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KG Chaitanya
M.V.Sc Student, Department of Veterinary Public Health and Epidemiology, College of Veterinary Science, Tirupati, Andhra Pradesh, India

T Madhava Rao
Registrar, Sri Venkateswara Veterinary University, Tirupati, Andhra Pradesh, India

A Jagadeesh Babu
Professor and Head, Department of Veterinary Public Health and Epidemiology, College of Veterinary Science, Tirupati, Andhra Pradesh, India

B Sreedevi
Professor and Head, Department of Veterinary Microbiology, College of Veterinary Science, Tirupati, Andhra Pradesh, India

Corresponding Author:
KG Chaitanya
M.V.Sc Student, Department of Veterinary Public Health and Epidemiology, College of Veterinary Science, Tirupati, Andhra Pradesh, India.

Isolation and characterization of enteroaggregative *Escherichia coli* (EAEC) from pigs and pork in and around Tirupati, Andhra Pradesh

KG Chaitanya, T Madhava Rao, A Jagadeesh Babu and B Sreedevi

Abstract
Enteroaggregative *Escherichia coli* (EAEC) is an important emerging enterovirulent pathogen having worldwide public health concern both in humans and animals. The present study conducted to study the prevalence of EAEC in pig feces including both diarrheic and non-diarrheic, pork and water samples used in pig rearing and during slaughter. A total of 430 samples (250 pig fecal samples, 120 pork and 60 water samples) were collected from different areas in and around Tirupati. All the samples were subjected to isolation of *E. coli* through conventional cultural methods and also subjected to *E. coli* specific E16S rRNA gene for species level identification. Out of 430 samples, *E. coli* was recovered from 165 (38.37%) samples (132 pig feces, 17 pork and 16 water samples). Further *E. coli* isolates were subjected to PCR for the detection of EAEC virulence genes *viz*., pCVD432 (*aat* AA), *aggR* and *astA* genes. On analysis none of the isolates carried *aat* AA and *aggR* genes, while *astA* gene was found in 98 (59.3%) *E. coli* isolates (69 pig feces, 12 pork and 17 water samples).

Keywords: Pigs, pork, enteroaggregative *E. coli*, pCVD432, *aggR*, *astA*

1. Introduction
Escherichia coli. (*E. coli*) are classified as motile, rod-shaped, non-spore forming, gram-negative and facultative anaerobic bacteria belonging to *Enterobacteriaceae*. The majority of *E. coli.* strains co-exist in the gastrointestinal tract of animals and humans as harmless commensal symbionts having greatest public health significance (Wani *et al.*, 2004) [19]. The *E. coli* strains are classifies into six major pathotypes based on clinical associations, phenotypic assays and identification of virulence characteristics such as enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli* (EHEC), diffusely adhering *E. coli* (DAEC), shiga toxin producing *E. coli* (STEC) and enteroaggregative *E. coli* (EAEC) (Nataro and Kaper, 1998; Estrada-Garcia *et al.*, 2005) [6].
Among different diarrheagenic *E. coli*, EAEC is an emerging enterovirulent pathogen that causes infection in both humans and animals (Manjushree *et al.*, 2019) [13]. Enteroaggregative *Escherichia coli* (EAEC) are characterized by the ability to aggregate intimately with each other, adhere to Human epithelial type-2 (HEp-2) cells, and forming stacked-brick like aggregates (Weintraub, 2007) [22]. The adherence pattern of EAEC is mediated by aggregative adherence fimbriae (AAF) coded on a large virulence pAA plasmid. Laboratory studies on prototype strains suggested that the aggregative adherence (AA) phenotype was encoded on 55-65 MDa plasmids, collectively called pAA (Vial *et al.*, 1988) [16]. In a subsequent report, a cryptic fragment of the pCVD432 plasmid was found to be useful as a specific and variably sensitive DNA probe, which could distinguish EAEC from other *E. coli* (Baudry *et al*., 1990) [2]. In addition, EAEC form mucoid biofilms and secrete cytotoxins (e.g., plasmid encoded toxin Pet) that are toxic to epithelial cells. The enterotoxins *viz*., enteroaggregative heat stable enterotoxin (EAST1) which is encoded by *aat* AA gene and transcriptional activator *aggR* genes were associated with EAEC (Croxen *et al.*, 2013) [5]. With the increasing incidence in humans, EAEC has gained importance. Since its initial discovery, EAEC has been identified in endemic and epidemic diarrheal diseases all over the world. Humans acquire EAEC infection through contaminated food and water.
Among all the food animals, high occurrence of EAEC strains were reported in pigs (32%) (Kagambega *et al*., 2012) [9] and it was one of the common enteric pathogen causing diarrhoea in new born and weaned pigs (Vu-khac *et al.*, 2007) [18] which leads to decreased weight gain and high mortality (Fairbrother *et al*., 2005) [7].

High risk of transmission of EAEC strain of *E.coli* to farm workers was observed during routine operations, handling of infected piglets and fecal contamination of water sources. Pork contamination may occur due to fecal contamination of the meat and poor hygienic practices during processing of carcasses (Kagambega *et al.,* 2012) [9]. Water contamination at pig rearing areas and slaughter houses may occur due to poor drainage and disposal of slaughter waste into the water sources enabling direct transfer of EAEC to humans.

2. Materials and Methods

2.1 Sample collection

Pig fecal samples (250) from young and adult pigs including both diarrhoeic and non-diarrhoeic pigs, pork sample (120), water samples (60) used in pig farming and water samples were collected from AICRP on pigs, Tirupati and different villages in and around Tirupati town, A.P. All the samples were collected in suitable aseptic containers and were transported to laboratory under chilled conditions and processed within 24h of collection.

2.2 Microbiological assay

In the present study, a total of 430 samples were initially screened for presumptive colonies of *E. coli* by overnight enrichment of samples in nutrient broth, followed by streaking on selective agars like MacConkey (MC) agar and Eosin Methylene Blue (EMB) agar (Fig 1) and incubation of plates at 37℃ for 24 h. The suspected *E. coli* colonies were selected and used for further biochemical tests like indole test, methylred test, voges-proskaur test, citrate test, nitrate, urease and TSI agar slant test. The broth cultures were examined by Gram staining, oxidase and catalase tests. The positive cultures were stored at -20℃ for further analysis.

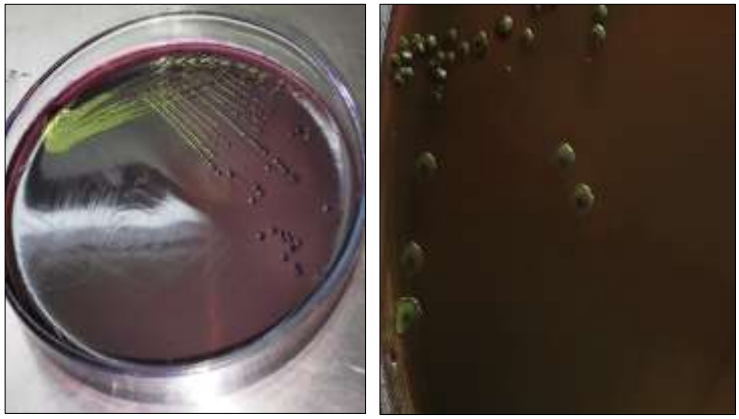


Fig 1: Plate showing green-metallic sheen on EMB agar

2.3 Extraction of DNA

From the isolates, chromosomal DNA was extracted using boiling and snap chilling method and plasmid DNA was extracted as per the procedure described by Hussain *et al.,* 2010 [8]. Briefly, bacteria from 1 ml broth culture were pellected by centrifugation at 5000 rpm for 10 min and pellet was re-suspended in 200µl of lysis buffer (20mM Tris HCl, 2 mM EDTA, 1% Triton-X). The bacteria were lysed in water bath by boiling for 10 min. The lysate was centrifuged again as before and the supernatant was used directly as template for PCR.

2.4 Polymerase chain reaction (PCR)

EAEC strains are highly heterogeneous and no virulence factor has been identified as common to all EAEC strains (Wani *et al.,* 2012) [20]. EAEC virulence factors were identified using specific primers as shown in Table 1. The components of the reaction mixture were finally optimized by using 3 µl of bacterial lysate as the template in a reaction mixture consisting of 12.5 µl of Master mix (2X), 1 µl of each primer and volume made up to 25 µl using nuclease free water. The cycling conditions for the genes pCVD432, *aggR* and *astA* genes were included in Table 2. The PCR products were visualized by gel electrophoresis in 1.5% agarose (Hi Media, India) containing SYBR green, in Tris–acetate–EDTA (TAE) buffer.

Table 1: Primers used for the identification of enteroaggregative *E. coli*

Target gene	Primer sequence 5'-3'	Amplicon size (bp)	Reference
CVD432	CTGGCGAAAGACTGTATCAT	630	Vijay *et al.* (2015) [17]
	CAATGTATAGAAATCCGCTGTT		
aggR	CTAATTGTACAATCGATGTA	308	Kahali *et al.* (2004) [10]
	ATGAAGTAATTCTTGAAT		
*ast*A	GCCATCAACACAGTATATCC	106	Manjushree *et al.* (2019) [13]
	GAGTGACGGCTTTGTAGTCC		

Table 2: PCR conditions for detection of virulence genes of EAEC

Genes	Initial denaturation (^{0}C/min)	Denaturation (^{0}C /sec)	Annealing (^{0}C /sec)	Extension (^{0}C /sec)	Final extension (^{0}C /min)	No. of cycles
pCVD432	94/3	94/60	55/45	72/60	72/7	30
*agg*R	94/3	94/60	42/60	72/60	72/7	30
*Ast*A	95/5	95/45	59.4/30	72/30	72/5	35

3. Results

An overall occurrence of *E. coli* was noticed in 165 (38.37%) out of 430 samples. Among the *E. coli* isolates obtained from different sources, prevalence in pig fecal samples (52.8%,

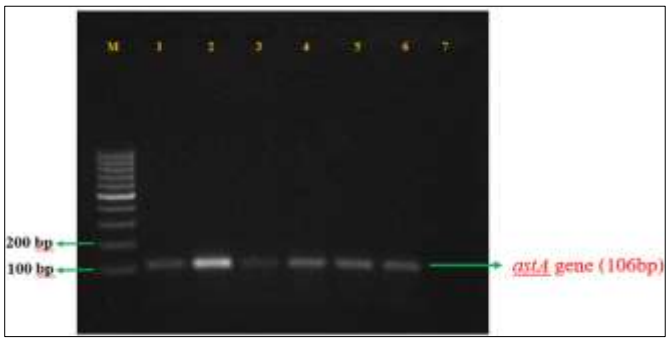
132/250) was highest followed by water samples (28.3%, 17/60) and then pork samples (13.3%, 16/120). None of the *E. coli* isolates carried pCVD432 (Fig 2) and *aggR* (Fig 3) genes while *astA* gene was found in 98 (59.3%) *E. coli* isolates (Fig 4). Among 98 *astA* positive isolates, 69 (52.2%), 12 (75%) and 17 (100%) were from pig fecal samples, pork and water samples respectively.

Lane M: Molecular weight marker (100 bp)
Lane 1: Positive control (EAEC 042) (630 bp)
Lane 2: Nuclease free water as negative control
Lane 3 to 7: None showing positive for *aat* AA gene

Fig 2: Detection of pCVD432 (*aat* AA) gene in *E. coli* isolates from different sources

Lane M: Molecular weight marker (100 bp)
Lane 1: Positive control (EAEC 042) (308 bp)
Lane 2 to 6: None showing positive for *aat* AA gene
Lane 7: Nuclease free water as negative control

Fig 3: Detection of *aggR* gene in *E. coli* isolates from different sources



Lane M: Molecular weight marker (100 bp)
Lane 1: Positive control for *astA* gene (106 bp)
Lane 2, 3 : Amplified DNA of *E. coli* from pig feces
Lane 4, 5 : Amplified DNA of *E. coli* from pork
Lane 6: Amplified DNA of *E. coli* from water
Lane 7: Nuclease free water as negative control

Fig 4: Detection of *astA* gene in *E. coli* isolates from different sources

4. Discussion

Foodborne diseases are widespread and growing public health problem both in developed and developing countries. Raw meat and meat products have been incriminated as a predominant cause of many foodborne infections and deaths in many parts of the world (Banerjee *et al.*, 2001) [1]. Changing in people's food habits, food processing industry, unsafe food storage conditions and poor hygiene practices are major potential cause for foodborne illness (Tassew *et al.*, 2010) [15]. Among the bacterial infections, *E. coli* is an important zoonotic pathogen and it may cause severe food poisoning in man. Among the animals, pigs are known to carry human pathogens that are excreted with fecal wastes and they are among the potential sources of *E. coli* inputs to the environment; a pig produces approximately 1 X 10^7 *E. coli* per gram of feces which corresponds to an *E. coli* flow rate per day that is 28 times higher than that of one human (Mieszkin *et al.*, 2009) [14] and that may contaminate pork because of the defective slaughter and storage conditions and pose a health risk to the consumers (Lee *et al*., 2009) [12].

In the present study out of 165 *E. coli* isolates screened, none of the *E. coli* isolates carried pCVD432 and *aggR* genes while *astA* gene was found in 98 (59.3%) *E. coli* isolates. Although EAEC possess *astA* gene, it can also be detected in other *E. coli* strains. Thus the *E. coli* isolates obtained from pig fecal samples, pork and water samples in the present study may be of *E. coli* strains other than EAEC. The absence of EAEC in pigs in the present study is in agreement with Cassar *et al.* (2004) [4] and Zhang *et al.* (2016) [23] who reported that pigs might not be the reservoir of EAEC. Absence of EAEC in pork in the present study is in agreement with Koo *et al.* (2012) [10] who reported that none of the pork samples were positive for EAEC virulence gene. Absence of EAEC in water samples used in pig farming and during slaughter in the present study is in agreement with Bibbal *et al.* (2014) [3] and Zhang *et al.* (2016) [23].

The EAEC strains were heterogeneous and diversified with a variety of virulence factors. The heterogeneity of EAEC is due to presence of a heavy molecular weight plasmid carrying many genes responsible for pathogenesis. This plasmid plays a role in acquisition or deletion of the virulence genes leading to heterogenecity with in the pathotype.

5. Conclusion

In conclusion, there is little evidence for the presence of the EAEC commonly associated with human diarrhoeal disease in formed animal species like pigs, in pork and water samples of pig farms and at the places of their slaughter in and around Tirupati, Andhra Pradesh, Although the findings from this study support the conclusions of Wasteson (2001) [21] and Cassar *et al.* (2004) [4], it should be noted that the isolates were from a cross-sectional, rather than a longitudinal, epidemiological survey. However, this cross sectional sample was collected only for a period of three months, so it seems that any seasonality associated with possible carriage of EAEC by farmed animals was a contributing factor to the lack of detection. Also, single isolates were made from fecal samples across EMB agar and it may be assumed that the *E. coli* organisms that formed the majority population of coliforms in any one animal at the time of sampling. If EAEC comprise a minority population in animals, other genetic approaches to test the entire *E. coli* fecal population may be a more appropriate approach. Further study is required to obtain conclusion evidence on the prevalence of EAEC in food animals in Tirupati, Andhra Pradesh.

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