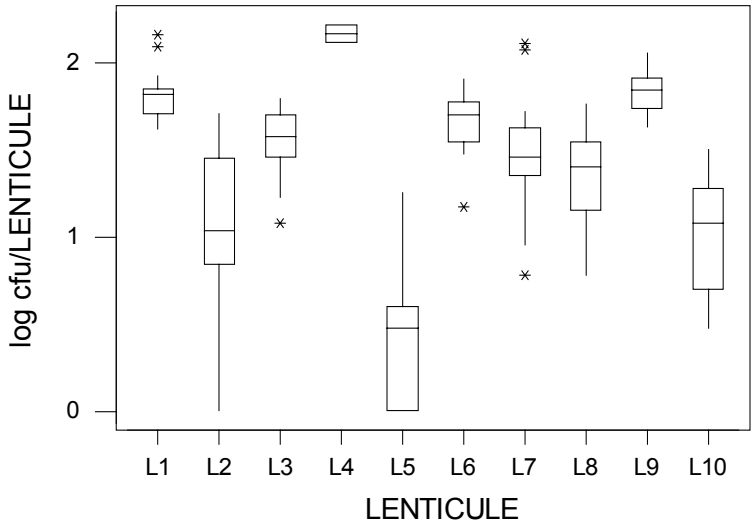
Nordstrom J, Rangdale R, Vickery M.C.L, Phillips A.M.B, Murray S.L, Wagley S, DePaola A. Evaluation of an alkaline phosphatase-labeled oligonucleotide probe for the detection and enumeration of the thermostable-related hemolysin (trh) gene of *Vibrio parahaemolyticus*. J Food Protection **69**:2770-2772

Annex I - Participating laboratories

INIAP/IPIMAR Av. Brasilia S/n 1449-006 Lisboa **PORTUGAL**	Finnish Food Safety Authority (Evira) Mustialankatu 3 FL-00790 Helsinki **FINLAND**
Centro Nacioal de Alimentacion Servicio de Microbiologia Alimentaria Crta/ Marjadahonda – Pozuelo Km, 5 28220 Marjadahonda Madrid **SPAIN**	Institute for food hygiene and bromatology Veterinary faculty Gerbiceva 60 1000 Ljubljana **SLOVENIA**
Tallinn Veterinary and food laboratory Vaike-Paala 3 11415 Tallinn **ESTONIA**	Veterinary and food laboratory Kreutzwaldi 30 Tartu 51006 **ESTONIA**
Cefas Weymouth Laboratory Barrack Road The Nothe Dorset DT4 8UB **UK**	Bornova Veterinary control and research institute Ankara road Bornova/ IZMIR-Turkey 35010 **TURKEY**
National Veterinary Research Institute Department of Hygiene of Food of Animal Origin Partyzantow 57 Pulawy 24-100 **POLAND**	National institute of public health and the environment (RIVM) Laboratory for Zoonoses and Environmental Microbiology P.O. Box 1 3720 BA Bilthoven **NETHERLANDS**
State veterinary and food institute Janoskova 1611/58 026 01 Dolny Kubin **SLOVAKIA**	IFREMER – EMP/Microbiologie-LNR Centre de Brest BP 70 29280 Plouzane **FRANCE**
CVI, Regional Veterinary Laboratory Rijeka Podmurvice 29 51000 Rijeka **CROATIA**	National veterinary laboratory J.Kairiukscio 10 LT-08409 Vilnius **LITHUANIA**
Istituo superiore di sanita v.le Regina Elena 299 00161 Rome **ITALY**	Federal institute for risk assessment FGr 42 Diedersdorfer Weg 1 12277 Berlin **GERMANY**

Croatian veterinary institute Regional veterinary laboratory split Poljicka ceste 33 21000 split **CROATIA**	Austrian agency for health and food safety Spargelfeldstrasse 191 A – 1220 Wien **AUSTRIA**

Annex II - Boxplots of CRL reference testing on individual LENTICULES (between LENTICULE variability)



Note. 1 LENTICULES 11 and 12 yielded no growth
2 LENTICULES 13 and 14 >300cfu/100µl