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RESISTANT STRAINS OF ENTERIC BACTERIA IN THE DAIRY FARM TOP SOIL IN SALEM DISTRICT, TAMILNADU

Kanakam Elizabeth Thomas*[1] Sivasubramanian Ramani[2], Devika Subramanian[3]

[1*]Department of Microbiology, SRMDC, SRM University, Ramapuram, India.

[2] Department of Food Science and Technology, Sejong University, Seoul, South Korea.

[3]Department of Bioinformatics, Bharathiar University, Coimbatore, India.

ABSTRACT

Indiscriminate use of antibiotics for food and animal production has resulted in a considerable rise of antimicrobial resistant strains of bacteria. The current study aims at finding out the prevalence of resistant strains of enteric bacteria from the top soil of dairy farms in Salem. **Materials and Method:** Top soil was collected from 10 randomly selected dairy farms in Salem. A total of 7 samples were obtained from each dairy farm. The roadside soil was collected from nearby each farm to be used as a control. All samples were uniformly processed by serial dilution. Resistant enteric bacteria were isolated (Antifungal agents were used to prevent growth of fungi) and identified according to standard protocol. Four commonly used antibiotics namely, Ampicillin, Tetracycline, Gentamycin and Chloramphenicol that are widely used in animal husbandry were selected for screening. **Result:** The roadside control samples contained a lesser amount of resistant Gram Negative Enteric Bacteria (GN-EB) (49 isolates) compared to the dairy farm topsoil (262 isolates), This result was validated by statistically significant differences for each category of the antibiotic. Through this study, it could be found that resistant strains of enteric bacteria are prevalent in dairy farm top soil.Our study supports the hypothesis that dairy farm topsoil can serve as a zoonotic mode of transmission of resistant strain and in addition, the observed high-level antibiotic resistances suggests that dairy farm topsoil serves as an environment in which clinically relevant resistance can develop.

KEYWORDS

Antibiotics, Multi-Drug-Resistance, Livestock, Veterinary, Humans, Topsoil, Dairy-farm, Anti-microbial, Gram Negative, Isolates and Environment.

Author for Correspondence:

Kanakam Elizabeth Thomas,
Department of Microbiology, SRMDC,
SRM University, Ramapuram, India.

Email: kannu.eliza@gmail.com

INTRODUCTION

Background

Use of antimicrobials in livestock has resulted in the considerable rise of antimicrobial resistant strains of bacteria, in turn, leading to the complications of the treatment of infectious diseases in livestock, companion animals and humans. Antibiotics are extensively used in agricultural settings and human as well as veterinary medicine such as the treatment

of infections, growth enhancement and prophylaxis in food animals, potentially leading to the selection of drugs and multidrug resistant bacteria[1,2] (Aarestrup FM., 1999; van den Bogaard AE and Stobberingh EE., 2000).

The selective pressure due to the use of antimicrobial agents at sub-therapeutic levels in dairy cattle could result in the natural selection of those strains that contain genes for antimicrobial resistance[3]. Such antibiotics are not only used in veterinary indication for therapy and prevention of bacterial infections but are also added continuously to animal feeds to promote growth, an increase in feed efficacy and decrease waste production. The antibiotics used for this purpose are commonly called feed savers, antimicrobial growth promoters or performance enhancers (APE). These APE's are found to be mainly active against gram positive bacteria[2,4].

Uncontrolled use of antibiotics in medicine and in farm animals has led to the selection of multiple antibiotic resistant bacteria in humans and cattle respectively[5]. Enteric bacteria such as *E.coli, Enterococcus faecalis* and *Salmonella* species are not only resistant to multiple antibiotics given to animals but also to antibiotics available for human use. Regarding agriculture in particular, if humans come in direct contact with infected fecal matter, transmission of antibiotic resistant bacteria is highly likely. Consequently, the antibiotic resistant gene when transferred to human microflora may reduce the efficacy of treatment for infectious diseases[6].

The tetracyclines continue to be one of the most widely used antibiotics in both human medicine and animal agriculture, as they are relatively inexpensive, can't be administered orally and have relatively fewer side effects[7,8].

Transfer of Resistant Bacteria from Animals to Humans

Most preliminary studies on the transfer of resistant bacteria from animals to human are focused on Gram Negative bacteria from food infections[9,10]. At the time of introduction of antibiotics (Murray Collections), isolates were fully susceptible to most of them. However, the level of antibiotic resistance among bacteria belonging to the normal flora of humans and animals increased as a result of continuous exposure to antibiotics[11]. Resistant zoonotic bacteria from the intestinal flora of food animals can contaminate the carcasses of slaughtered animals and reach the intestinal tract of humans via the food chain. Investigation of the prevalence of antibiotic resistance in certain indicator bacteria like *E.coli* and enterococci in the intestinal tract of different populations of animals and humans can help to compare their occurrence in different populations and to detect the possible transfer of resistant bacteria from animals to humans and vice-versa[12,13.]

The use of antibiotics not only causes an increase of resistance in pathogenic bacteria but also in the endogenous flora of humans and animals. Resistant zoonotic bacteria, other resistant strains from animals or their intestinal flora can infect or reach the human population not only by direct contact but also via food products of animal origin. There are high chances that these resistant bacteria can either colonize humans and/or transfer their resistance genes to other bacteria belonging to the endogenous flora of man[2].

The current study was conducted to determine the prevalence and distribution of antimicrobial resistant Gram Negative Entero Bacteria (GN-EB) from dairy farm topsoil in Salem District, Tamil Nadu.

MATERIAL AND METHODS

Soil sample collection

A modified method of Burgos *et al*. was used for the collection of the soil sample (24). Top soil was sampled from ten randomly selected dairy farms in Salem District, Tamil Nadu. The sampling and randomization strategies were as follows. For each dairy farm, three areas were selected. From each area, two samples were collected and one sample from the roadside as a control. A sterile spatula was used to collect the soil sample and sterile zip-pouch covers were used for the storage/holding of soil. A total of seven samples were obtained from each farm.

Isolation and Enumeration of GN-EB and Antimicrobial Resistance

One gram of each soil sample was transferred to 9 ml of sterile distilled water. The contents were serially diluted to 6 folds. MacConkey agar was used for the selective growth of GN-EB. From 10^{-3} dilution 0.1 ml was plated onto MacConkey agar without antibiotic, which was kept as MacConkey Control Plate and 0.1 ml of the sample was plated onto MacConkey agar with antibiotics purchased from HiMedia. The medium was chosen for the selective isolation and differentiation of enteric bacteria by their ability to ferment lactose. Pancreatic digests of gelatin and peptones (meat and casein) provide the essential nutrients and factors required for the growth of microorganisms. Sodium chloride helps to maintain the osmotic balance and the crystal violet and bile salts inhibit most species of gram-positive bacteria.

To isolate the resistant strains from the samples, the media is supplemented with the following antibiotics: Ampicillin (64 µg/ml), Tetracycline (32 µg/ml), Gentamycin (256 µg/ml) and Chloramphenicol (16 µg/ml). The inoculated plates were incubated at 37ºC for 24 hrs. The number of Colony Forming Units (CFU) with antibiotics and without antibiotics were counted and expressed as antimicrobial resistant GN-EB CFU/g of soil sample[14]. A standard t-test was carried out to test the significance of differences between the test and control.

Species Identification of Antibiotic Resistant GN-EB

Based on colony morphology, a few lactose fermenting colonies and Non Lactose fermenting colonies from each plate were selected for species identification. Colonies were identified by Gram staining, IMViC test, Oxidase test and TSI test as described by Mackie and McCartney (32).

RESULTS

The CFU/g of soil ranged from 2 x 10^5 to 7 x 10^5 for the 60 dairy farm topsoil samples. For roadside control soil sample, it ranged from 1 x 10^5 to 3 x 10^5**.** Out of 349 antibiotic resistant colonies that were grown from 10 Dairy farms (60 soil samples) on MacConkey agar plates with antibiotics, 262 GN-EB isolates were identified to species level (Table No.1). Antibiotic resistant GN-EB belonging to 8 gram negative bacterial species namely *Citrobacter* sp*; Klebsiella pneumonia;* other *Klebsiella* sp*;Escherichia coli; Pseudomonas aeruginosa, Pseudomonas fluorescens; Proteus mirabilis;* and other *Proteus* sp. were identified from the MacConkeyagar plates containing antibiotics. Compared to Ampicillin and Gentamycin, low levels of antibiotic resistance were observed in Chloramphenicol and Tetracycline. From the roadside control sample, 49 antibiotic-resistant GN-EB CFU's were isolated (Table No.1).

It was observed that the roadside control samples contained a significantly lesser amount of resistant GN-EB. In order to test the significance of differences between the dairy farm sample and the roadside control sample, two tailed p-values were calculated for each antibiotic by standard t-tests (Table No.2). The p-values <0.05 indicate that the differences can be considered statistically significant at 95% confidence intervals.

E.coli showed maximum resistance to Ampicillin and Gentamycin and also to other two antibiotics. The next higher level of resistance was shown by *Pseudomonas aeruginosa* against Ampicillin and Chloramphenicol. *Citrobacter* and *Proteus* did not show such high levels of resistance.

DISCUSSION

The study of prevalence of antimicrobial resistance in micro flora can be very useful in monitoring and understanding the process of antibacterial mediated selection in individual hosts as well as in general population[15,16] MacConkey agar supplemented with antimicrobial agents can be effectively used for the isolation of antibiotic resistant bacteria. The absence of therapeutics to treat emerging infectious diseases caused by antibiotic resistant organisms has heightened public apprehension. The resistance observed in zoonotic and nosocomial pathogens might just be the tip of the iceberg.

The pattern of antibiotic susceptibility of various

pathogens isolated from farm and poultry animals have been previously studied under various factors and at different locations[17-21]. Antibiotic- resistant *E.coli, Citrobacter, Enterobacter, Klebsiella* and *Pseudomonas*species are opportunistic pathogens, which can cause mastitis, enteritis, pneumonia and urinary tract infections in dairy cattle. Recently it was found that *Salmonella* and *E.coli* isolates from humans and animals contained the same antimicrobial resistance determinants[22].

In the current study, *E.coli* and *Pseudomonas* were the two major GN-EB detected in the dairy farm soil. They showed maximum resistance to all the four antibiotics. The high prevalence of Ampicillin, Tetracycline and Gentamycin resistant GN-EB as seen in the current study shows that the dairy farm topsoil can act as a reservoir of antibiotic resistant GN-EB. However, though Chloramphenicol resistant bacteria are lower in number compared to the other three antibiotics used in the study, their role as reservoirs cannot be ignored.

According to Sawant *et al*., 2007[14] beta-lactams and tetracyclines[23] are the most widely used antimicrobials in dairy herds. The absence of antimicrobial treatment records, lack of written systematic plans for treating sick animals, the failure to consult a veterinarian for treating sick animals and most often the failure to complete an antimicrobial treatment course- all have led to the emergence of antibiotic resistant bacteria[24-29]. The variation in the number of farms and cows that shed antimicrobial resistant GN-EB could be influenced by conditions such as 1) transition of animals from one environment to another 2) change in nutrition 3) severe weather conditions and 4) interaction with other animals in the herd[30,31].

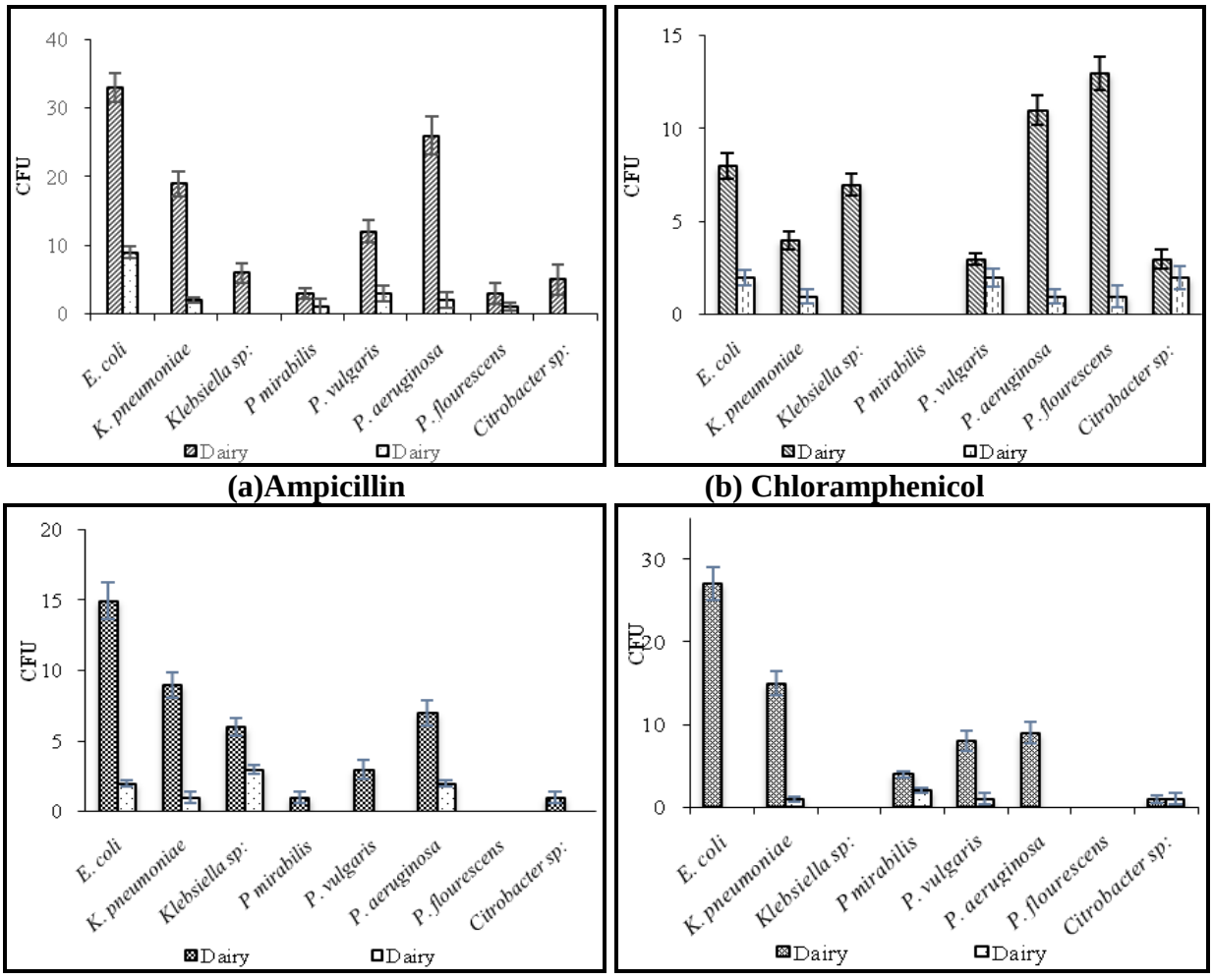
The requirement of antibiotics in veterinary therapy and bacterial infection in animals should be minimized by improving methods of animal husbandry, disease eradication, and optimal usage of existing vaccines. Thus, it can be seen that the increased use of antibiotics in veterinary medicine has led to closer analysis of resistance mechanisms in zoonotic pathogens, primarily in the soil of food animal and dairy farm environment.

Table No.1: Observed no. of CFU's of GN-EB isolated

Species	No. of CFU's of GN-EB isolated from topsoil on MacConkey agar supplemented with:															
	Ampicillin				Chloramphenicol				Tetracycline				Gentamycin			
	Dairy farm	SD	Roadside control	SD	Dairy farm	SD	Roadside control	SD	Dairy farm	SD	Roadside control	SD	Dairy farm	SD	Roadside control	SD
E. coli	33	2.1	9	0.8	8	0.7	2	0.4	15	1.3	2	0.2	27	2	-	-
K. pneumoniae	19	1.8	2	0.4	4	0.5	1	0.4	9	0.9	1	0.4	15	1.5	1	0.3
Klebsiella sp:	6	1.4	-	-	7	0.6	-	-	6	0.6	3	0.3	-	-	-	-
P mirabilis	3	0.8	1	1.2	-	-	-	-	1	0.4	-	-	4	0.4	2	0.3
P. vulgaris	12	1.6	3	1.1	3	0.3	2	0.5	3	0.60.5	-	-	8	1.2	1	0.7
P. aeruginosa	26	2.8	2	1.1	11	0.8	1	0.4	7	0.9	2	0.2	9	1.3	-	-
P. flourescens	3	1.5	1	0.5	13	0.9	1	0.5	-	-	-	-	-	-	-	-
Citrobacter sp:	5	2.2	-	-	3	0.5	2	0.6	1	0.4	-	-	1	0.5	1	0.7
Total isolates	107	14.2	18	5.1	49	4.3	9	2.8	42	5.15	8	1.1	64	6.9	5	2

Table No.2: Observed p-values obtained by standard t-test for each antibiotic

S.No	Antibiotic	t-score	p-value
1	Ampicillin	2.663	0.018
2	Chloramphenicol	3.142	0.0072
3	Tetracycline	2.298	0.037
4	Gentamycin	2.238	0.0419



(a)Ampicillin **(b) Chloramphenicol**

(c) Tetracycline **(d) Gentamycin**

Figure No.1: Comparison of isolates obtained from dairy farm top soil and roadside control which are resistant to (a, b. c, d)

CONCLUSION

From the above study it can be concluded that the top soil in dairy farms can be a reservoir of Antibiotic resistant strains of Enterobacteriaceae. There is a need for awareness among cattle breeders and dairy farm owners about the indiscriminate use of antibiotics on animals. There arises a need for strict monitoring and control on the use of antibiotics from FDA and various veterinary organizations, as antibiotic resistant strains of bacteria makes it difficult to treat patients and has to go for stronger antibiotics. However, as this study is limited to a small sample, further investigations is required.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest in the publication.

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