

1 **ALTERNATIVE MOLECULAR METHODS FOR IMPROVED DETECTION OF MENINGOCOCCAL**
2 **CARRIAGE AND MEASUREMENT OF BACTERIAL DENSITY.**

3 **Running title: *improved* methods for detection of meningococcal carriage**

4
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22 Key words: *Neisseria meningitidis*, pharyngeal carriage, diagnosis, broth culture, carriage
23 density, filter paper.

24

25 **Abstract**

26 **Background:** Conventional methods for detecting pharyngeal carriage of *Neisseria*
27 *meningitidis* (*Nm*) are complex. There is a need for simpler methods with improved
28 performance. We have investigated two alternative approaches.

29 **Methods:** Three pharyngeal swabs were collected from 999 pupils aged 10 to 18 years in
30 The Gambia. Carriage of *Nm* was investigated using three different methods: (i) plating on
31 Thayer-Martin selective medium and testing by conventional microbiological methods
32 followed by polymerase chain reaction (PCR) testing, (ii) seeding in Todd Hewitt broth (THB)
33 and, after overnight culture, testing by PCR, (iii) compression of the swab on filter paper
34 and, after DNA concentration, testing by PCR.

35 **Results:** PCR after culture in THB was more than twice as sensitive as conventional methods
36 in detecting *Nm* (13.2% versus 5.7%; $p < 0.0001$). PCR after DNA extraction from filter paper
37 had a similar sensitivity to that of conventional methods (4.9% vs 5.7%, $p = 0.33$). Capsular
38 genogroups detected by broth culture were W (21), B (12), Y (8), E (3), and X (2), and 68
39 meningococci had the capsule null intergenic region. The distribution of genogroups and of
40 capsule null organisms was similar with each of the three methods. Carriage density in
41 samples extracted from filter paper ranged from 1 to 25,000 DNA copies.

42 **Conclusions:** PCR of overnight broth culture doubled the yield of *Nm* carriage isolates
43 compared with conventional methods. This approach could improve the efficiency of
44 carriage studies. Collection on filter paper followed by quantitative PCR could be useful for
45 density measurement and for carriage studies in areas with limited resources.

46

47 **Introduction**

48 Infection with *Neisseria meningitidis* (*Nm*) is usually characterized by asymptomatic, or
49 minimally symptomatic, carriage of bacteria in the pharynx; meningococcal septicemia
50 and/or meningitis are rare events occurring in as few as 1:100 to 1:1000 colonized
51 individuals. For this reason, selective pressure induced by antimicrobials or by naturally-
52 acquired or vaccine-induced immunity is exerted primarily on bacteria carried by
53 asymptomatic carriers. Therefore, the epidemiology of meningococcal infections cannot be
54 understood fully without considering carriers (1, 2).

55 Carriage of *Nm* is relatively uncommon and thus determining carriage prevalence
56 requires large-scale surveys (3), which are usually conducted using microbiological
57 techniques developed several decades ago. Detection of meningococcal carriage involves
58 three main steps that could be improved, namely sample collection, transport to the
59 laboratory and identification of meningococci. Most studies have focused on the
60 optimization of methods for collecting pharyngeal swabs; these have shown that a per-oral
61 swab taken behind the uvula is the site most likely to yield a positive result, and that taking
62 two sequential swabs increases sensitivity (4-6). Until recently, little has been done to
63 improve methods of transport (7, 8), and we are aware of only two studies aimed at
64 improving identification of meningococci by using molecular methods (9, 10). PCR testing on
65 DNA extracted directly from a swab has been employed in some studies, but a recent
66 investigation in UK adolescents showed that this method was less sensitive than
67 conventional culture followed by PCR (11). We have developed two new methods for
68 detecting meningococcal carriage; molecular analysis of extracted DNA obtained either after
69 overnight culture in Todd-Hewitt broth (THB) or from a filter paper on which a swab had

70 been pressed, and compared the results obtained with these methods with those obtained
71 by conventional microbiology confirmed by PCR in a study of meningococcal carriage in 999
72 Gambian schoolchildren.

73

74 **Materials and methods**

75 ***Study design and participants***

76 Following sensitization of the community, and with permission from the educational
77 authorities, a cross-sectional carriage survey was undertaken from July 1st - 30th 2013, the
78 start of the rainy season, in children attending middle or high schools in the peri-urban area
79 of Fajikunda, The Gambia, West Africa. Healthy school attenders aged between 10 to 18
80 years were recruited sequentially until 1,000 had been enrolled. No children or parents
81 invited to join the study refused to participate. Written, informed consent was obtained
82 from 18-year old students. Assent and written consent from a parent or guardian was
83 obtained from those aged 10 to 17 years. A questionnaire which investigated potential risk
84 factors for meningococcal carriage was administered to all participants. Three pharyngeal
85 swabs were then collected from each student on the same occasion. One was streaked
86 directly onto a Thayer-Martin selective agar plate, a second was placed in THB and a third
87 smeared on a filter paper strip. Children were randomized prior to collection of the samples
88 to one of three groups, which indicated the order in which the three samples were to be
89 collected (figure 1), to ensure that first, second and third swabs had an equal chance of
90 being tested by each of the three laboratory methods.

91 The study was approved by the Scientific Coordinating Committee of the Medical Research
92 Council (MRC) Unit, The Gambia, by the Gambian Government/MRC Joint Ethics Committee
93 and by the Ethical Committee of the London School of Hygiene & Tropical Medicine.

94 ***Conventional microbiology***

95 The conventional methods employed to identify *Nm* by the MenAfriCar consortium have
96 been described in detail elsewhere (12) and are reported briefly here. Swabs were streaked
97 directly onto a modified selective Thayer-Martin agar plate in the field, and plates held in a
98 5% CO₂ jar until transported to the laboratory within six hours of collection. After 24 hours
99 of sub-culture on chocolate agar plates, an oxidase test and a Gram stain were performed.
100 All oxidase positive, Gram negative diplococci (OPGNDC) were tested for β-galactosidase
101 activity with ortho-nitrophenyl-β-D-galactopyranoside (ONPG) and for γ-glutamyl-
102 transferase (GGT) and butyrate esterase activity (Tributyryn). ONPG negative, GGT positive,
103 Tributyryn negative bacteria were then serogrouped by slide agglutination, initially with
104 serogroup A and W antisera and then, if negative, with X and Y antisera. DNA was
105 extracted from all OPGNDC isolates using Qiagen extraction of a bacterial suspension boiled
106 for 20 minutes and then tested with the multiplex real-time PCR (rt-PCR) described below.
107 In the conventional microbiology group, OPGNDC isolates which were ONPG negative, GGT
108 positive and tributyrin negative and also PCR positive (see below) were considered to be
109 *Nm*.

110 ***Broth culture***

111 Prior to the field study, the ability of two broth cultures to support the growth of *Nm* was
112 tested using aliquots spiked with serial dilutions of a serogroup A reference strain (ATCC®
113 13077™), starting with a dilution of approximately 1200 X 10⁸ CFU per ml. The first medium

114 tested was Mueller-Hinton broth (MHB - Oxoid, Basingstoke, UK) supplemented with VCNT
115 (Oxoid SR0091E) containing vancomycin (3mg/L), colistin (7.5mg/L), nystatin (1250U/L) and
116 trimethoprin (5mg/L)]. The second medium investigated was Todd-Hewitt broth (THB -
117 Oxoid, Basingstoke, UK) supplemented with 0.5% yeast, rabbit serum (B&K Universal Ltd,
118 Grimston, East Yorkshire, UK), to facilitate pre-incubation before arriving at the laboratory,
119 and the same antibiotic combination described above. DNA was extracted from broth
120 cultures using the Qiagen method according to the manufacturers' instructions (with elution
121 in 200 μ L). The Todd-Hewitt medium supported bacterial growth at higher dilutions than
122 the Mueller-Hinton broth (see Table 1) and this medium was used in the field study.

123 ***Filter paper cards***

124 The ability of filter paper strips (Whatman FTA MiniCard[®] WB 120055) to preserve *Nm* DNA
125 prior to DNA extraction was explored in the laboratory using serial dilutions of a suspension
126 of a serogroup A meningococcal reference strain (ATCC[®] 13077[™]); samples spiked with
127 different dilutions of bacterial suspension were spotted onto filter paper strips and held at
128 room temperature (18°C to 23°C) for 48 hours prior to DNA extractions using Qiagen. Next,
129 elution was done twice with a volume of 25 μ l, a lower volume than the usual 200 μ l in
130 order to concentrate DNA. In the laboratory, it was possible to detect approximately one
131 *porA* gene copy of *Nm* by rt-PCR. In the field, a swab was smeared directly onto a MiniCard
132 which was held at room temperature for several weeks prior to extraction. In the field, a
133 swab was smeared directly onto a MiniCard which was held at room temperature for 6-12
134 months prior to extraction as it is well known that DNA collected on FTA cards can be
135 preserved for years at room temperature. Using a punch, a small segment of the filter paper
136 (approximately 6 mm diameter) was obtained from the centre of the smear and DNA

137 extracted as described above. A second sample was obtained as close as possible to the
138 centre and tested using the same procedure as it was considered that bacteria might have
139 been concentrated off-center when the swab was compressed onto the MiniCard.

140 ***Multiplex rt-PCR for Nm detection***

141 Detection of *Nm* was undertaken by targeting both *porA* and *sodC* genes and the capsule
142 null intergenic region (*cnI*) simultaneously. Genogrouping of all *porA* and/or *sodC* positive
143 samples, considered as *Nm*, was done according to the method developed by Wang *et al*
144 (13) for genogroups A, W and Y, followed by B, C and X. All samples of *Nm* that could not be
145 characterized in this way underwent an H, E, Z multiplex PCR (see table S1). The cycling
146 conditions were the same for all tests: 1 cycle of 2 min at 50°C, 1 cycle of 10 min at 95°C, 50
147 cycles of 15 s at 95°C and 1 min at 60°C. The ABI 7500 fast cycler was used to perform the
148 reaction and the results analysed using the 7500 fast software. Samples were kept at 4°C
149 after amplification. For both the examination of THB samples and PCR confirmation of
150 isolates obtained by conventional microbiological methods, a stringent Ct threshold of 25
151 was used for both *porA* and *sodC* genes to select true positives. This threshold was shown to
152 be optimal by comparison with positive controls diluted at different concentrations and
153 plotted on standard curves. For genogrouping multiplex PCR, the conventional Ct of 35 was
154 used as the threshold for positivity and samples between 36 and 40 were retested after 10-
155 fold dilution as per Wang's method (13). For filter paper testing, using positive controls at
156 different dilutions plotted on standard curves, better results were obtained with the *porA*
157 monoplex rt-PCR than with the multiplex *porA/sodC/cnI* during our preliminary evaluation
158 and, therefore, we used only *porA* monoplex rt-PCR during the field study with a Ct
159 threshold of 35 as the criterion of positivity.

160 ***Measurement of bacterial density***

161 For calculation of bacterial density, reference strain Nm W ATCC® 35559™ was harvested
162 from an overnight culture and diluted in phosphate buffered saline to reach a 4.0 McFarland
163 concentration. Serial dilutions were made and DNA extraction done using Qiagen kits
164 according to the manufacturers' recommendations to build standard curves for DNA
165 quantification through rt-PCR. Standard curves of DNA measurements were made using a
166 Nanodrop™ spectrophotometer (ThermoScientific, USA) and PicoGreen® dsDNA
167 quantitation assay (Lifetechnologies, France) techniques. The average of both DNA
168 measurements was used when values were discrepant. Genome copies were estimated
169 using the formula: mass = number of DNA base pairs per genome X 1 mole/6.022 140 X 10²³
170 X 660 g per mole, where 6.022 140 X 10²³ is the Avogadro's number (molecules per mole)
171 and 660 g/mole is the average molecular weight of a double-stranded DNA molecule.
172 Extracted bacterial DNA was then subject to PCR as described above.

173 ***Statistical analyses***

174 The study was designed to have 80% power to detect an increase in carriage prevalence
175 from an estimated carriage prevalence of 5% in the conventional microbiology group to at
176 least 7% from paired samples employing one of the two novel approaches to carriage
177 detection; this required a sample size of 870. Data were analyzed using Stata v12.0.
178 Sensitivity was compared between methods using the exact McNemar's significance
179 probability. Differences in carriage prevalence by group (e.g. age, sex) were investigated
180 using chi-squared tests and logistic regression.

181

182 **Results**

183 One thousand students were recruited into the study; one child was excluded because of a
184 missing questionnaire leaving 999 for analysis. The majority (n=859) were aged 10-13 years,
185 the remainder (n=138) were 14- 18 years old; age was not recorded for two children. More
186 male than female students were enrolled (589 vs 410). Pharyngeal carriage of *Nm* was
187 detected in 143 students by one or other method (overall carriage prevalence 14.3%).
188 Carriage prevalence was similar in males (80/589 [15.3%]) and females (63/410 [13.5%])
189 (p=0.428). Carriage was not associated with age within the limited age range investigated
190 (p=0.519).

191 One hundred and eleven oxidase positive, gram negative diplococci were isolated using
192 the conventional microbiology approach; 49 were characterized as *Nm* by biochemical
193 methods (ONPG, GGT and Tributyrin tests). This diagnosis was confirmed by multiplex PCR
194 in all but three samples. An additional 21 OPGNDC isolates had a weak reaction with one of
195 the three biochemical tests and were tested by rt-PCR. Eleven were positive for *Nm* on rt-
196 PCR testing making a total of 57 *Nm* detected by conventional microbiology followed by rt-
197 PCR (carriage prevalence 5.7%). Several false positive reactions were seen using sero-
198 agglutination. For example, 13 *Nm A* were identified by sero-agglutination which were not
199 confirmed by *Nm A* specific genogrouping rt-PCR. Genogrouping of the 57 *Nm* isolates
200 identified the following genogroups in decreasing order of prevalence: *cnI* (21), W (11), B
201 (5), Y (3), E (2), C (2) and dual infection *cnI/W* (2) and *cnI/Y* (1). Ten samples could not be
202 classified through genogrouping PCR (NG). All samples positive by *sodC* and *porA* negative
203 were *cnI* or NG.

204 Overnight culture in THB followed by rt-PCR detected 132 carriers of *Nm* (carriage
205 prevalence 13.2%), a marked increase in sensitivity compared to the conventional method

206 ($p < 0.0001$). Sixty-eight of the 132 *Nm* isolates (51.5%) possessed only the *cnI* intergenic
207 region (see figure 2). Genogroups detected following broth culture were W (21), B (12), Y
208 (8), E (3) and X (2). Four additional samples (not classed as meningococci in the primary
209 analyses) had doubtful results, with a Ct between 25 and 30. No genogroups A, C, H or Z *Nm*
210 were detected. Eleven strains grown in THB were non groupable (NG) and 7 dual carriers
211 were identified. Overall, genogrouping results matched well between methods, but there
212 were a few discrepancies between the results obtained with THB culture compared to the
213 conventional method: two genogroups C and two E were not confirmed in THB. The
214 superiority of the THB culture over conventional microbiology in detecting both *cnI* strains
215 and different genogroups of meningococci is demonstrated in figure 2.

216 Forty-nine *Nm* carriers (carriage prevalence 4.9%) were identified using filter paper
217 minicards, a similar prevalence to the conventional method ($p = 0.33$). Among these, 18
218 carried the *cnI* gene with Ct values lower than or equal to 35. Genogrouping rt-PCR could
219 not be performed on the other samples due to the low volume of elution used to
220 concentrate the DNA for *Nm* detection and the fact that repeated tests were done to
221 verify positive tests. The density of carriage, as reflected in the number of DNA copies
222 obtained from the filter paper, varied between 1 and 25,000 DNA copies with a median
223 value of 59 copies (figure 3). There was no association between subject's age and carriage
224 density. Additional testing with a second punch taken away from the centre of the smear
225 allowed detection of one additional carrier. Median density from 13 second punch
226 samples (2.0 [95% CI 1.0-10.8]) was lower than that found in 48 first punch samples (58.5
227 [95% CI 35.0, 226.0]), emphasising the importance of taking the punch from the centre of

228 the smear. Only two of 49 subjects had > 10,000 copies of *porA* DNA and only seven had
229 > 1,000 copies.

230 The overlap in detection of carriage between the different methods is shown in the
231 Venn diagram (figure 4). Most of carriers detected by conventional microbiology (80.7%)
232 were also detected by broth culture and 47.4% were detected on filter paper. Forty-seven of
233 the 49 carriers detected by filter paper were also detected through THB culture and 27 by
234 conventional microbiology. Using both *porA* and/or *sodC* genes to define positivity, and
235 assuming detection by any method to be a true positive, the sensitivity of conventional
236 microbiology was 39.6%, that of the filter paper method 34% (using a *porA* monoplex rt-
237 PCR) and that of THB culture 91.6%.

238

239 **Discussion**

240 Detection of meningococcal carriage in children attending schools in Fajikunda, The Gambia
241 was enhanced more than two fold compared to conventional culture by culture overnight
242 in a selective medium and subsequent detection of meningococcal DNA. The genogroup
243 distribution of carriers detected using broth culture was similar to that seen using
244 conventional culture, suggesting that overnight culture did not have a major selective effect,
245 although genogrouping of a few strains did not match between methods. The enhanced
246 sensitivity of the broth culture approach probably reflects the fact that many carriers have
247 only a low density of bacteria on the surface of the pharyngeal mucosa, and that liquid
248 broth provides *Nm* with a better environment for immediate growth than solid Thayer-
249 Martin medium. The broth culture technique is cheap and easily implementable, with

250 collected samples cultured overnight, boiled in PBS and then stored at -20°C for later rt-PCR
251 analysis at a convenient time.

252 A limitation of this study is that the preservative medium made of skimmed milk,
253 (tryptone), glucose and glycerin [S(T)GG], commonly used for transport in pneumococcal
254 carriage studies, was not evaluated. However, in a qualitative and semi-quantitative
255 comparison of preservation in STGG with conventional direct plating in the field, O'Brien *et*
256 *al.* demonstrated that direct plating was slightly superior to STGG for recovery of *S.*
257 *pneumoniae* (14). In a recent study of 601 students in Portugal (10), a similar prevalence of
258 meningococcal carriage was obtained from culture and by direct *sodC* rt-PCR on samples
259 collected in STGG (13.3% vs 14.5% respectively) (Adam Finn, personal communication). Our
260 study demonstrated that broth can be used both as a transport and culture medium but
261 more studies are needed to define which medium is optimal. The use of rabbit serum is a
262 limitation of our method as this reagent has to be shipped frozen, a challenge for resource-
263 limited countries. Further studies are needed to define whether the efficiency of detection
264 is similar without this reagent and whether the efficacy of broths for short-term culture of
265 *Nm* can be improved further.

266 Collection of blood spots on filter paper strips with subsequent DNA extraction and
267 molecular analysis is a technique used widely in study of other infectious diseases including
268 malaria and HIV (15) but, as far as we are aware the filter paper technique has not been for
269 collection of meningococcal DNA although it has been used for detection of meningococcal
270 antigen in cerebrospinal fluid samples sent to a central laboratory (17). We tested filter
271 paper eluates on which a swab had been pressed by *porA* monoplex rt-PCR only rather than
272 by *porA* and *sodC* as this gave us better results during the evaluation on spiked samples so

273 that the comparison of the results obtained with the filter paper technique and
274 conventional microbiology reflect a comparison of two optimum techniques rather than a
275 comparison of directly similar PCR methods. Employing the optimum techniques for each
276 method, the filter paper approach had a similar sensitivity to that of the conventional
277 method. However, it has the advantage that samples can be stored at room temperature for
278 prolonged periods and readily transported to a central laboratory without degradation of
279 DNA prior to analysis, avoiding the need for a cold chain or transport medium with
280 temperature monitoring. Another advantage of the filter paper technique is that it does not
281 involve any culture pre-amplification and so allows direct measurement of the density of
282 bacteria present on the swab and hence an indication of the density of pharyngeal carriage
283 in an individual. We found a wide range of bacterial density in the filter paper samples with
284 a density distribution similar to that found in UK students (16). Since high density carriers
285 are likely to be more infectious than individuals carrying only a few bacteria, determination
286 of carriage density is likely to become an important end-point in future meningococcal
287 vaccine trials. Strips were stored for up to six months at room temperature before testing
288 and experience with malaria filter paper blood spots suggests that samples could be held for
289 up to six years without loss of DNA (19), especially when short DNA fragments are targeted.
290 The simplicity of the filter paper technique could make this a useful approach when carriage
291 surveys are needed in areas with few facilities or when there is a need to define the groups
292 with the highest bacterial densities; this warrants further investigation.

293 The increased yield of *Nm* detected using short term culture in THB needs confirmation,
294 but suggests that meningococcal carriage studies to date have considerably underestimated
295 the true level of pharyngeal carriage of *Nm*. Using this new technique, the size and cost of

296 carriage studies, for example those required to evaluate the impact of new meningococcal
297 vaccines, that are being developed could be reduced.
298

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304 and Melinda Gates Foundation, the Wellcome Trust and the Meningitis Research Foundation to the
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307

308 **Conflict of interest statement**

309 CLT reports receiving a consulting payment from GSK in 2013;

310

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364

365 **Tables**

366 **Table 1.** Comparative efficacy of Todd-Hewitt and Mueller-Hinton broths in supporting the
 367 growth of *N. meningitidis* in overnight culture. PCR positivity obtained with serial dilutions
 368 of a serogroup A reference strain of *N. meningitidis* is shown.

369

Average Log ₁₀ number of genomes copies by spectrophotometry	Average Todd-Hewitt broth <i>porA</i> Ct	Average Mueller- Hinton broth <i>porA</i> Ct
7.47	11.28	14.38
6.48	13.83	16.73
5.57	18.08	22.45
4.41	20.10	24.03
3.57	16.38	21.02
2.71	20.60	25.85
1.85	25.09	29.51
0.90	27.95	33.33
Approximately 1 copy	35.21	Undetermined
No copy	Undetermined	Undetermined

370

371

372

373 **Figure 1.** Summary of the methodology employed in the study.

374 **Figure 2.** Comparison of *N. meningitidis* positivity using *sodC* and *porA* and genogroup

375 characterization by real-time PCR on isolates after conventional microbiology versus Todd-

376 Hewitt Broth overnight culture.

377 **Figure 3:** Bacterial density distribution measured by copies of a fragment of the *porA* DNA

378 gene.

379 **Figure 4:** Venn diagram representing the positive swabs collected and tested by the three

380 methods (i) conventional microbiology and PCR after DNA extraction (ii) collection on filter

381 paper and direct PCR after DNA extraction (iii) Todd-Hewitt Broth overnight culture and

382 direct PCR after DNA extraction.

383 **Supplement**384 **Table S1**

385 Primers and probes used for real-time PCR

Targeted gene (species specific)	primer and probe name	5'-3' nucleotides sequence	dye	probe type
<i>sodC</i> (Nm)	sodC-F	GCA CAC TTA GGT GAT TTA CCT GCA T		
	sodC-R	CCA CCC GTG TGG ATC ATA ATA GA		
	sodC-probe	CAT GAT GGC ACA GCA A	NED	MGB
<i>porA</i> (Nm)	RT_porA-F	GCCGGCGTTGATTATGATT		
	RT_porA-R	AGTTGCCGATGCCGGTATT		
	RT_porA-P	CTCCGCCATCGTGTC	FAM	MGB
<i>Cnl</i>	RT_cnl-Fa	GAATTGCATAGGTTATCCAAAATCAC		
	RT_cnl-Fb	GAGTTGCACAGATTATCCAGAATCAC		
	RT_cnl-P	ATAAAACCGGTGCCGCC	VIC	MGB
	RT_cnl-R	TTTGCCCGATAACAATCTGAAAG		
A	F2531	AAAATTCAATGGGTATATCACGAAGA		
	R2624	ATATGGTGCAAGCTGGTTTCAATAG		
	RT_csaB_P	CTAAAAGTAGGAAGGGCACTT	VIC	MGB
W	F857	TATTTATGGAAGGCATGGTGTATG		
	R964	TTGCCATTCCAGAAATATCACC		
	Pb907i FAM	AAATATGGAGCGAATGATTACAGTAACTATAATGAA	FAM	BHQ
X	F173	TGCCCCAACCGTTTATTGG		
	R237	TGCTGCTATCATAGCCGCC		
	Pb196 CY5	TGTTTGCCACATGAATGGCGG	Cy5	BHQ

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B	F737	GCTACCCCATTTTCAGATGATTTGT		
	R882	ACCAGCCGAGGGTTTATTTCTAC		
	Pb839i CY5	AAGAGATGGGYAACAACTATGTAATGTCTTTATTT	Cy5	BHQ
C	F478	CCCTGAGTATGCGAAAAAATT		
	R551	TGCTAATCCCGCCTGAATG		
	Pb4951 FAM	TTTCAATGCTAATGAATACCACCGTTTTTTTGC	FAM	BHQ
Y	F787	TCCGAGCAGGAAATTTATGAGAATAC		
	R929	TTGCTAAAATCATTGCTCCATAT		
	Pb1099i HEX	TATGGT GTACGATATCCCTATCCTTGCCTATAA	HEX	BHQ
Z	cszC-1_Fwd1	CAGGCCGAAGAGCGTTATCA		
	cszC-1_Rev1	CGCCATTCAGGGCGATT		
	cszC-1_probe1	ACAGCTCTGGCCTTAG	Cy5	MGB
E	RT_cseE-F	GAGGCTGGCAATGACCAATT		
	RT_cseE-R	CCCAGCATATCGACAACCAA		
	RT_cseE-P	ATCTTATGTGAACGTGGCGC	FAM	MGB
H	RT_cshC-F	AAGCCCGTTCCAAGATCATG		
	RT_cshC-R	GCGGTTTGGAGAAATAATATGTGTT		
	RT_cshC-P	AATGTCAGCCGTAACCTT	VIC	MGB