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Prevalence of Antimicrobial Resistance Patterns of *Escherichia coli* Faecal Isolates of Cattle

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A B S T R A C T

A total of 154 of *Escherichia coli* (*E. coli*) isolates were isolated from faecal specimens of healthy cattle (n=100). Serotyping of 154 *E. coli* isolates indicated that 112 (72.72%) isolates were typeable, 28 (18.18%) untypeable and 14 (9.09%) were rough. The most predominant serotype observed was O22 followed by O56, O60, O120 and O1. Antibiogram pattern using a total of 25 different antibiotics indicated that high resistant was present against clindamycin, metronidazole and penicillin followed by cephalothin, neomycin, kanamycin, cephalexin, streptomycin, furazolidone and tetracycline antibiotics. The amplification of tet(A) and tet(B) resistant determinants by PCR resulted in generation of 372bp and 228, respectively. The tet(A) gene was predominant gene compared to tet(B) gene. This study using pheno-genotypic characterization indicated the presence of antimicrobial resistant *E. coli*isolates amongst healthy cattle, emphasizing that effective strategy should be applied to persist the efficiency along with ideal usage of novel antibiotics though minimizing the risk of antibiotic resistant bacteria.

Introduction

Antibiotic resistance is of great concern and various strategies have been conducted for its monitoring and investigation in the veterinary practices and public health. Antimicrobials are used frequently for curing, controlling and prophylactic of various transmissible diseases

in animals and humans. To successfully control infectious diseases of bovines and to prevent the possible hazard linked with bacterial resistance and therapeutic failure, it is essential to evaluate antibiotic resistance of pathogenic bacteria (Authier *et al.,* 2006). The improper and extensive use of antibiotic in veterinary practices is undoubtedly the main cause of bacterial resistance (Sorum and Sunde, 2001)

The intestinal tract of the animals and humans is considered as a main reservoir for the commensal bacteria, though *E. coli* represents one of the most significant bacterial commensal in the intestinal tract of animals and humans (Rosas *et al.,* 2006). However, some serotypes are able to causes illness in both humans and animals, like, haemorrhagic colitis, urinary tract infections (UTIs). The resistance of the *E. coli* against commonly practised antimicrobials in veterinary and human medicine has progressively increased. Amongst resistance to many antimicrobial agents, the resistant to tetracycline is commonly reported in zoonotic, pathogenic and non-pathogenic bacteria like *E. coli* (Authier *et al.,* 2006). The tetracyclines are widely used in animals and humans due to its broad spectrum activity and therapeutic efficacy with low side effects. The mechanism for resistance to tetracycline can be classified into four categories and 35 different tetracycline or oxytetracycline genes are involved (Chopra and Roberts, 2001).

Scientist are nowadays concerned on therapeutic usage of antimicrobial agents and concurrent development of antibiotic resistant pathogenic bacteria that might be of slipover to humans either through dealings with food, animal foodstuff chain and/or through food producing animals (Lanz *et al.,* 2003). However, the uses of antibiotics in various veterinary fields may potentially influence the antimicrobial resistance profile in both exogenous infectious bacteria and commensal bacteria like *E. coli* (Catry *et al.,* 2003).

Despite withdrawal of antimicrobial agent from the rations, the bacterial resistant can be still determined for a long time (Langlois *et al.,* 1988; Gellin *et al.,* 1989) in animals. Therefore, a commensal bacteria that poses antimicrobial resistance determinants constitutes a main resource of resistant genes for highly pathogenic bacteria (Moyaert *et al.,* 2006). The studies involved in the antimicrobial resistance are frequently targeted to assessing resistant phenotypes that might originate from diverse genetic determinants, however, these determinants might represent a particular epidemiological pattern. Consequently, the genotypic assessment of antibiotic resistance is considered as significant for monitoring antimicrobial resistant diseases.

Materials and Methods

Samples collection

A total of hundred faecal specimens (n=100) were collected aseptically and randomly from apparently healthy cattle of varying age groups located in different agro-climatic zones of the Punjab state, India. The faecal samples were collected into small sterile plastic bag wearing sterile gloves directly from animals per rectal and processed directly in the laboratory for further microbiological investigation.

Isolation, serotyping and phenotypic antibiotic resistant profile of the *E. coli* isolates

MacConkey lactose bile salt agar (MLA, HiMedia) was used for initial isolation. The faecal samples were streaked on freshly prepared MLA and incubated at 37ºC for 24 hours. Preliminary identification was carried

out on the basis of Gram reaction, morphology and colony characteristics. Subsequently, pure *E. coli* colonies were analysed by biochemical tests for confirmation as described by Quinn *et al.*, (1994). The confirmed pure isolates were stored at 4ºC using tryptone soya agar slants. The serotyping of *E. coli* isolates was carried out at the Central Research Institute, Kasauli, Himachal Pradesh, India. The phenotypic antibiotics sensitivity of the *E. coli* isolates was conducted using disc diffusion method on Mueller-Hinton agar against 25 antibiotics (HiMedia) as per modified Kirby-Bauer Method (Carter *et al.*, 1973). The breakpoint of inhibition zone was interpreted on the basis of zone size interpretation data provided by manufacturer.

Tetracycline resistant gene profiling

The multiplex polymerase chain reaction (mPCR) was conducted for genotypic characterization of 79 phenotypically tetracycline resistant faecal *E. coli* isolates. The purified isolated colonies of *E. coli* were inoculated into 5 ml Luria broth (LB broth) and incubated for 37º C for 24 hours and bacterial DNA was extracted by hot-cold lysis method. The PCR primers as described by Guillame *et al.*, (2000) were used to amplify the tetA) 372 bp and tet(B) 228 bp genes responsible for encoding tetracycline resistance. The mPCR assay was standardized in 25µl reaction mixture and the reaction mixture contained: 10mM dNTPs 1µl, 25mM Mgcl2 2.5µl, 10X PCR buffer 2.5µl, extracted DNA 5µl, primer [tet(A) and tet(B)] 1µl each, Taq DNA polymerase 1 µl and nuclease free water to make a final volume of 25.0µl. The PCR amplification was conducted using a thermal cycler (BiometraTgradient, Germany) with an initial denaturation at 94º C for 5 minutes, thirty-five PCR cycles each of denaturation at 94º C for 1 minute, annealing at 50º C for 1 minute, extension at 72ºC for 45sec followed by a step of final extension at 72º C for 7 minutes. The negative control contained of nuclease free water instead of DNA template while standards of tet (A) and (B) available at Department of Veterinary Public Health, GADVASU, Ludhiana were used as positive control. The PCR products were electrophoretically analysed in 2% Agarose gelin 1X TBE buffer having ethidium bromide (0.50µg/ml) at 80V for 1 hour. Agarose gels were visualized and photographed in Gel Documentation System (BioRad Pvt Ltd.).

Results and Discussion

Isolation and serotyping of the E. coli isolates

Out of 100 faecal samples, 154 isolates of *E. coli* were confirmed as per cultural and biochemical characterization. All the 154 isolates were got serotyped from Central Research Institute, Kasauli, Himachal Pradesh. Serotyping of 154 *E. coli* isolates of cattle indicated that 112 (72.72%) isolates were typeable, 28 (18.18%) were untypeable and 14 (9.09%) were rough. The most predominant serotype were found to be O22 (26 isolates) followed by O56 (7), O60 (5), O120 and O1 (4 each), O69, O1, O41, O59, O79, O87 and O147 (3 each), O21, O128, O16, O48, O105, O73, O85, O97, O159, O2, O25, O149, O5 and O88 (2 each) and O101, O110, O125, O13, O130, O17, O170, O28, O32, O96, O152, O20, O91, O103, O137, O100, O4 (1 each).

Nearly 19.48% of isolates are found to be pathogenic for humans like O22, O103, O128 and O91, whereas nearly 40% isolates from healthy cattle of German Democratic Republic were of pathogenic serotypes (Montenegro *et al.*, 1990). Various serogroups of faecal *E. coli* isolated from calves in India include O22, O24, O55, O62,

O86, O110, O128, O131, O157, O171, O172, O168, O5, O2 and O167 (Arya *et al.,* 2008). In the present study, none of the isolate out of 154 isolates of the bovine faecal sample was found to be of serotype O157. Singh *et al.*, 2007 and Kaur, 2007 also reported the absence of O157 in cattle in this region of the country. However, Kanwar (1999) found three O157 serotype out of 50 *E. coli* isolates from calf diarrhoea cases in the same geographical location. The absence of serotype O157 can be due to the reason that animals may not be shedding this serotype as samples were collected from apparently healthy animals. The various serogroups frequently reported in India with public health signifance are O24(Kaura *et al.,* 1991), O2, O5, O9, O62, O55, O86, O131, O157 and O172 (Wani *et al.,* 2003; Sharma *et al.,* 2004). Serogroups like O2, O55, O86, O128 and O157 have recurrently been linked with gastrointestinal illness among neonates and adults suffering from haemorrhagic diarrhoea/HC and/or HUS (Nishikawa *et al.*, 2002) which are also of public health concern.

Phenotypic antibiotic resistant profile of the E. coli isolates

The phenotypic characterization of faecal *E. coli* isolates to determine the drug resistance pattern against 25 antimicrobials resulted in various antimicrobial resistance profiles. The highest resistance was observed against clindamycin and penicillin as 154 (100%) isolates were resistant to both antibiotics, followed by metronidazole 152 (98.70%), neomycin 146 (94.81%), kanamycin 139 (90.26%), cephalothin 133 (86.36%), cephalexin 79 (51.30%), tetracycline 79 (51.30%), streptomycin 75 (48.70%), doxycycline 74 (48.05%), trimethoprim 60 (38.96%) and ampicillin 58 (37.66%) resistant isolates, and less frequently to the co-trimoxazole 56 (36.36%), nalidixic acid 54 (35.06%), polymyxin B 54 (35.06%), furazolidone 54 (35.06%), amoxycillin 51 (33.12%), gentamicin 42 (27.27%), ciprofloxacin 29 (18.83%), colistin 28 (18.18%), enrofloxacin 29 (18.83%), cefazolin 27 (17.53%), ceftriaxone 26 (16.88%), gatifloxacin 25 (16.23%) and chloramphenicol 15 (9.74%) isolates.

The high resistance against penicillin and metronidazole varying from 96.4-100% is also observed at other parts of world like Zambia, Malaysia (Radu *et al.,* 2001; Mubita *et al.,* 2008), whereas at Czech dairy farms, *E. coli* isolates exhibited highest resistance to tetracycline followed by resistance to streptomycin (Dolejska *et al.,* 2008). Indian *E. coli* isolates are usually found to have high resistance against metronidazole (100%) and penicillin (98.08%) antibiotics (Singh *et al.,* 2007; Kaur, 2007) as these are amongst the most commonly used drugs under field conditions for the treatment of animals. The *E. coli* isolates are also frequently resistant to streptomycin (85.7%), tetracycline (80.0%) as compared to trimethoprim (29.1%)and nalidixic acid (29.1%) antibiotics (Hoyle *et al.,* 2005). Kanwar (1999) also found *E. coli* isolates from calf diarrhea resistant to metronidazole (98.08%) and co-trimoxazole (34.62%) similar to the resistant pattern observed in present study. At eastern part of India, different resistance profile of ampicillin (25.4%) tetracycline (23.8%), streptomycin (14.3%), cephalothin (86.36%), co-trimoxazole (9.5%), nalidixic acid (6.4%) and neomycin (94.81%) was observed (Khan et al.,2002)as compared to northern India in the present study viz. 51.30%, 48.70%, 86.36%, 36.36%, 35.06% and 94.81% of the *E. coli* isolates presenting resistance against tetracycline, streptomycin, cephalothin, Co-trimoxazole, nalidixic acid and neomycin, respectively. The commensal *E. coli* isolated from food-producing animals may exhibit moderate to low level antibiotic resistance i.e. ampicillin (22.5%), cephalothin (20%),

amoxycillin (12.5%), ceftriaxone (0%), gentamicin (5%), kanamycin (12.5%), neomycin (15%), streptomycin (20%), tetracycline (70%), nalidixic acid (2.5%) and ciprofloxacin (0%) as observed by Knezevic and Petrovic (2008)at Serbia. The resistance of the isolates to Ceftriaxone was 16.88%, in contrary to that was reported by Srinivasan *et al.,* (2007) who reported 3.1% resistance. The resistance to gentamicin, cephalothin and trimethoprim can be less than 20% (Srinivasan *et al.,* 2007) or more than 20% as observed in current study. The resistance of the isolates to tetracycline (51.30%) is more in this part of India as compared to other parts where around 25-47% resistance against tetracycline may be present (Khan *et al.,* 2002; Srinivasan *et al.,* 2007; Gow *et al.,* 2008). However, very high resistance (up to 98%) to tetracycline may be present as reported by Donaldson *et al.,* (2006). The resistances of the isolates to chloramphenicol was less 9.74% as observed earlier also by Radu *et al.,* (2001) and may be due to the fact that chloramphenicol is not frequently used under field conditions for treatment purpose, whereas in country like Pennsylvania, USA, *E. coli* isolates exhibited very high resistance (98%) to chloramphenicol (Donaldson *et al.,* 2006; Sawant *et al.,* 2007). Though chloramphenicol is banned from use in food animals, florfenicol, a structural analog of chloramphenicol, approved by the Food and Drug Administration in 1996, is commonly used for treating bovine respiratory pathogens in this part of world.

Comparatively, the high resistance profile of antibiotic resistance against the frequently used antibiotics was detected in the *E. coli* isolates. Virtually, numerous antimicrobial drugs like penicillins, cephems, tetracycline and fluoroquinolones are being doled out with no control and might be easily obtained without prescription. The abuse and indiscrimination of antimicrobial agents either for curing of animal diseases, feed additive (growth promotion) as well as for preventive measures is not controlled. Therefore, the misuse of antibiotics is frequently associated with an incomplete course of treatment, or sub-therapeutic dose and mistake for drug of choice might be considered the principle causes of high prevalence of antimicrobial resistance. The abuses of antibiotics may lead to high occurrence of resistance amongst *E. coli* strains and enhance the possibility of development of multiple antibiotic resistance (MAR) posing public health risk. The isolation of multiple drug resistant *E. coli* from dairy farms in this area may make difficulty in success of antimicrobial therapeutic regimen.

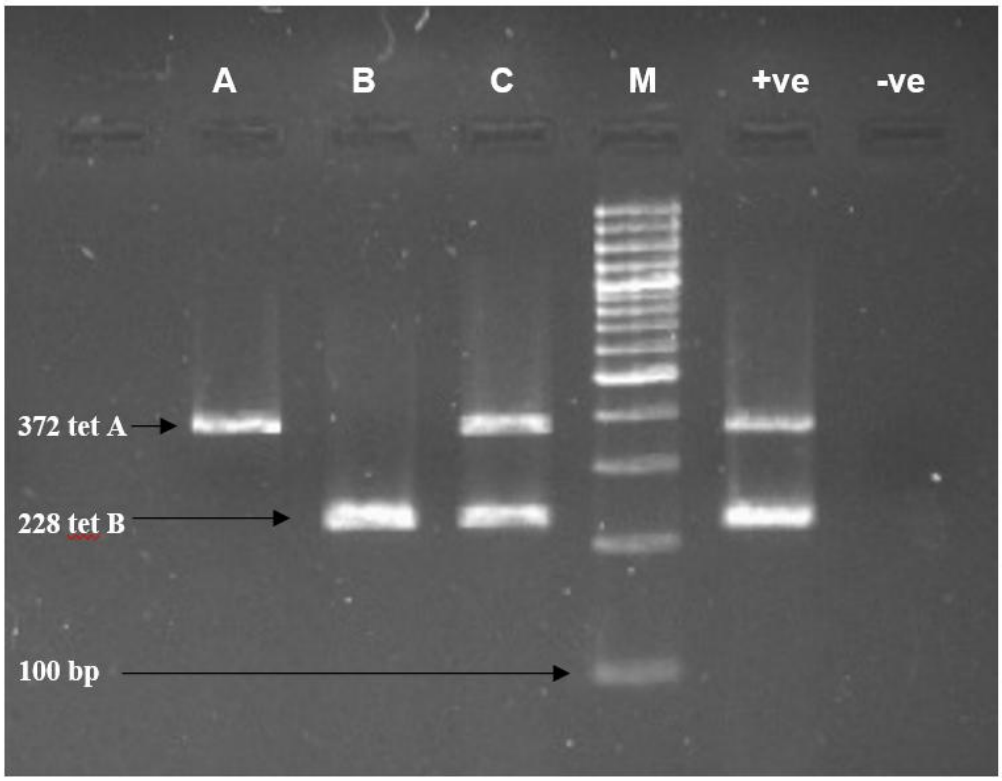
The high prevalence of antibiotic resistance patterns was also observed in calves which might be related to specific genes like adhesion genes that represents a high frequency in the micro flora of younger calves and may be found on the same plasmid carrying antimicrobial resistance genes (Dolejska *et al.,* 2008). The high level of resistance in young animals suggest that there is a correlation between resistance, animal age and acquirement of resistance commensal flora (Hoyle *et al.,* 2005; Donaldson *et al.,* 2006; Gow *et al.,* 2008). It can also be attributed to the fact that the younger calves might be more exposed to antibiotic resistant *E. coli* that spread into the dairies through the wastes, and consequently get picked up and reaches to intestinal tract of young calf, even without therapeutically use (Berge *et al.,* 2005).

Tetracycline resistant gene profiling

The genotypic characterization of tetracycline resistant tet(A) and tet(B) genes in faecal *E. coli* isolates revealed that the tet(A) gene was predominant gene as was detected in 22 (27.85%) isolates as compared to tet(B) gene

observed in 8 (10.26%) isolates, whereas 15 (18.99%) isolates carried both tet(A) and tet(B) gene. Lanz *et al.,* (2003) reported that the tet(A) gene is frequently reported at farms suffering from diarrhoea and enterotoxaemia whereas tet(B) gene was frequently constant prevalent until 1994 (Mayrand *et al.,* 2003); while at beginning of 1995 to 2000, tet(A) and tet(C) were the most dominant closely associated tetracycline resistance genes. The study conducted on three flocks with diverse histories of antimicrobial application exhibiting comparable results revealed that tet(B) was frequently dominant gene as compared to tet (A) and tet (C) (Lee *et al.,* 1993). Similarly, Sawant *et al.,* (2007) reported that tet(B) was predominant gene (93%) followed by tet(A) (7%). The tetracycline resistance *E. coli* may carry the tet(B) gene varying from 60-80% (Blake *et al.,* 2003; Wilkerson *et al.,* 2004).

Figure.1 Detection of tet(A) and tet(B) genes by multiplex PCR.
Lane A, B and C: tet(A) gene, tet(B) gene and both tet(A) & (B) gene, respectively; Lane M: 100 bp DNA ladder; Lane +ve: positive control; Lane –ve: negative control



The absence of both tet(A) or tet(B) gene in 34 out of 79 tetracycline resistant *E. coli* isolates emphasize the need for screening of these isolates for other known tet genes also. The tetracycline resistant *E. coli* isolates may carry unknown tetracycline resistant genes as observed earlier for *E. coli* isolated from humans or other animals (Miranda *et al.,* 2003; Wilkerson *et al.,* 2004). More than one tetracycline resistance genes may be found from the same E. coli isolate (Maynard *et al.,* 2003; Villedieu *et al.,* 2003). Nevertheless,

this did not have any influence on antibiotics resistance patterns of *E. coli* isolates. The high level resistance profile of tetracycline is not potentially influenced by number of genes (Villedieu *et al.*, 2003). Fairly, this might be as certain of tet genes are enclosed inside conjugative transposon. It's well known that conjugative transposons play a potential role and considered an important determinant for antibiotic resistance. The conjugative transposons are also infrequent in that their relocation activities are synchronised by tetracycline via a complex regulatory network. The carrying of single conjugative transposon may not effect as the same cell being able to receive other correlated or not correlated conjugative transposons (Chopra and Roberts, 2001). This may help *E. coli* in further dissemination of the genotypic tetracycline resistance as observed in other bacteria like *Neisseria*, *Haemophillus* and *Streptococcus* which are naturally competent to disseminate resistance genes (Roberts, 1998). The presence of tet genes can mobile genetic material like plasmid and transposon helping in the spreading or dissemination of these resistance genes (DePaola and Roberts, 1995).

The results of phenotypic resistance patterns of *E. coli* isolates are mostly influenced by gene determinants. However, the association between phenotypic and genotypic appearance of antibiotic resistance patterns might be influenced by numerous assortment inducing factors such as the usage of particular antibiotic (antibiotic-induced stress responses). Multiple phenotypic antibiotic resistance among *E. coli* isolates in relation to the frequency distribution of resistance genes indicate the presence of multi-gene resistance. These findings highlight the urgent requirement on restriction and regulation of antimicrobial agent in the veterinary practices so as to minimize the risk of development of resistant organisms especially multidrug resistance (MDR).

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