RESEARCH ARTICLE
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Journal of Veterinary and Animal Sciences
ISSN (Print): 0971-0701, (Online): 2582-0605

The occurrence of antibiotic residues in pooled raw cow milk samples of Palakkad, Kerala*

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Citation: Asif, M. H., Latha, C., Menon, K. V., Jolly, D., Nair S. N. and Sunanda C. 2020. The occurrence of antibiotic residues in pooled raw cow milk samples of Palakkad, Kerala. *J. Vet. Anim. Sci.* **51**(1): 34-39.

Received : 02.08.2019 *Accepted : 22.10.2019* *Published : 01-01-2020*

Abstract

Antibiotic residues in milk pose a potential risk to the health of consumers and are of public health significance due to the dissemination of antimicrobial resistance. The present study was conducted to assess oxytetracycline and enrofloxacin residues in pooled raw milk samples in Palakkad district. A total of 120 pooled raw milk samples collected from five co-operative societies in Palakkad district were screened by Microbial Inhibition Assay (MIA). The positive samples were subjected to charm assay to determine whether the level of antibiotic residues viz., oxytetracycline and enrofloxacin were within or above Maximum Residual Limit (MRL). Of the 120 samples screened using MIA, 14 samples (11.67 per cent) were found to be positive for antibiotic residues. The occurrence of tetracyclines and enrofloxacin residues in pooled raw milk samples was found to be respectively 3.33 and 4.16 per cent using charm assay. Statistical analysis using chi-square test for multiple proportions revealed that the occurrence of antibiotic residues between the co-operative societies did not differ significantly (p > 0.05). The occurrence of antibiotic residues in milk necessitates prudent use of antibiotics, observance of the withdrawal period and monitoring system for antibiotic residues in foods of animal origin.

Keywords: *Antibiotic residues, Tetracyclines, Enrofloxacin, MIA and Charm assay*

Milk is an indispensable part of the human diet because of the high bioavailability of nutrients. India's milk production increased at the rate of 6.62 per cent to 176.35 million tonnes in 2017-18 ranking the country first in global milk production (DAHDF, 2018). The use of antibiotics in livestock production for therapeutic, prophylactic and growth promotion purposes has increased over the decades along with the rise in demand for foods of animal origin. The non-prudent use of antibiotics

** Part of the MVSc thesis submitted by the first author to Kerala Veterinary and Animal Sciences University, Pookode, Wayanad, Kerala*

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in dairy animals, including unregulated extra-label or illegal drug applications, failure to adhere to the withdrawal period, frequent overdose and use of long-acting drugs results in the occurrence of antibiotic residues in milk. The Codex Alimentarius Commission (CAC), European Union (EU) and Food Safety, Standards Authority of India (FSSAI) have established Maximum Residue Limits (MRL) for veterinary drugs in various food matrices. The effect of antibiotic residues on consumer health and the emergence of antimicrobial resistance necessitates the establishment of monitoring and surveillance systems for foods. The present study was conducted to investigate the occurrence of antibiotic residues in pooled raw milk samples from Palakkad district of Kerala.

Materials and Methods

Collection of samples

A total of 120 pooled raw milk samples were collected in three to four visits to five selected co-operative societies in Palakkad district. About 150 ml of pooled raw milk samples from milk cans were collected in clean and sterile polypropylene tubes. The collected samples were brought under refrigerated conditions and were stored at -20 °C for further analysis.

Microbial Inhibition Assay (MIA)

The pooled raw milk samples were screened for antibiotic residues using MIA (Gaudin *et al*., 2004). The MIA was designed with four test organisms in four different media (Table 1). The test organisms were cultured in nutrient broth (HiMedia) at 37 °C for 24 hours. The bacterial suspension turbidity was adjusted to 0.5 McFarland standard and inoculated on to test media using sterile swabs. The milk samples were heated to 80 °C for five minutes before analysis. The sterile discs (HiMedia) of diameter six millimetres were dipped in the milk samples and placed in all four-test media using sterile forceps. Then the plates were incubated for 18 to 24 hours. After incubation, plates with a zone of inhibition of 12 mm in at least one plate were considered as positive for antibiotic residues.

Charm Rapid One Step Assay (ROSA)

The positive samples in MIA were subjected to Charm MRLBLTET and Charm ENRO (Charm Sciences Inc., USA) for detection of tetracycline group and enrofloxacin residues at or above Maximum Residue Limit (MRL) respectively. The test strips were thawed to room temperature and about 300 µL of milk sample was added to the milk chamber. The strips were incubated at 56 °C for eight minutes in Charm ROSA incubator. The strips with the TE line and the test line absent or lighter than the control line were considered positive for tetracycline and enrofloxacin residues respectively. The test results were interpreted visually and using Charm ROSA reader.

Results and Discussion

The MIA is a screening method for detection of antibiotic residues which inhibit the growth of standard test organisms in suitable solid or liquid media. These assays are characterised by their sensitivity, cost-effectiveness and ease of use but they lack specificity and require long incubation periods. The MIA evaluated in the present study consisted of the test organism *B. cereus* which showed high sensitivity for tetracyclines, *B. subtilis* for aminoglycosides and macrolides, *E. coli* for quinolones and *G. stearothermophilus* for penicillins, cephalosporins and sulphonamides (Nouws *et al*., 1999; Gaudin *et al*., 2004). The MIA was carried out using four organisms to

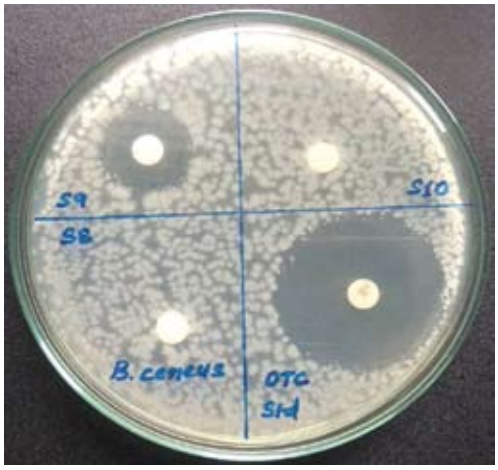

Fig. 1. Microbial Inhibition Assay with *Bacillus cereus* showing positive sample

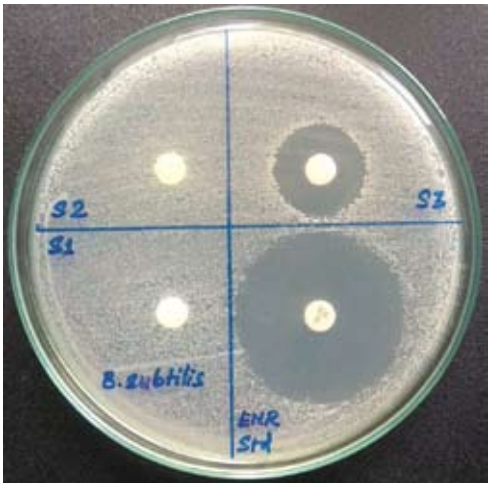

Fig. 2. Microbial Inhibition Assay with *Bacillus subtilis* showing positive sample

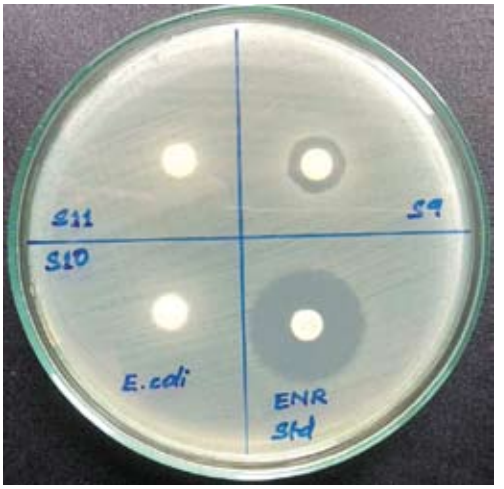

Fig. 3. Microbial Inhibition Assay with *Escherichia coli* showing positive sample

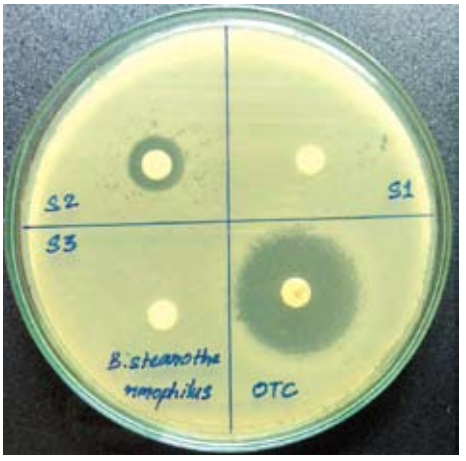

Fig. 4. Microbial Inhibition Assay with *Geobacillus stearothermophilus* showing positive sample

Fig. 5. Charm MRLBLTET test strips

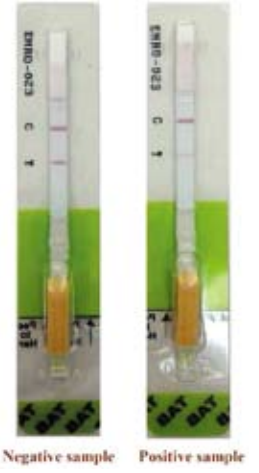

Fig. 6. Charm ENRO test strips

increase the sensitivity for detection of a wide range of antimicrobial groups. After incubation, the presence of a zone of inhibition measuring 12 mm or more around the test discs in at least one of the plates was considered as positive for antibiotic residues. The zone of inhibition reported for positive samples was two millimetres or more by Gaudin *et al*. (2004) with nine millimetres discs, and 22 mm by Nouws *et al*. (1999) with 14 mm diameter punch holes.

Out of 120 pooled raw milk samples screened for antibiotic residues using MIA, 14 samples (11.67 per cent) were positive as shown in Table 2. Statistical analysis using the chi-square test for multiple proportions revealed no significant difference ($p > 0.05$) in the occurrence of antibiotic residues between the five co-operative societies. Arora and Chhabra (2004) reported 23.8 per cent occurrence of antibiotic residues in raw milk samples from unorganised dairy farms of Indore, Madhya Pradesh using *B. subtilis* on Nutrient agar. A similar study conducted by Dinki and Balcha (2013) in Guwahati, Assam reported 23.3 per cent occurrence of antibiotic residues in milk samples using *B. subtilis* on Nutrient agar. Higher occurrence of antibiotic residues (49.75 per cent) than the present study was reported by Mangsi *et al*. (2014) in milk samples from Sindh province, Pakistan. The extra-label use, overdosage and misuse of

Table 1. The organisms and culture media used for Microbial Inhibition Assay

Sl. No.	Bacterial strains	Agar medium	Incubation
1	*Bacillus cereus* MTCC 430	Antimicrobial inhibitor test agar at pH 6	37 °C, 18-24 hrs
2	*Bacillus subtilis* MTCC 441	Antimicrobial inhibitor test agar at pH 7.2	37 °C, 18-24 hrs
3	*Escherichia coli*	Antimicrobial inhibitor test agar at pH 8	37 °C, 18-24 hrs
4	*Geobacillus stearothermophilus* MTCC 38	Diagnostic Sensitivity Test (DST) agar	55 °C, 18-24 hrs

Table 2. Occurrence of antibiotic residues by Microbial Inhibition Assay

Sl. No.	Co-operative society	No. of samples analysed	No. of positive samples	Per cent
1	Karakurissi (CS1)	20	2	10.00 [a]
2	Erattakulam (CS2)	25	4	16.00 [a]
3	Malakkulam (CS3)	25	4	16.00 [a]
4	Mambad (CS4)	25	2	8.00 [a]
5	Alathur (CS5)	25	2	8.00 [a]
Total		**120**	**14**	**11.67**

Figures bearing the same superscript do not differ significantly ($p > 0.05$)

Table 3. Occurrence of tetracyclines and enrofloxacin residues in pooled raw milk samples by Charm assay

Co-operative society	Total no. of samples	No. of Samples analysed	Tetracyclines positive samples		Enrofloxacin positive samples	
			No.	Per cent	No.	Per cent
Karakurissi (CS1)	20	2	0	0.00[a]	2	10.00[a]
Erattakulam (CS2)	25	4	1	4.00[a]	0	0.00[a]
Malakkulam (CS3)	25	4	2	8.00[a]	0	0.00[a]
Mambad (CS4)	25	2	1	4.00[a]	1	4.00[a]
Alathur (CS5)	25	2	0	0.00[a]	2	8.00[a]
Total	**120**	**14**	**4**	**3.33**	**5**	**4.17**

Figures bearing the same superscript do not differ significantly ($p > 0.05$)

antibiotics by farmers, para-veterinarians and veterinarians could lead to the occurrence of antibiotic residues. Noncompliance with regard to the withdrawal periods, lack of treatment records and a dearth of awareness among farmers could be attributed to the occurrence of antibiotic residues in milk.

Occurrence of tetracycline and enrofloxacin residues by Charm ROSA

Charm ROSA is a receptor-based screening assay for the detection of antibiotics by interaction with their binding proteins. Charm MRLBLTET test strips are used for detection of 14 β-lactam drugs and three tetracycline drugs at or below MRL of 100 ng/mL (EU/CAC) in milk. Enrofloxacin residues can be detected by using Charm ENRO test strips at or below MRL of 100 ng/mL (EU) in milk. The FSSAI has established tolerance limits for oxytetracycline and enrofloxacin residues in milk as 100 and 10 ng/mL respectively, under Food Safety and Standards (Contaminants, Toxins and Residues) Second Amendment Regulations, 2018. Salter *et al*. (2011) evaluated and validated Charm ROSA test for detection of β-lactam drugs and reported detection limit below US safe levels/

tolerance limits ranging from 5 – 20 ng/ml.

The overall occurrence of tetracycline group and enrofloxacin residues in pooled raw milk samples were 3.33 and 4.17 per cent respectively in co-operative societies of Palakkad district, Kerala (Table 3). Statistical analysis using the chi-square test for multiple proportions revealed no significant difference in the occurrence of tetracyclines and enrofloxacin residues between co-operative societies in Palakkad district in the milk samples collected. A previous study conducted by Kumarswamy *et al*. (2018) to screen individual raw milk samples from Thrissur, Kerala reported 1.82 and 1.82 per cent tetracyclines and enrofloxacin residues respectively using Charm assay. A study conducted by Schlemper and Sachet (2017) in Brazil, reported 4.04 per cent enrofloxacin residues in unpasteurised milk samples using Charm ROSA. Abbasi *et al*. (2011) reported 25.4 per cent of tetracyclines residues in bovine milk samples from Iran. Moharana *et al*., 2015 investigated the occurrence of enrofloxacin residues in milk from Chennai and reported 16.8 per cent positive samples. Pallavi (2017) reported 6.1, 9.8 and 7.9 per cent of milk samples from large, medium and small dairy farms were positive for enrofloxacin residues in Punjab.

The present study revealed the presence of tetracyclines and enrofloxacin residues in pooled raw milk samples of Palakkad district. The Charm assay confirmed the presence of antibiotic residues above MRL. The wide use of tetracyclines and enrofloxacin antibiotics for treating diseases in cattle because of easy availability and cost-effectiveness and noncompliance of withdrawal periods are the main reasons for the occurrence of residues in milk samples. The health consequences due to treatment failure and economic costs of antimicrobial resistance are a major threat to public health. Tetracyclines and quinolones have been classified under highly important and highest priority critically important antimicrobials respectively by WHO in 2017. The study indicates the necessity of regular monitoring of foods of animal origin for antibiotic residues which is attributed as one of the major reasons for antimicrobial resistance.

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