Indian Journal of Animal Sciences **85** (11): 1202–1204, November 2015/Short communication

A novel multiplex PCR assay for simultaneous detection of three important pathotypes of *Escherichia coli* from diarrhoeic piglets

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Received: 2015 April 18; Accepted: 2015 May 13

Key words: Diarrhoea, *Escherichi coli* pathotypes, Novel m-PCR, Piglets, Rapid detection

Diarrhoea in piglets is caused by different pathotypes of *Escherichia coli*. Detection of Enterotoxigenic *E. coli* (ETEC) and Shigatoxin producing *E. coli* (STEC) from diarrhoeic piglets by simplex PCRs were earlier reported from India (Rajkhowa *et al*. 2014, Rajkhowa and Sarma 2014). The present study reports the development of a novel multiplex PCR assay for the rapid, specific and simultaneous detection of 3 important pathotypes of *E. coli* (ETEC, atypical enteropathogenic *Escherichia coli* and STEC) from diarrhoeic piglets.

Bacterial strains and DNA preparations: Reference *E. coli* strains and non pathogenic *E. coli* strain as shown in Table 1 were used for the study. All strains examined by PCR were grown on MacConkey agar plates at 37º C. DNA was extracted from bacteria by resuspending one bacterial colony in 50 µl of sterile water, boiling the suspension for 5 min and centrifuging it at 10,000 *g* for 1 min. The supernatant was then used as the DNA template for PCR.

Oligonucleotide primers: The primers were designed using Primer 3 software. The sequences of these primers are shown in Table 2 along with their corresponding GenBank accession numbers and predicted product sizes. The specificity of each of the 8 primers was initially confirmed using BLASTN and the GenBank sequence database.

Amplification of m-PCR and analysis of its products: The PCR reaction mixtures contained 8 primers at a concentration of 20 pmol each, 3 µl of template, 10x PCR buffer, 2 mM of $MgCl_2$, 200 µM of each of the 4 deoxynucleotide (dNTPs) and 1 U Taq DNA polymerase in a final volume of 25 µl. The thermal cycling conditions consisted of an initial denaturation at 95ºC for 5 min, followed by 30 cycles of denaturation for 30 sec at 95ºC, annealing at 56ºC for 45 sec, extension for 45 sec at 72ºC with the final extension for 5 min at 72º C. The PCR amplicon was electrophoresed along with 100 bp molecular weight marker on a 2% agarose gel. In parallel, all the samples (field samples) were also tested using standard conventional microbiology procedures in order to not only confirm the method of m-PCR system but also evaluate its performance.

Sensitivity assays: The sensitivity of the m-PCR was tested using serial dilution of bacterial culture prepared from overnight cultures. The organism was decimally diluted (10^9, 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10,<10 cfu/mL) in sterile saline. DNA was extracted from 1 mL of each dilution and subsequently tested using the multiplex PCR method.

*Specificity assays:*The m-PCR specificity was determined by examining the ability of the test to detect and distinguish used bacterial strains (strains as shown in Table 1).

Detecting target genes in field samples: A total of 353

Table 1. Reference bacterial strains used in the study

Strains used	Positive for genes				
	est	*elt*	*stx1*	*eae*	*bfpA*
EDL933	-	-	+	+	-
E2348/69	-	-	-	+	+
MTCC-723	+	+	-	-	-
MG1655	-	-	-	-	-

Table 2. Oligonucleotide sequences used for multiplex PCR

Target genes	Oligonucleotide sequences	Amplicon size (bp)	GenBank accession number
est1	F: CATGACGGGAGGTAACATGA		
	R: GCACAGGCAGGATTACAACA	222	M25607
elt1	F: CCCTCACCCATATGAACAGG		
	R: TGCTCAGATTCTGGGTCTCC	308	S60731
stx1	F: GCGTGGAGGATGTCAAGAAT		
	R: CCTTCGCCACCACATTAACT	127	M17358
eaeA	F: CAACATGACCGATGACAAGG		
	R: ACGGATATCGAAGCCATTTG	535	Z11541

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E. coli strains isolated from diarrhoeic piglets of North-eastern states of India during the period of 2011–2014 were used. All DNA from field samples were processed simultaneously by single PCR and m-PCR to determine the comparative efficiency of the 2 methods. For confirmation of atypical EPEC, isolates positive for *eaeA* were subsequently tested by PCR for the presence of *bfpA* (Gunzburg *et al*. 1995) and *stx* (Rajkhowa and Sarma 2014) genes.

The current report describes development and evaluation of a novel m-PCR assay for rapid detection of 3 important pathotypes of *E. coli* namely ETEC (*est*$^+$, *elt*$^+$) , atypical EPEC (*eaeA*$^+$, *stx*$^-$, *bfpA*$^-$) and STEC (*stx*$^+$) from diarrhoeic piglets. Although there are several reports of use of monoplex PCRs for detection of important pathotypes of *E. coli* from piglets (Rajkhowa *et al*. 2014, Rajkhowa and Sarma 2014), these assays detect only single gene at a time therefore requiring several such simplex PCRs for detection of multiple genes which is labour intensive and time consuming. The target genes included in the m-PCR in the current study were selected because they are well characterized and were found prevalent in *E. coli* isolates of porcine origin (Ho *et al*. 2013, Mohlatlole *et al*. 2013).

Evaluation of the specificity of the m-PCR primers revealed that each primer pair was specific for the corresponding target genes (Fig. 1). No amplification products were obtained for strain MG 1655 examined thereby indicating that the multiplex PCR assays are highly specific for the detection of target genes of *E. coli* representing 3 pathotypes, viz. ETEC, atypical EPEC and STEC. There was complete agreement between the results of single and m-PCRs for all reference strains tested in the study indicating the high degree of specificity of the assay. The performance of the m-PCR in field studies with samples from diarrhoeic piglets further validated its use as an optimal tool for rapid detection of these 3 important pathotypes of *E. coli* from diarrhoeic piglets.

A potential advantage of the m-PCR developed in this study is that it has a high level of sensitivity. The experiments showed that the detection limit using the m-PCR was 10^2 cfu/mL for the used different strains of the organism for simultaneous detection of 3 target pathotypes of *E. coli* from diarrhoeic piglets. Although the infectious dose varies among pathogen types, it is generally believed that most bacterial pathogens are able to cause infection when more than 10^3 cfu/mL are ingested (US EPA 1992). Thus, the detection sensitivity of the multiplex assay described in this study is within the infectious dose of most pathogens. The m-PCR assay developed in this study was effective for the detection of the target genes of the organism. The results also demonstrated that amplification of the 4 sets of primers was efficient for producing 4 clear bands (Fig. 1).

The efficiency of the m-PCR was evaluated for the simultaneous detection of the target genes of *E. coli* representing 3 target pathotypes. The results showed that the efficiency was not influenced when detecting mixtures of multiple target genes in comparison with the individual target gene.

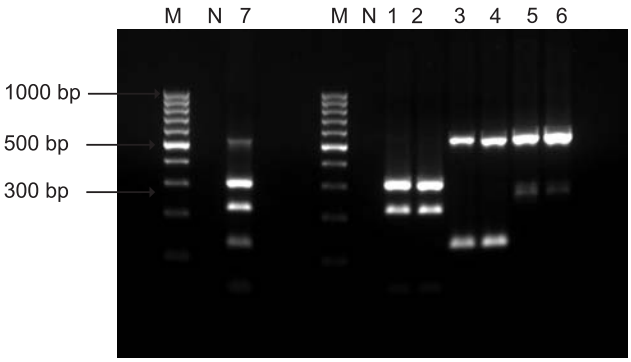

Fig. 1. Agarose gel showing multiplex PCR assays for detection of three important pathotypes of *Escherichia coli* from diarrhoeic piglets. Lane M, molecular marker (100 bp DNA ladder); N, negative control; lane 1, positive control for genes *est1* and *elt1*; lane 2, test sample positive for *est1* (222 bp) and *elt1* (308 bp) genes representing ETEC; lane 3, positive control for genes *eaeA* and *stx1*; lane 4, test sample positive for genes *eaeA* (535 bp) and *stx1*(127 bp) representing STEC; lane 5, positive control for *eaeA* gene; lane 6, test sample positive for *eaeA* (535 bp) gene representing atypical EPEC (*stx*$^-$ *bfpA*$^-$ *eaeA*$^+$); lane 7, mixture of all positive control strains showing band representing different pathotypes of *E. coli* from diarrhoeic piglets.

To evaluate the discriminating power of the method even further and to test its applicability, 353 *E. coli* strains isolated from diarrhoeic piglets were tested using the m-PCR and confirmed by monoplex PCR with the same 4 sets of primers. The m-PCR results were the same as the monoplex PCR results. Out of these 353 *E. coli* isolates, 115 (32.60%) isolates belonged to ETEC, 58 (16.43%) to STEC, and 33 (9.34%) to atypical EPEC whereas no target genes of our interest could be detected in rest of the isolates.

In conclusion, the multiplex-PCR assay that we developed showed the same results as those obtained using individual protocols, making them a useful tool for the simultaneous detection of 3 important pathotypes of *E. coli* from diarrhoeic piglets in future studies with larger numbers of samples.

SUMMARY

A novel multiplex PCR assay was developed and evaluated for rapid and simultaneous detection of 3 important pathotypes of *Escherichia coli* from diarrhoeic piglets. The targets selected for each pathotype were *est* and *elt* for ETEC, *eaeA* for atypical EPEC and *stx* for STEC isolates. The detection limit of the m-PCR assay was 10^2 cfu/mL for the simultaneous detection of all the target genes representing 3 pathotypes of *E. coli* from diarrhoeic piglets, viz. ETEC, atypical EPEC and STEC, and the assay was highly specific for detection of target genes of *E. coli* representing these 3 pathotypes. Out of 353 *E. coli* isolates from diarrhoric piglets screened by this assay, 115 (32.60%) isolates belonged to ETEC, 58 (16.43%) belonged to STEC and 33 (9.34%) belonged to atypical EPEC whereas no

target genes of our interest could be detected in rest of the isolates.

ACKNOWLEDGEMENT

The authors are thankful to the Ministry of Science and Technology, Department of Biotechnology (DBT), Government of India, New Delhi for sanctioning the project and providing financial support.

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