Occurrence of Extended Spectrum Beta Lactamase Producing

Escherichia coli in Hyena from Chhattisgarh, India

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Abstract

Present study was aimed to characterize Escherichia coli isolated from wound from a hyena. Pus swab collected from wound was processed for bacterial isolation and identification as per standard protocol. Isolate was then subjected to antimicrobial susceptibility test by disc diffusion method using commercial available antibiotics such as ampicillin, amoxycillin, amoxyclav, trimethoprim, chloramphenicol, ciprofloxacin, enrofloxacin, cefepime, cefotaxime, ceftriaxone, ceftriaxone/tazobactum, gentamicin and oxytetracycline. Further isolate was screened for presence of extended spectrum beta lactamase genes (blaSHV, blaTEM and blaCTX-M) by PCR. Bacterial culture, staining and biochemical properties revealed the presence of Escherichia coli. Escherichia coli was found susceptible to chloramphenicol and intermediate sensitive to ceftriaxone/tazobactum and gentamicin and oxytetracycline. Further isolate was screened for presence of extended spectrum beta lactamase genes (blaSHV, blaTEM and blaCTX-M) by PCR. Bacterial culture, staining and biochemical properties revealed the presence of Escherichia coli. Escherichia coli was found susceptible to chloramphenicol and intermediate sensitive to ceftriaxone/tazobactum and gentamicin and resistant to ampicillin, amoxycillin, amoxyclav, trimethoprim, ciprofloxacin, enrofloxacin, cefotaxime, cefepime, ceftriaxone and oxytetracycline. Escherichia coli isolate was found positive for blaCTX-M genes whereas blaSHV and blaTEM genes were not detected. In conclusion, investigation of present study revealed the presence of multi drug resistant and extended spectrum beta lactamase producing Escherichia coli in hyena.

Keywords
Hyena, Multi drug resistant Escherichia coli, ESBL, Chhattisgarh

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Introduction

Antimicrobial resistance is not only threat to human and livestock health but also have huge wildlife concern because close interaction between wildlife, livestock and human can lead to interspecies transmission of pathogenic bacteria including strains that exhibit antimicrobial resistance (Lloyd, 2007). One of the most common antimicrobial resistance mechanisms in Enterobacteriaceae is production of extended spectrum beta lactamases (ESBL) enzymes. During last few decades, evolution and dissemination of beta-lactam resistance in Escherichia coli (E. coli) and other gram negative bacteria became a
challenge worldwide. Beta-lactam resistance in *E. coli* can be acquired through generation of ESBL; acquisition of genes encoding ESBL (CTX-M) or due to high level expression of chromosome encoded beta lactamases (*bla*) genes (Pfeifer et al., 2010). During 1980s and 1990s, the majority of the ESBLs were the SHV or TEM types (Peterson and Bonomo, 2005) but later ESBL pandemics occurs in *E. coli* due to widespread occurrence of CTX-M beta lactamases (Pitout, 2012).

The study of wildlife as guard of antimicrobial resistance has recently acquired more consideration globally (Huijbers et al., 2015) and *E. coli* has been described as principal indicator of the selective pressure in wildlife (van den Bogaard and Stobberingh, 2000). However, there is inadequate information pertaining to existence of antimicrobial resistance in wildlife in India and; Chhattisgarh in particular. Therefore, present study was made to investigate the existence of multidrug resistant (MDR) *E. coli* in hyena from Chhattisgarh, India.

**Materials and Methods**

**Sample processing**

Pus swab collected from wound in a hyena was brought in the laboratory of Veterinary Microbiology, Veterinary College Anjora from Directorate of wildlife and forensic science, Chhattisgarh Kamdhenu Vishwavidyalaya, Durg, Chhattisgarh (India).

Swab was initially enriched in sterile normal saline for 2 hours. Enriched swab was then inoculated into brain heart infusion broth and incubated for 24 hours at 37°C. Broth culture was examined for morphology of organism by Gram’s staining and then subjected to isolation of specific bacteria.

**Isolation and identification of bacteria**

Standard methodology was used for isolation and identification of bacteria (Holt et al., 1994). Broth culture depicting Gram negative bacilli was streaked on to Mac Conkey Lactose (MLA) agar and incubated at 37°C for 24 hrs. Pink colonies on MLA was picked up and was then inoculated on eosine methylene blue (EMB) and 10% sheep blood agar and incubated at 37°C for 24 hrs. Bacterial isolate was identified through a series of biochemical tests viz. Catalase, oxidase, motility, indole production, methyl red, Voges Proskauer, citrate utilization, urease and H2S production in triple sugar iron agar (TSI) slant.

**Antimicrobial susceptibility test**

The antimicrobial susceptibility test was done in Muller Hinton agar using disc diffusion method as described by Bauer et al., (1966). Antibiotic discs of commercially available antimicrobials such as ampicillin (10µg), amoxyccillin (30µg), amoxyclav (30µg), trimethoprim (10µg), chloramphenicol (30µg), ciprofloxacin (5µg), enrofloxacin (10µg), cefepime (30µg), ceftriaxone (30 µg), ceftriaxone/tazobactum (30/10 µg), gentamicin (10µg), oxytetracycline (30µg) and cefotaxime (10µg) procured from Himedia laboratories were used. The diameter of zone of inhibition (in mm) was measured and categorized as susceptible, intermediate or resistant.

**Extraction of genomic DNA**

Extraction of bacterial genomic DNA was performed by snap chill method (Nagappa et al., 2007). Briefly, a single colony was inoculated into nutrient broth and incubated at 37°C overnight. Then, 1.5 ml of the bacterial culture was centrifuged at 8000 rpm followed by three times washing of pellet in PBS.
Pellet was then eluted in 100 μl nuclease free water and kept in to boiling water bath for 10 minutes which is followed by quick chilling in ice. Concentration and purity of extracted DNA was determined by 0.8% agarose gel electrophoresis and then stored at -20°C till further use.

**Polymerase Chain Reaction (PCR) for detection of ESBL gene**

Detection of the ESBL gene sequence coding for the TEM, SHV, CTX-M enzymes (Table 1) were performed by the multiplex PCR as described by Akpaka et al., (2010) with slight modifications. Amplified PCR products were electrophoresed on 1.5 % agarose gel stained with ethidium bromide (0.5μg/ml). The images of ethidium bromide stained DNA bands were analysed under UV transilluminator (Biometra) and digitized using a Gel Documentation System (Gel Doc™XR, Biorad, USA). Molecular reagents used during present study were procured from Thermo Scientific (USA) and Bangalore Genei (India).

**Results and Discussion**

The bacterial isolate obtained from wound from hyena during present investigation was morphologically gram negative, rods in shape, non-spore forming with peritrichous flagella. Isolate was lactose fermenter which produced pink colonies on MLA and greenish metallic sheen on EMB agar. On Blood agar, colonies were non haemolytic. Yellow slant and yellow but without H₂S production was observed in TSI slant. Isolates showed positive reaction for catalase, indole and methyl red, but negative test for oxidase, urease, citrate and Voges-Proskauer. Cultural, staining and biochemical characteristics of bacterial isolate during present study was in accordance and confirmation with the *E. coli* as reported by earlier workers (Forbes et al., 2007; Mohammed and AL-Khyat, 2008).

Present study reported MDR *E. coli* from hyena. Antimicrobial susceptibility test of *E. coli* revealed that organism was highly sensitive to chloramphenicol; intermediate sensitive to ceftriaxone/tazobactum and gentamicin and resistant to ampicillin, amoxycillin, amoxyclav, trimethoprim, ciprofloxacain, enrofloxacain, cefotaxime, cefepime, ceftriaxone and oxytetracycline. In a similar type of study, Jobbins and Alexander (2015) and Pesapane et al. (2013) reported MDR *E.coli* in faeces of hyena and they also observed that multidrug resistance was significantly higher in carnivores suggesting the life history and food chain may be the key to understanding the exposure patterns and transmission dynamics. Wasyl et al., (2018) reported MDR *E. coli* from wild boar exhibiting resistance against 11 of 14 tested antimicrobials including sulfamethoxazole, ampicillin, trimethoprim and tetracycline. In accordance with the present finding, earlier workers have reported resistance of *E. coli* towards aminoglycosides, fluoroquinolones and ceftriaxone (Sharma et al., 2016) in human and towards ampicillin, enrofloxacain, trimethoprim and amoxyclav in canine (Wong et al., 2015). Increased sensitivity of *E.coli* towards chloramphenicol during present study corroborates with observation of Kibret and Abersa (2011). Similarly, Sood (2016) reported 83% of the MDR *E. coli* isolate sensitive to chloramphenicol. Re-emergence of chloramphenicol sensitivity might be due to decreased frequency of its use in recent years, thereby not giving the bacteria much possibility to develop resistance.

During present investigation, *E. coli* isolate from hyena was found to harbour *bla*CTX-M gene (Fig.1) whereas other two ESBL genes (*bla*SHV and *bla*TEM) were not detected by PCR amplification. Present report was in agreement with the findings of Darwich et al., (2019) who reported higher prevalence of
beta lactamase genes (bla SHV 20%, blaCTX-M, 18%) in wild animals. Likewise, Wasyl et al., (2018) detected ESBL genes (blaCTX-M) producing E. coli in wild boar. On contrary, Alonso et al., (2017) described lower prevalence of betalactamase producing E. coli in wild mammals as compared to wild birds.

Emergence of MDR E.coli in hyena need to be understand. It is well documented that use of antibiotics is the most direct mechanism for the evolution of antimicrobial resistant pathogens in livestock and man. Horizontal transfer via transposon and plasmids facilitate resistant genes to spread rapidly through bacterial populations (Peterson and Kaur, 2018). 30-90% of the antibiotics administered in animals and man are excreted unmetabolized which causes environmental diffusion of resistant bacteria and present a direct source for onward transmission to wild animals (Marshall and Levy, 2011). As wildlife is not directly exposed to any antimicrobial agent, therefore fecal contamination of water or soil with MDR bacteria can lead to selection pressure. Besides socioeconomic factors such as forest fragmentation (Goldberg et al., 2008), research and tourism (Goldberg et al., 2007), urbanization and intensification of agriculture and livestock (Jones et al., 2008) may lead to close interaction of wild animals with livestock and human, thereby leading to emergence of antimicrobial resistance in wildlife. In ordinary circumstances, hyenas are extremely timid around humans and sometimes implicated in the killing of livestock (Heptner and Sludskii, 1992), which makes close interface between livestock-human and hyena facilitating the rapid interspecies dissemination of resistant pathogens. It is well supported with the observations of Dorado-Garcia et al., (2018) and Weiss et al., (2018) who reported similar ESBL genes in human and wild life settings; and identical MDR pattern of E. coli in livestock, wildlife and human, respectively. Similarly, Rwego et al., (2008) also demonstrated high rate of E. coli transmission between livestock and humans.

**Table.1** Details of ESBL gene primers

| Gene  | Primer sequence                                             | Length | References               |
|-------|-------------------------------------------------------------|--------|--------------------------|
| blaSHV| 5' - ATG CGT TAT ATT CGC CTG TG - 3'                        | 747-bp | (Paterson et al., 2003)  |
|       | 5' - TGC TTT GTT ATT CGG GCC AA - 3'                        |        |                          |
| blaTEM| 5’ – TCG CCG CAT ACA CTA TTC TCA GAA TGA - 3’              | 445-bp | (Akpaka et al., 2010)    |
|       | 5’ – ACG CTC ACC GGC TCC AGA TTT AT – 3’                    |        |                          |
| blaCTX-M| 5’ – ATG TGC AGY ACC AGT AAR GTK ATG GC – 3’              | 593-bp | (Boyd et al., 2004)      |
|       | 5’ – TGG GTR AAR TAR GTS ACC AGA AYC AGC GG-3’             |        |                          |
**Fig. 1** *Escherichia coli* isolate positive for ESBL gene *bla*CTX-M. Lane 1: 100 bp ladder, Lane 3: Positive control, Lane 8: Positive for *bla*CTX-M genes, Lane 4, 5, 6, 7: Negative *E. coli* isolates

In conclusion, antimicrobial resistance has emerged as a serious problem in both veterinary and human medicine around the globe. Occurrence of MDR *E. coli* with extended spectrum beta lactamase activity in a hyena needs immediate attention and therefore periodic monitoring of antimicrobial susceptibility both in wildlife and surrounding human dwellings is recommended.

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