

Original Research Article

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## Evaluation of *Escherichia Coli* as a Microbial Tool in Residue Monitoring of Enrofloxacin in Milk

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### ABSTRACT

Antimicrobials are used in dairy cattle to treat disease and to improve feed efficiency. The widespread use of antimicrobials has created potential residue problems in dairy products. The problems associated with the antimicrobial residues in milk includes the risk of allergic reactions after consumption and transfer of antimicrobial resistant bacteria from livestock to human through contaminated food products. This study aims to monitor Enrofloxacin antimicrobial drug residue in milk by a simple microbial screening method. Two hundred milk samples comprising of 100 cow and 100 buffalo milk were collected from organized dairy farms. Milk samples were extracted for the antibiotic Enrofloxacin and were examined for the presence of Enrofloxacin antibiotic residues by microbial tube test method using the indicator organism *Escherichia coli*. The results of this study revealed 5 positive buffalo milk samples (5%) for enrofloxacin antimicrobial drug residues. This study explains that microbial screening method has a unique advantage of a high-throughput, economical and simple tool that can be used to screen the existence of antibiotic residues in foods of animal origin.

#### Keywords

Antibiotic residues,  
Milk, Microbial  
screening,  
Microbial assay,  
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### Introduction

Antibiotics are used in veterinary practice at therapeutic levels to treat diseases and to prevent infection and at sub therapeutic levels to increase feed efficiency. Thus the frequent use of antibiotics may result in drug residues at different concentration levels in animal

products such as milk or meat. The antibiotic residues may cause potential health hazards such as allergic reaction, carcinogenicity and bacterial resistance to antibiotics (Khaskheli *et al.*, 2008). Quinolones are a recently developed antimicrobial drug used for fighting infections in animals (Schenck *et al.*, 1998).

Quinolones are new antimicrobials has excellent activity against both Gram-positive and Gram-negative organisms and anaerobes and synthesized from 3-Quinolone carboxylic acid (Elizabetha *et al.*, 2011).

The use of fluoroquinolones in both medical and veterinary practice has generated concern due to increased antimicrobial resistance. Thus efficient methods are required to monitor the food supply to ensure the presence of antibiotic residues at permissible or violative levels in order to assure food safety (Schneider *et al.*, 2004).

The presence of violative levels of residues in foods are illegal and may be subjected to financial penalties in many countries. Thus, there is a need for antibiotic residue detection methods to detect and reduce residues in foods of animal origin (Vishnuraj *et al.*, 2016).

The International organisations such as Codex Alimentarius Commission, European Agency for the Evaluation of Medicinal Products (EMA), Office International des Epizooties (OIE), Food and Drug Administration in the United States, the Bureau of Veterinary drugs in Canada and the Veterinary Products Committee of the Ministry of Agriculture, Fisheries and Foods in the United Kingdom controls the use of drugs in animals. Limits have been established for drug residues in foods in the form of tolerances or maximum residue limits (MRLs). The term MRL may be defined as the maximum concentration of marker residue expressed in parts per million (ppm) or parts per billion (ppb) that is legally permitted as acceptable daily intake (ADI) (Mitchell *et al.*, 1997). The World Health Organisation (WHO) and the Food Agriculture Organisation (FAO) have set standards for acceptable daily intake and Maximum residue limits (MRLs) in foods. Regulatory limits for antibiotic residues are

also been imposed on the dairy industry. The different methods were developed to detect antibiotic residues in milk and are applied in laboratory analysis. The antibiotic residue detection method consists of screening and chromatographic methods. The microbial assay screening method is based on the susceptibilities of bacteria to antibiotics (Kebede *et al.*, 2014).

The microbiological assays for the detection of antibiotic residues is a highly sensitive screening method. It is recommended as official and conventional method because of their simplicity (Kebede *et al.*, 2014). Thus the present study adopted a simple microbial assay method to detect the existence of Enrofloxacin antimicrobial residue in milk.

## **Materials and Methods**

### **Reagents and reference standards**

All chemicals used were of analytical grade reagent of Merck. Certified Reference Material standards of Enrofloxacin with purity of 99.99 % were obtained from M/S Neospark Drugs and Chemicals Private Limited, Hyderabad, India.

### **Stock and working standards solution**

Enrofloxacin stock standards of 1mg/mL concentration were prepared in methanol separately. Working standards of enrofloxacin were prepared by diluting with water to get 200, 100 and 50 µg/kg concentration each for microbial analysis.

### **Sample collection**

A total of 200 milk samples comprising 100 cow and 100 buffalo milk were collected from organized dairy farms in and around Chennai, Tamil Nadu, India. The milk samples were kept in the refrigerator until further use.

### **Preparation of culture medium**

The following culture media were used: nutrient agar and nutrient broth obtained from HIMEDIA. Nutrient broth (1.3g) was weighed in a conical flask and 100 ml of distilled water was added, as per the manufacturer's instructions.

It was then sterilized in an autoclave at a pressure of 15 mm Hg and a temperature of 121°C for 15 minutes, after which it was cooled to about 50°C. Then Bromocresol Purple indicator (0.002g/l) was added to the autoclaved nutrient broth medium (Tharanya *et al.*, 2018).

### **Preparation of bacterial culture**

The bacterial culture used was *Escherichia coli* (MTCC 40), obtained from Microbiological Type Culture Collection (MTCC). The freeze-dried bacterial culture was activated according to the instructions given by MTCC.

Single colony obtained from the Petri plate (Fig.1) was inoculated into 5ml nutrient broth and incubated at 37°C for 18- 24 h. The broth suspension was adjusted with sterile physiological saline to a concentration approximately equal to 0.5 McFarland standard equivalent, to  $1.5 \times 10^8$  CFU/ml. The microbiological works were done under biosafety cabinet (Tharanya *et al.*, 2018).

### **Microbiological tube test method**

The antimicrobial drug residues in milk samples were screened using microbiological tube test method with a suitable indicator organism for the antibiotic studied. The Indicator organism *Escherichia coli* MTCC 40 is used to detect the Enrofloxacin residue in milk (Hee-Jung Cho *et al.*, 2008).

### **Standardisation of Enrofloxacin by microbial assay**

Working standards 200 ppb, 100 ppb and 50 ppb from Stock standard solution were prepared by diluting in water as per the MRL levels set by European Union to obtain the Limit of Detection (LOD) of Enrofloxacin with the indicator organism *Escherichia coli*.

### **Procedure**

Microbiological tube test was performed as per the method used by Tharanya *et al.*, (2018). 1800µl of nutrient broth with pH indicator bromocresol purple was taken in a test tube and then 100µl of extracted milk sample and 100µl of test bacterium *Escherichia coli* were pipetted into the test tube and mixed thoroughly. The test tubes with positive growth controls containing culture organism in the broth culture and a negative control containing only the broth were also taken. The test tubes were incubated at 37°C for 18–24h. The test tubes with purple colour after the incubation were recorded as positive for antimicrobial residues and those that turned yellow or turbid were negative for antimicrobial residues.

### **Sample extraction for enrofloxacin**

Extraction was done as per the method described by Sureshkumar *et al.*, (2018). Four grams of the milk samples was weighed using a weighing balance and stored in a sample bottle. The milk samples were shaken well with 16 ml of 5 % aqueous trichloroacetic acid (TCA) in an orbital shaker for 30 minutes. Samples were then centrifuged at 5000rpm for 15 min using Remi Centrifuge. The supernatant obtained was filtered through Whatman filter paper No.1 and collected separately. The collected aqueous supernatant was subjected to liquid-liquid extraction by adding 32 mL of dichloromethane in a

separating funnel. The organic fraction-I was collected separately in the beaker and left out aqueous fraction was once again extracted with 10 mL of dichloromethane. The obtained organic fraction-II was added to the fraction I.

The resultant final organic extract was passed through sodium sulphate bed and collected in a beaker and concentrated in a hot plate under the fume hood. Finally, the dried extract was reconstituted with 100 µl of water and subjected to microbial analysis for enrofloxacin.

### Statistical analysis

The results were expressed as percentages. The collected data were statistically analysed by chi-square test using Statistical Package for Social Sciences (SPSS<sup>®</sup> ver,20.0 for Windows<sup>®</sup>).

### Results and Discussion

The limit of detection (LOD) for the antibiotic Enrofloxacin was found to be 50 µg/kg which is below the accepted maximum residue level (MRL) for the tested antibiotic (100µg/kg) set by the European Union shown in Fig.2.

A positive result was indicated by color change, i.e. test tubes that remained purple after the incubation were recorded as positive and those that turned yellow or turbid were recorded as negative for antimicrobial residues.

Out of 100 buffalo milk samples analyzed, 5 buffalo milk samples (5 %) were found to be positive for Enrofloxacin antibiotic drug residues against the test organism *Escherichia coli* and 95 buffalo milk samples (95%) were negative for Enrofloxacin residue shown in (Table.1 and Fig. 3 & 4 ).The analysed 100 cow milk sample were negative for

Enrofloxacin. The positive milk samples for Enrofloxacin residues were compared within cow and buffalo milk. It was statistically significant at 5 % level ( $P < 0.05$ ) shown in the Table.2.

Milk is a rich source of proteins, vitamins, and minerals such as calcium, magnesium, phosphorous, potassium, and zinc and a food of high nutritional value and consumed by all populations. Thus, milk and milk products must be safe, without microbiological, physical or chemical contaminants that is intended for human population (Trombete *et al.*, 2014). Direct contamination of milk may occur during processing, storage and transportation from air and water. Feed given to animals is also a source of indirect contamination and therefore man will be the ultimate consumer of these antibiotic residues (Nisha *et al.*, 2008).

The dairy cows are treated with drugs for mastitis through intramammary infusion and hence the drug and their metabolites may persist at unacceptable levels and thus the consumers may be exposed to them. Failure to observe the mandatory withdrawal period, illegal or extra-label use of drugs and incorrect dosage are the reasons for the presence of antibiotic residues in milk. Also the prolonged drug excretion or use of unauthorized drug may result in residues of these substances in milk and tissues (Asredie *et al.*, 2015).

The study of Cho *et al.*, (2008) in the 120 chicken egg samples of Republic of Korea detected fluoroquinolones residue in 2 chicken eggs samples by microbial assay using *Escherichia coli* as the reference organism which is in accordance with the present study in which 5 milk samples were positive for Enrofloxacin out of 100 buffalo milk samples.

**Table.1** Microbial screening of milk using *Escherichia coli* for Enrofloxacin

Milk sample	Sample Number (n)	Enrofloxacin	
		No. of positive samples	% of positive samples
Cow	100	0	0
Buffalo	100	5	5

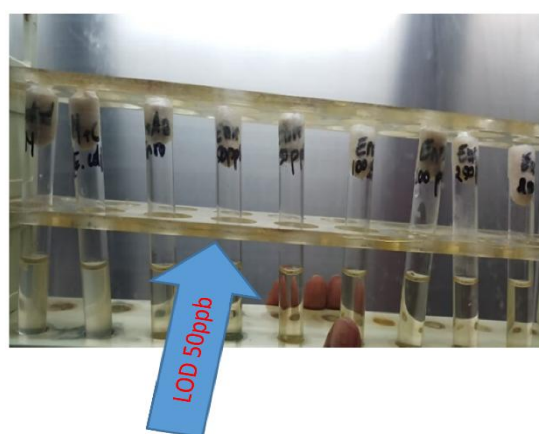
**Table.2** Statistical analysis of positive milk samples for Enrofloxacin by microbial screening

Milk sample	Sample Number (n)	Enrofloxacin		Chi-square value
		No. of positive samples	% of positive samples	
Cow	100	0	0	5.13*
Buffalo	100	5	5	

\*Significant statistically at 5% level ( $P < 0.05$ )

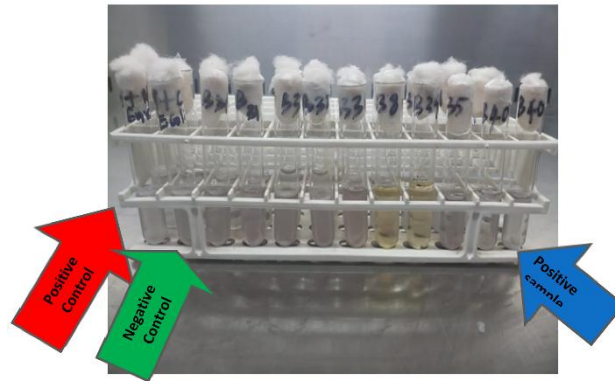


**Fig.1** Bacterial colony growth of *Escherichia coli* (MTCC 40) bacteria on petriplate from the activated freeze dried culture obtained from MTCC Chandigarh



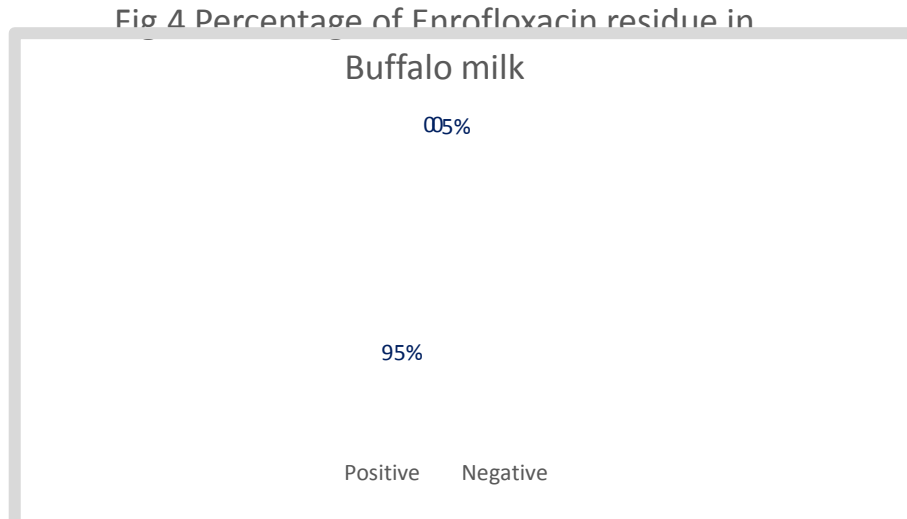


**Fig.2** Standardization of Enrofloxacin to assess Limit of Detection (LOD)



- Positive control – Media + Antibiotic
- Negative control – Media + Culture
- Positive sample

**Fig.3** Microbial assay of antibiotic Enrofloxacin in milk samples



**Fig.4** Percentage of Enrofloxacin residue in Buffalo Milk

The microbial screening of animal feeding stuffs was performed based on the growth inhibition of *Bacillus subtilis*, *Bacillus cereus*, *Kocuria varians*, *Escherichia coli* on agar medium for detection of  $\beta$ -lactams, tetracyclines, aminoglycosides, macrolides and quinolones antibiotic residues (Monika *et al.*, 2007) which is in accordance with the present study in which *Escherichia coli* is

used for inhibiting the growth of Quinolone antibiotic. Microbial inhibitor tests are widely used for screening antibiotics in raw milk as they are inexpensive, user friendly and have high sample throughput (Beltran *et al.*, 2015). Raw milk is investigated by various microbiological screening tests in order to check the fulfilment of established tolerances for antimicrobial drugs since they are cheap,

easy to perform on a large scale and possess non-specific spectrum in sensitivity (Nouws *et al.*, 1999). Hence there is a need for rapid screening procedures for the analysis of antibiotic residues and instant grading and prohibition of food containing antibiotics more than MRL. This can be achieved by the development of a simple and economic field test to identify drug residue in edible animal products. Thus the present study paved way for a rapid screening procedure in milk by adopting a simple and economic microbial assay.

The presence of antibiotic residues in animal tissues is due to the negligence of withdrawal time. Insufficient period of time given for the drug to be eliminated from food. Hence there is a need for appropriate screening tests to detect residue status in milk. Therefore the veterinarians and producers should stick to maximum residue limits (MRL) and prescribed withdrawal times of antimicrobial agents to reduce antimicrobial residues in milk. The individuals and organisations should be made aware of the problem of the residues through education by personnel, organizations and literatures and governmental agencies.

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