

Detection & characterization of Shiga toxin producing *Escherichia coli* (STEC) & enteropathogenic *Escherichia coli* (EPEC) in poultry birds with diarrhoea

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Received November 25, 2009

Background & objectives: Limited information is available on shiga toxin producing *Escherichia coli* (STEC) in animals and birds from India. An outbreak of acute diarrhoea in poultry birds at Aizawl, Mizoram was investigated for detection and characterization of STEC and enteropathogenic *E. coli* (EPEC).

Methods: *E. coli* was isolated and identified from rectal swabs, intestinal contents, heart blood and spleen of 19 poultry birds that died due to acute diarrhoea during the outbreak. Phenotypic characterization was done by standard bacteriological and biochemical techniques. All the isolates were serotyped based on their somatic antigens. Virulence genes (*stx*₁, *stx*₂, *eaeA* and *hlyA*) were detected by multiplex PCR assay.

Results: A total of 42 *E. coli* isolates were obtained, of which 24 belonged to 3 serogroups (O64, O89 and O91) and the remaining 18 were untypable (UT). Altogether, 14 (33.33%) isolates carried at least 1 virulence gene, of which 10 (23.81%) and 4 (9.52%) were recorded as STEC and EPEC, respectively. Of the 10 STEC isolates, one carried only *stx*₂, one carried *stx*₂ and *hlyA*, four carried *stx*₁, *stx*₂ and *hlyA*, two carried *stx*₁, *eaeA* and *hlyA* genes and two carried *stx*₁ and *eaeA*. Of the four EPEC isolates, two carried *eaeA* and *hlyA*, one carried only *eaeA* gene and 1 carried only *hlyA* gene.

Interpretation & conclusions: This is the first report on the involvement of STEC in poultry in India.

Key words EPEC - India - poultry - STEC - virulence genes

The morbidity and mortality associated with several large outbreaks of gastrointestinal diseases caused by Shiga toxin-producing *Escherichia coli* (STEC) indicating the threat of these organisms to public health¹. These are commonly recovered from food animals and were found responsible for severe gastrointestinal and systemic diseases such

as haemorrhagic colitis (HC) and haemolytic uremic syndrome (HUS) leading to diarrhoea, especially among the infants in the developing countries^{1,2}. STEC strains produce one or both of two major types of Shiga toxins, designated Stx₁ and Stx₂. The Stx₂ is associated with an increased risk of developing HUS³. Although, in India, reports are available on isolation, identification

and characterization of STEC in human and animals⁴⁻¹⁰, there appears to be no information on association of STEC in poultry.

An outbreak of acute diarrhoea in broiler chickens aged 6-8 wk was reported in Aizawl, Mizoram in 2007. We investigated this outbreak for detection and characterization of pathogenic organism in the broiler chickens with diarrhoea.

Material & Methods

Collection of samples: An outbreak of diarrhoeal disease in a flock of 150 broiler chickens housed under intensive care system at Aizawl, Mizoram in 2007 was attended. A total of 49 birds were affected, of which 19 died. These 19 dead birds were brought to the pathology/microbiology laboratory, College of Veterinary Sciences & Animal Husbandry, Aizawl, Mizoram. During post-mortem examination, rectal swabs, intestinal contents, heart blood and pieces of spleen were collected aseptically for isolation and identification of causative agents.

Screening of the specimens for *Coccidia* and Group A rotavirus: In addition to screening for *Coccidia*, the faecal samples were also screened for the presence of group A rotavirus by RNA-PAGE analysis¹¹ with certain modifications. In brief, RNA was extracted from faecal samples followed by electrophoresis in 7.5 per cent polyacrylamide gel using Laemmli's discontinuous buffer system. Silver staining of the gel was done as described by Svensson *et al*¹².

Bacteriological screening of clinical specimens: The clinical samples (heart blood, liver, spleen, rectal swab, intestinal contents) were immediately inoculated on 10 per cent sheep blood agar and MacConkey's agar (HiMedia, Mumbai, India) plates and incubated at 37°C for 18-24 h. Pure and a single population of bacterial colonies were recorded from heart blood and spleen samples. Five randomly selected colonies from MacConkey's agar and 10 per cent sheep blood agar plates were picked up and subcultured on eosin methylene blue (EMB) agar (HiMedia, Mumbai, India) plates to observe the characteristic metallic sheen of *E. coli*. The well separated pure colonies were picked up on nutrient agar slants as pure culture and subjected for standard morphological and biochemical tests¹³.

Serotyping of *E. coli*: The 42 *E. coli* isolates were serotyped based on their somatic (O) antigens at National Salmonella and Escherichia Centre, Central Research Institute, Kasauli, India.

Preparation of *E. coli* DNA for PCR assay: For rapid detection of virulence genes, isolated bacterial cultures were inoculated into 2 ml Luria Bartani (L-B) broth and incubated at 37°C under constant shaking for 18 h. After incubation, 1 ml broth culture was taken in a 1.5 ml microcentrifuge tube and centrifuged at 5867 g for 10 min. The pellet was washed twice in sterile normal saline solution (NSS) (0.85% NaCl) and resuspended in 400 µl of nuclease-free sterile distilled water and boiled for 10 min followed by immediate chilling. Cell debris was removed by centrifugation at 2292 g for 5 min. The supernatant was used as template DNA for PCR.

Detection of virulence genes by multiplex PCR: A multiplex PCR was carried out using 4 sets of oligonucleotide primers for *stx*₁, *stx*₂, *eaeA* and *hlyA* genes (Table I). The PCR protocol was followed as per the method described by Paton and Paton¹ with some modifications. In brief, the multiplex PCR mixture of 25.0 µl contained 1X PCR buffer, 1.5 mM of MgCl₂, each primer within the 4 primer sets at a concentration of 40 nM, 200 µM each of dNTPs, 1.0 U of *Taq* DNA polymerase and 2.0 µl of template DNA. The PCR reaction was performed in a thermal cycler (Thermo Electron, Germany) using the following standard cycling procedure: an initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 94°C for 45 sec, primer annealing at 59°C for 45 sec and extension at 72°C for 1 min and a final extension at 72°C for 6 min.

Amplified products were separated by agarose gel (2% agarose in 1X Tris-borate-EDTA buffer) electrophoresis at 5v/cm for 2 h and stained with ethidium bromide (0.5 µg/ml)¹⁴. Standard molecular size marker (100 bp DNA ladder) was included in each gel. DNA fragments were observed by ultraviolet transilluminator and photographed in a gel documentation system (Alpha Imager, Germany).

Table I. Oligonucleotide primers used in multiplex PCR reaction

Sl No.	Primers	Primer sequences
1	<i>stx</i> ₁ F	5'-ATA AAT CGC CAT TCG TTG ACT AC-3'
	<i>stx</i> ₁ R	5'-AGA ACG CCC ACT GAG ATC ATC -3'
2	<i>stx</i> ₂ F	5'-GGC ACT GTC TGA AAC TGC TCC -3'
	<i>stx</i> ₂ R	5'-TCG CCA GTT ATC TGA CAT TCT G -3'
3	<i>eaeA</i> F	5'-GAC CCG GCA CAA GCA TAA GC -3'
	<i>eaeA</i> R	5'-CCA CCT GCA GCA ACA AGA GG -3'
4	<i>ehxA</i> F	5'-GCA TCA TCA AGC GTA CGT TCC -3'
	<i>ehxA</i> R	5'-AAT GAG CCA AGC TGG TTA AGC T-3'

Source: Ref. 1. F, forward; R, reverse

The PCR was performed three times to ensure the repeatability of the technique and to make sure that isolates were correctly assigned to respective patterns.

Results

Epidemiological details and postmortem observations: Out of 150 birds in the flock, 49 were affected and 19 died within one week time with an overall mortality and case fatality rate of 12.67 per cent (19/150) and 38.78 per cent (19/49), respectively. Prior to death, the affected birds were anorexic and emaciated, dull and depressed with ruffled feathers and showed progressive somnolence with closed eyes. Majority of the birds were shivering and huddled near the source of heat. Clinically ill birds showed profuse watery diarrhoea and severe dehydration. On post-mortem, besides the generalized septicaemic lesions, severe lesions of enteritis accompanied with focal necrotic lesions in the mucosa of the small intestine were prominent in majority of the cases. Spleens and livers were swollen and congested with haemorrhagic or necrotic foci. Major parasites including round worm, tape worm and *Coccidia* sp. were not recorded during post-mortem examination/screening of faecal samples.

RNA-PAGE: All the faecal and rectal swab samples screened by RNA-PAGE for detection of group A rotavirus were also found negative.

Bacterial isolation and characterization: The bacteriological examination of heart blood, liver, spleen and intestinal contents revealed the presence of Gram-negative bacilli. In biochemical tests, the isolates were identified as *E. coli*. The haemolytic pattern in 10 per cent sheep blood agar showed narrow zone of haemolysis, typical for the STEC. A total of 42 isolates of *E. coli* were recorded from 19 clinical samples, which belonged to 4 serogroups, *viz.*, O64 (16), O89 (3), O91 (5) and UT (18).

Multiplex PCR for virulence genes: Multiplex PCR assay yielded amplified products of ~180 bp, ~255bp, ~384bp and ~534 bp specific for *stx*₁, *stx*₂, *eaeA* and *hlyA* genes, respectively. Out of 42 isolates, 14 (33.33%) carried at least one virulence gene, of which 10 (23.81%) and 4 (9.52%) were detected as STEC and EPEC, respectively. Of the 10 STEC isolates, one carried only *stx*₂, one carried *stx*₂ and *hlyA*, four carried *stx*₁, *stx*₂ and *hlyA*, two carried *stx*₁, *eaeA* and *hlyA* genes and two carried *stx*₁ and *eaeA*. Similarly,

Table II. Virulence genes profile of *E. coli* strains isolated from poultry birds with diarrhoea

Serogroup	No. of isolates	Stx ₁	Stx ₂	eaeA	hlyA
O64	2	+	+	-	+
O64	1	-	+	-	-
O64	2	+	-	+	+
O64	1	-	-	+	+
O89	1	+	+	-	+
O91	1	-	-	+	-
O91	1	+	+	-	+
UT	1	-	-	-	+
UT	2	+	-	+	-
UT	1	-	+	-	+
UT	1	-	-	+	-
Total	14	8	6	7	9

+, positive; -, negative; UT, untypable

out of 4 EPEC isolates, one carried *eaeA* and *hlyA*, two carried only *eaeA* gene and one carried only *hlyA* gene (Table II).

Discussion

During the investigation of the present outbreak of acute diarrhoea in a poultry flock, the clinical symptoms and post-mortem study indicated the involvement of systemic infection by some enteric pathogens. Because of absence of group A rotavirus, *Salmonella* spp. or any other diarrhoea causing parasites and isolation of pure haemorrhagic *E. coli* from heart blood as well as intestinal contents warranted for further investigation of virulence genes of *E. coli* isolates.

In India, there is limited information regarding the STEC in animals including cattle⁵, sheep^{4,9}, fish⁷, beef⁸ and human faeces^{6,8} available. Wani *et al*⁴ and Farooq *et al*¹⁰ reported the association of EPEC in chickens in Kashmir, India.

In the present study, a total of four different serogroups of *E. coli* were isolated, of which O64 was the predominant. Contrary to our result, other workers^{4,15} from Jammu and Kashmir reported the predominance of O9, O8, O60 and O25. Mishra *et al*¹⁶ found O2, O19, O20 and O78 as predominant serogroups amongst the *E. coli* isolates from 250 clinical specimens from poultry in India. These findings indicate the variable distribution of different serogroups of *E. coli* in different geographical regions in India.

The present study showed a higher percentage of *E. coli* isolates carrying at least one virulence gene. Farooq *et al*¹⁰ reported 19.81 per cent *E. coli* isolates

from different avian species containing at least one virulence gene. In another study, Wani *et al*⁴ reported that only 12 out of 426 (2.82%) *E. coli* isolates from poultry and pigeon carried at least one virulence gene. In both the cases, the apparently healthy birds or birds with no history of diarrhoea were tested, which may be the reason for recording of low percentage of *E. coli* with virulence genes. However, in the present study, all the *E. coli* isolates were recovered from clinically infected birds. It also justifies the association of STEC and/or EPEC isolates as the main cause of present outbreak.

Detection of STEC in poultry in the present study is probably the first report in India. Absence of STEC is also reported by Kobayashi *et al*¹⁷ in faecal samples from 199 broiler chickens, 32 pigeons and 86 gulls in Finland. Schroeder *et al*¹⁸ also failed to isolate any STEC in retail chicken and turkey obtained from Washington, DC, USA. In all these cases, *E. coli* was recovered from apparently healthy birds, either from live birds or after slaughter. Parreira and Gyles¹⁹ reported the association of STEC with 53 per cent of avian pathogenic *E. coli* (APEC) isolates: 35 per cent from lesions of avian cellulitis, 32 per cent from avian septicemia, 13 per cent from swollen head syndrome (SHS) in chickens and 20 per cent from diseased turkeys. None of the 5 isolates from healthy chickens were positive for *stx* genes¹⁹. These reports indicate that STEC may be closely associated with diarrhoeal disease in poultry in the present study.

Of the 14 *E. coli* isolates positive for at least one virulence gene, 7 (50) possessed *eaeA*, which is in agreement with the findings of other workers¹⁷, who reported a higher percentage (57 and 40% of chicken and gulls, respectively) of *E. coli* isolates carrying *eaeA* gene. However, in contrast to our study, Wani *et al*⁴ reported only 2.49 per cent of *E. coli* isolates from chicken carrying *eaeA* gene.

Prevalence of EPEC was 9.52 per cent among *E. coli* isolates from chickens in the present study, while other studies reported 2.74 per cent⁴ and 15.56 per cent¹⁰. Of the four EPEC isolates in this study, one carried both *eaeA* and *hlyA* genes under the serogroup O64. Wani *et al*⁴ reported 1.49 per cent isolates of *E. coli* from chicken carrying both *eaeA* and *hlyA* genes. Kobayashi *et al*¹⁷ reported that *eae* *E. coli* strains were highly prevalent among gulls (40.0%), pigeons (7.0%) and broiler chickens (57.0%), which lacked *hlyA* gene. All these reports indicate the variable distribution of *eaeA* and *hlyA* gene in *E. coli* isolates from birds.

Our result is in agreement with that of by Parreira and Gyles¹⁹ reporting 53 per cent APEC with *stx* gene sequences: one isolate carried *stx*₂ sequence, two carried both *stx*₁ and *stx*₂ sequences, and the remaining 49 isolates carried only *stx*₁ sequences. In conclusion, our findings provide the information about the involvement of STEC in diarrhoea in poultry in India.

Acknowledgment

The authors thank the Director, National Salmonella & Escherichia Center, CRI, Kasauli, HP, India, for serotyping the isolates; the Dean, CVSc&AH, CAU, Selesih, Aizawl, Mizoram for providing necessary funds and facilities to carry out the investigation.

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