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Mary Murphy
Health Service Executive-South Western area, Cherry Orchard Hospital, Ballyfermot, Dublin 10., Centre for Food Safety

Anne Carroll
Public Health Laboratory, Dublin.

Paul Whyte
Health Service Executive-South Western area, Cherry Orchard Hospital, Ballyfermot, Dublin 10., Centre for Food Safety.

Micheál O'Mahoney
Health Service Executive-South Western area, Cherry Orchard Hospital, Ballyfermot, Dublin 10., Centre for Food Safety

Wayne Anderson
University College Dublin, Ireland

See next page for additional authors

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Authors
Mary Murphy, Anne Carroll, Paul Whyte, Micheál O'Mahoney, Wayne Anderson, Eleanor McNamara, Seamus Fanning, and Ciara Walsh

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Version 1

Development and assessment of a rapid method to detect *Escherichia coli* O26, O111 and O157 in retail minced beef.

Mary Murphy[1, 2], Anne Carroll[1], Paul Whyte[2], Micheál O' Mahony[2, 3], Wayne Anderson[3], Eleanor McNamara[1] and Séamus Fanning[2].

Public Health Laboratory[1], Health Service Executive-South Western area, Cherry Orchard Hospital, Ballyfermot, Dublin 10., Centre for Food Safety[2], School of Agriculture, Food Science and Veterinary Medicine, University College, Belfield, Dublin 4., Food Safety Authority of Ireland[3], Abbey Court, Lower Abbey Street, Dublin 1, Ireland.

Corresponding Author: Professor Séamus Fanning,
Centre for Food Safety,
School of Agriculture, Food Science and
Veterinary Medicine,
University College,
Belfield, Dublin 4,
Ireland.
Tel. (+353-1) 716 6082,
Fax (+353-1) 716 6091.
sfanning@ucd.ie

ABSTRACT

A molecular-based detection method was developed to detect *Escherichia coli* O26, O111 and O157 in minced (ground) beef samples. This method consists of an initial overnight enrichment in modified tryptone soya broth (mTSB) and novobiocin prior to DNA extraction and subsequent serogrouping using a triplex PCR. This method has a low limit of detection and results are available within 24 hours of receipt of samples. Once optimized, this rapid method was utilized to determine the prevalence of these *E. coli* serogroups in six hundred minced beef samples all of which were previously examined by immunomagnetic separation (IMS) and selective plating for *E. coli* O26 and O111. Using IMS, two *E. coli* O26 isolates were detected. No *E. coli* O111 were recovered. The multiplex PCR technique described here did not detect *E. coli* O111 nor O157 in any of the samples, however six minced beef samples were positive for *E. coli* O26 using our method, only two of these were previously detected by IMS and culture. Application of molecular methods are useful to support culture-based approaches thereby further contributing to risk reduction along the food chain.

Keywords: non-O157 *E. coli*; *E. coli* O157, O26 and O111; serogroup-specific PCR; rapid detection in food.

55 **INTRODUCTION**

56 *Escherichia coli* O157 was initially implicated in human disease in 1982 (Riley et
57 al., 1983) and since then the reported incidence of verocytotoxigenic *Escherichia*
58 *coli* (VTEC) related illness has increased worldwide. In addition to *Escherichia*
59 *coli* (*E. coli*) O157, other serogroups of *E. coli* such as O111 and O26 have
60 emerged as important etiological agents of gastrointestinal disease in humans
61 (Tarr and Neill, 1996). There is evidence of these infections in Ireland including
62 an outbreak of *E. coli* O26 which was reported in a crèche in 1999 (McMaster et
63 al., 2001). However, the true incidence and clinical significance of non-O157
64 serogroups in Ireland still remains to be determined. Routine surveillance to
65 detect these non-O157 *E. coli* in foods, animals and the environment need to be
66 introduced so that public health measures can be implemented. Development of
67 rapid diagnostics capable of detecting a wide range of *E. coli* serogroups
68 associated with disease in humans is a priority.
69
70 In recent years, a great deal of work has been carried out to ascertain the most
71 sensitive and specific methods for isolating and identifying *E. coli* O157. The
72 current method of choice for the detection of *E. coli* O157 in foods is enrichment
73 followed by concentration using Immunomagnetic separation (IMS), with
74 subsequent plating onto the selective Cefixime-Tellurite Sorbitol MacConkey
75 agar (CT-SMAC). This procedure was adapted to allow for the detection of *E.*
76 *coli* O26 and O111 in foods with the use of serogroup-specific beads. However, a

77 major obstacle in detecting *E. coli* O26 and *E. coli* O111 by this method is the
78 lack of suitable selective agars.
79
80 CT-SMAC is the selective agar of choice used to detect *E. coli* O157 from many
81 food matrices as the majority of *E. coli* O157 are non-sorbitol fermenting.
82 However sorbitol fermenting *E. coli* O157 strains capable of causing haemolytic
83 uremic syndrome (HUS) have been reported (Bettleheim et al., 2002; Karch and
84 Bielaszewska, 2001; Karch et al., 1993). While *E. coli* O26 and O111 have
85 demonstrated abilities to ferment both sorbitol and lactose (Batson et al., 2002;
86 Hiramatsu et al., 2002), non-sorbitol fermenting *E. coli* O26 and O111 have also
87 been isolated (Batson et al., 2002; Hiramatsu et al., 2002; Louie et al., 1998).
88 Batson et al. (2002) reported on the carbohydrate fermenting ability of forty-one
89 isolates of *E. coli* O26 and twenty-two isolates of *E. coli* O111. These authors
90 reported that all islolates could ferment lactose, 92.5% of the *E. coli* O26 isolates
91 and only 64.7% of the *E. coli* O111 fermented sorbitol.
92
93 As sugar fermenting characteristics are not consistent, it is difficult to choose (or
94 formulate) a selective agar for the detection of all of these serogroups in food
95 matrices containing high numbers of background microflora. The situation has
96 improved somewhat, with the introduction of Rhamnose MacConkey agar
97 (Hiramatsu et al., 2002) as a selective media for *E. coli* O26 and with the
98 development of other chromogenic agars. However, laboratories remain over-
99 reliant on the fermentation characteristics of these micro-organisms which could

100 result in pathogenic isolates going undetected. Taking this into consideration
101 along with the fact that culture methods are time-consuming, a PCR based
102 method could be valuable in providing additional information to identify specific
103 *E. coli* serogroups.
104
105 In this paper, we report on the development of a triplex PCR method to detect *E.*
106 *coli* O26, O111 and O157 in a single tube reaction following enrichment. Our
107 method was applied retrospectively to analyze 600 minced beef samples for the
108 three serogroups. This food matrix was previously investigated by microbiological
109 methods (Murphy et al., 2005), and in this case failed to identify a number of
110 positive samples. Application of our method is discussed as a convenient tool to
111 support risk reduction measures along the food chain.
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123 **MATERIALS & METHODS**

124 *Preparation of bacterial strains for inoculum.*

125 A single colony of *E. coli* O26 (NCTC 8781) or *E. coli* O111 (NCTC 9111) or
126 *E. coli* O157 (NCTC 12900), were inoculated into three separate falcon test-
127 tubes containing 4 ml of nutrient broth (Oxoid, Basingstoke, UK) and incubated at
128 37°C for 4 h. Serial dilution of 4 h cultures were prepared in 9 ml Maximum
129 Recovery Diluent (MRD; Oxoid) down to a dilution of 10^{-9}. One hundred micro-
130 liters of 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} serial strain dilutions were plated directly
131 onto nutrient agar (Oxoid) and incubated at 42°C for 16 h to determine the cell
132 numbers in the initial inoculum. All inoculations were carried out in duplicate.

133

134 *Inoculation of minced beef samples to determine limit of detection.*

135 Minced beef was purchased at a local supermarket and stored at 4°C prior to
136 inoculation. One ml of the 10^{-7}, 10^{-8} and 10^{-9} serial dilutions of each strain were
137 added to separate 25 g minced (ground) beef samples containing 225 ml of
138 modified Tryptone Soya Broth (mTSB; Oxoid) with novobiocin (20 mg/L), (LabM
139 Ltd., Lancashire, UK) and incubated at 42°C overnight (16 h). An uninoculated
140 sample of minced beef was also included as a control.

141

142 *DNA Extraction*

143 DNA was extracted from the overnight (16 h) enrichments both immediately and
144 after storage for approximately 7 days at –20°C, using the Dneasy tissue kit
145 (Qiagen, Hilden, Germany) according to manufacturer's instructions with minor

146 modifications. Briefly, 25 µl of each sample were added to eppendorf tubes
147 containing 180 µl of the kit tissue lysis buffer (ATL). Twenty micro-liters of
148 proteinase K (supplied in the kit) was added to each tube and mixed. All samples
149 were heated for approximately 135 min at 55°C and were mixed occasionally
150 during incubation to maintain homogeneity. Following incubation, samples were
151 vortexed for 15 seconds and the Protocol for 'Isolation of total DNA from animal
152 tissues', was followed from step 3, of the Dneasy tissue kit instructions (Qiagen).
153
154 *Triplex-PCR mediated serogrouping*
155 All spiked samples were examined for the presence of *E. coli* O26, O111 and
156 O157 using the triplex PCR. This triplex reaction was designed by combining
157 previously published primers by Paton and Paton (1998) for the *rfb* genes of *E.*
158 *coli* O157 and O111 and primers published by Debroy et al. (2004) for *E. coli*
159 O26 *wzx* gene. All primers were tested against several bacterial species other
160 than *E. coli* and also against different serogroups of *E. coli* (Table 1).
161
162 *Triplex-PCR*
163 Samples (5 µl) of each purified template DNA [approx. 100 ng] were amplified in
164 50 µl reaction mixtures containing 5 µl of dNTP mixture (consisting of 3 mM each
165 of dATP, dCTP, dGTP, and dTTP), 2 µl of forward and reverse primer mix (5
166 pmol/µl each primer–see Table 2), 5 µl of 10 x buffer (750 mM Tris-HCL, pH 8.8,
167 200 mM $(NH_4)_2SO_4$, 0.1% (v/v) Tween 20), 5 mM $MgCl_2$ and 2.5 U Red Hot *Taq*
168 DNA Polymerase (Abgene, Surrey, U.K.). Thermal cycling consisted of 36 cycles,

169 each of 95°C for 30 sec, 60°C for 60 sec and 72°C for 60 sec, and a final
170 extension at 72°C for 10 min. Each amplification reaction included a positive
171 control and a no-DNA template control. Amplified DNA products were resolved
172 by 2% (w/v) agarose gel electrophoresis in 1 x Tris-Borate-EDTA (TBE) buffer
173 containing 0.5 mg/ml ethidium bromide at 100 V and the results visualized and
174 photographed using the Gel Doc 2000 system (Bio-Rad, Hercules, CA, U.S.A.).
175
176 *Purification and pooling of DNA templates.*
177 In a previous study, six hundred retail minced beef samples (25 g each), were
178 examined by IMS and culture for the presence of *E. coli* O26 and O111 (samples
179 were not examined for *E. coli* O157 (Murphy et al., 2005)). Two ml aliquots of
180 each beef sample were also frozen at -70°C after an overnight enrichment (for 16
181 h) in mTSB and novobiocin. Each frozen enrichment broth was thawed rapidly at
182 50°C (Ternent et al., 2004) and 1 ml aliquots were pooled into lots containing five
183 representative meat samples. DNA was purified from each pool using the DNA
184 extraction protocol previously outlined and the recovered DNA pools were then
185 subjected to the triplex PCR. Individual samples from any positive pool were
186 subsequently tested separately, to identify the positive sample(s). Further work to
187 determine the virulence status of all positive samples was undertaken using real-
188 time PCR for *vt1*, *vt*2, *eae* and *hly*A.
189
190
191

192 *'Real-Time' PCR for detection of vt1, vt2, eae and hlyA-encoding genes*

193 Five micro-liters of purified template genomic DNA were added to the following
194 25 µl PCR mix containing 1 X SYBR-1 green PCR master mix (Bio-Rad
195 Laboratories) and 50 ng of each primer to target the *vt*1, *vt*2 (Gannon et al.,
196 1992), *eae* (Beebakhee et al., 1992) and *hly*A genes (Paton and Paton, 1998).
197 Samples were amplified in an I-Cycler (Bio-Rad, Hercules, CA, U.S.A), at 95°C
198 for 2 min followed by 30 cycles each of 45 sec at 95°C, 45 sec at 58°C, and 60
199 sec at 72°C and a final extension step of 72°C for 10 minutes. Amplification was
200 monitored by the accumulation of SYBR-1 Green as the amplification reaction
201 progressed and data captured was later analyzed to produce the corresponding
202 melt curve.

203

204 **RESULTS**

205 In this study, a triplex PCR was developed to detect *E. coli* O26, O111 and O157
206 in a single reaction. The specificity of the PCR was tested using a panel of
207 micro-organisms (Table 1). This triplex PCR method was used in conjunction
208 with the Dneasy animal tissue kit to detect *E. coli* O26, O111 and O157 from
209 minced (ground) beef, after an initial 16 h enrichment. The limit of detection for
210 the assay was determined to be 10 colony forming units (CFU) for *E. coli* O111,
211 15 CFU for *E. coli* O26, <10 CFU for *E. coli* O157 in 25 g of minced beef (Fig.
212 1a). The uninoculated minced beef sample was determined to be negative for *E.*
213 *coli* O157, O26 and O111 by this method. Results were available in less than 24
214 h. No difference in the limit of detection was observed between spiked broths

215 examined directly after enrichment and the enrichment broths that were frozen
216 for a period before testing (data not shown). Enrichment time trials were also
217 undertaken, that included times of 4 h, 6 h and overnight (16 h) enrichments.
218 Data showed that an overnight enrichment (16 h) was required to obtain the low
219 limits of detection described above (data not shown). These data correlated with
220 a recent study by O'Hanlon et al. (2004) wherein these authors showed that a
221 longer enrichment time allowed for the multiplication of slower growing
222 serogroups, thereby reducing the possibility of false negative results being
223 reported.
224
225 Compared to IMS and culture (Murphy et al., 2005) this molecular approach
226 identified six *E. coli* O26 isolates, only two of which were previously detected. No
227 *E. coli* O157 nor *E. coli* O111 were detected using this rapid method. The latter
228 case correlates with the IMS and culture results where *E. coli* O111 was not
229 detected (Murphy et al., 2005). All six *E. coli* O26 isolates were tested for the
230 presence of the virulence factors *vt*1, *vt*2, *eae* and *hly*A by real-time PCR. Table
231 3 showed that isolate-4 (Fig. 1b, 1c) contained both *vt*2 and *hly*A and isolate-6
232 contained the *eae* gene alone (Fig. 1c and Table 3). The remaining four isolates
233 did not contain any virulence factors as determined by this method (Table 3).
234
235
236

237 **DISCUSSION**

238 A study, was recently carried out on the ‘Prevalence and characterization of
239 *Escherichia coli* O26 and O111 in retail minced beef in Ireland’ (Murphy et al.,
240 2005), which highlighted the need for an alternative method to detect these
241 micro-organisms in food. IMS and culture is the technique routinely used
242 worldwide for the detection and identification of *E. coli* O157 in food matrices.
243 However, this technique is time-consuming and is sub-optimal when used for the
244 detection of other *E. coli* serogroups such as *E. coli* O26 and O111, increasing
245 the risk of reporting false negative results.
246
247 In our study, a triplex PCR was developed to detect the three *E. coli* serogroups
248 in minced beef after an initial overnight enrichment step (16 h) followed by DNA
249 purification. The presence of these micro-organisms in minced beef was
250 determined within twenty-four hours, reducing the time required when compared
251 to standard methods by up to four days. Speed of detection is a very important
252 factor in outbreak senarios and routine food surveillance. Our method could
253 detect less than 15 CFU of all the serogroups tested, which is comparable to the
254 limit of detection for the IMS and culture technique (Safarikova et al., 2001) and
255 to data previously reported from studies using real-time PCR (O’Hanlon et al.,
256 2004; Sharma et al., 2002). While, the molecular method described in this paper
257 compliments the methodologies of O’Hanlon et al. (2004) and Sharma et al.
258 (2002), a useful feature is its capability to detect all three serogroups in a single
259 triplex reaction whether virulent or non-virulent. The additional real-time PCR

260 allows for further characterization of any positive samples for *vt*1, *vt*2, *eae* and
261 *hly*A genes.
262
263 Using the protocol described, serogrouping results are available within eight
264 hours after enrichment therefore, if these broths were stored at 4°C employing
265 suitable biosecurity measures, positive samples can be cultured, if required. A
266 unique finding of our study relates to large longitudinal surveillance studies. In
267 this case culture enrichments may be frozen and tested at a later stage in bulk or
268 in pools, a point which would contribute positively to reducing the financial
269 burden associated with these studies.
270
271 Analysis of the six hundred frozen enrichment broths demonstrated the ability of
272 this method to detect target sequences in samples negative for IMS and culture.
273 As these isolates were detected among high numbers of background micro-flora
274 in enriched minced beef, it would be reasonable to assume that all these isolates
275 were viable, an important point when applying PCR methods. As one of the *E.*
276 *coli* O26 isolates detected using the rapid method contained *vt*2 and *hly*A genes
277 and the other isolate contained the *eae* gene alone, these organisms could be of
278 potential public health significance, should they be ingested by a vulnerable
279 individual. This observation demonstrates the advantage of combining the
280 approach described here along with conventional approaches, emphasizing the
281 value of molecular methods.

282 Implementation of the method outlined in this study would be of value in food
283 surveillance and public health laboratories. Results of analysis are available in
284 less than twenty-four hours. It has a low limit of detection and it is less labour
285 intensive compared to the more traditional methods applied. Our method could
286 be extended to include a wider range of serogroups or other food-borne
287 pathogens of public health significance.

288

289

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397 **Figure Legends to Illustrations**

398 **Fig. 1a.** Results of the limit of detection dilution assay for *Escherichia coli (E.*
399 *coli)* O157, *E. coli* O111 and *E. coli* O26 in minced beef using this rapid
400 technique. Lane M = molecular weight marker VIII (Roche); Lane 1 = *E. coli* O26
401 10^{-7} (15 CFU/ml); Lane 2 = *E. coli* O26 10^{-8} (0 CFU/ml); Lane 3 = *E. coli* O111
402 10^{-7} (10 CFU/ml); Lane 4 = *E. coli* O111 10^{-8} (0 CFU/ml); Lane 5 = *E. coli* O157
403 10^{-7} (40 CFU/ml); Lane 6 = *E. coli* O157 10^{-8} (4 CFU/ml); Lane 7 = *E. coli* O157
404 10^{9} (0 CFU/ml); Lane 8 = Control (sample); Lane 9 = multiplex positive control
405 consisting of equivalent template DNA from three independent *E. coli* serogroups
406 [including O26, O111 and O157]; Lane 10 = No template PCR control.
407 A 2% (w/v) agarose gel in 0.5 X Tris-Borate-EDTA (TBE) buffer containing 0.5
408 mg/ml ethidium bromide.

409

410 **Fig. 1b.** Melt curve graph of SYBR-1 green real-time PCR assay for *vt*2. Melting
411 temperature (T_m), 89°C. *E. coli* O157 positive control (NCTC 12900), (red peak);
412 Isolate 4, (green peak). Other isolates and the negative control are shown as flat
413 lines.

414

415 **Fig. 1c.** Melt curve graph of SYBR-1 green real-time PCR assay for *eae* and
416 *hlyA* genes. Melting temperature (T_m) of 88°C for *eae* and 87°C for *hlyA*. *E. coli*
417 O157 positive control, (pink peak, *hlyA;* green peak*, eae*); Isolate 4, (red peak,
418 *hlyA*); Isolate 6, (blue peak, *eae*). Other isolates and the negative control are
419 shown as flat lines.

Table 1. List of the micro-organisms used to test the specificity of the primers, with their corresponding reactions.

Micro-organism	Source	*O26 *wzx*	*O111 *rfb*	*O157 *rfb*
Clostridia perfringens	NCTC 8237	-	-	-
Vibrio parahaemolyticus	NCTC 10885	-	-	-
Salmonella poona	NCTC 4840	-	-	-
Staphylococcus aureus	NCTC 6571	-	-	-
Listeria monocytogenes	NCTC 11994	-	-	-
Bacillus cereus	NCTC 7464	-	-	-
Pseudomonas aeruginosa	NCTC 10662	-	-	-
Citrobacter freundii	NCTC 9750	-	-	-
Enterobacter faecalis	NCTC 775	-	-	-
E. coli O126	Clinical Isolate	-	-	-
E. coli O128	Clinical Isolate	-	-	-
E. coli O142	Clinical Isolate	-	-	-
E. coli O124	Clinical Isolate	-	-	-
E. coli O44	Clinical Isolate	-	-	-
E. coli O55	Clinical Isolate	-	-	-
E. coli O111	NCTC 9111	-	+	-
E. coli O26	NCTC 8781	+	-	-
E. coli O157	NCTC 12900	-	-	+

* target present (+), absent (-).

Table 2. Oligonucleotide primers used to identify *Escherichia coli* serogroups.

Oligonucleotide primers	Gene	Sequence (5' to 3')	Size	% G+C	Amplicon (bp)
O111-F	*rfb* E_{O111}	TAGAGAAATTATCAAGTTAGTTCC	[24-mer]	29	406
O111-R		ATAGTTATGAACATCTTGTTTAGC	[24-mer]	29	
O157-F	*rfb* E_{O157}	CGGACATCCATGTGATATGG	[20-mer]	50	259
O157-R		TTGCCTATGTACAGCTAATCC	[21-mer]	43	
O26-F	*wzx* E_{O26}	GCGCTGCAATTGCTTATGTA	[20-mer]	45	152
O26-R		TTTCCCCGCAATTTATTCAG	[20-mer]	40	

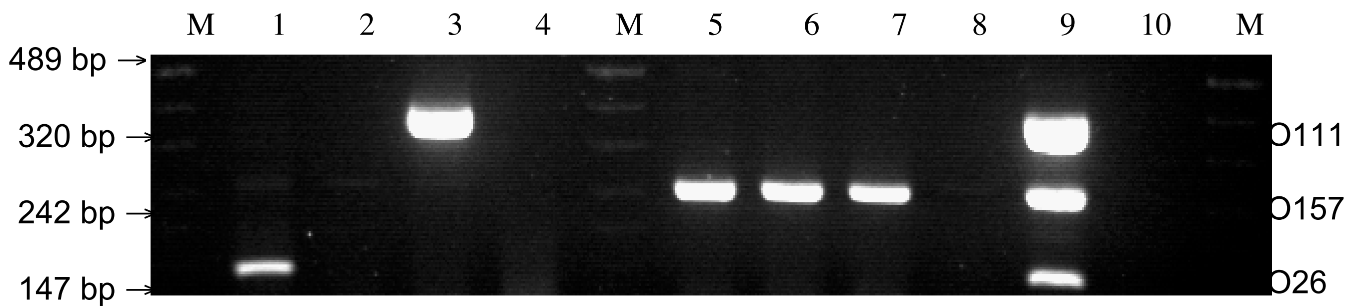
Table 3. Characteristics of the *Escherichia coli* O26 isolates detected in raw minced beef utilizing the triplex PCR.

Isolate number	Package type	Detection Method		*Virulence Markers			
		IMS	New PCR Technique	vt1	vt2	*eae*	*hly*A
1	Prepacked	Detected	Detected	-	-	-	-
2	Prepacked	Detected	Detected	-	-	-	-
3	Prepacked	Not Detected	Detected	-	-	-	-
4	Loose	Not Detected	Detected	-	+	-	+
5	Loose	Not Detected	Detected	-	-	-	-
6	Prepacked	Not Detected	Detected	-	-	+	-

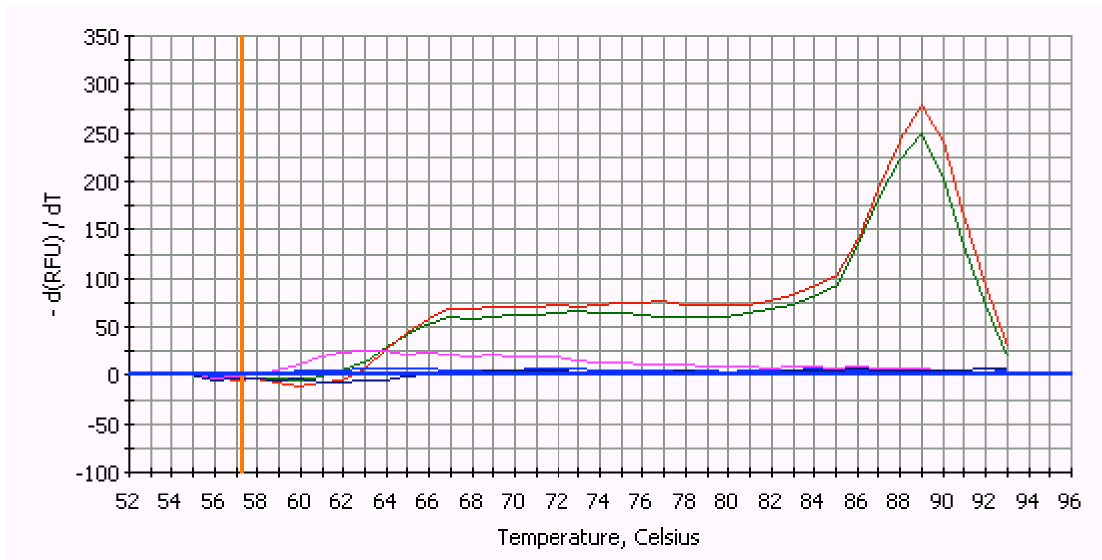
* virulence target present (+), absent (-).

Figures

[a].



[b].



[c].