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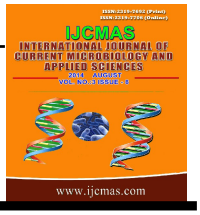

Original Research Article

Plasmid Diversity and Transferable Antimicrobial Drug Resistance, in *E.coli* Isolates from Calf Diarrhoea

Vandana Gupta[1]*, Ashish Roy[2], Sourabh Gupta[2] and Megha Katare[2]

Department of Veterinary Microbiology College of Veterinary Science & A.H., Rewa, M.P. India
[2]Department of Veterinary Microbiology College of Veterinary Science Gujarat, India
**Corresponding author*

Keywords

E.coli, Transconjugates Plasmid, Antibiotic resistance

A B S T R A C T

The present study was undertaken to analyze the plasmid profile of *E.coli* isolates, to ascertain the transferable nature of antibiotic resistance, among the isolates from calf diarrhoea cases. Nineteen transconjugates obtained on conjugation showed the transferable nature of antibiotic resistance against many antibiotics *viz*. kanamycin, cephalexin, cephaloridine, amikacin, tetracycline, enrofloxacin and co-trimoxazole. Plasmid profile analysis of wild as well as transconjugates revealed the presence of multiple (1 to 4) plasmids which were autotransferable in nature except for 2 transconjugates in which no transfer of small sized plasmid from wild strain was seen. Plasmid sized varied from 3kbp to 56kbp.The present conjugation study revealed that all the tested strains appeared to harbor conjugative plasmid

Introduction

E.coli is one of the serious pathogen that can cause tremendous therapeutic problem by developing resistance against antibiotics. The drug resistance in bacterial population is may be due to a genetic and non genetic mechanism (Choudhary 1988). Regarding genetic mechanism most drug resistant microbes emerged as a result of genetic changes and subsequent processes by antimicrobial drugs. The drug resistance may be chromosomal DNA or plasmid DNA mediated. The plasmid mediated drug resistance is caused due to the presence of drug resistance gene(s) harboring on the plasmid DNA. These genes confer the drug resistant phenomenon in the host organism (Meyer's et al.1976).Transferable multiple drug resistance has been reported among the *E.coli* isolated from disease outbreaks. This character is mostly coded by auto transferable plasmids. Plasmid associated resistance genes have been discovered for a majority of known antimicrobials including the quinolones and fluroquinolones (Hawkey, 2003; Neu, H.C. 1992) and it is not uncommon for a single plasmid to simultaneously mediate resistance to five or six antimicrobials. This ability to sequester multiple resistance genes is of particular concern to modern medicine. The present

study was undertaken to determine the plasmid mediated transferable drug resistance.

Materials and Methods

***Escherichia coli* isolates**: Twenty *E.coli* isolates were selected on the basis of Tetracycline/ Kanamycin resistance. All the isolates were obtained from the Department of Veterinary Microbiology, Anand Veterinary College.

Antibiogram: Antibiotic sensitivity test was done as per the standard disc diffusion method of Bauer *et al*1966.

1.1 Transfer of Plasmid 'R' determinant by conjugation:

Transferable drug resistance by conjugation was studied as per the method described by Kaliannan and Gupta 1975. BHI Brain Heart Infusion broth and Mac Conkey Agar were used in this study.

Strains

a) Wild strain- Twenty strains were selected for conjugation study based on their Tetracyclin/Kanamycin resistance (Table-1)

b) Field strain – *E.coli* K-12 Nal^r L^+ (ECF) was used as recipient strain and was nalidixic acid resistant.

The selected test strains and recipient strain were grown overnight in 10ml BHI broth at 37ºC. To obtain a young culture, 0.2ml of the overnight broth culture was inoculated in 10ml fresh BHI broth and incubated at 37ºC for six hrs. For conjugation, 0.5ml of both test strains as well as recipient strain from fresh cultures were inoculated in 10ml BHI broth and then incubated at 37ºC for 24 hrs. The mixed broth culture was then streaked on MCA containing tetracycline or kanamycin (30mcg) and nalidixic acid (30mcg), depending on the resistance pattern of both donor and recipient strains. The single isolated pink color colonies were then selected as transconjugants for further study. ECF fail to grow on either media containing tetracycline/kanamycin and nalidixic acid. The transconjugates obtained on conjugation were numbered by adding "T" to the wild parent strains.

In vitro transfer of Multiple antimicrobial drug resistance of Transconjugate

In vitro antibiotic sensitivity test of transconjugates was performed as per the method of Bauer *et al*

Antibiotic Discs

Ampicillin(A,10µg), Amikacin (Ak,30µg), Cephalexin (Cp,30µg), Cephaloridine (Cr,10µg), Ciprofloxacin (Cf, 5µg), Co-trimoxazole (Co,25µg), Enrofloxacin (Ex,10µg), Kanamycin (K,30µg), Tetracycline (T,30µg).

Strains

Nineteen transconjugates obtained by conjugation were then subjected to *in vitro* antibiotic sensitivity test as per the method of Bauer *et al.*

Each conjugate strain was grown in BHI broth overnight. Sterile plates of MH agar medium were seeded with about 1ml of inoculum. Antibiotic discs were placed on inoculated agar surface at about 2 cm apart. Plates were incubated at 37ºC overnight and the zones of inhibition were measured. The measurements were compared with zone size interpretative table supplied by manufacturer and zones were graded as sensitive and resistant. The recipient (ECF),

K-12 Nal^r L^+ was also tested for its antibiotic sensitivity pattern.

Plasmid profile: The plasmid DNA was prepared from *E.coli* isolates as well as from their transconjugates by Alkaline Lysis Method with SDS: according to Sambrook *et al* 1989.

Plasmid isolation Plasmid isolation was performed by Alkaline Lysis Method with SDS: Minipreparation according to Sambrook *et al* 1989 with some modifications.

Test strains

a) Selected wild strains of *E. coli* (Table 1)
b) Transconjugates of the above isolates

Reference strains

a) V517 (E382)
b) *E.coli* K-12 Nal^r L^+ (ECF)

Procedure

a) Single colony of the test strain was inoculated in 10ml Luria Broth and grown at 37^{o}C overnight for 18 hrs.
b) After the incubation, Chloramphenicol was added in culture at the concentration of 170 µg/ml for amplification of plasmid and incubated for six hrs.
c) The cells were pelleted by centrifuging at 11000 rpm for ten min at 4^{o}C.
d) The supernatant was discarded and the pellet was resuspended in 100 µl of ice-cold alkaline lysis solution I by vigorous vortexing.
e) To the above suspension, 200 µl of freshly prepared alkaline lysis solution II was added and mixed rapidly by inverting 15 times and then stored on ice for 15 min.
f) Alkaline lysis solution III (150 µl) was added and mixed by inverting the tubes 4-6 times and incubated on ice for three min.
g) Chromosomal DNA was sedimented along with the cell debris by centrifugation at 11000 rpm for 10 min.
h) The supernatant (containing plasmid) was transferred to a fresh tube.
i) An equal volume of phenol:chloroform (1:1) was added, vortexed briefly and centrifuged at 11000 rpm for 10 min at 4^{o}C.
j) The upper aqueous phase was transferred to a fresh tube.
k) The plasmid DNA was precipitated by adding 2.5 volumes of chilled absolute ethanol and stored at room temperature for two min.
l) The plasmid DNA was pelleted by centrifugation at 11000 rpm for 15 min at 4^{o}C.
m) The plasmid DNA pellet was washed twice with one ml of 70% ethanol followed by centrifugation at 11000 rpm for 15 min at 4^{o}C.
n) The pellet was air dried for 30 min to three hrs.
o) The dry DNA pellet was dissolved in 50µl of TE buffer.
p) Before running the DNA on Agarose gel electrophoresis, RNAse treatment was given by adding 1 µl of RNAse (at a concentration of 20 µg/ml) in 50 µl of the dissolved DNA, followed by incubation at 37^{o}C for half an hour in a waterbath.

Agarose gel electrophoresis: Ten microliters of Plasmid DNA was loaded in each well in 0.8% agarose gel and electrophoresis was carried out at 80 volts for minimum of 2hrs in 0.5X TBE buffer. The amplified product was visualized under UV light and documented by gel documentation system. The molecular size was determined by comparing plasmid DNA extracts of sample strains with known

plasmid V517 on agarose gel using a software gene tool.

Results and Discussion

Eighteen out of 19 isolates *viz,* AU2, AU6, AU5, AU12, AU22, OF25, AU29, AU34, OF24, AU24, JU16, VC1, VC2, AU23, AU13, AU27, AU39 and AU38 were able to transfer en bloc resistance against Cephalexin, Cephaloridine and Kanamycin (CpCrK) to the recipient strain ECF (K-12 Nal^r L^+).In 7 transconjugates *viz.* AU2T, AU6T, AU12T, AU22T, OF25T, OF24T and AU24T en bloc transfer of Amikacin, Cephalexin, Cephaloridine, Kanamycin, Tetracycline and Enrofloxacin (AkCpCrExKT) was seen. In 2 isolates AU5 and AU29, en bloc transfer of Amikacin, Cephalexin, Cephaloridine, Kanamycin and Tetracycline, (AkCpCrKT) to their respective transconjugates was achieved. In AU34T, Cephalexin, Cephaloridine, Enrofloxacin, Kanamycin and Tetracycline (CpCrExKT) was transferred. In JU16T transfer of Amikacin, Cephalexin, Cephaloridine, Enrofloxacin and Kanamycin (AkCpCrExK) was seen. Transfer of Cephalexin, Cephaloridine, Kanamycin (CpCrK) was seen in seven transconjugates *viz.* VC1T, VC2T, AU23T, AU13T, AU27T, AU39T and AU38T. Cephalexin and Kanamycin (CpK) were found to be transferred in JU13T. In 11 transconjugates *viz.* AU2T, AU12T, OF25T, AU34T, OF24T, AU24T, JU16T, AU3T, AU13T, AU27T and AU39T, ampicillin resistance was not transferred, similarly in 2 transconjugates OF25T and OF24T Co-trimoxazole resistance was not transferred. The result of transfer of multiple antimicrobial drug resistance is being shown in table 2

The plasmid profile analysis was carried out for all 19 wild isolates as well as their transconjugates. All the 19 isolates revealed possession of plasmids numbering from one to four. In the present study the different molecular size plasmids observed ranged from 4Kbp to 58Kbp. Eight of wild strains contained large plasmid of molecular weight ranging from 54Kbp to 56Kbp.

Medium sized plasmids ranging from 17Kbp to 32Kbp is found in 8 strains. Small sized plasmids ranging from 3Kbp to 8Kbp is isolated from maximum of 13 strains. Two plasmids in each AU29 (55kbp, 5.8kbp), AU6 (55kbp, 5.3kbp) and AU23 (20kbp, 4.9kbp) were isolated.

Eight isolates showed the presence of one plasmid. In seven wild strains, three plasmids were isolated. In one isolate i.e. AU12 four (54kbp, 5.5kbp, 3.9kbp and 2.8kbp) plasmid were found. All these plasmids found to be auto transferable through conjugation and posses Mdr.

Transconjugates obtained during conjugation study were also analyzed for plasmid profile and all transconjugates obtained, plasmid profile observed was similar to that of wild parent strain and this correlates with transfer of Mdr, except for AU29T in which only 1 plasmid was transfer out of 2 found in wild parent strain, similarly in AU12T only 3 plasmids were transferred out of 4 found in wild strain. Similar work related to detection of plasmid profile and their transferable natures among the *E.coli* isolates have been done by several workers.

Jones *et al.* 1978 identified a large plasmid of 80Mda conferring resistance against ampicillin, tetracycline, and streptomycin and also identified a small plasmid of 5.5Mda. Franklin *et al.*1981 identified three plasmid of molecular weight 55Mda coding for antibiotic resistance.

White *et al. 2000* identified plasmid of 225kbp responsible for florfenicol resistance in *E.coli* isolates associated with bovine diarrhoea.

Laz *et al. 2001* isolated plasmid DNA having molecular size of 28.4kbp to 39kbp from drug resistant mutants of 20 *E.coli* isolates, and also found multiple drug resistance against ampicillin, tetracycline, chloramphenicol, erythromycin, kanamycin, streptomycin and nalidixic acid.

Sherley *et al.* 2004 reported 72 plasmids varying in size from 32Kbp to 250Kbp in clinical isolates of *E.coli.* It also carried multiple drug resistance plasmids against tetracycline, trimethoprim and Chloramphenicol. Shiraki *et al.* 2004 studied the plasmid profile analysis from *E.coli* isolates and showed the presence of three large plasmids of 33Mda, 50Mda and 86Mda, out of which 33Mda and 50Mda plasmids were transferred to nine recipient strains.

Table 1 List of isolates along with their antimicrobial resistance pattern, and marker antibiotic selected for conjugation study.

S. No.	Isolate No.	Antibiotic resistance Pattern	Selected for antimicrobial Resistance to Tetracycline/ Kanamycin
1	AU2	AkACpCrExKT	T
2	AU6	AkCpCrExKT	T
3	AU5	AkCpCrKT	T
4	AU12	AkACpCrCf ExKT	T
5	AU22	AkCpCrCfExKT	T
6	OF25	AkACpCrCoExKT	T
7	AU29	AkCpCrKT	T
8	AU34	ACpCrExKT	T
9	OF24	AkACpCrCoExKT	T
10	AU24	AkACpCrExKT	T
11	JU16	AkCpCrExK	K
12	VC1	CpCrK	K
13	VC2	CpCrK	K
14	AU23	CpCrK	K
15	AU13	ACpCrK	K
16	VC5	CpCrK	K
17	AU27	ACpCrK	K
18	AU39	ACpCrK	K
19	JU13	CpK	K
20	AU38	ACpCrK	K

Note- Ampicillin(A,10μg), Amikacin (Ak,30μg), Cephalexin (Cp,30μg), Cephaloridine (Cr,10μg), Ciprofloxacin (Cf, 5μg), Co-trimoxazole (Co,25μg), Enrofloxacin (Ex,10μg), Kanamycin (K,30μg), Tetracycline (T,30μg).

Table 2 Result of conjugation for transfer of Multiple antimicrobial drug resistance by wild strain isolates to the recipient *E.coli* (K-12 $Nal^r L^+$)

Sr. No.	Isolate No.	Conjugation results\ Transconjugates	Donar Character Drug resistance Pattern	Transferable Character Drug resistance pattern
1.	AU2	AU2T	AkACpCrExKT	AkCpCrExKT
2.	AU6	AU6T	AkCpCrExKT	AkCpCrExKT
3.	AU5	AU5T	AkCpCrKT	AkCpCrKT
4.	AU12	AU12T	AkACpCrCfExKT	AkCpCrCfExKT
5.	AU22	AU22T	AkCpCrCfExKT	AkCpCrCfExKT
6.	OF25	OF25T	AkACpCrCoExKT	AkCpCrExKT
7.	AU29	AU29T	AkCpCrKT	AkCpCrKT
8.	AU34	AU34T	ACpCrExKT	CpCrExKT
9.	OF24	Of24T	AkACpCrCoExKT	AkCpCrExKT
10.	AU24	AU24T	AkACpCrExKT	AkCpCrExKT
11.	JU16	JU16T	AkCpCrExK	AkCpCrExK
12.	VC1	VC1T	CpCrK	CpCrK
13.	VC2	VC2T	CpCrK	CpCrK
14.	AU23	AU23T	CpCrK	CpCrK
15.	AU13	AU13T	ACpCrK	CpCrK
16.	AU27	AU27T	ACpCrK	CpCrK
17.	AU39	AU39T	ACpCrK	CpCrK
18.	JU13	JU13T	CpK	CpK
19.	AU38	AU38T	ACpCrK	CpCrK

Three out of 9 strains carried plasmids of 33Mda, 50Mda and 86 Mda and two carried plasmid of 33Mda, 86Mda. Thirty three Mda plasmid was detected in 2 strains. Three plasmids of 33, 50 and 61Mda were present in one isolate. Shome *et al.* 2005 isolated plasmids from 70 *E.coli* strains from piglet diarrhea, the number varied from 2 to 7 with molecular size ranging from 2Kbp to 26.4Kbp. Analysis of plasmid profiles of the isolates revealed 35 different patterns of multiple plasmids with a common one in the molecular size of 23.1Kbp.

Miles *et al.*2006 isolated one or more plasmids, ranging in size from 2Kbp to ≥ 12Kbp among seven avian and eight human tetracycline resistant *E.coli* isolates. Pignato *et al*. 2009 revealed the presence of single plasmid from 10 transconjugates of *E.coli* with a size of 125kbp, 54kbp and 60 kbp from waste waters, exhibiting multiple drug resistance patterns. Yah 2010 isolated significant number of plasmid encoded multidrug resistant salmonella and shigella isolates from diarrheal cases among humans.

The molecular weight of plasmid ranged from 1.1kbp to 4.7 kbp. Goren *et.al.* 2010 identified the interspecies transfer of 105kbp carbapenem-resistant plasmid from Klebsiella to *E.coli*, conferring the resistance to all cephalosporins, monobactums and carbapenems.

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