Original Research Article

Antibiogram and Biofilm Phenotypic Characterization of *E. coli* Isolates from Milk and Environmental Sources

Bobade Sumedha[*], R.M. Gade, S. Rajurkar, A. Raut, P. Uike and A. Bhoyar

Vasantrao Naik College of Agricultural Biotechnology, Yavatmal (M.S.), India

*Corresponding author

A B S T R A C T

Escherichia coli is responsible for a wide variety of intestinal infections, showing increasing antimicrobial resistance. Antimicrobials resistance in biofilm-forming isolates contributes to bacterial persistence which may lead major public health concern and treatment problems. The aim of this study was to study the antimicrobial resistance profile of *E. coli* with reference to biofilm production to study the possible relationship among *E. coli* isolates from cattle and their farm environment. Out of 64 samples 34 (53.12%) samples were confirmed as *E. coli*, whereas 16 (47.05%) isolates were found to be Biofilm producer on Congo red Agar. The recovered isolates (18) were further studied for Antibiotics sensitivity patterns against 6 antibiotics. The highest number of isolates was resistance to Tetracycline (66%) and Ampicillin (66%). The isolates were susceptible to other antibiotics like Chloramphenicol (77.78%), Ciprofloxacin (77.78%), Streptomycin (88.89%). All isolates were sensitive to Gentamycin. The different Antibiotic resistivity patterns have been observed among the isolates. *E. coli* is an indication of poor hygienic practices in dairy. These organisms originate from the cow's environment and infect the udder may enter the food chain by faecal contamination and pose potential public health hazards. Biofilm production by these pathogenic organism make resistant to antibiotics and there is possibility of public health threat from such drug resistance strains of *E. coli*.

Introduction

Escherichia coli is an important pathogen in bovine, capable of causing intestinal and extra intestinal infections which constitute a public health hazard. Environmental survival of *Escherichia coli* may play an important role in the persistence and dissemination of this organism on farms. Cattle are an important reservoir of *E. coli* organisms. Infection may also occur through consumption of unpasteurized milk and other foods, person-to-person transmission and direct contact with infected cattle or their manure (Rahn *et al.*, 1997). *E. coli* is commensal microbe which is the major part of normal aerobic microbial population of the intestine of humans and warm blooded animals. Its presence is considered as major indicator of faecal contamination in food and water (Karmali *et al.*, 2010). There is strong evidence that the use of antimicrobials can lead to the appearance and rise of bacterial resistance both in human and animals. These are disseminated in environment such as farm animals and derived foods, domestic and even

in wild animals, healthy humans, waste water, vegetables and other sources (Ben Sallem *et al.,* 2011). Attachment of pathogenic bacteria to food contact surfaces and the subsequent biofilm formation represent a serious threat to the food industry, since these bacteria are more resistant to antimicrobials or possess more virulence factors (Pavlickova *et al.,* 2017).

Ability to adhere to different surfaces, and formed biofilms have been important features associated with *E. coli* virulence (Bello *et al*., 2013). Additionally, resistance to antimicrobials in biofilm-forming isolates contributes to bacterial persistence which may lead to chronic infections and treatment problems (Cergole-Novella *et al*., 2015).

As a matter of fact, *E. coli* from livestock is exposed to a great selective pressure because in some countries, more than half of the antimicrobial agents are used in food-producing animals. Antibiotics have helped in reducing diseases in animal husbandry; however, there is a growing awareness of public health concerns associated with the use of antibiotics. Antibiotic is widely used to protect the infectious diseases caused by *E. coli.* More uses of antimicrobial agents are believed to enhance resistance of bacteria and it may contribute to antimicrobial agent resistance in humans acquired through the food chain. Therefore the disk diffusion method can be used to study the resistivity pattern of *E. coli* (Guerra *et al*., 2003). Several mechanism have been proposed to explain this high resistance of Biofilm including restricted penetration of antimicrobial agent into Biofilms, slow growth owing to nutrient limitation, expression of genes involved in general stress response and emergence of Biofilm specific phenotypes (Ito *et al.,* 2009).

Bacterial biofilm cause chronic infection because they show increased tolerances to antibiotics and disinfectant chemicals as well as other component of the body defence system (Hoiby *et al*., 2010). The Congo Red Agar method is fast, reproducible, and presents an advantage that the colonies remain viable in the medium for further analysis. Therefore the method was chosen in an attempt to improve its ability to identify biofilm production of *E. coli.* There are fair chances of contamination from animal product with intestinal of fecal of animals there may serve as a source of infection of human being. Thus, it is an important to study pathogenic characteristic of *E. coli* of animal origin.

The main aim of this study was to isolate and characterize *E. coli* and to investigate the correlation between antibiotic resistance against 6 antibiotics, and biofilm formation in *E. coli* recovered from bovine and their farm environment, according to their origin.

Materials and Methods

A total of 64 samples from 10 different sources viz. soil (5), fecal (11), manure (7), drainage (2), drinking water (6), tap water (2), fodder- dry fodder (12) and green fodder (4), cotton seed cake (6), milk (9) samples were collected in sterile container, labeled and transported to the laboratory for analysis. The samples were stored in cold condition for further analysis.

The isolation and identification of *E. coli* were performed as per the guidelines of Cowan and Steel (1970) and Cruickshank *et al.,* (1975) and Rappaport *et al.,* (1953). The isolates were further confirmed by biochemical reaction.

Biofilm production on congo red agar plate

The isolates were further analyzed for biofilm production on Congo red medium, prepared as per the Berkhoff and Vinal (1986) and *E. coli* isolates were streaked on the CR medium

and incubated at 37ºC for 3 days. The colonies were examined daily for color change. The *E. coli* isolates which produced intense orange or brick red colonies were considered as CR positive and those which produced grayish white colonies and remained so throughout the incubation period were recorded as CR negative.

Antibiotic sensitivity tests

Antimicrobial susceptibility testing was done by the disc diffusion method using Mueller–Hinton agar (Hi Media Laboratories, Mumbai, India).

Susceptibility of *E. coli* isolates to 6 commercially available antimicrobial disk was determined following disc diffusion method (Bauer *et al*., 1966). The antimicrobial agent used as ampicillin (AMP), tetracycline (TE), streptomycin (S), gentamycin (GEN), chloramphenicol (C), ciprophloxacin (CIP).

Results and Discussion

The present study was undertaken to investigate comparison between antibiotic sensitivity test and biofilm production among *E. coli* isolated from bovine origin.

Prevalence of *E. coli*

Out of 64 samples collected 34 (53.12%) isolates were identified as *E. coli* (Table 1). The confirmed isolates were screened for biofilm production and Antibiotic resistivity pattern.

Congo red agar test (CRA) was used to differentiate invasive and non invasive *E. coli*. In the present study *E. coli* isolates were screened for biofilm production on 0.3% Congo Red agar. Out of 34 isolates 16 were found to be biofilm producer on congo red (Table 2).

Out of 34 samples 18 *E. coli* isolates were studied for Antibiotic Sensitivity Test. Eighteen *E. coli* isolates from various sources were tasted against 6 antibiotics. The highest resistance was found for tetracycline (66%) and ampicillin (66%). The isolates were susceptible to some antibiotics like Chloramphenicol (77.78%), Ciproflloxacin (77.78%), Streptomycin (88.89%). All isolates were sensitive to Gentamycin. The different antibiotic patterns have been observed (Figure 1). The highest resistivity pattern reported in tetracycline and lowest in streptomycin and no resistance in Gentamycin.

Table.1 Frequency of prevalence of *E. coli* isolated

Sr.No	Source Soil	No. of sample collected	No. of positive sample
1.	Soil	5	3
2.	Fecal	11	8
3.	Manure	7	3
4.	Drainage	2	1
5.	Drinking water	6	3
6.	Green Fodder	4	1
7.	Tap water	2	1
8.	Dry fodder	12	7
9.	Milk	9	6
10	Cotton seed Cake	6	1
Total		**64**	**34(53.12%)**

Table.2 Summary of biofilm producing *E. coli* isolate

Sr. No	Source	No. of isolate	Biofilm Production
1.	Soil	3	1
2.	Fecal	8	6
3.	Manure	3	2
4.	Drainage	1	1
5.	Drinking water	3	1
6.	Green Fodder	1	1
7.	Tap water	1	1
8	Dry fodder	7	2
9.	Milk	6	1
10.	Cotton seed Cake	1	0
Total		34	16(47.05%)

Fig.1 Antibiotic resistivity pattern of *E. coli* isolates
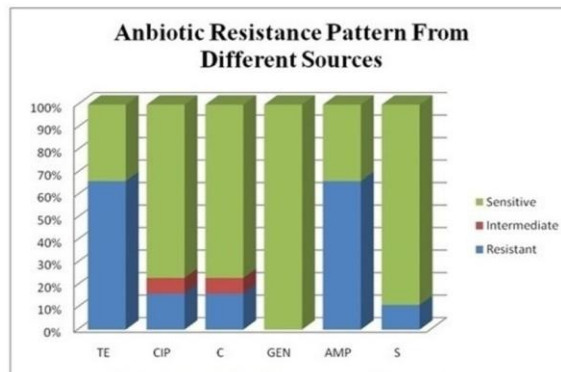

Figure : Antibiogram of *E.coli* isoaltes from different sources.

Out of 64 samples 34 (53.12%) samples were confirmed as *E. coli*, whereas 16 (47.05%) isolates were to be Biofilm producer on Congo red Agar. Thakre *et al.,* (2016) recorded the overall prevalence of *E. coli* (53.12%) isolated from fecal samples of cattle. Alam *et al*., (2013) reported an overall prevalence of *E. coli* of 69.23% with highest in the diarrhoeic stool sample (91.61%) the finding from our study showed higher prevalence was found in fecal samples. The prevalence was observed to be 85.71% and 20% of the fecal and soil samples (Parul *et al*., 2014), while in our study prevalence of 72.72% from fecal and 60% of soil was recorded.

The biofilm production study show positivity in 47.05% *E. coli* isolates. Thakrey *et al*., (2016) and Warke *et al*., (2017) reported 82.08 % and 77.67% biofilm producer isolates of fecal and environmental samples from cattle farm on Congo red agar. Parul *et al*., 2014 recorded percent positivity of

44.28% for feces and 5% for soil was reported on Congo red dye assay while in our study it was found to be 54.5% and 20% respectively. The antibiogram of pathogenic strains showed high level of sensitivity to Ciprofloxacin (93%), Gentamycin (89%) and low level of sensitivity against Ampicillin (8%) and Streptomycin (5%). All isolates were 100% resistant to Tetracycline while in this study the highest resistance was found for tetracycline (66%) followed by ampicillin (66%) and sensitive to Ciprofloxacin (77.78%), Streptomycin (88.89%), Gentamycin (100%).

The multidrug resistance pattern among six antibiotics suggest the contribution to the spread of various drug resistance strains of *E. coli*. Tadesse *et al.,* 2018 studied 24 raw cow milk samples from dairy farms (27.91%) were found to be positive for *E. coli* and highly resistant to ampicillin (70%), chloramphenicol (50%), and kanamycin (50%) and susceptible to gentamicin (100%), tetracycline (60%), and ciprofloxacin (90%) from our study resistivity in Ampicillin and tetracycline (66%), and susceptibility to chloramphenicol (77.78%), Ciprofloxacin (77.78%) and Gentamycin (100%) was recorded the variation of multidrug resistance recorded in the current study might be due to biofilm production and high antimicrobial use in dairy cattle.

In our study high prevalence, biofilm production and resistivity pattern was found in fecal sample. Animal feces are potential source of antibiotic resistant bacteria. If released into the environment, resistant strains may contaminate water and food sources and can be a potential threat to human health (Roy *et al*., 2009). Nsofor and Iroegbu, (2013) reported resistant rate of 100% to Gentamycine, 89% streptomycin, 77% to chloramphenicol, 34% to ampicillin, 34 % tetracycline. The results of this study showed highest resistivity to Ampicillin (66%) and lowest to streptomycin (11%), Chloramphenicol (16%) and 100 % sensitivity to Gentamycin.

Multiple antibiotic resistant strains can be transported from animals to humans by food chain represents public health hazard due to the fact that foodborne outbreaks would be difficult to treat.

In conclusion, the findings of this study suggest that biofilm producing strains of *E. coli* from milk and environmental samples from cattle farm can be an important reservoir for various multidrug resistant determinants. The presence of foodborne pathogens in milk can be due to direct contact with contaminated sources in the dairy farm environment and to excretion from the udder of an infected animal. The presence of pathogenic *E. coli* are of prime importance due to their public health implications, which enter into the food chain through the consumption of contaminated milk or through farm runoff water, soil which greatly influenced by the application of manure. Antimicrobial resistance is more common in biofilm forming *E. coli* and can be a source of transferring antimicrobial resistant bacteria to human. Biofilm production is a common phenomenon and it is one of the important mechanisms of antimicrobial resistance among the foodborne pathogen. Antimicrobial resistance is more common in biofilm forming *E. coli* and can be a source of transferring antimicrobial resistant bacteria to human. Bacteria gradually become resistant to routinely used antibiotics may also lead to a failure of antimicrobial therapy. Antimicrobial resistance is a global health concern in both human and animals. Therefore there is need of implementation of effective hygienic measures for food safety at farm level as well as steps must be taken to control the overuse of antibiotics.

References

Alam, M J, Rahman, M P, Siddique, M F R Khan and Rahman. M B. (2010). Antibiogram and plasmid profiling of *E. coli* isolates. *Int. J. BioRes*. 1(3): 01-07.

Bauer, A.W. Kirby, W.M.M., Sheris, Jc. and M. Truck. (1966). Antibiotic susceptibility testing by a standardized single disk method. *Am J Clin Pathol.* 145: 225-230.

Bello, B.K, Adebolu, T.T and Oyetayo, V.O. (2013). Antibiogram and plasmid profile of *Escherichia coli* isolates in Well Water In Akure, South Western Nigeria. *IOSR Journal Of Pharmacy*. 3(7): 30-37.

Ben Sallem, R., Ben Slama, K., Estepa, V., Jouini, A., Gharsa, H., Klibi, N., Sáenz, Y., Ruiz Larrea, F., Boudabous, A. and Torres, C. (2011) Prevalence and characterisation of extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* isolates in healthy volunteers in Tunisia. Eur. J. Clin. Microbiol. *Infect. Dis*. 31: 1511–1516.

Berkhoff, H.A., and A.C. Vinal (1986). Congo red medium to distinguish between invasive and non-invasive *E. coli* pathogenic for poultry. *Avian Dis*. 30: 117-121.

Cergole-Novella M., Pignatari, A. and Guth B., (2015) Adhesion, biofilm and genotypic characteristics of antimicrobial resistant *Escherichia coli* isolates. *Brazilian Journal of Microbiology*. 46(1): 167-171.

Cowan, S.T. and K.J. Steel (1970). Manual for the identification of Medical Bacteria. The Syndics of the Cambridge Univ. Press, Bentley House 200, Euston Road, London, U.K

Cruickshank, R., J.P. Duguid, B.P. Marmoin and R.H.A. Swain (1975). Medical Microbiology: 2, 12th edn. Churchill Livingstone, Edinburgh, London and New York.

Guerra, B., Avsaroglu, M. D., Junker, E., Schroeter, A., Beutin, L., and Helmuth, R. (2007). Detection and characterisation of ESBLs in German *Escherichia coli*, isolated from animal, foods, and human origin between 2001-2006. *Int. J. Antimicrob. Agents* 29(2): 270-271.

Hoiby N., Djarnsholt T., Givskov N., Molin S., Ciofu O., Antibiotic resistance of bacterial biofilm (2010). *Internationl J. of Antimicrobial Agents*. 35 (4):322-332.

Ito A., Taniuchi A.,May T.,Kawata K. and Okabe S., (2009). Increased Antibiotics Resistance Of *Escherichia coli* in Mature Biofilms. *Appl. Environ. Microbiol*. 75: 4093-4100.

Karmali, M.A., Gannon, V. and Sargeant, J.M. (2010) Verocytotoxin-producing (VTEC).*Vet Microbiol*.140: 360–370.

Nsofor C.A. and Iroegbu C.U. (2013). Antibiotic Resistance Profile of *Escherichia coli* Isolated from Five Major Geopolitical Zones of Nigeria. *J. Bacteriol. Res*. 5(3):29-34.

Parul, Basanti Bist, Barkha Sharma and Udit Jain (2014) Virulence associated factors and antibiotic sensitivity pattern of *Escherichia coli* isolated from cattle and soil. *Veterinary World* 7(5): 369-372.

Pavlickova S., Klancnik A., Dolezalova M., Mozina S.S. and Holko I., (2017) Antibiotic resistance, virulence factors and biofilm formation ability in *Escherichia coli* strains isolated from chicken meat and wildlife in the Czech Republic. *J. Environ Sci Health* 52(8):570-57.

Rahn K., Renwick S. A., Johnson R. P., Wilson J. B., Clarke R. C., Alves D.,

Mcewen S., Lior H. and Spika J. (1997) Persistence of *Escherichia coli* O157:H7 in dairy cattle and the dairy farm environment. *Epidemiol. Infect.*, 119:251-259.

Rappaport F. and Sark G.J. (1953) Culture medium to determine motility and to differentiate intestinal pathogens. *Am.J.Clin.Pathol*. 23:948.

Roy K, Lebens M, Svennerholm A, and Teneberg S (2009). Enterotoxigenic *Escherichia coli EtpA* mediates adhesion between flagella and host cells. *Nature* 457: 594–598.

Tadesse H. A., N. B. Gidey, K. Workelule, H. Hailu, S. Gidey, A. Bsrat, and H. Taddele (2018) Antimicrobial Resistance Profile of *E. coli* Isolated from Raw Cow Milk and Fresh Fruit Juice in Mekelle, Tigray, Ethiopia. *Veterinary Medicine International,* 1-7.

Thakre T., Warke S., Bobade. S and D.R. Kalorey. (2016). Characterization of *E. coli* pathotypes of bovine and livestock farm environment origin. *Indian J. Anim. Res.*, 1-5.

Warke, S., Bobade, S., and Ingle, V. (2017). Isolation and Molecular Characterization of ETEC and NTEC *Escherichia coli* from cattle farm with reference to virulence marker gene. *Int. J. Liv. Res.*, 7(10): 1-7.