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Basant
Ph.D. Scholar, Department of Animal Biotechnology, Division of Veterinary Biotechnology, ICAR-Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh, India

BN Shringi
Head, Department of Veterinary Microbiology and Biotechnology, College of Veterinary and Animal Science, Rajasthan University of Veterinary and Animal Sciences, Bikaner, Rajasthan, India

Sudesh Kumar
M.V.Sc. Animal Biotechnology, Department of Veterinary Microbiology and Biotechnology, College of Veterinary and Animal Science, Rajasthan University of Veterinary and Animal Sciences, Bikaner, Rajasthan, India

Parma Ram Gorachiya
Ph.D. Livestock Products Technology, College of Veterinary and Animal Science, Rajasthan University of Veterinary and Animal Sciences, Bikaner, Rajasthan, India

Rekha Panwar
M.V.Sc. Department of Veterinary Microbiology and Biotechnology, College of Veterinary and Animal Science, Rajasthan University of Veterinary and Animal Sciences, Bikaner, Rajasthan, India

Corresponding Author
Basant
Ph.D. Scholar, Veterinary Biotechnology, Division of Veterinary Biotechnology, ICAR-Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh, India

Antimicrobial resistance profile of *Escherichia coli* isolates from mastitic milk samples

Basant, BN Shringi, Sudesh Kumar, Parma Ram Gorachiya and Rekha Panwar

Abstract
Antimicrobial resistance is increasing at an alarming rate and becoming major threat to human as well as animals. The present study aimed to isolation and proteomic based identification of *Escherichia coli* (*E. coli*) isolated from clinical mastitic milk samples and antimicrobial susceptibility pattern towards commonly used antimicrobial agents in mastitis. A total of 28 mastitic milk samples from cattle were evaluated for incidence of *E. coli* and antibiogram. Out of 28 milk samples, 10 (35.71%) were identified as *E. coli*. All the 10 isolates of *E. coli* were tested for *in vitro* sensitivity towards 23 antibacterial drugs. The highest resistance (*i.e.,* 100%) was attributed towards oxacillin, vancomycin, tetracycline, clindamycin, erythromycin, rifampicin, and co-trimoxazole. It was concluded that microbiological and antibiogram studies are necessary for treatment and control of the disease.

Keywords: *E. coli*, resistance profile, AMR

Introduction
Antimicrobial resistance (AMR) is one of the most important health concerns. AMR has an important health and financial association for humans, animals and the environment (WHO, 2018) [30] and according to one OIE report, this burden will dramatically increase the number of human deaths in the future (O'Neill, 2016) [20]. Antimicrobial resistance in bacterial pathogen is a global health threat and need efforts to improve this worldwide challenge associated with high morbidity and mortality (Velez *et al*., 2016) [28]. Resistance of important bacterial pathogens to common antimicrobial therapies and emergence of multidrug-resistant bacteria are increasing at an alarming rate (Akova, 2016) [1]. The declining effectiveness of antibiotics imposes potentially large health and economic burdens on societies and antibiotic resistance is the next great global challenge and significant action to combat it is required. The use of antibiotics in food animals play a major role in human health, as antibiotic-resistant bacteria can be transmitted between humans and animals through contact, food products and from the environment (Landers et al., 2012) [16]. New Delhi Metallo β-Lactamase-1 (NDM-1) and Expanded Spectrum β-Lactamases (ESBL) producing gram-negative bacteria (Ghatak *et al.,* 2013) [10] isolated in milk samples obtained from cattle with mastitis have been reported (Eisenberger *et al.,* 2018) [7]. Vancomycin-resistant *Staphylococcus aureus* (VRSA) strains in samples obtained from surgical site (Bhattacharyya *et al.,* 2016) [2] and multidrug resistant *E. coli* was also isolated from the milk samples with clinical mastitis (Todorvic *et al.,* 2018) [26].
E. coli, is a common universal inhabitant of gastrointestinal tract and can act as a commensal or a pathogen, being commensal bacteria, it is always exposed to antibiotic stress when an individual is treated with an antibiotic such exposures also increase the chances of *E. coli* to become an MDR. Most *E. coli* strains are harmless, but some pathogenic strains (enteropathogenic *E. coli*) are mainly responsible for bloody diarrhea, gastroenteritis, dysentery and urinary tract infections in humans and animals (Levine, 1987) [17]. The occurrence of multidrug resistant bacteria is a serious problem in the treatment of bacterial infections (Bolhuis *et al.,* 1997) [3]. Increased use of antibiotics has also raised their enrolment in transfer of resistance genes to gut bacteria (Van *et al.,* 2001) [27]. Therefore, it is important to study resistance profiles of *E. coli*. The prevalence of resistance in commensal *E. coli* is an indicator for the selective pressure by antibiotics used and resistance to be expected in commensal bacteria. The prevalence of resistance in commensal *E. coli* is a good indicator for the selective pressure by antibiotics used and resistance problem to be expected in pathogenic bacteria (Salehi and Bonab, 2006) [23].

Materials and Methods

Sampling

In the present investigation, a total 28 milk samples were collected from the veterinary clinics complex, cvas, Bikaner, Rajasthan. The samples were collected aseptically and placed in sterile container, taking all precautions to avoid contamination.

Isolation and species level confirmation

The procedure for isolation and identification of bacterial culture was followed as per the standard protocols (Carter *et al.,* 1994) [4]. For primary cultivation each isolate was streaked on MacConkey agar plates in primary, secondary, and tertiary fashion in order to obtain isolated colonies of bacteria. These petri plates were incubated for 24 hrs at 37˚C. After the revival organism isolated colonies were further streaked on to Eosin Methylene Blue (EMB) agar. Besides this, MALDI-TOF MS was used for species level conformation also used as per the method described by Singhal *et al.,* (2015) [24].

Antibiotic sensitivity test

To determine resistance profile of *E. coli* isolated from clinical samples disc diffusion method was used as per Bauer *et al.,* (1966) and according to guidelines of Clinical Laboratory Standard Institute (CLSI) against 23 antibiotics of different classes.

1. In brief, the isolates were inoculated in sterile 5 ml nutrient broth, incubated for 18 hr at 37ºC and then the opacity was adjusted to 0.5 McFarland opacity standards with Normal saline solution.
2. Each inoculum was then spread over the surface of Mueller-Hinton agar with the help of sterile swab.
3. Plates were allowed to dry for 10 min at 37oC and then antibiotic discs were carefully placed on the surface with enough space around each disc for diffusion of the antibiotic.
4. Plates were incubated for 24 hrs at 37ºC and the zone of inhibition of growth of the organism around each disc was measured in millimeters.

Result

Isolation and identification of *E. coli*

A total 10 (35.71%) isolates of *E. coli* were isolated from 28 milk samples of cattle with clinical mastitis on the basis of cultural characteristics and biochemical tests. *E. coli* isolates revealed characteristic rose pink colonies (lactose fermenting type) on MacConkey agar plates. Pink colonies were further streaked on Eosin Methylene blue (EMB) agar on which all ten isolates of *E. coli* produced greenish metallic sheen colonies on EMB agar and after that the MALDI-TOF MS used for species level conformation in which results were expressed in percent probability, where 90-100%, 80-90%, 70-80% and 0% represents excellent peaks, moderate peaks, lower peaks and no peaks respectively. This technique is consistent with 16S rRNA gene sequencing. One of the major advantages of using MALDI-TOF technology for bacterial identification is the time required to analyse, which is reduced from 24-48 hr to less than an hour.

In the antibiogram study of the *E. coli* isolates, the highest resistance (*i.e.,* 100%) was shown against oxacillin, vancomycin, tetracycline, clindamycin, erythromycin, rifampicin, and co-trimoxazole. The resistance against cefuroxime, nitrofurantoin, ceftazidime, kanamycin, and chloramphenicol was also reported to be high, *i.e.,* 90%, 70%, 60%, 60%, and 50%, respectively. Among the cephalosporin, the highest resistance was shown against 2nd generation cephalosporin drug, *i.e.,* cefuroxime (90%) while among the cell wall synthesis inhibitors, the highest resistance was shown against oxacillin and vancomycin drug (100% each). Among the 30S ribosomal protein synthesis inhibitors, the highest resistance was shown against tetracycline (100%) while amongst the 50S ribosomal protein synthesis inhibitors, the highest resistance was found to be against clindamycin and erythromycin (100% each). RNA synthesis inhibitor and antimetabolite antibiotic drug, *i.e,* Rifampicin and co-trimoxazole each were reported to be fully resistant (100%) against *E. coli* isoaltes of mastitic milk origin.

Among cephalosporins, the 4th generation cephalosporin drug, *i.e,* cefipime was found to be fully sensitive (100%) against *E. coli* isolates. In the same way, the polypeptide drug, *i.e,* polymyxin B and DNA synthesis inhibitor drug, *i.e,* levofloxacin was also reported to be 100% sensitive against *E. coli* isolates.

Table 1: Antibiogram obtained for *E. coli* isolates:

Mechanism	Class of antibiotic	Antibiotics	Sensitive (%)	Intermediate (%)	Resistance (%)
	1st gen. Cephalosporin	Cephalothin (CEP)	60	40	0
Cell wall synthesis inhibitor	2nd gen. Cephalosporin	Cefuroxime (CXM)	0	10	90
	3rd gen. Cephalosporin	Ceftazidime (CAZ)	20	20	60
	4th gen. Cephalosporin	Cefipime (CPM)	100	0	0
	Penicillinase stable	Oxacillin (OX)	0	0	100
	Aminopenicillin	Ampicillin (AMP)	80	20	0
	Carboxypenicillin	Ticarcillin (TI)	90	0	10
	Polypeptide	Polymyxin-B(PB)	100	0	0
	Monobactums	Azetreonam (AT)	40	20	40
	Carbapenems	Meropenam (MRP)	80	20	0
	Glycopeptides	Vancomycin (VA)	0	0	100
Protein synthesis inhibitor (30-S)	1st gen. aminoglycoside	Kanamycin (K)	40	0	60
	2nd gen. aminoglycoside	Gentamicin (GEN)	60	10	30
	3rd gen. aminoglycoside	Amikacin (AK)	60	0	40
	Natural tetracycline	Tetracycline (TE)	0	0	100
Protein synthesis inhibitor (50-S)	Lincosamides	Clindamycin (CD)	0	0	100
	Macrolides (50-S)	Erythromycin (E)	0	0	100
	Phenicoles (50-S)	Chloramphenicol (C)	50	0	50
DNA synthesis inhibitor	2nd gen. quinolone	Ciprofloxacin (CIP)	90	0	10
	3rd gen. quinolone	Levofloxacin (LE)	100	0	0

RNA synthesis inhibitor	RNA synthesis inhibitor	Rifampicin (RIF)	0	0	100
Antimetabolite antibiotics	Sulphonamide	Nitrofurantoin (NIT)	30	0	70
	Combination of sulpha + trimethoprim	Co-trimoxazole (COT)	0	0	100

Discussion

There are several mechanisms behind emergence of antibiotic resistance in microbes. It can either be inherited/acquired or non-inheritant/adaptive mechanisms. Adaptive mechanisms include expression of efflux pumps, reduced number of porins and other cell surface modifications created by stress of low-level antibiotics (Bhattacharya *et al.,* 2016) [2]. The most common reason behind emergence of AMR is huge and unmonitored use of antibiotics in human as well as veterinary medicine (Mia *et al.,* 2017) [19]. By limiting the use of antibiotics especially as a growth promoter in livestock and by searching some potent alternatives to antibiotics can only tackle this global health threat. Gram negative bacteria are more prone to become multidrug resistance. Potent solution to combat this global health challenge is much required action of present time.

Various antibiotics used for Antibiotic sensitivity test (AST) in this study has also been reported by other workers viz. Farooq *et al.,* (2008) [8], Ranjan *et al.,* (2011) [22], Kisku and Samad (2013) [14] Preethirani *et al.,* (2015) [21], and Verma *et al.,* (2018) [29]. There has been evidence of increase in resistance to wide range of drugs in *E. coli* from animals in India and abroad (Dhakal *et al.,* 2007 [6], Sumathi *et al.,* 2008 [25], Kalmus *et al.,* 2011 and Jeykumar *et al.,* 2013) [13, 12].

Prudent use of antibiotics should be considered in dairy farm since many strains are resistant to common antibiotics as described in this study. Potential drug resistant pathogens in otherwise normal dairy may be a serious concern for public health. Current findings warrants further studies with the isolated strains of bacteria. The present study has demonstrated the existence of alarming levels of resistance of *E. coli* to commonly used antimicrobial agents in the study farms and the results are suggesting a possible development of resistance from prolonged and indiscriminate usage of some antimicrobials. It is therefore, very important to implement a systemic application of an *in vitro* antibiotic susceptibility test prior to the use of antibiotics in both treatment and prevention of intra-mammary infections.

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Competing interests

Authors have declared that no competing interests exist.

Authors' contributions

This work was carried out in collaboration among all authors. Author B. designed the study, wrote the protocol, conducted the experiments and wrote the first draft of the manuscript. Other authors managed the analyses of the study and contributed in conducting experiments. All authors read and approved the final manuscript.

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