Indian J Med Res 149, May 2019, pp 671-676
DOI: 10.4103/ijmr.IJMR_1394_17

Quick Response Code:

A novel ready-to-use dry-reagent polymerase chain reaction for detection of *Escherichia coli* & *Shigella* species

Mukti Nath Mishra[1], Raghavendra D. Kulkarni[2], Jeevanandam Mohanraj[1], S. Durairaju Nisshanthini[1], G.S. Ajantha[2], Arun Chandrasekhar[1], Prachee Kenge[1] & Shama Bhat[1]

[1]Department of Molecular Diagnostics, Bhat Biotech India Pvt. Ltd., Bengaluru & [2]Department of Microbiology, SDM College of Medical Sciences & Hospital, Dharwad, India

Received August 24, 2017

***Background & objectives*: Polymerase chain reaction (PCR) has wide acceptance for rapid identification of pathogens and also for diagnosis of infectious conditions. However, because of economic and expertise constraints, a majority of small or peripheral laboratories do not use PCR. The objective of the present study was to develop a dry-reagent PCR assay as an alternative to conventional PCR to assess its applicability in routine laboratory practice using *malB* gene for identification of *Escherichia coli* as a model.**

***Methods*: A total of 184 isolates were selected for the study comprising clinical isolates of *E. coli* and non-*E. coli* including *Shigella* sp. and a few other control strains. The DNA was isolated from all the isolates. The isolated DNA as well as the overnight grown bacterial cultures were subjected to both conventional wet PCR and dry-reagent PCR.**

***Results*: The genomic DNA isolated from *E. coli* showed amplification of *malB* gene in both conventional wet and dry-reagent PCR and the band was observed at 491 bp. In dry-reagent PCR, the overnight grown *E. coli* cells also showed positive result. The non-*E. coli* strains other than *Shigella* sp. showed negative in both conventional wet and dry-reagent PCR. *Shigella* sp. showed positive in both conventional wet and dry-reagent PCR.**

***Interpretation & conclusions*: Considering the elimination of genomic DNA isolation step, and similar results with the conventional wet PCR, dry-reagent PCR may be a good alternative for the conventional wet PCR.**

Key words ABC transporter - dry-reagent PCR - *Escherichia coli* - *malB* gene - maltose/maltodextrin transporter ATP-binding gene - pathogens - *Shigella* sp.

Escherichia coli a commensal Gram-negative bacillus, has various pathotypes causing gastrointestinal and most other pyogenic infections such as wound infections, septicaemia and meningitis. *E. coli* is the most common cause of urinary tract infections and now is placed in the list of important hospital pathogens[1]. It is routinely identified by standard biochemical and physiological tests. PCR assays though are available for molecular identification of *E. coli*[2-5], these are not commonly used by the small laboratories because of

high cost of testing and lack of expertise. Therefore, an effort was made to develop a dry-reagent mix for PCR targeting *malB* gene commonly used to identify *E. coli*[4,5]. The use of dry-reagent mix with an enzyme resistant to PCR inhibitors has helped us to eliminate the step of DNA extraction from bacterial isolates. This feature enabled the use of this assay directly on bacterial suspensions as well as on isolated DNA for the identification of *E. coli*. The specificity of this test was assessed using a number of bacterial isolates other than *E. coli* (non-*E. coli*). Here, we report the development of optimized 'ready-to-use' dry-reagent mix dispensed in individual PCR tubes.

Material & Methods

The study was conducted at Bhat Biotech India Pvt. Ltd., Bengaluru, and department of Microbiology, SDM College of Medical Sciences and Hospital, Dharwad, India, from June 2015 to June 2017. All clinical isolates were identified to species level by commonly used conventional methods[6]. A total of 184 isolates were used in this study of which 104 were phenotypically confirmed *Escherichia coli*, 73 phenotypically confirmed non-*E. coli* and seven control strains including two *E. coli* and five non-*E. coli* (Table).

Bacterial genomic DNA isolation: A well-isolated single colony of the isolate from brain-heart infusion agar plates was inoculated into 1 ml of sterile Luria-Bertani (LB) broth (HiMedia, Mumbai) and incubated overnight at 37°C. The genomic DNA from all the isolates was extracted using standard protocol[7]. The eluted DNA was used for both conventional and dry-reagent PCR assays.

Primer selection and PCR conditions: Primers designed for this study were selected from *E. coli* (accession number LT615379) targeting 491 bp amplicon of *malB*, sugar ABC transporter and maltose/ maltodextrin import ATP-binding protein *malK* genes. The sequences and location of forward and reverse primers in *E. coli* chosen were as follows, forward primer 5'-GATGCGTGCACCTGTTTTTA-3' (4242866 - 4242885 bp) and reverse primer 5'-ACACCACGAATTCACCTTCA-3' (4243337 - 4243356 bp) (Sigma-Aldrich, USA).

Conventional PCR: The DNA isolated from all 184 bacterial isolates was tested with conventional wet-reagent mix in PCR[8]. PCR reaction mix was prepared to a final volume of 50 µl containing 32 µl of nuclease-free water, 10 µl of 5X PCR reaction buffer, 2 µl each of 1 µM forward and reverse primer, 1 µl of 10 mM deoxyribonucleotide triphosphates (dNTPs), 1 µl of Taq DNA polymerase (2.5 units/µl) (Fermentas, Vilnius, Lithuania) and 2 µl of isolated DNA as

Table. A list of non-*Escherichia coli* isolates and control strains used in the present study

Name of the organism	Number of isolates tested
Gram-negative	
Acinetobacter sp.	3
A. baumannii	3
Citrobacter diversus	1
C. freundii	3
Enterobacter sp.	1
E. cloacae	1
Klebsiella pneumoniae	4
Proteus mirabilis	4
P. vulgaris	3
Pseudomonas sp.	1
P. aeruginosa	4
Salmonella sp.	1
S. Typhi	7
Shigella boydii	2
S. dysenteriae	2
S. flexneri	5
S. sonnei	4
Vibrio cholerae	2
Staphylococcus aureus	3
Gram-negative	
Coagulase-negative Staphylococci	4
Methicillin-resistant Staphylococci	4
Streptococcus sp.	3
Enterococcus sp.	4
E. faecalis	4
Gram-negative	
ATCC *Escherichia coli* 25923	1
E. coli DH5α	1
K. pneumoniae 600703	1
ATCC *P. aeruginosa* 27853	1
Gram-positive	
ATCC *Enterococcus faecalis* 29212	1
ATCC *S. aureus* 25922	1
Acid fast	
Mycobacterium tuberculosis H37Rv	1

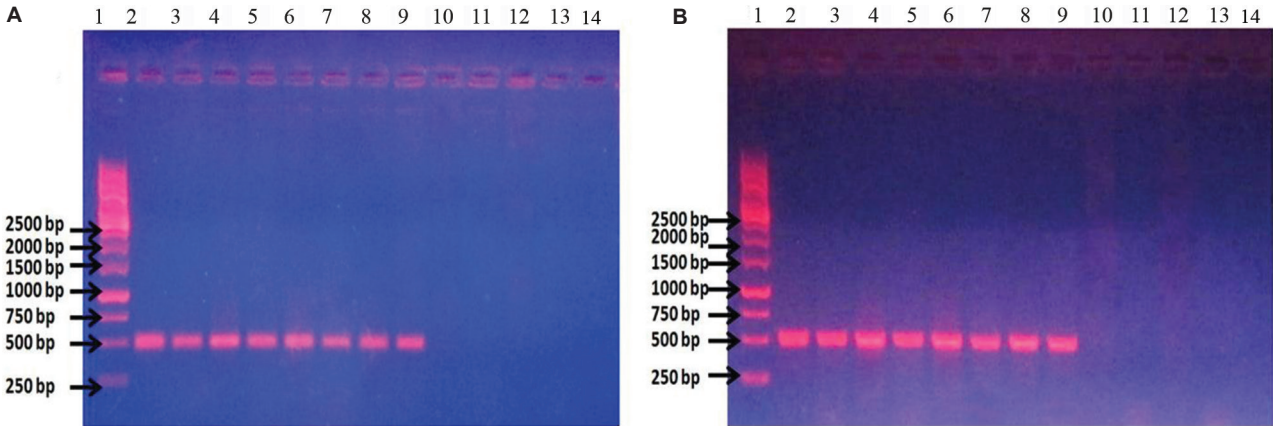

Fig. 1. (**A**) Conventional wet mix PCR with *malB* primers. Lane 1: ladder; lanes 2 and 3: *Escherichia coli* DH5α DNA; lanes 4 and 5: uropathogenic *E. coli* (UPEC) U110 DNA; lanes 6 and 7: UPEC U111 DNA; lanes 8 and 9: UPEC U310 DNA; lanes 10 and 11: *Pseudomonas* sp. DNA; lanes 12 and 13: *Klebsiella pneumoniae* DNA; lane 14: blank. (**B**) Dry-reagent PCR with *malB* primers. lane 1: ladder; lane 2: *E. coli* DH5α DNA; lane 3: *E. coli* DH5α culture; lane 4: UPEC U110 DNA; lane 5: UPEC U110 culture; lane 6: UPEC U111 DNA; lane 7: UPEC U111 culture; lane 8: UPEC U310 DNA; lane 9: UPEC U310 culture; lane 10: *Pseudomonas* sp. DNA; lane 11: *Pseudomonas* sp. culture; lane 12: *K. pneumoniae* DNA; lane 13: *K. pneumoniae* culture; lane 14: blank.

template. PCR reaction mix was short spun and mixed well. The PCR amplification was performed in DNAmp thermocycler (Bhat Biotech India Pvt. Ltd., Bengaluru) and QB-96 (Quanta Biotech, UK) thermocycler. The thermocycling programme was initiated at 97°C for three minutes, followed by 35 cycles comprising denaturation at 94°C for 45 sec, annealing at 57°C for 45 sec, extension at 72°C for 45 sec and final extension at 72°C for 10 min. The PCR products and 1 kb DNA reference ladder (Fermentas,Vilnius, Lithuania) were resolved in 1.5 per cent agarose gel and viewed under ultraviolet (UV) transilluminator (Zenith Engineers, Agra) and photographed.

Dry-reagent mix PCR: The dry-reagent mix PCR was tested with the DNA isolated from all the 184 isolates and also directly with bacterial isolates including test and control. The DNA extracted from the bacterial isolates was used as template, and the test was performed as per the protocol suggested. To the dry PCR tube, 2 µl of template DNA and 48 µl of PCR grade water were added and spun down. The amplification was performed in a QB-96 thermocycler. The PCR conditions were same as that of conventional PCR. The amplified PCR products were analyzed by agarose gel electrophoresis and the DNA was visualized in UV transilluminator and photographed.

All the procedures for dry PCR were the same as above except for the template. Here, 5 µl of bacterial culture in LB broth was added to the dry-reagent PCR tube which served as template followed by 45 µl of PCR grade water and was short spun. The advantage being elimination of DNA extraction step from bacterial culture and the bacterial suspension was directly added to the dry-reagent PCR tube. The designed primers were verified with NCBI BLAST (*https://blast.ncbi.nlm.nih.gov/Blast.cgi*) and the multiple sequence alignment was performed with Multalin version 5.4.1[9] to confirm the homology between *E. coli* (accession no. LT615379) and *Shigella* sp. (accession no. CP014768).

Results

The study group included 177 isolates from clinical samples and seven control strains belonging to different species. Of these isolates, 106 (104 clinical isolates and 2 control strains) were phenotypically confirmed as *E. coli* and 78 (73 clinical isolates and 5 control strains) were non-*E. coli* bacterial isolates (Table). The genomic DNA isolated from these 184 isolates was used as template for conventional wet PCR. The conventional PCR was considered as the gold standard for determining sensitivity and specificity of our primer and dry-reagent master mix. All the 104 *E. coli* isolates and two control strains amplified the target by both conventional wet PCR and dry-reagent PCR (Fig. 1A & B), while 60 non-*E. coli* clinical isolates and five controls did not show amplification. All the 13 different species of *Shigella* showed the presence of the target DNA sequence of 491 bp in both conventional wet-reagent and dry PCR (Fig. 2A & B). It was found that *malE* gene, ABC transporter and maltose/maltodextrin transporter ATP-binding gene of

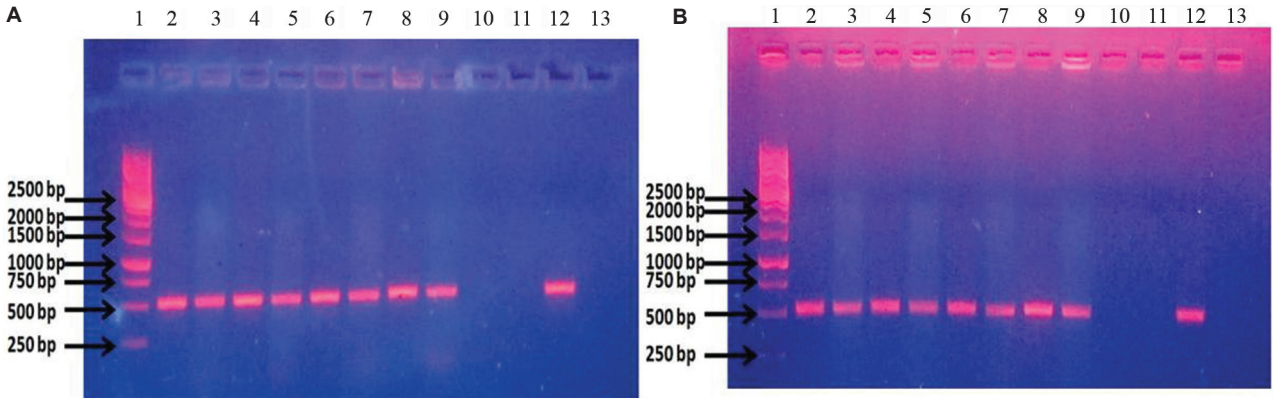

Fig. 2. (**A**) Conventional wet mix PCR with *malB* primers. Lane 1: ladder; lanes 2 and 3: *Shigella flexneri* DNA; lanes 4 and 5: *S. sonnei* DNA; lanes 6 and 7: clinical isolate of *Shigella* DNA; lanes 8 and 9: uropathogenic *Escherichia coli* (UPEC) U110 DNA; lanes 10 and 11: *Klebsiella pneumoniae* DNA; lane 12: *malB* clone as template for positive control; lane 13: blank. (**B**) Dry-reagent PCR with *malB* primers. Lane 1: ladder; lane 2: *S. flexneri* DNA; lane 3: *S. flexneri* culture; lane 4: *S. sonnei* DNA; lane 5: *S. sonnei* culture; lane 6: clinical isolate of *Shigella* DNA; lane 7: clinical isolate of *Shigella* culture; lane 8: UPEC U110 DNA; lane 9: UPEC U110 culture; lane 10: *K. pneumoniae* DNA; lane 11: *K. pneumoniae* culture; lane 12: *malB* clone as template for positive control; lane 13: blank.

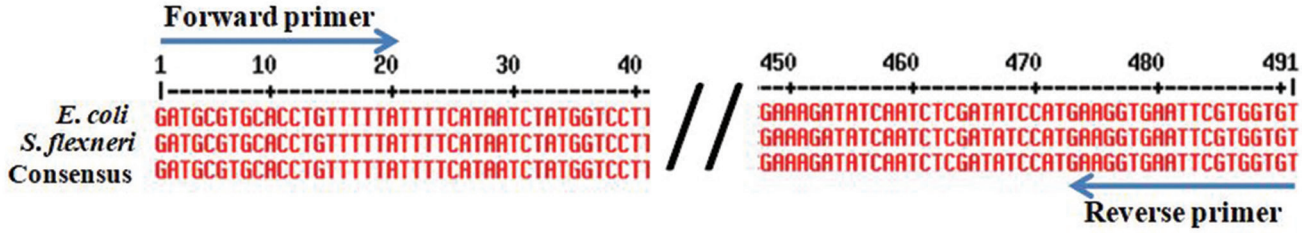

Fig. 3. Multiple sequence alignment of *Escherichia coli* (accession no. LT615379) and *Shigella* sp. (accession no. CP014768) and their consensus sequence. Arrow marks indicate the forward and reverse primer.

Shigella showed high sequence similarity with *malB* sequence of *E. coli* (Fig. 3).

Discussion

Dry-reagent PCR is not a popular technique in spite of having number of merits. Dry-reagent PCR has been successfully employed for the rapid detection of *Mycobacterium* species, *M. ulcerans, Salmonella* Typhi, *Vibrio cholerae, Clostridium* sp., *Staphylococcus* sp., *Acinetobacter* sp. and *Yersinia pestis*[10-20]. Dry-reagent mix can also be used for quantitative PCR[21,22], real-time reverse transcription PCR[23] and isothermal PCR[24]. Carbohydrate polymers have been used to stabilize PCR mix which forms glassy matrices and provide room temperature stability[25] without compromise or decline in their efficacy even after a year of storage at 20°C[26]. Several techniques of freeze-drying PCR reagents providing phenomenal stability have been used by different workers[10,12,14,15,20-24].

The dry-reagent PCR assay for *malB* gene showed 100 per cent agreement with conventional PCR assay. The 13 strains of *Shigella* were also identified in the PCR. This gene is conserved across diverse lineages of *E. coli* and is not shared by other Gram-negative bacteria except *Shigella* sp., based on BLAST analysis. We considered this region to be appropriate because of its conservation, as well as the rarity of *Shigella* as a cause of extraintestinal infections[5]. Genome comparisons showed that *Shigella* shared a common backbone sequence with *E. coli*[27], and the nucleotide homology was nearly 90 per cent[28]. Molecular evolutionary studies have shown that enteroinvasive *E. coli* (EIEC) and *Shigella* can be regarded as a single pathovar of *E. coli*[29].

The lyophilization was not used in our study to convert the reaction mixture in dry format, thus reducing cost of freeze-drying. The significant modification was the elimination of DNA extraction step. DNA extraction is critical for the success of any molecular assay. Poor-quality DNA often gives erroneous results. Most laboratories use either column-based or phenol:chloroform-based DNA extraction. Column-based DNA extraction is

commercially available; however, it is expensive. The phenol:chloroform extraction though cheap is time-consuming and hazardous. Dry-reagent PCR master mix was user-friendly by further reducing the stages of storing and thawing of reagents before use and preparation of master/reaction mixture. Therefore, chances of activity loss and contamination of reagents were eliminated.

In conclusion, the dry-reagent PCR developed in this study was speedy, less cumbersome and user-friendly without compromising the sensitivity and specificity. As there is no need to establish a dedicated PCR laboratory, peripheral laboratories may also use this molecular assay.

***Financial support & sponsorship*:** Authors acknowledge the financial assistance provided by the Bhat Biotech India Pvt. Ltd., Bengaluru.

***Conflicts of Interest*:** None.

References

1. Peleg AY, Hooper DC. Hospital-acquired infections due to Gram-negative bacteria. *N Engl J Med* 2010; *362* : 1804-13.
2. Caeiro JP, Estrada-Garcia MT, Jiang ZD, Mathewson JJ, Adachi JA, Steffen R, *et al.* Improved detection of enterotoxigenic *Escherichia coli* among patients with travelers' diarrhea, by use of the polymerase chain reaction technique. *J Infect Dis* 1999; *180* : 2053-5.
3. Holland JL, Louie L, Simor AE, Louie M. PCR detection of *Escherichia coli* O157:H7 directly from stools: Evaluation of commercial extraction methods for purifying fecal DNA. *J Clin Microbiol* 2000; *38* : 4108-13.
4. Candrian U, Furrer B, Höfelein C, Meyer R, Jermini M, Lüthy J. Detection of *Escherichia coli* and identification of enterotoxigenic strains by primer-directed enzymatic amplification of specific DNA sequences. *Int J Food Microbiol* 1991; *12* : 339-51.
5. Hill J, Beriwal S, Chandra I, Paul VK, Kapil A, Singh T, *et al.* Loop-mediated isothermal amplification assay for rapid detection of common strains of *Escherichia coli*. *J Clin Microbiol* 2008; *46* : 2800-4.
6. Bergey DH, Buchanan RE, Gibbons NE; American Society for Microbiology. *Bergey's manual of determinative bacteriology,* 6th ed. Baltimore: Williams & Wilkins; 1974.
7. Sambrook J, Fritsch EF, Maniatis T. *Molecular cloning. A laboratory manual,* 2nd ed. New York: Cold Spring Harbor Laboratory Press; 1989.
8. Mullis KB, Faloona FA. Specific synthesis of DNA *in vitro* via a polymerase-catalyzed chain reaction. *Methods Enzymol* 1987; *155* : 335-50.
9. Corpet F. Multiple sequence alignment with hierarchical clustering. *Nucleic Acids Res* 1988; *16* : 10881-90.
10. Klatser PR, Kuijper S, van Ingen CW, Kolk AH. Stabilized, freeze-dried PCR mix for detection of mycobacteria. *J Clin Microbiol* 1998; *36* : 1798-800.
11. Aziah I, Ravichandran M, Ismail A. Amplification of ST50 gene using dry-reagent-based polymerase chain reaction for the detection of *Salmonella* typhi. *Diagn Microbiol Infect Dis* 2007; *59* : 373-7.
12. Chua AL, Elina HT, Lim BH, Yean CY, Ravichandran M, Lalitha P. Development of a dry reagent-based triplex PCR for the detection of toxigenic and non-toxigenic *Vibrio cholerae*. *J Med Microbiol* 2011; *60* : 481-5.
13. Seise B, Pollok S, Seyboldt C, Weber K. Dry-reagent-based PCR as a novel tool for the rapid detection of *Clostridium* spp. *J Med Microbiol* 2013; *62* : 1588-91.
14. Al-Talib H, Yean CY, Al-Khateeb A, Hasan H, Ravichandran M. Rapid detection of methicillin-resistant *Staphylococcus aureus* by a newly developed dry reagent-based polymerase chain reaction assay. *J Microbiol Immunol Infect* 2014; *47* : 484-90.
15. Nagaraj S, Ramlal S, Kingston J, Batra HV. Thermostabilization of indigenous multiplex polymerase chain reaction reagents for detection of enterotoxigenic *Staphylococcus aureus*. *J Microbiol Immunol Infect* 2018; *51* : 191-8.
16. Kulkarni RD, Mishra MN, Mohanraj J, Chandrasekhar A, Ajantha GS, Kulkani S, *et al.* Development of a dry-reagent mix-based polymerase chain reaction as a novel tool for the identification of *Acinetobacter* species and its comparison with conventional polymerase chain reaction. *J Lab Physicians* 2018; *10* : 68-72.
17. Siegmund V, Adjei O, Racz P, Berberich C, Klutse E, van Vloten F, *et al.* Dry-reagent-based PCR as a novel tool for laboratory confirmation of clinically diagnosed *Mycobacterium ulcerans*-associated disease in areas in the tropics where *M. ulcerans* is endemic. *J Clin Microbiol* 2005; *43* : 271-6.
18. Siegmund V, Adjei O, Nitschke J, Thompson W, Klutse E, Herbinger KH, *et al.* Dry reagent-based polymerase chain reaction compared with other laboratory methods available for the diagnosis of buruli ulcer disease. *Clin Infect Dis* 2007; *45* : 68-75.
19. Qu S, Shi Q, Zhou L, Guo Z, Zhou D, Zhai J, *et al.* Ambient stable quantitative PCR reagents for the detection of *Yersinia pestis*. *PLoS Negl Trop Dis* 2010; *4* : e629.
20. Lee SV, Tai ES, Mutalib AR, Khairani-Bejo S, Bahaman AR. Rapid detection of pathogenic leptospires by lyophilized reagent-based polymerase chain reaction. *Trop Biomed* 2011; *28* : 497-505.
21. Kamau E, Alemayehu S, Feghali KC, Juma DW, Blackstone GM, Marion WR, *et al.* Sample-ready multiplex qPCR assay for detection of malaria. *Malar J* 2014; *13* : 158.
22. Babonneau J, Bernard C, Marion E, Chauty A, Kempf M, Robert R, *et al.* Development of a dry-reagent-based qPCR to facilitate the diagnosis of *Mycobacterium ulcerans* infection in endemic countries. *PLoS Negl Trop Dis* 2015; *9* : e0003606.
23. Takekawa JY, Hill NJ, Schultz AK, Iverson SA, Cardona CJ, Boyce WM, *et al.* Rapid diagnosis of avian influenza virus

in wild birds: Use of a portable rRT-PCR and freeze-dried reagents in the field. *J Vis Exp* 2011. pii: 2829.

24. Chen HW, Weissenberger G, Ching WM. Development of lyophilized loop-mediated isothermal amplification reagents for the detection of leptospira. *Mil Med* 2016; *181* : 227-31.
25. Ramanujam R, Koelbl J, Ting E, Jolly J, Burdick B. Room-temperature-stable PCR reagents. *PCR Methods Appl* 1993; *3* : 75-6.
26. Wolff C, Hörnschemeyer D, Wolff D, Kleesiek K. Single-tube nested PCR with room-temperature-stable reagents. *PCR Methods Appl* 1995; *4* : 376-9.
27. Fukiya S, Mizoguchi H, Tobe T, Mori H. Extensive genomic diversity in pathogenic *Escherichia coli* and *Shigella* strains revealed by comparative genomic hybridization microarray. *J Bacteriol* 2004; *186* : 3911-21.
28. Brenner DJ, Fanning GR, Steigerwalt AG, Orskov I, Orskov F. Polynucleotide sequence relatedness among three groups of pathogenic *Escherichia coli* strains. *Infect Immun* 1972; *6* : 308-15.
29. Lan R, Alles MC, Donohoe K, Martinez MB, Reeves PR. Molecular evolutionary relationships of enteroinvasive *Escherichia coli* and *Shigella* spp. *Infect Immun* 2004; *72* : 5080-8.

For correspondence: Dr Shama Bhat, Bhat Biotech India Pvt. Ltd., 11-A, 4th Cross, Veerasandra Industrial Area, Electronic City Phase II, Bengaluru 560 100, Karnataka, India
e-mail: bhatbiotech@gmail.com