Culture independent real-time PCR reveals extensive polymicrobial infections in hospitalized diarrhoea cases in Kolkata, India

A. Sinha,[1] S. SenGupta,[1] S. Guin,[1] S. Dutta,[1] S. Ghosh,[1] P. Mukherjee,[1] A. K. Mukhopadhyay,[1] T. Ramamurthy,[1] Y. Takeda,[2] T. Kurakawa,[3] K. Nomoto,[3] G. B. Nair,[1] and R. K. Nandy[1*]

National Institute of Cholera and Enteric Diseases (NICED), Kolkata 700010,[1] Collaborative Research Centre of Okayama University for Infectious Diseases in India, NICED, Kolkata 700010,[2] and Yakult Central Institute for Microbiological Research, Kunitachi, Tokyo, Japan[3]

Running title: Polymicrobial diarrhoeal infection

Key words: Real-time PCR, Diarrhoea, Polymicrobial infection

*Correspondence:

Dr. Ranjan Kumar Nandy
National Institute of Cholera and Enteric Diseases,
P-33, C. I. T Road, Scheme XM, Beliaghata,
Kolkata - 700 010, India.
Tel: 91-33- 2363- 3373
Fax: 91-33-2350-5066
E-mail: nandyrk@icmr.org.in; nandy_rk@hotmail.com

Abstract

Culture independent identification of diarrhoeal etiologic agents was performed using DNA harvested from diarrhoeal stool specimens with SYBR Green based real-time PCR targeting *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Campylobacter spp.*, *Shigella spp.*, and 3 different pathotypes of diarrhoeagenic *Escherichia coli*. Conventional culture dependent methods detected bacterial enteropathogens in 68 of 122 diarrhoeal stool specimens. Of 68 specimens, 59 (86.8%) had single pathogen while the remaining 9 (13.2%) had polymicrobial infections with multiple pathogens. Reanalysis of the 68 specimens by culture independent real-time PCR methods showed 25 (36.8%) specimens contained single pathogen while 43 (63.2%) specimens contained mixed infections with multiple pathogens. The prevalence of such high level of polymicrobial infections would not have been detected if real-time PCR was not utilized. Culture dependent analysis assigned 54 of the 122 selected archived specimens as 'no known aetiology'. However, reanalysis of these samples by real-time PCR showed presence of single or multiple pathogens among 34 (63%) of these specimens. Estimation of relative pathogen load by real-time PCR in the stool specimens indicated the inability of conventional culture dependent methods to detect the pathogens was related to lower colony forming units of the pathogen as reflected by lower Ct values. Detection of high levels of polymicrobial infection by real-time PCR indicate that in the settings like Kolkata and around, which is endemic for cholera and other enteric diseases, the concept of one pathogen one disease might need to be re-evaluated.

Introduction

Globally, about two billion cases of diarrhoeal diseases occur every year. It is considered as the second leading cause of death in children less than five years old, killing about 1.336 million children every year [1]. India contributes about 77% of the child deaths in southeast Asia and 18% of the global child deaths due to diarrhoea [1]. The irony lies in the fact that most diarrhoeas are treatable and most of the diarrhoeal deaths are preventable. Diarrhoea should thus be attended rapidly and effectively to detect the causal aetiology and to avoid significant morbidity and mortality as well as to prevent secondary transmission.

Polymicrobial infections in diarrhoeal diseases have been reported extensively in countries where sanitation is compromised and where availability of safe drinking water is restricted [2-8]. In some cases, polymicrobial infections have been considered as a major factor contributing to the severity of diarrhoea [4]. Despite using all modern days bioassay based tools, various hospital and community based diarrhoeal surveillance studies have consistently been unable to detect a causal aetiology in about 30% of the specimens [8-12]. This has stressed the need for more sensitive, specific and rapid detection assays for identifying pathogens from diarrhoeal stools.

Culture dependent methods to identify the enteric pathogens as pure culture followed by characterization through various biochemical tests are considered as gold standard. But it takes considerable time to confirm the aetiology. Further to this an enormous number bacterial species that resides in the human gut are yet to be cultured. In spite of being able to culture hundreds of enteric bacteria, 80- 90 % of gut flora still remains as unculturable. Culture independent techniques for identifying and to characterize these uncultivable floras are currently being perused. In the post genomic era, culture independent rapid detection assays have been developed of which real-time PCR based assays have gained much interest

79   [13-19]. This study is a part of such a trend in identifying enteropathogens directly from stool

80   specimens.

81   **Materials and methods**

82   **Archived diarrhoeal stool specimens and DNA extraction**

83   Stool specimens were collected from hospitalized diarrhoeal patients after obtaining informed

84   consent and the study was approved by the Institutional Ethical Committee. Samples were analyzed

85   by culture dependent methods for the detection of bacterial, viral and parasitic

86   enteropathogens [8]. In brief, diarrheal stool specimens were streaked on selective plates,

87   colonies grew on the plates were tested through limited number of biochemical tests for

88   presumptive identification. Confirmation of the pathogens were done afterwards through

89   pathogen specific tests. The *ompW* PCR were performed for the species confirmation of *V.*

90   *cholerae*. Strains of *V. parahaemolyticus*, *Shigella* spp and *Salmonella* spp were serotyped

91   using commercially available antisera (Denka Seiken, Tokyo, Japan, BioRad, Marnes-la-

92   Coquette, France). *V. cholerae* O1 strains were serotyped using antisera prepared in NICED.

93   Three different lactose-fermenting colonies isolated from each sample were picked from

94   MacConkey agar plate and included in the multiplex PCR assay for the detection of different

95   diarrhoeagenic *E. coli* that include enterotoxigenic *E. coli* (ETEC, inclusive of both heat-

96   labile and heat-stable enterotoxin producers), enteropathogenic *E. coli* (typical and atypical

97   EPEC) and enteroaggregative *E. coli* (EAEC). The diarrhoeal stool specimens were stored

98   frozen at -80 °C in aliquots of 500 µl each. A total of 122 specimens were selected from the

99   archive of which 68 had aetiologies of bacterial pathogens and remaining 54 were assigned as

100   'no known pathogen' (Fig. 1). Among the 68 specimens, *Vibrio cholerae*, *Vibrio*

101   *parahaemolyticus*, *Campylobacter spp.*, *Shigella spp.*, enterotoxigenic *Escherichia coli*

102   (ETEC), enteropathogenic *E. coli* (EPEC) and enteroaggregative *E. coli* (EAEC) were

103   identified among 29, 11, 8, 14, 7, 1 and 9 specimens, respectively. Of the 68 specimens, 59

104   were indentified to contain single pathogen and 9 were with mixed pathogens. One aliquot of

105   the selected specimens were thawed and used for DNA extraction by QIAmp DNA stool mini

106   kit (Qiagen, USA). The real-time PCR reanalysis for abovementioned pathogens were

107   performed using 1 µl of DNA solution.

108   **Bacterial strains and culture condition**

109   Bacterial strains for *V. cholerae* O1 (N16961 and O395), *V. parahaemolyticus* (KXV139),

110   *Campylobacter spp.* (*C. jejuni* IDH1138, *C. coli* IDH797, *C. fetus* IDH1156), *Shigella spp.* (*S.*

111   *sonnei* 500228, *S. boydii* 500202, *S. flexneri* ATCC12022), ETEC (500205), EPEC (11044)

112   and EAEC (2075) were used to validate SYBR Green real-time PCR based species specific

113   detection assay. Luria broth (LB) supplemented with 1%, 3% and 0.5% NaCl was used for

114   culturing *V. cholerae*, *V. parahaemolyticus*, and *Shigella spp.*, respectively. Diarrheagenic *E.*

115   *coli* was also cultured in LB supplemented with 0.5% NaCl. *Campylobacter spp.* was

116   cultured for 48 h at 37 °C in brain heart infusion agar plates supplemented with 5% serum

117   under microaerophilic conditions.

118   **SYBR Green real-time PCR with pure culture**

119   The 10 pairs of real-time PCR primers used in this study for the detection of *V. cholerae*, *V.*

120   *parahaemolyticus*, *Campylobacter spp.*, *Shigella spp.*, ETEC, EAEC and EPEC. *V. cholerae*

121   O1 antigen coding region specific primers were used as described by Hoshino *et al*, [20].

122   Primers for *V. parahaemolyticus* and *Campylobacter spp.* were used as described by

123   Kurakawa *et al* [21]. Primers for invasion plasmid antigen H (*ipaH*) were used as specific

124   primers   for   *Shigella   spp.*   [22].   One   set   of   ETEC   primers   (5'-

125   GGCGACAAATTATACCGTGC-3' and 5'- AAACATATTTGGTGCTGTCGC-3') specific

126   to labile toxin (*lt*) gene was developed, while another set specific to stable toxin (*st*) were

127   used as described by Fukushima *et al* [16]. Reverse primers specific to virulence gene *aggR*

128   of EAEC (5´-TCGGAAAAGAAGCTTACAGCC-3´) and virulence gene *eaeA* of EPEC (5´-

129   CAGAGATCGCGACTGAAGC-3´) were developed and used in combination with respective

130   pathogroup specific forward primers [16]. Validation of the species specific detection of

131   enteropathogens (*V. cholerae*, *V. parahaemolyticus*, *Campylobacter spp.*, *Shigella spp.*,

132   ETEC, EPEC and EAEC) by real-time PCR was made using boiled lysate as source of DNA

133   template prepared from pure culture and SYBR Green as detecting dye. Boiled lysate was

134 prepared by suspending one loopful of pure culture in 200 µl of PBS, boiled for 10 min,

135 centrifuged for 5 min at 10000 x g and debris free 1 µl clear supernatant was used directly for

136 real-time PCR. PCR primers were adjusted to a final concentration of 0.6 pmole/ µl in a 20 µl

137 of reaction volume with 1X Power SYBR Green master mix (Applied Biosystems, USA).

138 The real-time PCR was performed in a 7900 HT Fast real-time PCR machine (Applied

139 Biosystems). During pre-PCR, tubes were heated to 50 °C for 2 min followed by 95 °C for 10

140 min. Subsequently, complete 35 cycles of PCR were performed using 94 °C for 20 s, 55 °C

141 for 20 s and with an extension step of 72 °C for 50 s. Fluorescence signals were measured at

142 the extension step of each of the cycle. Amplicon specificity was established through melting

143 (T_m) curve analysis [23]. The T_m values of the amplicons generated against DNA from each

144 of the included pathogens with respective primers are presented in Fig. 2. Single peak for the

145 amplicons specific to O1 *wb* of *V. cholerae* O1 (Fig. 2A), 23S rDNA of *V. parahaemolyticus* (B),

146 *lt* of ETEC (C), *st* of ETEC (D), *eaeA* of EPEC (F), 16S rDNA of *Campylobacter spp.* (G) and *epaH*

147 of *Shigella spp.* (H) is evident. The *aggR* amplicon of EAEC, agave dual peak (Fig. 2 E) which

148 may be considered due to difference in GC content (high G:C content in one area versus

149 another) within the amplicon. Specificity of *aggR* amplification was further confirmed by

150 visualization of single band in the agarose gel electrophoresis. All PCR assays used in this

151 study produced single amplicon when analyzed through agarose gel electrophoresis. Ability

152 to detect specific pathogen was established as this assay could produce single amplicon even

153 with mixed DNA template based on the usage of specific set of primers with similar melt

154 curve generating same T_m as compared to a situation when tested individually with purified

155 DNA template (Fig. 2). For all assays, negative controls were included that comprised of

156 PCR grade water as well as lysates prepared from heterologous organisms.

157 **Detection and relative quantification of pathogens by real-time PCR**

158 Bacterial suspensions from pure culture were made and subsequently dilution plating was

159 performed using 10 folds diluted suspensions to estimate number of colony forming units

160 (CFU)/ ml of the suspension. From each of the serial dilution tubes as generated for dilution

161 plating, 100 µl of suspension was taken out to prepare boiled lysate and 1 µl of which was

162 used in the real-time PCR. Threshold cycle (Ct) values obtained for each of the dilutions were

163 plotted against normalized CFUs and organism specific standard curve was generated. DNA

164 extracted from diarrhoeal stool specimens was used directly for detecting enteropathogens

165 and pathogen specific Ct values were recorded. Obtained pathogen specific Ct values were

166 plotted on standard curve for an estimation of the load of the pathogen when present in the

167 diarrhoeal stool in the form of single or multiple pathogens and expressed as CFU/ ml

168 equivalence.

169 **Results**

170 **Bacterial enteropathogen detection by real-time PCR**

171 Real-time PCR assay successfully detected *V. cholerae*, *V. parahaemolyticus*, *Campylobacter*

172 *spp.*, *Shigella spp.*, ETEC, EAEC and EPEC when boiled lysate prepared from respective

173 strains were used. The melt curve analysis of the product obtained in the real-time PCR assay

174 is presented in Fig. 2. Detection of specific melting curve with characteristic T_m for each

175 species confirmed specificity of real-time PCR detection. Amplification was possible only

176 with homologous combinations of pathogen and its primer pairs. A linear relationship was

177 established between the Ct value and number of viable cells included in the assay that ranged

178 between 10^9 CFU/ ml and 10^4 CFU/ ml and such relationship was subsequently utilized to

179 estimate pathogen load equivalence in the stool specimens (Fig. 3).

180 **Application of real-time PCR for pathogen detection in stool specimens and estimation**

181 **of pathogen load**

182 Of 68 specimens, 59 were previously identified to contain sole pathogen and 9 had mixed

183 pathogens by culture dependent methods (Fig. 1). Reanalysis of the 59 specimens by culture

184 independent real-time PCR showed presence of mixed pathogens in 34 specimens and 25

185 contained sole pathogen (Fig. 1). In fact, all pathogens detected by culture based assays were

186 also detected in respective specimens by real-time PCR. Detection of additional pathogens

187 through real-time PCR assay resulted in an increase of mixed infections from ca.13% to ca.

188 50%.

189 Reanalysis of these 68 specimens by real-time PCR showed matching detection of culture

190 based aetiologies with a pathogen load equivalence ranging between 10^9 and 10^6 CFU/ ml (Ct

191 values ranged between 13 and 23). Interestingly, Ct value for the pathogens that remained

192 undetected by the culture dependent methods ranged between 25 and 30 that corresponded to

193 pathogen load equivalence ranging between 10^5 and 10^4 CFU/ ml. A comparative analysis on

194 the pathogen detection among the 68 specimens by real-time PCR against culture dependent

195 methods is presented in Table 1.

196 The culture independent real-time PCR detection of pathogens was subsequently extended

197 to 54 specimens, which were assigned as "no-known pathogen" by culture dependent

198 methods. The presence of pathogens was detected by real-time PCR in 34 of 54 specimens

199 which were originally assigned as "no-known pathogen" (Fig. 1, Table 2). Of the 34

200 specimens, 25 and 9 had single and mixed pathogens, respectively. Analysis of pathogen

201 specific Ct values obtained with real-time PCR positive 34 specimens showed pathogen load

202 equivalence that ranged between 10^5 and 10^4 CFU/ ml equivalence.

203 **Discussion**

204 This study was initiated to detect bacterial enteropathogens directly from stool specimens

205 and that targeting pathogen specific virulence genes or rDNA regions. This was an effort to

206 understand the inadequacy, if any, of culture dependent methods in comparison to culture

207   independent assays. Culture independent real-time PCR based reanalysis of 68 specimens

208   (including sole and mixed pathogens) revealed detection of all aetiologies that were identified

209   by culture dependent methods thereby validating the real-time PCR methods. Interestingly,

210   real-time PCR detected additional pathogens in most of these specimens. In fact, many of the

211   samples, which were reported to contain sole pathogen, were shown to have multiple

212   pathogens following reanalysis by real-time PCR (Fig. 1, Table 1).

213   The real-time PCR assay revealed an interesting relationship between pathogen load and

214   aetiologies as detected by culture dependent methods. The culture dependent methods based

215   aetiologies was detected in specimens with pathogen load 10^6 or more CFU/ ml. Analysis

216   also revealed 10^4 CFU/ ml equivalence was the limit for detecting pathogens by the real-time

217   PCR assay. This relationship also remained valid with specimens that were identified to have

218   mixed pathogens by culture dependent methods; pathogen load ranged between 10^6 and 10^7

219   CFU/ ml equivalence. Approximately one third of hospitalized diarrhoeal cases yielded

220   mixed infections by culture dependent methods as shown in several studies in impoverished

221   settings including recently in Kolkata [3,4,6-8]. While culture dependent methods showed

222   presence of mixed pathogens among 9 (13.2%) cases, real-time PCR based detection

223   increased the percentage of mixed pathogens to 50% (Fig. 1). Comparative analysis of

224   pathogen detection by culture dependent vs. culture independent real-time assay is presented

225   in Fig. 4. It is evident from Fig. 4 that good number of specimens contained multiple

226   pathogens. In fact, in some cases presence of 4 enteropathogens were also detected. Therefore,

227   the real-time PCR based reanalysis established that mixed infections are much higher than

228   previously conceived.

229   Diarrhoeal surveillance studies have shown that approximately 30% of the specimens do

230   not yield any known aetiologies in diverse geographic settings. A recent study conducted in

231   Kolkata showed that 27.9 % of the stool specimens from hospitalized diarrhoea patients did

232   not yield any pathogen despite examining the samples for 26 known diarrhoeal pathogens [8].

233   In this study we therefore extended our analysis to examine 54 specimens that were assigned

234   as 'no known pathogen' by culture dependent methods. However, when examined by real-

235   time PCR 34 of these 54 specimens showed presence of one or more pathogens (Figs. 1 and

236   4). The density of the pathogens present in these specimens ranged between 10^4 and 10^5

237   CFU/ ml equivalence. Existence of pathogen below 10^6 CFU/ ml equivalence, a load below

238   the detection limit, to be considered as basis for under detection of aetiologies by culture

239   dependent assays. This study therefore unequivocally confirmed the ability of culture

240   independent real-time PCR to detect enteric pathogens at lower densities in stool specimens

241   where culture dependent methods failed to detect the same. Considering reanalysis of 122

242   specimens, real-time PCR detected 102 specimens with one or more pathogens in contrast to

243   68 specimens with aetiologies by culture dependent methods.

244   Detection of multiple pathogens in single diarrhoeal stool specimen indicates that the

245   subjects living in this impoverished setting are assaulted by multiple enteric pathogens at any

246   given time. Therefore, the concept of one pathogen one disease might need to be re-evaluated.

247   This study has been carried out with limited number of bacterial enteropathogens. Inclusion

248   of real-time PCR based detection methods for other viral and parasitic pathogens may further

249   enhance the melange of pathogens harboured by subjects living in poorly hygienic conditions.

250   Relative distribution of the pathogens as detected by culture based methods (Table 1 and Fig.

251   4) should not be construed as true representation of their degree of associations among

252   clinical cases in settings of Kolkata and around as selection of these specimens were only

253   made for a comparative analysis between culture dependent and independent assays.

254   Polymicrobial infections are common in settings of low resource countries. This is in stark

255   contrast to what is seen in the sanitized developed country settings where the aetiology of

256   diarrhoea is due to single pathogen. As majority of the patients came from low income group

257   living in poorly hygienic conditions, detection of multiple pathogens in diarrheal stool

258   specimens indicated gross contamination in food and water that they consumed. Synergistic

259   action of microorganisms impacting each other in the polymicrobial infection situations has

260   already been reported for wound infections as well as diarrhoeal cases caused by either

261   EAEC or EPEC [24,25]. Preferential association between enteric pathogens present as mixed

262   infection has also been demonstrated recently [26]. Consumption of grossly contaminated

263   food and water by the majority of the patients living under impoverished conditions lead to

264   infection by multiple pathogens and subsequently to hospitalization. However, the

265   significance of contrasting densities (about 100 folds more of one pathogen as compared to

266   another) of enteric pathogens in mixed infection needs to be addressed in greater detail

267   through a case-control field study to portray actual scenario. Clinical findings of the status of

268   patients having mixed infection will be described in a distinct work. Detection of

269   polymicrobial infections with pathogens in lower densities by the real-time PCR assay raised

270   a concern on likely existence of potentially good number of human carrier. Polymicrobial

271   infections among hospitalized patients thus clearly emphasized the need to pursue more

272   exploratory approach to understand the epidemiological, inter-microbial interactions and

273   clinical implications of the presence of more than one pathogens.

282 **Author's Contribution**

283 AS prepared DNA from the faecal samples, performed all Real time PCR assays quantified

284 the load of pathogens present in the stool specimens. SSG, SG, SD, SG and PM isolated the

285 different bacterial pathogens microbiologically from the stool specimens. TK and KN

286 designed some of the primers for this study. AS, AKM, TR, YT, GBN, RKN analyzed the

287 results and wrote the paper. All authors read and approved the final manuscript.

288 **References**

289 1. Black RE, Cousens S, Johnson HL *et al*. Global, regional, and national causes of child

290 mortality in 2008: a systematic analysis. *Lancet* 2010; **375**: 1969-1987.

291 2. Schultsz C, van den EJ, Cobelens F *et al*. Diarrheagenic *Escherichia coli* and acute and

292 persistent diarrhea in returned travelers. *J Clin Microbiol* 2000; **38**: 3550-3554.

293 3. Chowdhury F, Begum YA, Alam MM et al. Concomitant enterotoxigenic *Escherichia*

294 *coli* infection induces increased immune responses to *Vibrio cholerae* O1 antigens in

295 patients with cholera in Bangladesh. *Infect Immun* 2010; **78**: 2117-2124.

296 4. Faruque AS, Mahalanabis D, Islam A, Hoque SS. Severity of cholera during concurrent

297 infections with other enteric pathogens. *J Diarrhoeal Dis Res* 1994; **12**: 214-218.

298 5. Chen SY, Tsai CN, Chao HC *et al*. Acute gastroenteritis caused by multiple enteric

299 pathogens in children. *Epidemiol Infect* 2009; **137**: 932-935.

300 6. Fischer Walker CL, Sack D, Black RE. Etiology of diarrhea in older children,

301 adolescents and adults: a systematic review. *PLoS Negl Trop Dis* 2010; **4**: e768.

302 7. Nimri LF, Elnasser Z, Batchoun R. Polymicrobial infections in children with diarrhoea

303 in a rural area of Jordan. *FEMS Immunol Med Microbiol* 2004; **42**: 255-259.

304 8. Nair GB, Ramamurthy T, Bhattacharya MK *et al*. Emerging trends in the etiology of

305 enteric pathogens as evidenced from an active surveillance of hospitalized diarrhoeal

306 patients in Kolkata, India. *Gut Pathog* 2010; **2**: 4.

307   9. Torres ME, Pirez MC, Schelotto F et al. Etiology of children's diarrhea in Montevideo,

308   Uruguay: associated pathogens and unusual isolates. *J Clin Microbiol* 2001; **39**: 2134-

309   2139.

310   10. Samal SK, Khuntia HK, Nanda PK *et al*. Incidence of bacterial enteropathogens among

311   hospitalized diarrhea patients from Orissa, India. *Jpn J Infect Dis* 2008; **61**: 350-355.

312   11. Germani Y, Morillon M, Begaud E *et al*. Two-year study of endemic enteric pathogens

313   associated with acute diarrhea in New Caledonia. *J Clin Microbiol* 1994; **32**: 1532-1536.

314   12. el Sheikh SMel Assouli SM. Prevalence of viral, bacterial and parasitic enteropathogens

315   among young children with acute diarrhoea in Jeddah, Saudi Arabia. *J Health Popul*

316   *Nutr* 2001; **19**: 25-30.

317   13. Jothikumar NGriffiths MW. Rapid detection of *Escherichia coli* O157:H7 with

318   multiplex real-time PCR assays. *Appl Environ Microbiol* 2002; **68**: 3169-3171.

319   14. Reischl U, Youssef MT, Wolf H, Hyytia-Trees E, Strockbine NA. Real-time

320   fluorescence PCR assays for detection and characterization of heat-labile I and heat-

321   stable I enterotoxin genes from enterotoxigenic *Escherichia coli*. *J Clin Microbiol*

322   2004; **42**: 4092-4100.

323   15. Lin S, Wang X, Zheng H et al. Direct detection of *Campylobacter jejuni* in human stool

324   samples by real-time PCR. *Can J Microbiol* 2008; **54**: 742-747.

325   16. Fukushima H, Tsunomori Y, Seki R. Duplex real-time SYBR green PCR assays for

326   detection of 17 species of food- or waterborne pathogens in stools. *J Clin Microbiol*

327   2003; **41**: 5134-5146.

328   17. Holland JL, Louie L, Simor AE, Louie M. PCR detection of *Escherichia coli* O157:H7

329   directly from stools: evaluation of commercial extraction methods for purifying fecal

330   DNA. *J Clin Microbiol* 2000; **38**: 4108-4113.

331   18. Iijima Y, Asako NT, Aihara M, Hayashi K. Improvement in the detection rate of

332   diarrhoeagenic bacteria in human stool specimens by a rapid real-time PCR assay. *J*

333   *Med Microbiol* 2004; **53**: 617-622.

334   19. Tatavarthy ACannons A. Real-time PCR detection of Salmonella species using a novel

335   target: the outer membrane porin F gene (ompF). *Lett Appl Microbiol* 2010; **50**: 645-

336   652.

337   20. Hoshino K, Yamasaki S, Mukhopadhyay AK *et al*. Development and evaluation of a

338   multiplex PCR assay for rapid detection of toxigenic *Vibrio cholerae* O1 and O139.

339   *FEMS Immunol Med Microbiol* 1998; **20**: 201-207.

340   21. Kurakawa T, Kubota H, Tsuji H *et al*. Development of a sensitive rRNA-targeted

341   reverse transcription-quantitative PCR for detection of *Vibrio cholerae/ mimicus*, *V.*

342   *parahaemolyticus/ alginolyticus* and *Campylobacter jejuni /coli*. accepted in

343   Microbiology and Immunology

344   22. Luscher DAltwegg M. Detection of *Shigellae*, enteroinvasive and enterotoxigenic

345   *Escherichia coli* using the polymerase chain reaction (PCR) in patients returning from

346   tropical countries. *Mol Cell Probes* 1994; **8**: 285-290.

347   23. Ririe KM, Rasmussen RP, Wittwer CT. Product differentiation by analysis of DNA

348   melting curves during the polymerase chain reaction. *Anal Biochem* 1997; **245**: 154-160.

349   24. Keskimaki M, Mattila L, Peltola H, Siitonen A. Prevalence of diarrheagenic

350   *Escherichia coli* in finns with or without diarrhea during a round-the-world trip. *J Clin*

351   *Microbiol* 2000; **38**: 4425-4429.

352   25. Bowler PG, Duerden BI, Armstrong DG. Wound microbiology and associated

353   approaches to wound management. *Clin Microbiol Rev* 2001; **14**: 244-269.

354   26. Lindsay B, Ramamurthy T, Sen Gupta S *et al*. Diarrheagenic pathogens in

355   polymicrobial infections. *Emerg Infect Dis* 2011; **17**: 606-611.

356

357   TABLE 1. Real-time PCR based reanalysis of diarrhoeal stool specimens with aetiologies by

358   culture dependent methods

Aetiology	Number of specimens[a] with designated pathogen when analyzed by					
	Culture dependent methods[b]			Culture independent methods[c]		
	Single (%)	Mixed (%)	Total	Single (%)	Mixed (%)	Total
V. cholerae	23 (33)	6 (8.8)	29	2 (2.9)	31 (45.5)	33
V. parahaemolyticus	9 (13.2)	2 (2.9)	11	3 (4.4)	27 (39.7)	30
Campylobacter spp.	3 (4.4)	5 (7.3)	8	1 (1.4)	10 (14.7)	11
Shigella spp.	12 (17.6)	2 (2.9)	14	10 (14.7)	12 (17.6)	22
ETEC	6 (8.8)	1 (1.4)	7	4 (5.8)	9 (13.2)	13
EPEC	1 (1.4)	-	1	1 (1.4)	3 (4.4)	4
EAEC	5 (7.3)	4 (5.8)	9	4 (5.8)	12 (17.6)	16

375   [a]Total number of diarrhoeal stool specimens analyzed were 68 by both culture dependent and culture

376   independent methods

377   [b]Enteropathogens detection was performed on freshly collected stool specimens following

378   conventional techniques as described [8].

379   [c]Enteropathogens detection was performed through real-time PCR assay using archived specimens

380   stored at -80 °C.

382 TABLE 2. real-time PCR based reanalysis of diarrhoeal stool specimens with 'No known

383 pathogen' by culture dependent methods

384

Aetiology	Number of specimens[a] with designated pathogen when analyzed by					
	Culture dependent methods[b]			Culture independent methods[c]		
	Single (%)	Mixed (%)	Total	Single (%)	Mixed (%)	Total
V. cholerae	-	-	-	3 (5.5)	2 (3.7)	5
V. parahaemolyticus	-	-	-	7 (12.9)	4 (7.4)	11
Campylobacter spp.	-	-	-	-	3 (5.5)	3
Shigella spp.	-	-	-	4 (7.4)	8 (14.8)	12
ETEC	-	-	-	9 (16.6)	5 (9.2)	14
EPEC	-	-	-	-	-	-
EAEC	-	-	-	2 (3.7)	-	2

400 Out of 54 specimens 34 were found to contain bacterial pathogen and 20 specimens remained as 'No

401 known pathogen'

402 [a]Total number of diarrhoeal stool specimens analyzed were 54 by both culture dependent and culture

403 independent methods

404 [b]Enteropathogens detection was performed on freshly collected stool specimens following

405 conventional techniques as described [8].

406 [c]Enteropathogens detection was performed through real-time PCR assay using archived specimens

407 stored at -80 °C.

408

Diarrheal stool
specimens
(n=122)

Culture
dependent

Sole
(n=59)

Mixed
(n=9)

No known
pathogen
(n=54)

Culture
independent

Mixed
(n=9)

Sole
(n=25)

Mixed
(n=34)

Sole
(n=25)

Mixed
(n=9)
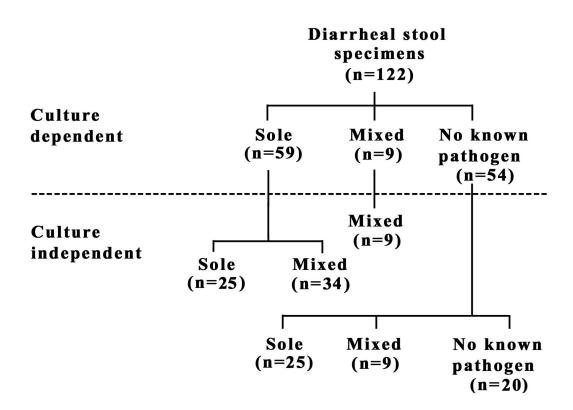
No known
pathogen
(n=20)

Fig.1 Comparative analysis for detecting pathogens by culture
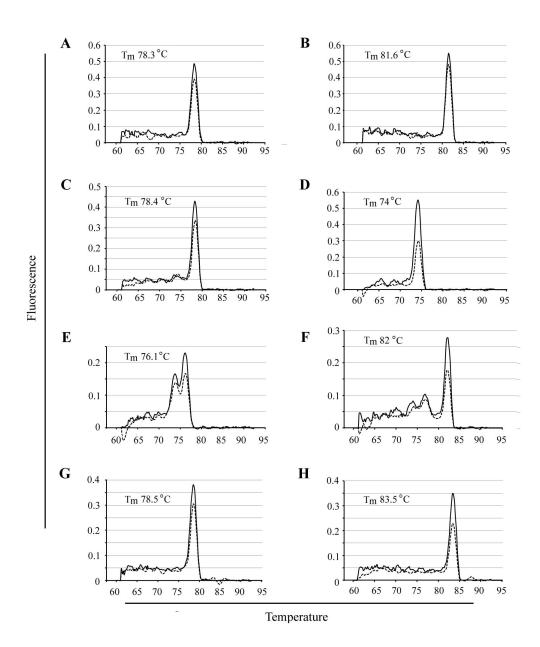**dependent and independent methods**

409

410

411

Fig. 2 Melting curve analysis of real-time SYBR Green PCR amplicons of (A) *wb* of *V. cholerae* O1, (B) 23S rDNA of *V. parahaemolyticus*, (C) *lt* of ETEC, (D) *st* of ETEC, (E) *aggR* of EAEC, (F) *eaeA* of EPEC, (G) 16S rDNA of *Campylobacter spp.* and (H) *epaH* of *Shigella spp.* Temperature of melting (T_m) of the amplicons specific to 7 different pathogens are indicated. In all the panels bold line (——) represents analysis of PCR amplicons obtained against DNA from each of the abovementioned pathogens used individually with respective primers whereas broken line (-----) represents analysis of PCR amplicons obtained against mixture of 1:100 times diluted DNA (all abovementioned 7 pathogens) with pathogen specific primers.

413

414

Fig. 3 **Standard curve generated by plotting varying number of**
***V. cholerae* viable cells against corresponding Ct value**
obtained in a real-time PCR assay carried out in 20 μl
reaction volume with SYBR Green as detecting dye.
Estimation of actual counts of viable cells were made by
dilution plating method using 10-fold serial dilution of
the cells. The average Ct value for duplicate samples
obtained per dilution was used for plotting.

Number of specimens with detected pathogens

25 20 15 10 5 0   0 5 10 15 20 25

A   B

n = 68   n = 54

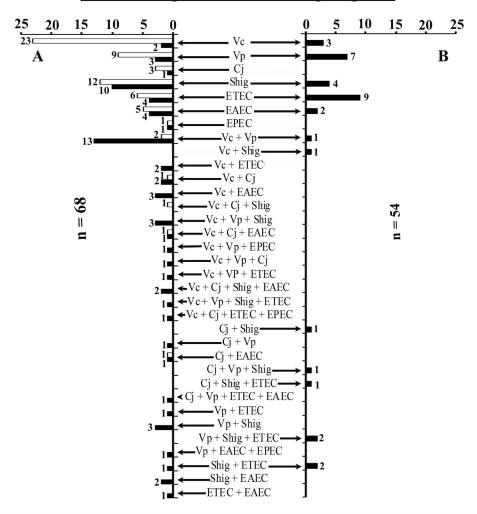| Category	A (white)	A (black)	B (black)
Vc	23	2	3
Vp	9	3	7
Cj	3	3 / 1	
Shig	12 / 10		4
ETEC	6 / 4		9
EAEC	5 / 4		2
EPEC	1 / 1		
Vc + Vp	2 / 1	13	1
Vc + Shig			1
Vc + ETEC		2	
Vc + Cj	2 / 1		
Vc + EAEC		3	
Vc + Cj + Shig	1		
Vc + Vp + Shig		3	
Vc + Cj + EAEC	1 / 1		
Vc + Vp + EPEC	1		
Vc + Vp + Cj	1		
Vc + VP + ETEC	1		
Vc + Cj + Shig + EAEC	2		
Vc + Vp + Shig + ETEC	1		
Vc + Cj + ETEC + EPEC	1		
Cj + Shig			1
Cj + Vp	1		
Cj + EAEC	1 / 1		
Cj + Vp + Shig			1
Cj + Shig + ETEC			1
Cj + Vp + ETEC + EAEC	1		
Vp + ETEC	1		
Vp + Shig	3		
Vp + Shig + ETEC			2
Vp + EAEC + EPEC	1		
Shig + ETEC	1		2
Shig + EAEC	2		
ETEC + EAEC	1		

Fig. 4 Detection of pathogens (A) in 68 diarrhoeal stool specimens with aetiology by culture dependent and culture independent real-time PCR assays and (B) in 54 diarrheal stool specimens with 'no known pathogen' by culture dependent assay but with aetiology when analyzed by real-time PCR assays. White bar (□) and black bar (■) represents detection of pathogens by culture based assays and culture independent real-time PCR assays, respectively. Number on the side of each bar represents specimens with respective aetiology. Abbreviation: n, total number of specimens analyzed; Vc, *V. cholerae*; Vp, *V. parahaemolyticus*; Cj, *Campylobacter spp.*; and Shig, *Shigella spp.*