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Research Article

High Prevalence of Antibiotic Resistance in Escherichia coli Isolated from Fecal Sample of Cows and Assessment of Antibacterial Efficacy of Indigenous Medicinal Plants from Assam, India

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Abstract

Cow dung is the most common organic manure in agriculture in Assam, India. *Escherichia coli* were isolated from cow dung samples in between January to May, 2013. The isolates were tested for antibiotic sensitivity to multiple antibiotics and production of ESBLs as per the NCCLS guidelines, 2002 and genotypically through PCR for the presence of *bla* genes. *E. coli* were isolated in 80% of the total 100 samples, of which 15% showed resistance to multiple drugs. Of these 80 isolates, 59 (73.75%) were found to be resistant to at least one of the 3GCs. PCR data revealed blaTEM in 25/28 isolates, blaSHV in 6/28 isolates, blaCTX-M in 8/28 isolates and blaAmpC in 27/28 isolates; both blaTEM and blaSHV were present in 21.42%, blaTEM and blaCTX-M in 28.57%, blaSHV and blaCTX-M in 10.71%. ESBLs are the main cause of resistance to beta-lactam antibiotics in *E. coli*. ESBL mediating resistance to 3GC was found in 35% of isolates. The leaves of *Holarhenna antidysenterica* wall, and flowers of *Woodfordia fructicosa* (L.) Kurz were collected at the same time and its antibacterial activity was assessed against antibiotic resistant isolates. Methanolic crude extracts from these medicinal plants exhibited inhibitory activity against TEM+SHV encoding enteric isolates from cow. Detection of enteric ESBL producers in cows in Assam, India in high numbers imply the risk of dissemination of antibacterial resistance into the environment being relatively high and thus, warrant preventive measures.

Keywords: Cow dung; SHV; TEM; CTX-M; Medicinal plants

Abbreviations

ESBL: Extended Spectrum β-lactamases; IMViC: Indole, Methyl red, Voges Proskauer and Citrate utilization test; dNTP: deoxynucleotides; TEM: Temoniera; SHV: Sulphydryl variable; CTX-M: Cefotaximase, Munich; DMSO: Dimethyl Sulphoxide; 3GC: 3rd Generation Cephalosporin; NCBI: National Centre for Biological Information; BLAST: Basic Local Alignment Search Tool; NCCLS: The National Committee for Clinical Laboratory Standards

Introduction

Antimicrobial resistance has been recognized as one of the world's most pressing public health problems [1,2]. The intensive use and, particularly, the misuse of antibiotics have led to the development and selection of resistant bacteria in different settings. Beyond the use of drugs for therapeutic purposes in the human and animal settings, antibiotics are also used extensively as prophylactic agents and as animal growth promoters in agriculture, except in European Union countries, where their use is banned since 2006 in animal feed [3]. Therefore, resistant bacteria are not only confined to the human clinical setting, such as hospitals, where they were first recognized and studied. They have also increased significantly in the community and in both farm and companion animals. Animals may act as reservoirs of resistant bacteria that can be transmitted to humans, or vice versa, by direct contact or indirectly, via the food chain [3-6]. Moreover, the growth of global trade and travel allows resistant microorganisms to be spread rapidly to distant countries and continents [7]. The consequences of antimicrobial resistance represent a growing threat. When infections often fail to respond to standard treatments, they result in prolonged illness and greater risk of death, and more chances for the resistant microorganism to spread. Furthermore, the costs associated with the length of hospitalization and the use of last generation antibiotics are significantly increased [8,9]. The use in human medicine of third generation cephalosporin's (3GCs, e.g. cefotaxime, ceftazidime, ceftriaxone) is generally believed to have been a major selective force in the emergence of extended-spectrum β-lactamases (ESBLs) [8]. Shortly after the introduction of cefotaxime, transferable plasmid-mediated resistance was noted, initially in Germany, with the identification of a mutant β-lactamase SHV-2 that conferred high-level resistance to all of these agents [8]. The following year, in France, a similar mutated variant of TEM β-lactamase (TEM-3) was observed [8] and this led to the adoption of the term Extended Spectrum β-lactamase (ESBL). The small but gradually increasing use of 3GCs in food animal production may be linked to the recent emergence of ESBLs in bacteria associated with cattle, poultry and pigs [10]. It is speculated that emergence of ESBL bacteria in food producing animals may present a risk of resistant

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strains being transmitted to humans through the food chain. Hence, it asserts importance of studying the prevalence of ESBLs in the veterinary population and their dissemination through the food chain to humans, if any [10].

In the North-Eastern part of India, particularly Assam, cow is the primary source of milk. Hence it is important to identify the most commonly infecting pathogens as well as commensal microorganisms and their resistance patterns to the commonly prescribed antibiotics, as well as molecular mechanism of resistance in resistant isolates. Production of ESBLs is the most common mechanism of resistance to 3G Cephalosporin in Gram negative bacteria. This study was designed with the aim of analyzing the antibiotic susceptibility pattern of *E. coli* isolated from cow-dung in Guwahati area of India. ESBL producers were detected both phenotypically as well as genotypically. The findings will aid in surveillance study and also act as a guide for prophylactic therapy.

In vitro antimicrobial activity of locally available medicinal plants *Holarhenna antidysenterica* Wall and *Woodfordia fructicosa* (L.) Kurz were selected based upon traditional knowledge gathered by interviewing local practitioners were assessed against the ESBL producing enteric isolates. While *Woodfordia fructicosa* (L.) Kurz finds its application in treating ailments that may be of bacterial origin, for example boils, diarrhea, dysentery, fever, cough, menstrual disorders, urinary disorders, wounds, swellings, cuts, skin diseases [11]. Bark of *Holarrhena antidsenterica* Wall is used in Ayurveda as an anti-microbial, anti-inflammatory and analgesics, other useful parts used as medicine are root and leaf. In addition the plant has been reported to possess antihelminthic, appetizing, antidiarrhoel and astringent properties [12]. This plant is found throughout tropical India and is an important plant used in indigenous systems of medicine as a remedy for bronchitis, hematuria, spermatorrhoea, epilepsy, asthma, piles, leprosy, eczema, diarrheal, fevers and jaundice [13]. Medicinal plants can be a source of chemical compounds which are able to restrict or stop the growth of microorganisms thereby finding immense application in pharmaceutical research. There is an ever-increasing demand for plant-based therapeutics in both developing and developed countries. Compounds of plant origin are believed to be safe without side effects [14].

Materials and Methods

Ethical clearance

Ethical clearance was obtained from Institutional Ethical Committee of Gauhati University.

Collection of bacterial samples

One hundred cow-dung samples were collected from different cowsheds from various locales within Guwahati region for a period of four months (January to May 2013). The samples were collected into sterile zip lock bags and transferred to the laboratory immediately. The isolation of bacteria was carried out within 4-10 hours from the time of collection.

Isolation and identification

One mg of the sample was added to 10ml of autoclaved saline distilled water and vortexed. An aliquot of 1.0 mL was transferred into the next test tube containing 9.0mL of the sterile distilled water and diluted serially in one-tenth stepwise to 10^{-2} dilution. With a sterile loop this suspension was streaked on a MacConkey agar and incubated at 37^{O}C for 24 hours. Pink, dry, irregular shaped colonies inferred as *E. coli* were selected for the study. Further, the *E. coli* isolates were confirmed by Gram's staining and by standard biochemical test (IMViC tests) [15].

Antibiotic susceptibility profile

The antibiotic susceptibility was determined by the Kirby Bauer's disc diffusion method on Mueller-Hinton agar by inoculating plates with a bacterial suspension compared with a 0.5 McFarland standard, according to the NCCLS guidelines for veterinary samples, 2002 [16]. The antibiotics tested were – Erythromycin (15 µg), Imipenem (10 µg), Kanamycin (30 µg), Co-trimoxazole (25 µg), Ampicillin (10µg), Chloramphenicol (30 µg), Aztreonam (30 µg), Cefotaxime (30µg) and Ceftazidime (30µg) (HiMedia Laboratories Pvt. Ltd., Mumbai, India).

Phenotypic detection of ESBL Producers

All isolates were tested for the detection of ESBL production by the confirmatory method of NCCLS guidelines for veterinary samples, 2002 [16] using cefotaxime (30 µg) and ceftazidime (30 µg) and a disc of cefotaxime plus clavulanic acid (30/10 µg) and ceftazidime plus clavulanic acid (30/10 µg) (HiMedia Laboratories Pvt. Ltd., Mumbai, India) placed at a distance of 20 mm on a lawn culture (0.5 McFarland inoculum size) of suspected ESBL producing clinical isolate on Mueller-Hinton Agar (Hi-Media, Mumbai). *Escherichia coli* ATCC 25922 were used as the negative control and *Klebsiella pneumoniae* ATCC 700603 was used as the ESBL positive control. ESBL production was inferred if the inhibition zone was ≥5 mm towards the cefotaxime plus clavulanic acid disc or ceftazidime plus clavulanic acid disc in comparison to the third generation cephalosporin disc alone. Only those isolates identified phenotypically as ESBL producer were selected for genotypic detection of ESBL encoding genes.

Genotypic detection of ESBL encoding genes

To 100µL of sterile Millipore water 4 to 5 colonies of the overnight bacterial culture grown on Luria Bertani Agar (HiMedia Laboratories Pvt. Ltd., Mumbai, India) was taken with a loop. This suspension was boiled at 100^{O}C in a water bath for 10 minutes. Equal volume of Chloroform: isoamyl alcohol (24:1 v/v) was added and the samples were then centrifuged at 12000 rpm for 10 minutes. The upper layer was used as the crude DNA for PCR amplification [17].

PCR analysis was carried out for detection of β-lactamase encoding genes - TEM, SHV, CTX-M and AmpC. Primers obtained were - for *bla*TEM 5'-CTTCCTGTTTTTGCTCACCCA-3' and

5'-TACGATACGGGAGGGCTTAC-3', for *bla*SHV 5'-TCAGCGAAAAACACCTTG-3' and

5'-TCCCGCAGATAAATCACC-3' [17], for blaCTX-M 5'-ATGTGCAGYACCAGTAARGT-3' and

5'-TGGGTRAARTARGTSACCAGA-3' [18], and for blaAmpC5'-AATGGGTTTTCTACGGTCTG-3' and

5'-GGGCAGCAAATGTGGAGCAA-3' [19].

Amplification reactions were performed in 25uL volume containing 250 pg of template DNA. For TEM, SHV, CTX-M and AmpC amplification the reaction volume contained 1X PCR buffer, 0.8 mM of $MgCl_2$, 0.2 mM dNTP mix (Invitrogen, North America), 1

µl of each primer (10 µM) with 1 U *Taq* DNA Polymerase (Invitrogen, North America) per tube. *K. pneumoniae* ATCC 700603 was used as the positive control for SHV and for TEM; CTX-M and AmpC in-house positive controls confirmed by PCR-sequencing were used. All PCR amplifications were carried out in Veriti Thermal Cycler (Applied Biosystems, USA).

The cycling conditions for amplification were as follows: for *SHV* gene, initial denaturation at 94°C for 2 min and 30 cycles of 30 sec at 94°C, 30 sec at 46°C and 45 sec at 72°C, followed by 5 min at 72°C. For *TEM* gene initial denaturation of 2 min at 94°C and 30 cycles of 1 min at 94°C, 1 min at 52°C, and 1 min at 72°C, followed by 7 min at 72°C. For *CTX-M* gene, initial denaturation at 94°C for 3 min and 30 cycles of 45 sec at 94°C, 30 sec at 48°C and 60 sec at 72°C, followed by 5 min at 72°C and for *AmpC* gene initial denaturation at 95°C and 30 cycles of 30 sec at 95°C, 30 sec at 51°C and 1 min at 72°C, followed by 2 min at 72°C.

The resulting PCR products were analyzed by electrophoresis with Ethidium Bromide supplemented 1.5 per cent agarose gel in Tris-Acetate-EDTA buffer. The electrophoresis was run at 80-100 Volt till the sample reached 3/4th of the gel. The gels were observed and photographed in a Gel Documentation system (Gel logic, Carestream). A molecular weight standard (Quick–load™100bp DNA ladder; Invitrogen, North America) was included on each gel.

Two PCR amplicon samples for each gene were sequenced and compared to sequences available in National Centre for Biological Information (NCBI) through BLAST program.

Screening of plant antimicrobials

Flowers of *Woodfordia fructicosa* (L.) Kurz, and leaves of *Holarhenna antidysenterica* Wall. Were collected from Guwahati region, washed, shade dried and finely grounded to powder. The plant powder was used for the preparation of crude methanolic extract by soxhlet extraction [20] and aqueous extract was prepared by maceration in autoclaved distilled water [21]. The extracts were allowed to dry and then stored at 4°C until further use. Dimethyl Sulfoxide (DMSO) was used to dissolve the extracts to test the antibacterial efficacy which was done by well diffusion method on Muller Hinton Agar [22]. The inoculated plates were incubated for 16-18 hours at 37°C and the zone of inhibition was recorded. The presence of any measure of a zone of clearance indicated antibacterial activity of the plant extract.

Results and Discussion

A total of 80 non-duplicate bacterial isolates were cultured on MacConkey Agar from the 100 fecal samples collected and were identified to be *E. coli*. All the 80 isolates, in our study, were tested for susceptibility to antibiotics and β lactam-β lactamase inhibitor combinations as per NCCLS guidelines, 2002. These isolates tested showed resistance to Erythromycin – 80/80 (100%), Aztreonam - 76/80 (95%), Ampicillin – 60/80 (75%), Ceftazidime – 59/80 (73.75%), Kanamycin – 58/80 (72.5%) and Cefotaxime – 56/80 (70%) (Figure 1).

While the ESBL producing isolates tested showed a high degree of resistance to Erythromycin–28/28 (100%), Aztreonam–25/28 (89.28%), Ampicillin – 25/28 (89.28%), Kanamycin–21/28 (75%),

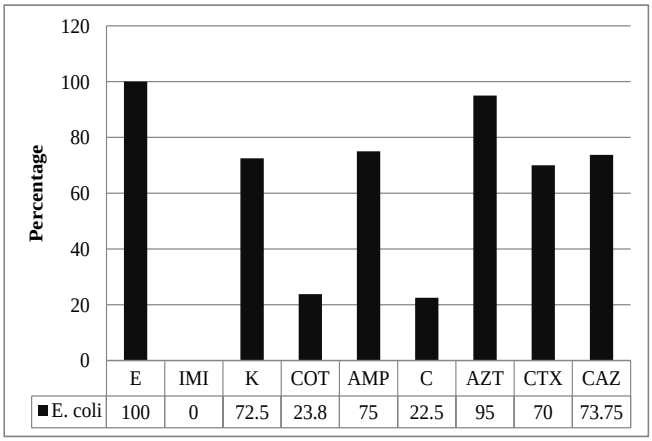

Figure 1: Bar Diagram showing the percentage resistance of all the enteric *E. coli* isolates to the antibiotics tested (Key – E – Erythromycin, IMP – Imipenem, K – Kanamycin, COT – Co-trimoxazole, AMP – Ampicillin, C– Chloramphenicol, AT – Aztreonam, CTX – Cefotaxime, CAZ – Ceftazidime).

Cefotaxime–24/28 (85.71%) and Ceftazidime–28/28 (100%). The susceptibility profile of Imipenem 80/80 (100%), Co-trimoxazole 61/80 (72.5%) and Chloramphenicol 62/80 (77.5%) indicated to be the most effective antibiotics. Of the 80 isolates, 59 (73.75%) were found to be resistant to at least one of the 3GCs.Of these 59 isolates thus tested for ESBL production, 28 were inhibited by ceftazidime/clavulanic acid. These isolates were tested by placing the ceftazidime and ceftazidime/clavulanic acid discs at a distance of 20 mm and a > 5 mm increase in the zone diameter towards cefotaxime/clavulanic acid was observed as per NCCLS guidelines, 2002, of these 28 isolated, 8 were also inhibited by cefotaxime/clavulanic acid. Thus, among these 59 isolates, 28 (47.45%) were found to be ESBL producer by Double-disc synergy test. Hence, out of the 80 isolates screened for ESBL production, 28 (35%) were found to be ESBL producers.

To understand the antibiotic resistance changes over a period of time, a study carried out by the Centre for Disease Control showed a significant increase in resistance in *E. coli* isolates from animals to 11 of 15 antimicrobials inclusive of Ampicillin, Tetracycline and Streptomycin [22]. Prevalence of ESBL was more in females (85.71%, 24 out of 28) than in males (14.29%, 4 out of 28). Of the 80 isolates, 59 (73.75%) were found to be resistant to at least one of the 3GCs. Overall high resistance was observed against commonly prescribed antibiotics. ESBL mediating resistance to 3GC was found in 35% of isolates. The prevalence rate is higher than the reported figure of *E. coli* in France (5.8%, 2008) [23] and Turkey (1.25%, 2008) [24]. This prevalence appeared to be higher than several studies; for example Duan and others, 2006 [25] reported a 3.1% prevalence of ESBL producers among *E. coli* isolates from cattle in Hong Kong; it was found that1.5% resistance to cefotaxime in *Enterobacteriaceae* members isolated from cattle in Japan [26]. Thus it can be concluded that the rate of occurrence of ESBLs has vastly increased in the last few years.

The ESBL positive strains confirmed phenotypically were further tested for the presence of blaSHV, blaTEM, blaCTX-M and blaAmpC genes to identify their resistance mechanism. The amplified product of blaSHV and blaTEM gene had a fragment size 471bp and 717bp corresponded to 100bp molecular weight marker and the positive

control (Figure 2). Of the 28 samples, 6 (21.42%) were positive for blaSHV, 25 (89.29%) were positive for blaTEM; presence of both blaTEM and blaSHV genes in 6 (21.42%) isolates and 27 (96.42%) were positive for blaAmpC with a fragment size of 192bp (Figure 3) while 8 (28.57%) were positive for blaCTX-M with a fragment size of 569 bp (Figure 4); a comparison of the presence of two or more ESBL genes in a single isolate is given as Table 1. Figure 5 shows the bar diagram representation of ESBL genes present in the enteric *E. coli*

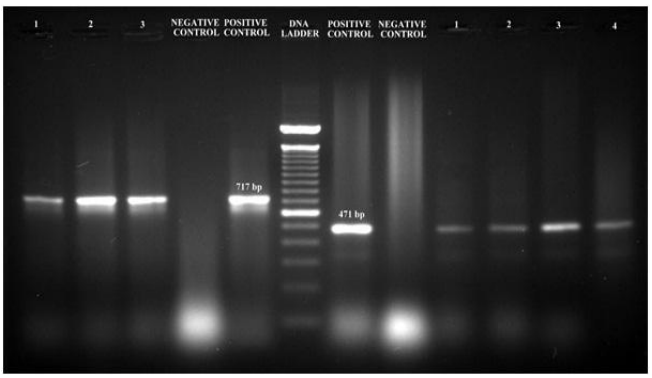

Figure 2: PCR amplified fragments blaTEM (on left of the ladder); Positive control in-house control (*E. coli;* Resistant to Aztreonam, Cefoxitin, ampicillin, Cefotaxime, Ceftazidime, Phenotype confirmed), Negative control – *E. coli* ATCC 25922, 1-3 – bacterial isolates (*E. coli)*, and blaSHV (on right of the ladder); Positive control – *K. pneumonia* ATCC 700603, Negative control – *E. coli* ATCC 25922, 1-4 bacterial isolates (*E. coli)* on agarose gel (1.5%).

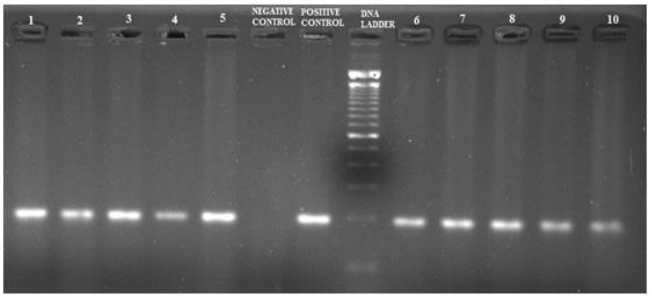

Figure 3: PCR amplified fragments of blaAmpC on 1.5% agarose gel; Positive control – in-house control (*E. coli;* Resistant to Aztreonam, Cefoxitin, ampicillin, Cefotaxime, Ceftazidime*)*, Negative control – autoclaved distilled water, Samples 1 to 10 – bacterial isolates of *E. coli*.

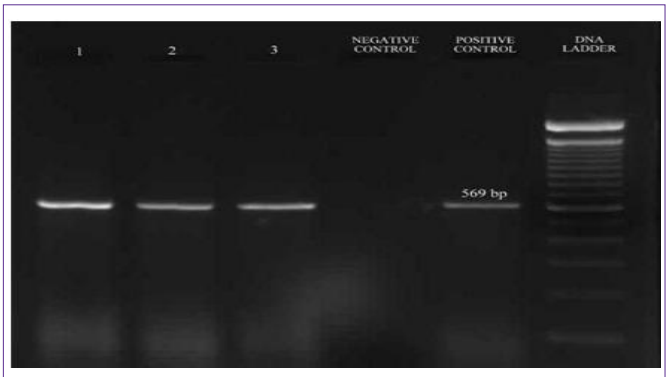

Figure 4: PCR amplified fragments on 1.5% agarose gel of blaCTX-M; Positive control - in-house control (*E. coli;* Resistant to Aztreonam, Cefoxitin, ampicillin, Cefotaxime, Ceftazidime), Negative control - autoclaved distilled water, Samples 1 to 3 – bacterial isolates of *E. coli.*

Table 1: Table representing presence of two or more *bla* genes in the *E. coli* isolates from cow dung (n=28).

*Bla*genes	Percentage (no. of isolates)
SHV + TEM	21.42(6)
SHV + CTX-M	10.71(3)
TEM + CTX-M	28.57(8)
TEM + AmpC	85.71(24)
SHV+ TEM + CTX-M	10.71(3)

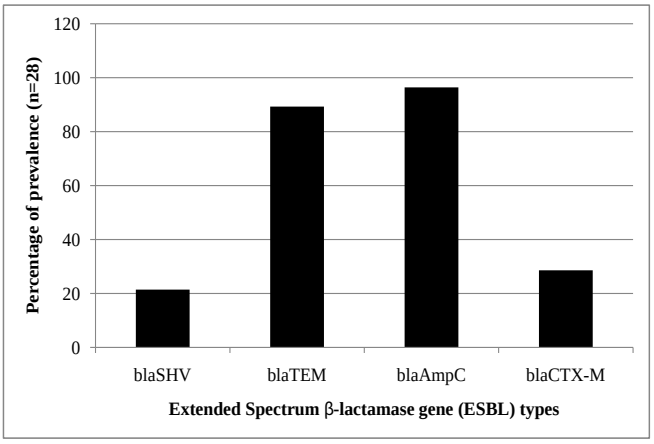

Figure 5: Bar diagram representing the percentage of ESBL gene detected in the *E. coli* isolates.

isolates from cows. It is important to detect the underlying genetic behavior for a phenotypically confirmed ESBL producing isolates to understand the present scenario of dissemination of CTX-M, TEM, SHV or AmpC genes in a veterinary population and whether the trend changes overtime needs to be monitored. High rates of ESBL producers carrying CTX-M, TEM and SHV genes in food animals and the high genetic diversity among these isolates have been reported [27] while the presence of ESBL genes have also been reported from different locations in three dairy farms stating the possibility for intramammary infections in cows [28].

Cow dung is used as an organic fertilizer for the cultivation of food crops in agricultural field, on a large scale as well as in kitchen gardens. The occurrence of ESBL producing *enteric E. coli* in the fecal microflora of cows (in our study) represents an obvious risk for contamination of raw food products and also that of animal origin furthering the dissemination of ESBL producing isolates into the environment. More recently, reports have also raised concern about the dissemination of ESBL producing *E. coli* in healthy food producing animals in several countries. Therefore, the impact of healthy farm animals as a possible reservoir for ESBL producing *Enterobacteriaceae* on the food processing chain need to be assessed. Given the relatively high occurrence of ESBL producers in faecal samples from cows in our study, it is striking, that the rate of ESBL producers has alarmingly increased over the last few years.

Both methanol and aqueous extracts of *Woodfordia fructicosa* (L.) Kurz flowers showed a zone of inhibition against TEM + SHV positive isolates in comparison to *Holarhenna antidysenterica* Wall. (Table 2) with comparable inhibitory zones between both the plant samples. The results observed from the screening of plant specimens

Table 2: Table representing diameter of the zone of inhibition (in mm) of the medicinal plants tested against three *E. coli* isolates possessing TEM and SHV genotype.

Plant	Parts used	Solvent	Concentration (mg/mL)	Average Zone of Inhibition (mm)*
Holarrhena antidysenterica Wall	Leaves	Methanol	400	11.0
		Aqueous	300	-
Woodfordia fructicosa (L.) Kurz	Flowers	Methanol	400	10.3
		Aqueous	400	10.3

*Note – Inclusive of well diameter (6 mm)

for their antibacterial efficacy indicates the need for the assessment of medicinal plants against ESBL producing isolates and the possibility of finding newer antibacterial agents to tackle the emerging antimicrobial resistance in *Enterobacteriaceae*. Considering the rich diversity of plants, it is expected that screening and scientific evaluation of plant extracts for their anti-microbial activity may provide new anti-microbial substances. Hence the present investigation clearly reveals the antibacterial nature of the medicinal plants and suggests that the plants could be exploited in the management of diseases caused by these bacteria in both human and animal systems. In this study, we aimed to provide an informative basis on the presence of ESBLs in cows in Assam, India and the possibility of exploring newer antimicrobial agents from plant samples indigenous to North Eastern India and Assam.

ESBLs are the main cause of resistance to beta-lactam antibiotics in *E. coli*. Due to the clinical importance of the detection of ESBLs, screening and confirmatory methods have been routinely used to investigate the production of these enzymes in *E. coli* species. As their occurrence has been increasing, it becomes essential to evaluate their occurrence in this population. Expanded spectrum cephalosporins are β–lactams with a broad spectrum of activity against most Gram negative bacteria. However they are sensitive to hydrolysis by ESBLs. The dissemination of these enzymes is currently a global problem [29,30]. It is also well known that plasmids carrying genes encoding ESBLs may also carry genes encoding resistance to non β–lactam antibiotics such as aminoglycosides, chloramphenicol and trimethoprim-sulphamethoxazole [31]. Relatively very little is known about the epidemiology of ESBLs in veterinary medicine. There have been a few studies reporting ESBLs from farm animals and pets [32-34]. In fact plasmid encoded ESBLs which are once rarely detected of animal origin bacteria are more frequently being observed in the recent years. The factors leading to the emergence of ESBLs among bacteria of animal origin are not fully elucidated.

Conclusion

To our knowledge, this is the first report describing genetic basis of ESBLs in *Enterobacteriaceae* detected from food animals in Assam, India. Larger scale surveillance studies in veterinary are needed to track the evolution and epidemiology of ESBLs. Development of newer antibiotic candidates for dealing with the ever emerging bacterial resistance mechanisms of ESBL positive organisms has become the need of the hour. Moreover, strict drug policies need to be implemented to limit the irrational use of antibiotics. Surveillance studies need to be done from time to time to keep track of the extent of resistance acquired by the organisms in a particular area and also to identify any new mechanisms developed, if any.

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