

Characterisation of porcine enteropathogenic *Escherichia coli* isolated in northeastern India

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Abstract

Introduction: Enteropathogenic *Escherichia coli* (EPEC) is one of the main pathotypes causing gastroenteritis, particularly in young immunocompromised hosts. The study reports the prevalence, characterisation, and molecular epidemiology of EPEC from piglets in northeastern India. **Material and Methods:** A total of 457 faecal samples were collected, from which 1,286 *E. coli* strains were isolated and screened by PCR. The resultant EPEC strains were serotyped and phenotypically characterised for resistance against 15 antimicrobials. Also, the phylogenetic sequence was analysed for 11 selected strains. **Results:** A total of 42 strains (3.26%) belonged to atypical EPEC, of which, 15 (35.71%, and 2.29% of the 654 strains from this farm type) were isolated from organised and 27 (64.29%, and 4.27% of the 632 strains from this farm type) from unorganised farms; further, 5 (11.90% of the EPEC strains and 1.51% of the 330 strains from this breed) were isolated from the indigenous breeds and 37 (88.10%, and 3.87% of the 956 strains from this breed) from crossbred piglets. Serogroups O111 (11.9%) and O118 (7.14%) were the most prevalent of the 10 present. Sequence analysis of a length of the *eaeA* gene of 11 isolates of the region showed them to have 100% homology with each other and their identity ranged from 99.4% to 99.7% with GenBank reference sequences. All the EPEC isolates were multi-drug resistant, showing the highest resistance to amoxicillin (80.9%) and cephalixin (76.19%). **Conclusion:** The study highlighted the association of EPEC with piglet's diarrhoea in northeastern India. EPEC isolates belonged to many serotypes and phenotypically all were multi-drug resistant with close genetic homology.

Keywords: piglets, EPEC, virulence, serotypes, drug resistance.

Introduction

Escherichia coli is a Gram-negative, rod-shaped, flagellated, nonsporulating, facultative anaerobic bacterium belonging to the *Enterobacteriaceae* family and it is classified into several pathotypes based on its virulence factors (13, 31). Its strains range from pure commensals that may turn pathogenic in immunocompromised animals to types combining of virulence determinants sufficient for pathogenicity in different hosts including humans, mammals, and birds, and causing debilitating and sometimes fatal diseases (4). Diarrhoeagenic *E. coli* strains are among the most

common aetiologic agents of diarrhoea (15, 24) and one of the pathotypes is the enteropathogenic *E. coli* (EPEC) producing attaching and effacing lesions caused by the intimate attachment of bacteria to the intestinal epithelial cells and effacement of enterocyte microvilli (1, 5). A protein called intimin mediates the bacterial attachment to outer cell membranes and is encoded by the *eae* gene, which is one of the genes used for the molecular diagnosis of EPEC and a key marker of EPEC infections (14).

EPEC strains are divided into typical (tEPEC) and atypical (aEPEC) subgroups depending on the respective presence or absence of the EPEC adherence

factor plasmid carrying the *bfp* operon (28, 30). Atypical EPEC are more closely related to shigatoxigenic strains and appear to be emerging pathogens (9, 30). It has been reported that atypical EPEC strains were commonly implicated in diarrhoea manifestation and have been isolated from different animal species and humans (1, 8). Infection results in production losses and mortality in piglets, seriously hampering the livestock improvement programme besides causing considerable harm to the economic viability and profitability of the swine industry. Another major concern is the emergence of drug resistant *E. coli*, one of the reasons for which is the indiscriminate use of antimicrobials, especially in food-producing animals, particularly pigs and poultry. As compared to humans and cattle, only a limited number of surveys are available regarding porcine EPEC. Keeping in mind the importance of porcine diarrhoeagenic *E. coli*, the present study aims to detect and molecularly characterise EPEC strains from piglets of organised and unorganised farms of northeastern India.

Material and Methods

Faecal samples. A total of 457 fresh faecal samples were collected from piglets under nine weeks of age from organised (n = 225) and unorganised (n = 232) farms in the North Eastern Region Indian states of Manipur (n = 108), Meghalaya (n = 124), Mizoram (n = 120), and Nagaland (n = 105) during the period of 2013–2015. Samples were collected from diarrhoeic (n = 339) and apparently healthy (n = 118) animals including crossbred (n = 327) and indigenous (n = 130) piglets. For this study, the pig farms were categorised as organised and unorganised farms according to their rearing schemes. Organised pig farms were considered to be those rearing more than 50 pigs and practising an intensive system of farming, whereas; unorganised farms were semi-intensive and backyard in scale, raising 5–10 pigs.

Isolation and identification. Faecal samples were directly streaked on MacConkey's agar plates (HiMedia, Mumbai, India) for *E. coli* isolation and incubated at 37°C overnight. Four pink colonies were taken randomly from each plate, streaked on eosin methylene blue agar plates and subsequently incubated overnight at 37°C. Colonies with the characteristic metallic sheen were studied for their morphological characteristics and subjected to biochemical tests such as indole, methyl red, Voges-Proskauer, citrate utilisation and hydrogen sulphide production on triple sugar iron (12). Isolates were stored as pure culture on semi-solid LB agar at 4°C and in glycerol stock at -20°C.

All the *E. coli* isolates possessing the *eaeA* gene were serotyped on the basis of their somatic antigen at

the National *Salmonella* and *Escherichia* Centre, Central Research Institute, Kasauli, Himachal Pradesh (India).

Molecular detection of EPEC. Template DNA was prepared by a boiling and snap chilling method as per standard procedure. Molecular characterisation of *E. coli* isolates was performed by multiplex PCR as described by Paton and Paton (22) targeting the *eaeA* (384 bp), *stx₁* (180 bp), *stx₂* (253 bp), and *hlyA* (534 bp) genes. PCR was performed with a total volume of 25 µL reaction mixture containing 1 × PCR buffer, 100 mM of dNTP, 1.5 mM of MgCl₂, 1U of Taq polymerase, 20 pM of forward and reverse primers, and 4 µL of DNA template. The PCR performed in a Mastercycler Gradient thermal cycler (Eppendorf A.G., Hamburg, Germany) involved an initial denaturation step at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 45 sec, annealing at 61°C for 45 sec and extension at 72°C for 45 sec; and a final extension step at 72°C for 10 min. The PCR products were separated by electrophoresis on 1.5% agarose gels containing ethidium bromide (0.5 µg/mL) in Tris-borate buffer and using the GeneRuler 100 bp DNA ladder (Fermentas, Vilnius, Lithuania) as a molecular size marker. The amplicons were visualised and photographed by an AlphaImager, gel documentation system (ProteinSimple, San Jose, CA, USA). All the *eaeA*-positive isolates were further subjected to a specific PCR for the detection of the *bfpA* gene (426 bp) as described by Wieler *et al.* (32) with an initial denaturation step of 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 57°C for 1 min and extension at 72°C for 1 min, and a final extension step at 72°C for 5 min. PCR products were separated by electrophoresis on 1.5% agarose gels and amplicons were visualised by the AlphaImager, gel documentation system.

Antimicrobial sensitivity assay. An *in vitro* antimicrobial susceptibility test of all EPEC isolates against 15 antimicrobial drugs (Table 1) was performed on Mueller-Hinton agar plates according to the criteria of the Clinical Laboratory Standards Institute (7). Zones of inhibition were measured using the zone size interpretative chart furnished by the manufacturer (HiMedia, India). *E. coli* ATCC 25922 was used as a control organism.

Cloning and sequence analysis. Extracted PCR products were gel purified and cloned in pTZ57R/T cloning vector using an InsTAclone PCR Cloning Kit (Thermo Scientific, Vilnius, Lithuania) and sequencing was performed at the DNA sequencing facility of the Department of Biochemistry, at the University of Delhi, India, using a 3730xl automated sequencer (Applied Biosystems, Foster city, CA, USA). Sequencing data were analysed using the MegAlign programme (DNASar, Madison, WI, USA). Phylogenetic and bootstrap analyses were performed using neighbour joining and seqboot programmes. The

obtained sequences were compared with other reference sequences from different sources, retrieved from the GenBank database.

Results

Isolation. A total of 1,286 *E. coli* were isolated from 457 faecal samples, with a higher rate of isolation from piglets from organised farms (n = 654) than unorganised farms (n = 632) of the North Eastern Region of India. A higher number of isolates (n = 1,042) were recovered from diarrhoeic samples than from samples of apparently healthy piglets (n = 244).

PCR-based screening for virulence genes. Of the 1,286 *E. coli* isolates screened by multiplex PCR assay for the presence of the *eaeA* virulence gene, 42 (3.26%) were found to possess *eaeA* gene (EPEC) and all were isolated only from diarrhoeic piglets. All 42 were found to be negative for the *bfpA* gene and classified as atypical EPEC. Of these 42 EPEC isolates, 15 (35.71%) were isolated from organised (15/654; 2.29%) and 27 (64.29%) from unorganised farms (27/632; 4.27%). Further, 37 (88.10%) were isolated from crossbred piglets (37/956; 3.87%) and 5 (11.90%) (5/330; 1.51%) were from the local indigenous breed.

Serotyping of EPEC. The result of serotyping the EPEC isolates based upon their somatic antigens revealed that out of 42, 20 isolates (47.62%) belonged to 10 different serogroups, 8 (19.05%) were untypable (UT), and 14 (33.33%) were recorded as rough strains. Serogroup O111 was found to be the most prevalent (5/20, 25.00%; 11.90% of all EPEC isolates) followed by O118 (3/20, 15.00%; 7.14% of all EPEC isolates) and O1, O4, O55, and O126 (each 2/20, 10.00%; 4.76%). Only one isolate each belonged to serotypes O26, O84, O91, and O114 (each 1/20, 5.00%; 2.38%).

Antimicrobial drugs susceptibility. All the EPEC isolates showed resistance to at least three antimicrobial drugs (Table 1), resistance to amoxicillin

(80.90%) being the most frequent, followed by cephalaxin (76.19%), enrofloxacin (57.14%), ampicillin (54.76%), piperacillin (38.09%), nalidixic acid (23.8%), cefixime (16.66%), gentamicin (11.90%), ceftazidime (11.90%), aztreonam (9.52%), cefotaxime (9.52%), streptomycin (7.14%), ciprofloxacin (4.76%) and ceftriaxone (4.76%). Conversely, all the isolates were sensitive to imipenem and nearly all were to ceftriaxone and ciprofloxacin (95.23%).

Phylogenetic analysis of sequences. Annotated partial nucleotide sequences of the *eaeA* gene of selected isolates were deposited in GenBank and were designated the accession numbers listed in Table 2. The sequences of these 11 isolates from different areas of the North Eastern Region states of India were compared with 9 sequences retrieved from GenBank, representing the same gene from different sources and countries. Reference sequences for comparison were selected from the isolates catalogued under *Enterobacteriaceae*. Sequence analysis of our 11 isolates from different states of northeastern India showed 100% homology with and zero divergence from each other and one reference sequence from a pig of Indian origin (KF650779). When compared with other reference sequences from different sources, retrieved from the GenBank database, the identity of our sequences ranged from 99.4% to 99.7% (Figs 1, 2). The highest of these sequence homologies (99.7%) were with sequences isolated from the faeces of a pig (AB647529), bird (AB647586) and human (AB647615) from Japan, human (KP197126), and chicken intestine (KP197099) from China, and cheese (HQ834728) from Brazil. Also, the *eaeA* sequences of *E. coli* in the present study showed 99.7% homology with those of *E. alberti* isolated from a chicken and human from China. Except for one *eaeA* sequence from a human (AF530555) from Australia, which was placed in a different cluster showing 88.3% identity, all the 11 sequences of the present study were grouped under one cluster along with other sequences retrieved from the GenBank database (Fig. 1).

Table 1. Antimicrobial drugs resistance pattern of EPEC from diarrhoeic faecal samples from piglets

Antibiotic disc and content	Sensitive (%)	Resistant (%)
Ampicillin (AMP) 10 µg	19 (45.23)	23 (54.76)
Amoxicillin (AMX) 30 µg	8 (19.04)	34 (80.90)
Aztreonam (AT) 30 µg	38 (90.47)	4 (9.52)
Cephalexin (CN) 30 µg	10 (23.80)	32 (76.19)
Ceftazidime (CAZ) 30 µg	37 (88.09)	5 (11.90)
Cefixime (CFM) 5 µg	35 (83.33)	7 (16.66)
Ceftriaxone (CTR) 30 µg	40 (95.23)	2 (4.76)
Cefotaxime (CTX) 30 µg	38 (90.47)	4 (9.52)
Ciprofloxacin (CIP) 5 µg	40 (95.23)	2 (4.76)
Enrofloxacin (EX) 10 µg	18 (42.85)	24 (57.14)
Gentamicin (GEN) 10 µg	37 (88.09)	5 (11.90)
Imipenem (IPM) 10 µg	42 (100.00)	0 (0.00)
Nalidixic acid (NA) 30 µg	32 (76.19)	10 (23.80)
Piperacillin (PI) 10 µg	26 (61.90)	16 (38.09)
Streptomycin (S) 10 µg	39 (92.85)	3 (7.14)

Table 2. NCBI accession numbers for nucleotide sequences of the *eaeA* gene of different EPEC strains

No.	Organism	Serotype	GenBank accession number
01	<i>E. coli</i>	O1	KP987181
02	<i>E. coli</i>	O4	KP987182
03	<i>E. coli</i>	Rough	KP987183
04	<i>E. coli</i>	O1	KP987184
05	<i>E. coli</i>	Untypable	KP987185
06	<i>E. coli</i>	Untypable	KP987186
07	<i>E. coli</i>	O1	KP987187
08	<i>E. coli</i>	Untypable	KP987188
09	<i>E. coli</i>	Rough	KP987189
10	<i>E. coli</i>	O111	KP987190
11	<i>E. coli</i>	O111	KP987191

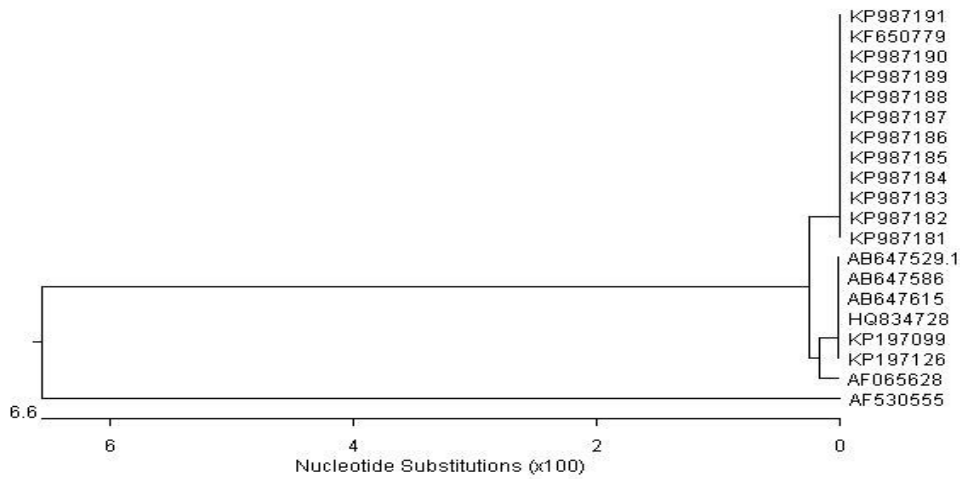


Fig. 1. Phylogenetic analysis of nucleotide sequences of the *eaeA* gene of *E. Coli*

		Percent Identity																					
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20		
Divergence	1	█	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	99.7	99.7	99.7	99.4	88.3	99.7	99.7	99.7	100.0	1	KP987181
	2	0.0	█	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	99.7	99.7	99.7	99.4	88.3	99.7	99.7	99.7	100.0	2	KP987182
	3	0.0	0.0	█	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	99.7	99.7	99.7	99.4	88.3	99.7	99.7	99.7	100.0	3	KP987183
	4	0.0	0.0	0.0	█	100.0	100.0	100.0	100.0	100.0	100.0	100.0	99.7	99.7	99.7	99.4	88.3	99.7	99.7	99.7	100.0	4	KP987184
	5	0.0	0.0	0.0	0.0	█	100.0	100.0	100.0	100.0	100.0	100.0	99.7	99.7	99.7	99.4	88.3	99.7	99.7	99.7	100.0	5	KP987185
	6	0.0	0.0	0.0	0.0	0.0	█	100.0	100.0	100.0	100.0	100.0	99.7	99.7	99.7	99.4	88.3	99.7	99.7	99.7	100.0	6	KP987186
	7	0.0	0.0	0.0	0.0	0.0	0.0	█	100.0	100.0	100.0	100.0	99.7	99.7	99.7	99.4	88.3	99.7	99.7	99.7	100.0	7	KP987187
	8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	█	100.0	100.0	100.0	99.7	99.7	99.7	99.4	88.3	99.7	99.7	99.7	100.0	8	KP987188
	9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	█	100.0	100.0	99.7	99.7	99.7	99.4	88.3	99.7	99.7	99.7	100.0	9	KP987189
	10	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	█	100.0	99.7	99.7	99.7	99.4	88.3	99.7	99.7	99.7	100.0	10	KP987190
	11	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	█	99.7	99.7	99.7	99.4	88.3	99.7	99.7	99.7	100.0	11	KP987191
	12	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	█	100.0	100.0	99.7	87.9	100.0	100.0	100.0	99.7	12	AB647529.1
	13	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.0	█	100.0	99.7	87.9	100.0	100.0	100.0	99.7	13	AB647586
	14	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.0	0.0	█	99.7	87.9	100.0	100.0	100.0	99.7	14	AB647615
	15	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.3	0.3	0.3	█	87.9	99.7	99.7	99.7	99.4	15	AF065628
	16	13.0	13.0	13.0	13.0	13.0	13.0	13.0	13.0	13.0	13.0	13.0	13.3	13.3	13.3	13.3	█	87.9	87.9	87.9	88.3	16	AF530555
	17	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.0	0.0	0.0	0.0	0.3	█	100.0	100.0	99.7	17	HQ834728
	18	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.0	0.0	0.0	0.0	0.3	13.3	0.0	█	100.0	18	KP197099
	19	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.0	0.0	0.0	0.0	0.3	13.3	0.0	0.0	█	19	KP197126
	20	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.3	0.3	0.6	13.0	0.3	0.3	0.3	█	20

Fig. 2. Comparative analysis of the nucleotide sequences of the *eaeA* gene of NE- Indian isolates with other published sequences showing the percentage homology

Discussion

The study provides a systematic report of the prevalence of aEPEC strains causing infection in piglets along with molecular, serological and antimicrobial profiles of EPEC isolates and phylogenetic analysis of selected strains. Our findings showed all the isolated EPEC to be atypical, due to the lack of a *bfp* plasmid gene, as proven by the specific PCR result. Atypical EPEC strains have diverse virulence factors and form a heterogeneous group that may have acquired the locus of enterocyte effacement (LEE) through horizontal transfer or may be previously tEPEC strains that have lost the adherence factor plasmid. Whether typical or atypical EPEC are more pathogenic is still insufficiently investigated, but nevertheless it has been proven that in addition to virulence factors coded in the LEE, intimin, *E. coli* secreted proteins, translocated intimin receptor, and type 3 secretion system, aEPEC strains can also express enteroaggregative heat-stable toxin 1, enterohaemolysin, afimbrial adhesion, and other factors (7, 29). Some researchers (10, 16) reported higher detection of atypical than typical strains from animals and poultry; however, low detection of aEPEC from piglets (1.6%) and infants (0.6%), was also reported (3), and these contrasting results indicate that the distribution of typical and atypical EPEC varies.

Diverse serotypes were detected in this study that differed from the findings of other authors (3, 11). The recovery of EPEC in the O1 and O111 serogroups from piglets may be an indication of potential emerging serogroups in India as they do not classify into the commonest porcine pathogenic serogroups (11). Therefore, it is uncertain, whether these particular serogroups are specific to pigs. Atypical EPEC are adapted to both human and animal hosts, and hence proper diagnostic procedure should be followed when food-borne infection is suspected (19, 30). In this study, the recovery of EPEC belonging to O26, O55, O111, and O114 is corroborated by the World Health Organisation report of established EPEC serogroups (5). A high number of EPEC isolates (19.0%) remained untypable, which indicates the occurrence of a wide spectrum of *E. coli* serogroups in India, as also reported by Dutta *et al.* (10) in diarrhoeic piglets.

Pork is the major source of protein for human consumption in northeastern India, where people in the countryside rear 5 to 10 pigs near their houses and in most cases share a source of water, hence there is probably a high risk of transmission of EPEC infection in the region. In our findings, the variation in EPEC infection being higher in unorganised farming and in crossbred may possibly be because the local piglets of nondescript breed possess better innate protection against natural infection than crossbred animals. The weaning age of local piglets is generally 10 weeks rather than the much shorter 6 weeks weaning period of crossbred piglets on organised farms; hence, maternal

immunity might play an important role in resisting the infection in piglets. Lack of scientific literature regarding the prevalence of EPEC; prevented us from comparing our results to precedents. However, similar findings were reported in a prevalence study of diarrhoeagenic bacterial and viral pathogens (17, 18).

Antimicrobial drug resistance in enteric bacteria is a serious problem globally in both human and veterinary medicine. Food animals such as pigs may act as a reservoir or a potential source for dissemination of resistant bacterial populations in the environment. *Enterobacteriaceae* resistance to cephalosporins is mainly due to the production of extended-spectrum beta-lactamases which have been increasingly detected in food animals and have gained considerable attention worldwide (27). Imipenem is a carbapenem class of beta-lactams that is known to have a wide spectrum of activity against Gram-positive and Gram-negative bacteria and hence, any decrease in susceptibility to it should be taken seriously. None of the isolates in the study were found to be resistant to imipenem, which concurs with the findings of researchers from other countries (2, 20). There is a variation in resistance to streptomycin, ampicillin, cefotaxime, ceftriaxone, and ceftazidime when compared to the findings of other researchers (21, 23, 25). This variation might be due to the EPEC investigated in this study having acquired the drug resistance gene through horizontal transfer because of indiscriminate use of antibiotics. Imipenem is not in use in northeastern India and other drugs such as ceftriaxone, cefotaxime, and ciprofloxacin have limited usage due to their high cost. This probably explains the bacterium's higher susceptibility of these drugs in the region. However, amoxicillin, cephalixin, enrofloxacin, and ampicillin are commonly used in veterinary practices in the North Eastern Region of India since they are easily available, which may contribute to the selection of EPEC-resistant variants. Multiple antimicrobial resistance in EPEC may partly result from the spread of genetic elements including plasmids, transposons, and integrons (26), and if it does, this may complicate any future drug therapy. Hence, regular monitoring of antimicrobial resistance among enteric pathogens, and particularly the food-borne kind, is a priority matter as resistant genes can be transferred to other pathogenic microorganisms.

The 100% sequence homology among the EPEC isolates of our study suggests that genetically similar strains are widely distributed in the region; the sequence diversity is less than that of other reported sequences retrieved from the NCBI database. More studies need to be carried out on the *eaeA* gene and its molecular epidemiology, as there is a paucity of published *eaeA* sequences of porcine origin. Analysis of phylogenetic trees together with epidemiological data can be used to determine the relationship between different isolates at different point of time or space and to thereby track the origin of a disease.

It may be stated that EPEC with different serotypes are persistently associated with piglet diarrhoea in northeastern India and more prevalent in the unorganised farming system. Based upon the sequence analysis, the selected isolates are uniquely placed in a separate clade. Taking into account the large pork consumption and close contact between the human and pig populations, the research findings warrant a more critical appraisal of EPEC and more emphasis on epidemiological study of all pathotypes with particular attention to antimicrobial drug resistance.

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