Biological Forum – An International Journal **15(8a): 23-27(2023)**

ISSN No. (Print): 0975-1130
ISSN No. (Online): 2249-3239

Antimicrobial Resistance of Microbial Flora of cervical Mucosal Discharge Associated with Reproductive Performance of Cow Estrus

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(Received: 07 June 2023; Revised: 25 June 2023; Accepted: 23 July 2023; Published: 15 August 2023)
(Published by Research Trend)

ABSTRACT: The use of antimicrobial agents is becoming increasingly widespread and alarming, not only in India but throughout the world. Controlling the misuse of antimicrobials is a challenge that often leads to the development of bacterial resistance and increased costs associated with chronic disease and healthcare services. Investigating AMR in bacteria isolated from cattle cervix prior to insemination is the main goal of this study. Cervical mucosal discharge (CMD) were collected aseptically from 18 cows between 3 to 6th parity prior to their first insemination and transported to the laboratory in the nutrient broth for carrying microbiological examination. After culturing on agar plates, bacterial isolates were identified by conventional and molecular methods. Antimicrobial susceptibility test was determined by disc diffusion technique against 12 antibiotics drugs. Bacteria isolated were mostly *E. coli* (61.11%), *Staphylococcus* spp. (11.11%), Mixed Infection (16.67%) and Candida spp. (11.11%). *E. coli* bacteria are most commonly associated with the cervical mucosal cases. The present results revealed that *E. coli* were highly sensitive against ciprofloxacin, gentamycin, and levofloxacin followed with cotrimoxazole (81.8%), nitrofurantoin (72.7%), oxytetracycline (72.7%) and ampicillin/sulbactum (63.6%). In conclusion, bacteria from the cow cervix showed low resistance to most antibiotics and antibacterial resistance may increase with increasing parity. It was also observed that the isolated *E. coli* have average resistance of 30% to seven antimicrobials. The highest resistance rates were seen with cefixime (81.8%) followed with trimethoprime (72.7%), ampicillin/sulbactum (36.4%), cotrimoxazole (18.2%), nalidixic acid (36.4%), nitrofurantoin (27.3%) and oxytetracycline (27.3%). The current study suggested that CMD should be evaluated more carefully when there are infertility problems.

Keywords: Antimicrobial resistance, Cervical swabs, antibiotics.

INTRODUCTION

The dairy industry's major goal is to provide milk for the consumer market. Over the past several decades, milk yield of cows has increased markedly. Milk production of individual cows depends on their ability to become pregnant because the lactation cycle is initiated and renewed by pregnancy. In an effort to gain the greatest efficiency and lifetime productivity, dairy cattle are inseminated and pregnancy is established while dairy cows are lactating. Reproductive performance of dairy cows influenced a herd's profitability and good heat detection and conception rates provided opportunities for management control (Grohn and Rajala-Schultz 2000).

Poor reproductive performance is an important production-limiting factor. There are many factors that directly or indirectly influence the reproductive performance (RP) of cows. Cow infertility is affected by many specific and nonspecific pathogens of the genital tract. Widespread resistance to antimicrobial substances is causing a severe problem globally when trying to treat some bacterial infections (O'Neill, 2014). Cervical mucous discharge (CMD) is a mechanical barrier against pathogen of the uterus. Normally a cow in estrus discharges a viscous liquid from the vulva. The healthy liquid is clear, originates from the cervix and has no bad odour. CMD of cows and heifers with abnormal appearance in estrous cycle is one of the factors that farmers or artificial inseminators consider it as a RP suppressor (Mahmoudzadeh *et al.*, 2001). With the trend of decreasing profit in dairy farming reported worldwide, it is necessary to identify where efficiency improvements can be made (Bishop, 1964).

Therefore, the aim of the present study was to describe whether abnormal cervical mucus discharge (ACMD) or pathogens such as bacteria and fungi in CMD have effects on RP of cows and heifers in estrus.

MATERIAL AND METHODS

A. Ethical approval

All the trial was conducted after the approval from the Institutional Animal Ethical Committee (IAEC), Sardar Vallabhbhai Patel University of Agriculture & Technology, Meerut-250110 (Registration No. 250/GO/Re/SL/19/CPCSEA).

B. Animals

Eighteen cows between 3 to 6th parity of approximately similar health status and body weight were selected for the study. The experimental animals were maintained under identical and optimal conditions of management and nutrition during the entire study period. The experimental animals were inseminated and divided into early pregnant and non-pregnant groups consisting of 9 animals each. The animals were synchronized using GPG protocol.

C. Sampling of cervical mucous discharge

The cervical mucous discharge (CMD) swab from each animal was taken 5 to 30 min before AI. After restraining the animal and securing its tail, the perineal region was washed, cleaned with soap 3-4 times and dried out. Swabs for CMD were obtained using a sterile double guarded swab device (Hi-media, India). Sterile swab was inserted through the vagina into the lumen of the cervical canal, guided by palpation per rectum. The swab was rotated three-four times against the mucosa while moving the swab backward and forward and then it was withdrawn under rectal guidance by means of the conventional artificial insemination technique. All the swabs were placed in transport medium and transported at ambient temperature to the laboratory in Department of Veterinary Microbiology, SVPUAT for carrying microbiological examination within 3 h of sampling.

D. Microbiological Examination

Swabs were streaked onto Brain heart infusion agar (BHI), MacConkey lactose agar (MLA) and Sabouraud dextrose agar (SDA) (Hi-media, India) plates. Brain heart infusion agar and MacConkey agar plates were incubated at 37°C for 24 hours in aerobic incubator. Sabouraud dextrose agar plate that was plated for mould sp. and yeast sp. isolation was incubated at 37°C and 25ºC, respectively. Identification of the bacteria was based on colony morphology and microscopic morphology, biochemical and growth characteristics of the isolates (Verma *et al.,* 2018). The identified colonies were stored in glycerol stock at -20℃ until required molecular and for resistance testing, when they were cultured for 24 h on blood agar at 37℃ to allow pure colonies to be sub-cultured for a further 24 h.

E. Antibiotic susceptibility test

Antibiotic susceptibilities of the strains isolated from the samples in the study were determined by Kirby-Bauer Disc Diffusion Method according to Verma *et al.* (2018). The antibiotic discs were Ampicillin/Sulbactam (AMS)-30/15mcg, Cefixime (CFM)-5mcg, Ciprofloxacin (CIP)-5 mcg, Co-Trimoxazole (COT) - 25mcg, Gentamicin (GEN) - 10mcg, Levofloxacin (LE)-5mcg, Nalidixic Acid (NA)-30mcg, Nitrofurantoin (NIT)-300mcg, Oxytetracycline (O)-30 mcg & Trimethoprime (TR)-5mcg. The results were recorded as susceptible (S) or resistant (R).

F. Genomic DNA extraction

The DNA was isolated as per Christa Ewers *et al.* (2006). Briefly, 2 ml of overnight culture of *E. coli* were taken in sterilized eppendorf and centrifuge at 10,000 rpm for 30 mins. Supernatant was discarded and pellets were mixed with 200 µl of NSS and vortexed. The eppendorfs were boiled in boiling water for 10 mins and immediately keep in ice for 5 mins. This step was repeated for 3 times followed by centrifugation for 10,000 rpm for 30 mins. Supernatant was taken in fresh eppendorf tube and stored at -20˚C until used for PCR amplification.

G. Oligonucleotide primers

The genus specific PCR for the *E. coli* used was uidA-Forward gene 5'- CTGGTATCAGCGCGAAGTCT-3' and uidA-Reverse gene 5'-AGCGGGTAGATATCACACTC-3' (Anbazhagan *et al.,* 2010).

H. Polymerase Chain Reaction

All reactions were carried out in a final volume of 25 µl. PCR mixture consisted of 1X PCR buffer (50 mm KCl, 20 mM Tris HCl), 1.5 µl of 25 mM MgCl2, 0.5 µl of 10 mM deoxynucleotide triphosphat (dNTP) mixture, 0.5 µl of 20 µM primers, 1.5U Taq DNA polymerase and DNA template 5 µl. The DNA was run in the thermocycler (Bio-rad, Germany). Amplification conditions were as follows: Initial denaturation at 95ºC for 4 min, followed by 30 cycles of 1 min at 95ºC, 1 min at 55ºC, 1 min at 72ºC and final extension at 72ºC for 10 min. The PCR products were analysed by agarose gel electrophoresis on 1.5% agarose gel containing 1 µg/ml ethidium bromide and bands were visualized under Gel doc system (Biorad, Germany).

I. Statistical Analysis

Data were evaluated with SPSS version 20. Comparison between different isolates' resistance to an antimicrobial was performed using the chi-square test. The threshold for statistical significance was $p < 0.05$.

RESULTS AND DISCUSSION

The purpose of this study was to examine the occurrence of antimicrobial resistance in the bacteria from cervical mucous discharge of cow. During the study period none of the cows exhibited any overt clinical signs of diseases. A total of 18 cervical mucosal discharge (CMD) swabs were collected from cow.

A. Prevalence of bacteria

The bacteria isolated in this study were mostly *E. coli*, *Staphylococcus* spp., *Candida* spp., and some mixed infection (Gram positive rods & Gram-negative rods) (Table 1) isolates in agreement with previous reports (Griffin *et al.,* 1974; Dohmen *et al.,* 1995; Sheldon *et al.,* 2002). However, parallel to the finding of

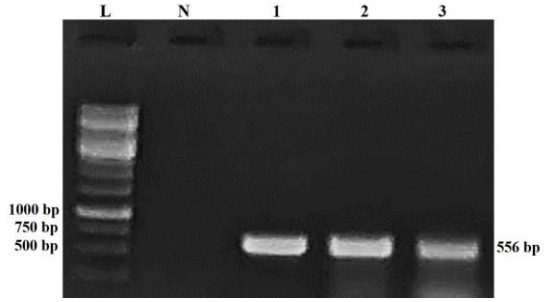
Panangala *et al*. (1978) our findings indicated that the most frequently isolated bacteria were *E. coli* and *Staphylococcus* spp. This may prove that *E. coli* and *Staphylococcus* spp. present in normal flora of animals but they may also play a role in genital tract infections. Overall, our data indicated that vaginal bacterial flora in cows affected by metritis was dominated by strains of *E. coli*, supporting previous observations (Ambrose *et al.,* 1986). Bacteria found within the microbiota are thus likely to be contaminants from the environment (Mixed infection), the cow's skin (*Staphylococcus* spp.), or faecal material (*E. coli*), rather than representing a stable flora autochthonous to the reproductive tract.

B. Confirmation by PCR

All elevenstrains identified by cultural methods as *E. coli* were further identified by PCR protocols and found positive for the *E. coli* with the product size of 556 bp (Fig. 1).

C. Antimicrobial resistance determination

All isolates showed a varying degree of sensitivity to different antibiotics (Table 2), regardless of the reproductive status of the animals from which they originated. All the isolated *E. coli* were 100% susceptible to ciprofloxacin, gentamycin, and levofloxacin followed with cotrimoxazole (81.8%), nitrofurantoin (72.7%), oxytetracycline (72.7%) and ampicillin/sulbactum (63.6%). It was also observed that the isolated *E. coli* have average resistance of 30% to seven antimicrobials. The highest resistance rates were seen with cefixime (81.8%) followed with trimethoprime (72.7%), ampicillin/sulbactum (36.4%), cotrimoxazole (18.2%), nalidixic acid (36.4%), nitrofurantoin (27.3%) and oxytetracycline (27.3%). These finding were agreed to the records of Barman *et al.,* (2013) where most of the organisms were highly sensitive to enrofloxacin and moderately sensitive to ampicillin, oxytetracycline, and gentamicin. A similar pattern of antibiotics was also found for fluoroquinolones, ciprofloxacin and gentamicin (Singh *et al*., 2018, Kumar *et al.,* 2018). Highest sensitivity of 92% and 90% was recorded for ciprofloxacin and gentamicin, respectively in another study (El-Kader and Shehata 2001). The Chi-square score trend and indicates the highly significant value 39.004 (P<0.001) (Table 3). The fact remains that development of resistance to antibiotics is a serious threat to both human and animal health and that usage of antibiotics for non-therapeutic purposes is at best controversial and at worst imprudent. It is known that using antibiotics allows resistant bacteria to be selected and that the genes for resistance can spread to other bacteria, including pathogens. Antibiotics play a major role for the treatment of uterine infections either through intrauterine or systemic route. However, the emergence of bacterial resistance is the key factor responsible for their restricted use over a period of time (Barman *et al.,* 2013). The use of accurate antibiogram is a required necessity to solve such problems.



Lane L: 1kb Ladder; Lane N: NSS (Negative Control); Lane 1-3: Samples

Fig. 1. Molecular confirmation of *E. coli* isolates.

Table 1: Distribution of the microorganisms isolated from cows (n=18).

Sr. No.	Microorganism	Isolates	Percent (%)
1.	*E. coli*	11	61.11
2.	*Staphylococcus* spp.	02	11.11
3.	Mixed infection	03	16.67
4.	*Candida spp.*	02	11.11
	Total	**18**	**100**

Table 2: Pattern of antimicrobial resistance of *E. coli.*

Sample	Antibiotics (Zone of inhibition) (in mm)									
	AMS	CFM	CIP	COT	GEN	LE	NA	NIT	O	TR
E-1	23	R	28	23	19	25	21	19	20	27
E-2	24	R	20	13	19	27	22	22	21	R
E-3	23	R	25	21	19	25	21	22	19	R
E-4	25	21	22	25	23	25	11	25	22	29
E-5	R	23	21	25	16	24	19	19	25	29
E-6	21	R	22	17	18	21	R	15	R	R
E-7	R	R	22	R	16	23	R	R	18	R
E-8	R	R	20	R	16	17	R	R	R	R
E-9	R	R	19	15	19	27	22	22	21	R
E-10	21	R	22	17	18	20	R	R	R	R
E-11	23	R	25	21	19	22	21	22	19	R

R: Resistant

Table 3: Statistical data analysis of antibiotics pattern against *E. coli.*

Antibiotics		Sensitive	Resistance	Total
Ampicillin/Sulbactum (AMS)	Count	7	4	11
	%within Antibiotics	63.6%	36.4%	100.0%
Cefixime (CFM)	Count	2	9	11
	%within Antibiotics	18.2%	81.8%	100.0%
Ciprofloxacin (CIP)	Count	11	0	11
	%within Antibiotics	100.0%	0.0%	100.0%
Co-Trimoxazole (COT)	Count	9	2	11
	%within Antibiotics	81.8%	18.2%	100.0%
Gentamicin (GEN)	Count	11	0	11
	%within Antibiotics	100.0%	0.0%	100.0%
Levofloxacin (LE)	Count	11	0	11
	%within Antibiotics	100.0%	0.0%	100.0%
Nalidixic Acid (NA)	Count	7	4	11
	%within Antibiotics	63.6%	36.4%	100.0%
Nitrofurantoin (NIT)	Count	8	3	11
	%within Antibiotics	72.7%	27.3%	100.0%
Oxytetracycline (O)	Count	8	3	11
	%within Antibiotics	72.7%	27.3%	100.0%
Trimethoprime (TR)	Count	3	8	11
	%within Antibiotics	27.3%	72.7%	100.0%
Total	Count	77	33	110
	%within Antibiotics	70.0%	30.0%	100.0%

Chi-Square value is 39.004 (P<0.05)

CONCLUSIONS

The bacteria isolated in this study from all animals were mostly *E. coli*, Staphylococcus spp., Candida spp. and mixed infection and were evenly distributed among individuals and farms. *E. coli* were more common followed with *Staphylococcus* in cows. A higher concentration of antibiotics was required to slow bacterial growth in isolates from cows which might suggest that resistance in the cervical flora could develop in response to the antibiotics.

FUTURE SCOPE

Further studies are required on cervical mucus discharge which should be evaluated more carefully when there are infertility problems. Future, researches need to be conducted more experiments on uterine pathogens and their effects on the reproductive performance and their clinical treatments in larger herds.

Author contributions. MKS and SK designed the experiment, Shambhavi masters student who did her work. AKV and AT help for the animal experimentation and in collection of the sample, HV executed the microbiological work, KR executed the microbial work of the bacterial isolates, AG help in the statistical analysis of the data by using the SPSS.

Acknowledgement. The authors are highly thankful to the Dean, College of Veterinary & Animal Sciences and Hon'ble Vice Chancellor, Sardar Vallabhbhai Patel University of Agriculture & Technology, Meerut for providing financial assistant and necessary facilities to carry out this work.

Conflict of Interest. None.

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How to cite this article: Shambhavi, Manish Kumar Shukla, Harshit Verma, Suresh Kumar, Ashutosh Tripathi, Atul Kumar Verma, Atul Gupta and Koushlesh Ranjan (2023). Antimicrobial Resistance of Microbial Flora of cervical Mucosal Discharge Associated with Reproductive Performance of Cow Estrus. *Biological Forum – An International Journal*, *15*(8a): 23-27.