



Detection and Analysis of Veterinary Drug Residues in Milk from the Valley of Kashmir

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ABSTRACT

Background: Irrational and indiscriminate use of drugs (antibiotics and anthelmintics) leads to increased occurrence of drug residues in animal foods like milk causing deleterious effects on the health and environment. It leads to the increased emergence of multi drug resistant (MDR) microbes and aggravates the problem of antimicrobial and anthelmintic resistance.

Methods: A study was conducted in 2021 to detect and analyze the presence of residues of antibiotics and anthelmintics in milk procured from the markets of Kashmir Valley. A total of 60 samples collected from different sources were randomly taken and analyzed for drug residues using the technique of reverse phase high performance liquid chromatography.

Result: The study revealed that 6.67% samples of milk were positive for Ivermectin while 10% of the overall samples of milk were detected to be positive for Fenbendazole. However none of the milk sample studied was found to be positive for Oxytetracycline, Tetracycline, Enrofloxacin and Ceftriaxone antibiotic residues.

Key words: Anthelmintics, Antibiotics, Drug residue, MDR, Reverse phase high performance liquid chromatography.

INTRODUCTION

Owing to the indiscriminate and overuse of antibiotics and anthelmintics in veterinary practice chances arise that some quantities of drugs or their metabolites (residues) may remain in edible tissues or in animal products (meat, milk, eggs, honey) causing deleterious effects in humans as potential consumers of such food (Sanders, 2007). Due attention should be paid to sensitivity of antimicrobial agents to regulate residues of veterinary drugs routinely used in veterinary practice (Vitomir *et al.*, 2011). The drug resistance is a growing menace and some of the reasons contributing to the emergence of antimicrobial resistance are the superfluous and unnecessary usage of antimicrobial drugs, inappropriate dose, inadequate duration of therapy, use of irrational antimicrobials fixed dose drug combinations (Soulsby, 2005). The spread of multiple drug resistant (MDR) pathogenic bacteria has been recognized by the World Health Organization for Animal Health (OIE), the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) as a serious and emerging global human and animal health problem and the presence of drug residues in foods like milk can further aggravate the problem. Results have been reported in Britain in 1963 when 11% of milk samples tested were found to have penicillin (Garrod, 1964). Controversial studies conducted in 1988 involving more sensitive detection methods showed that 75% of North American consumer milk had detectable levels of Tetracyclines, Sulphamethazine and other antibiotics (Brady and Katz, 1988; Kimbrell, 1990; Place, 1990).

Antibiotic use in livestock in India

Currently, there is little accurate data available in India on antimicrobial usage in food animals or resistant infections

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linked to animals and its effect on public health. A recent study estimating global use of antibiotics in poultry, swine and cattle in 2010 shows that India accounts for 3% of global consumption and is among the top consumers worldwide, along with China, the United States, Brazil and Germany. Estimates for 2030 show an overall increase of about two-thirds in animal antibiotic consumption worldwide. The WHO's list of Critically Important Antimicrobials is made up of antibiotics which are critically important for human health and their usage must be minimized in the veterinary sector. These include ampicillin, amoxycillin, cefadroxil, chlortetracycline, doxycycline, erythromycin, flumequine, gentamycin, vancomycin, oxytetracycline, spiramycin, sulfadiazine, sulfadimethoxine (Yann and Sivaraman, 2018). Recently a

survey published in The Hindu on July, 29th, 2020, conducted by the Centre for Science and Environment (CSE) reported that antibiotics are extensively misused in dairy sector in India. The leading national newspaper also published that as per FSSAI survey 41% milk samples are of poor quality, 7% samples are even unfit to consume in India. Keeping all the above facts in consideration a study was conducted in 2021 to detect and assess the residues of antibiotics and anthelmintics commonly used in veterinary practice in milk samples collected randomly from markets of Kashmir Valley.

MATERIALS AND METHODS

Analysis of veterinary drug residues in milk

A total of 60 milk samples were taken randomly from different sources.

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|-----------------------------------|-----------|
| (i) Local Shops | 20 number |
| (ii) Vendors and mini dairy farms | 20 number |
| (iii) Pasteurized packet milk | 20 number |

The samples were processed and prepared to be subjected to Reverse Phase High Performance Liquid Chromatography (RP-HPLC) at Department of Food Technology University of Kashmir, using Agilent HPLC 1260 series consisting of quaternary pump, PDA detector, manual sample injection system and zorbax C18 column with column dimension 150 mm length and 5 μ m pore size. The HPLC grade standards of the drugs to be studied and analyzed were procured from Sigma Aldrich. The drugs studied and analyzed were selected on the basis of their frequency of use in veterinary practice. This was another part of the current study in which the frequency of various drugs (antibiotics and anthelmintics) was studied and the antibiotics and anthelmintics having the highest frequency of usage were selected for study. The drugs selected and analyzed were Tetracycline, Oxytetracycline, Enrofloxacin, Ceftriaxone, Ivermectin and Fenbendazole. Gentamicin drug though initially selected was dropped from the study finally because it could not be validated in the procedure developed.

Stock solution of 1 mg/ml of each standard was prepared in mainly methanol solvent. Following dilutions of each drug standard: 5 μ g/ml, 10 μ g/ml, 15 μ g/ml, 20 μ g/ml, 25 μ g/ml, 30 μ g/ml, 35 μ g/ml, 50 μ g/ml, 100 μ g/ml etc was prepared for validation of the HPLC technique for the determination of the drug residues. Different methods were tested for optimum chromatographic separation parameters like resolution, repetition, precision and calibration. Finally following two methods (listed in Table 1 and Table 2) were selected for the final analysis of drug residues in milk samples.

Surveillance of antibiotic and anthelmintic residues in milk samples

The standardized RP-HPLC protocol was followed for the analysis and detection of antibiotic and anthelmintic drug residues in milk and meat samples. Total 60 samples of milk samples taken randomly from Kashmir Valley were

subjected to chromatographic analysis and the concentrations of residues detected were analyzed.

Comparison of residue levels in field samples with Codex and EU MRLs

Antibiotic and Anthelmintic residues in each of milk and meat samples were compared with the MRLs prescribed by European Union Commission (EU, 2010) and Codex Alimentarius Commission (Codex, 2015), to evaluate the concentrations of antibiotic and anthelmintic residues in them.

RESULTS AND DISCUSSION

Antimicrobials if not used judiciously, may have impact on human, animal and environmental health in a One Health context, as up to 90% of the antibiotic parent compounds can be directly excreted in milk and meat leading to antibiotic resistance development in the environment (Daniel *et al.*, 2014; Boeckel *et al.*, 2017).

Besides, a 2015 study revealed that global agricultural antibiotic usage will increase by 67% from 2010 to 2030, mainly from increase in use in developing BRIC countries. This is a matter of concern as antibiotic resistance is considered to be a serious threat to human and animals and growing levels of antibiotics or antibiotic-resistant bacteria in the environment could increase the numbers of drug-resistant infections. However, legislation and other restrictions on antibiotic usage in farm animals are now being introduced across the globe (OECD, Paris, 2019). In 2017, the World Health Organization strongly suggested reducing antibiotic use in animals used for food purposes. The occurrence of antimicrobial residues in foods of animal origin, together with failure to observe the instructions and precautions in their usage (dosage and waiting period) or poor livestock production practices, can have deleterious consequences for consumer health

Table 1: Specific HPLC method conditions for antibiotics.

Antibiotics	
Solution D	Orthophosphoric acid added to Millipore water with pH adjusted to 3
Solution C	Methanol added with 1% Tetrahydrofuran (THF)
Detection wavelength	280 nm
Flow rate	1 ml/min
Injection volume	20 μ l
Runtime	15 mins

Table 2: Specific HPLC method conditions for anthelmintics.

Anthelmintics	
Solution	Acetonitrile, Methanol and phosphate buffer in the ratio of 51:25:24
Detection wavelength	245 nm
Flow rate	1 ml/min
Injection volume	20 μ l
Runtime	15 min

(Hsieh *et al.*, 2011). The regulations for veterinary drugs define a risk assessment protocol to assess their active ingredients and set maximum residue limits (MRLs). Indeed, sustainable livestock systems in developing countries must agree the demand for animal products without compromising people's future nutritional needs or harming the environment.

The origin of residues in meat and milk

As intramammary infusions deliver high concentrations of antibiotics directly into the mammary gland, the majority of intramammary infusions contain penicillin or other beta-lactam antibiotics and many testing methods are particularly sensitive for this class of antibiotics, it is not astonishing that beta-lactam antibiotics are the most routinely detected residues in milk in most countries. Sulfa drugs are less commonly detected while tetracyclines, aminoglycosides, macrolides and other classes of antibiotics are hardly detected in milk (Heeschen and Suhren, 1996). While several factors have lead to the residue problem such as poor treatment records or inability to identify treated animals, most violations occur from the usage of a drug in some manner that does not comply with the labeling (Sundlof, 1989; Paige, 1994). This occurs basically through not observing withdrawal times as well as "extra-label" use of the drug. Treatments involving any other method than what is written on the product label (e.g., different species, increased dosage, different route of administration, different frequency of treatment) are recognized as extra-label usage and withdrawal times are difficult or impossible to determine in these circumstances (McEwen *et al.*, 1991). Excessive usage of antibiotics has caused xenobiotic residue occurrence in milk and milk products including meat and due to this milk and other food products like meat adulterated with antibiotics beyond a safe level are deemed to be unfit for human use (Hillerton *et al.*, 1999).

Detection of antibiotic and anthelmintic drug residues in milk samples from market

The above validated methods (Fig 1 and Fig 2) proved successful to detect and quantify the given veterinary drug residues thus indicating that the applied method was appropriate for the detection of antibiotic and anthelmintic residues in milk therefore the standardized and validated method was used for the analysis of total of 60 milk samples randomly collected from the market. Some of the representative chromatograms of anthelmintic residues in milk have been depicted in Fig 3, 4 and 5. The results are summarized in Table 3.

Comparison of levels of antibiotic and anthelmintic residues in milk samples with MRLs

The concentration of antibiotic and anthelmintic residues determined in each of milk and meat samples was compared with the tolerance limit (MRL) set forth by the International Regulatory Authorities MRLs given by European Union Commission (EU, 2010) and Codex Alimentarius Commission of WHO (Codex, 2015). The results are shown below in Table 4.

Drugs are used frequently in animal husbandry and the presence of drugs in foods like milk and meat is a health risk according to international food and drug regulation rules. Worldwide consumption of antimicrobials in food animal production was estimated at 63151 (± 1560) tons in 2010 out of which India shared 3 % and was ranked at 4th position (Boeckel *et al.*, 2015). This is an indicator that how higher chances of drug accumulation within animal tissues (meat and milk) were becoming inevitable particularly in a rural Country like India where Food and Drug Regulatory rules regarding veterinary area are very poor. Antibiotic residues in food have a deleterious effect on health and lead to the emergence of antibiotic resistant genes (Hassan, 2014). The occurrence of antibiotic residues in milk causes hindrance in the preparation of fermented dairy

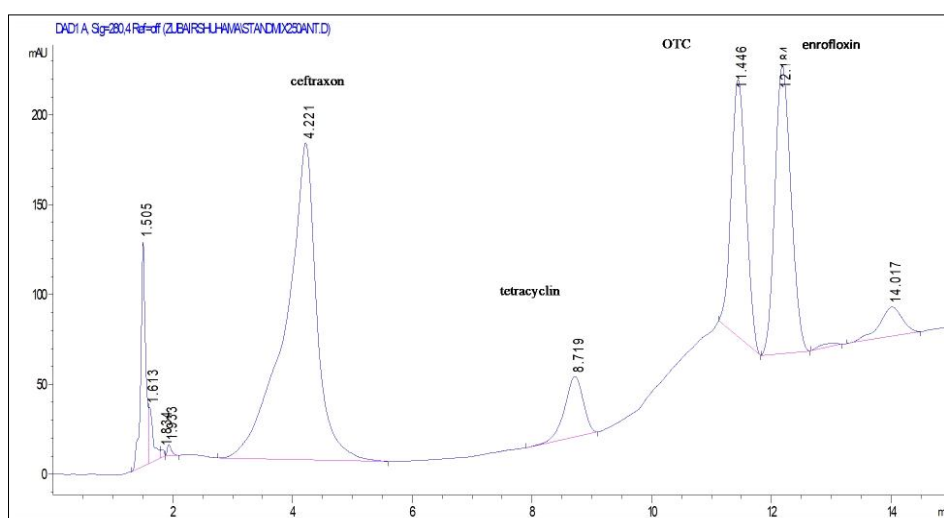


Fig 1: Chromatogram of mixed antibiotic standards.

products (Mayra-Makinen, 1995; Hays, 2003; Jones, 2009). The detection and analysis of drug residues in foods like milk, meat, chicken is a topic of urgent nature due to its impact on one health. Boultif *et al.* (2024) studied ELISA

based monitoring and quantification of tetracycline residues in fresh and powdered cow milk from Algeria. Al-Kindi *et al.* (2023) and Debbarma *et al.* (2025) have studied the antibiotic residues in Oman and Guwahati,

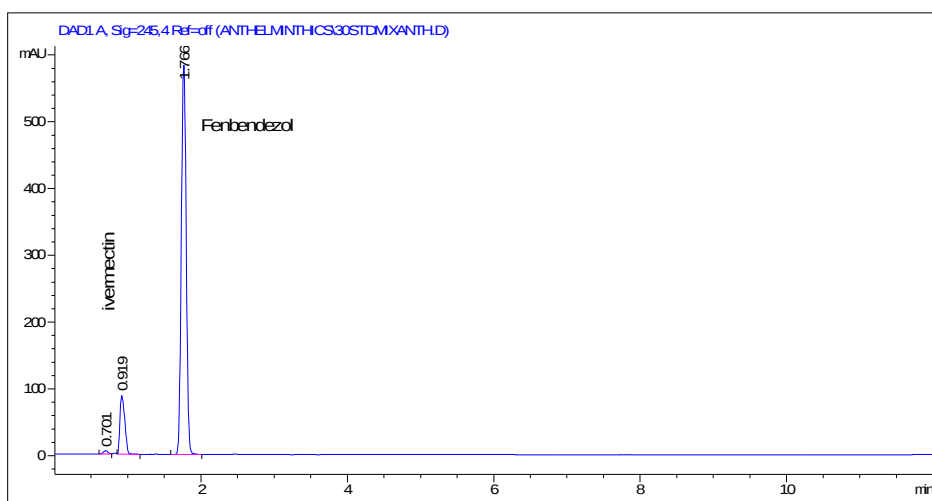


Fig 2: Chromatogram of standard mix of anthelmintics.

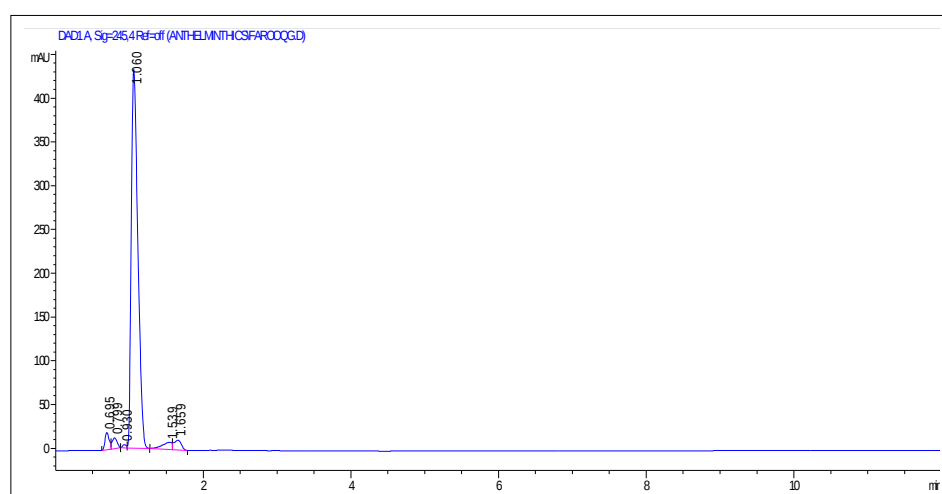


Fig 3: HPLC detection for Anthelmintics in milk (Ivermectin).

Table 3: Distribution of positive milk samples for each analyte.

Analytes	Number of positive samples			
	Local shops (n = 20)	Vendors and mini dairy farms(n = 20)	Pasteurized and packed milk(n = 20)	Total (n = 60)
Oxytetracycline	Nil	Nil	Nil	Nil
Tetracycline	Nil	Nil	Nil	Nil
Enrofloxacin	Nil	Nil	Nil	Nil
Ceftriaxone	Nil	Nil	Nil	Nil
Ivermectin	4 (20%)	Nil	Nil	4 (6.67%)
Fenbendazole	5 (25%)	1 (5%)	Nil	6 (10%)

Values in parenthesis indicate percentage.

India. It is extremely difficult under rural conditions in India to observe withdrawal periods in terms of veterinary drugs administered for disease treatment.

Detection and analysis of antibiotics and anthelmintics in market milk

In the present study none of the milk sample was detected positive for tetracycline, Oxytetracycline, enrofloxacin and ceftriaxone residues.

Anthelmintics (Ivermectin and Fenbendazole)

Ivermectin is a potent antiparasitic agent derived from naturally occurring fermentation products (Miwa *et al.*, 1983). Ivermectin is a member of a class of naturally occurring macrocyclic lactones called avermectins. Tissue distribution of ivermectin residues in cattle and sheep have been investigated (Tway *et al.*, 1981). Although ivermectin has a wide safety range in anthelmintic treatment (Ayres and

Table 4: Comparison of Antibiotic and Anthelmintic residue levels with EU and Codex MRLs.

Analyte	Number of samples violating MRLs			
	MRLs for milk		No of samples violating MRLs	
	EU (2010) (µg/Kg)	Codex (µg/L)	EU	Codex
Oxytetracycline	100	100	Nil	Nil
Tetracycline	100	100	-	-
Enrofloxacin	100	-	Nil	-
Ceftriaxone	100	100	-	-
Ivermectin	10	26	<u>1</u>	Nil
Fenbendazole	100	23	Nil	Nil

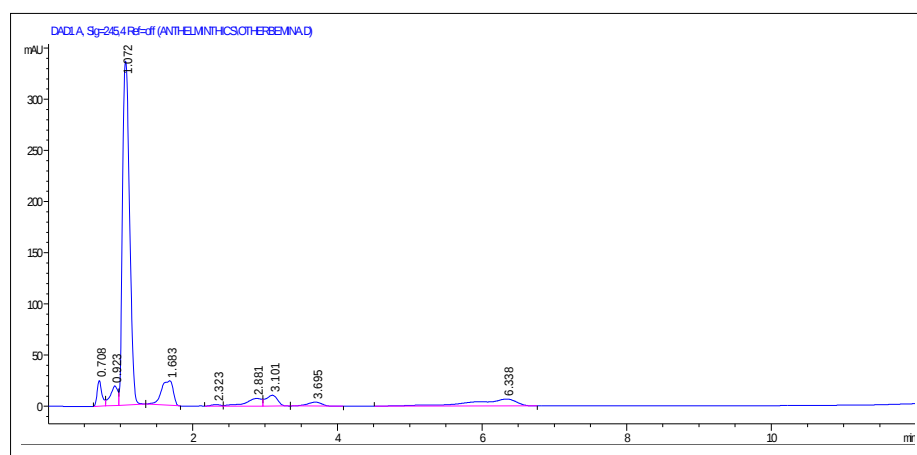


Fig 4: HPLC detection for Anthelmintics in milk (Ivermectin).

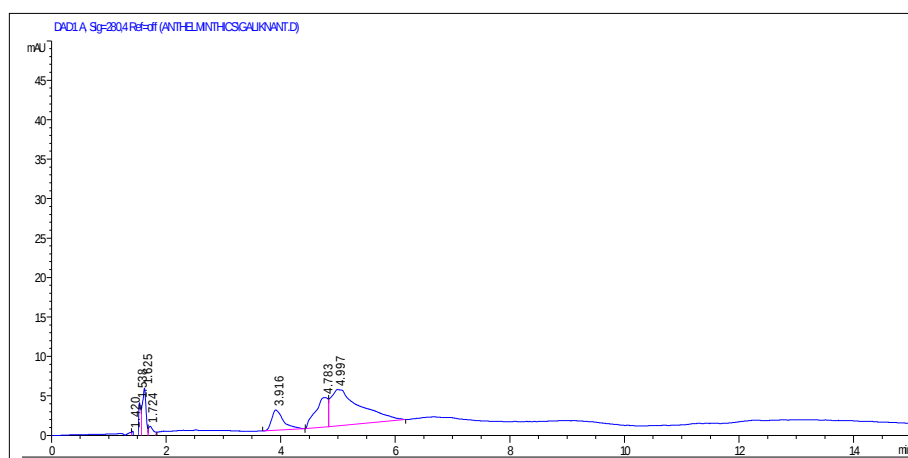


Fig 5: HPLC Detection of anthelmintics in milk (Fenbendazole).

Almeida, 2002) some of its excretion occurs through the mammary gland (Flajs *et al.*, 2005). The presence of ivermectin residues in foods may cause mutagenic effects in some mammalian species (Ema, 2004). The Codex alimentarius/European Union has set the residue limit of ivermectin in milk at 10 µg/L (FAO/WHO, 1993). Fenbendazole belonging to benzimidazole class of anthelmintics are mostly used in veterinary sector. In the present study 4 samples of milk amounting to 6.67% and 6 samples amounting to 10% of milk samples were found positive with Ivermectin and Fenbendazole residues respectively (Table 3). Alvinerie *et al.* (1987) in France subcutaneously gave ivermectin in a cow and studied the presence of ivermectin in milk for about three weeks through high performance liquid chromatography. This confirmed that avermectins which are very highly used in veterinary medical sector as ectoendoparasiticides can cause risk of health hazard to milk consumers of such treated cows as the drug is bound to protein and adipose tissues leading to its delayed excretion. The ivermectin residues upto the level of even 60% in bulk milk have been studied by Hanan *et al.*, (2016) in Egypt. Kochetkov *et al.* (2017) studied determination of Fenbendazole in milk by Liquid Chromatography and found the concentration of Fenbendazole versus time after treatment. The European Medicines Agency (EMA) in 2011 published a report on the use of Panacur, a liquid suspension of fenbendazole and the results warned that excessive use of panacur may cause anthelmintic resistance.

CONCLUSION

Judicious use of drugs particularly antibiotics and anthelmintics can lessen the occurrence of drug residues in animal foods like milk and meat and indirectly to say that rational use of drugs in veterinary practice can lessen the occurrence of antimicrobial and anthelmintic resistance and thus contribute overall to one health context. The selection of the drugs to be analyzed in milk samples was made on the basis of studying and surveying the rationality of drug use and thus observing the most commonly used antibiotics and anthelmintics. None of the open market milk samples was detected positive for antibiotic residues while 6.67 % of the milk samples were found to be positive for Ivermectin and 10% of the milk samples positive for Fenbendazole residues. However of the total milk samples analyzed only 1 milk sample crossed the limits of MRL as set forth by the European Union and Codex Alimentarius Commission.

Conflict of interest

All authors declared that there is no conflict of interest.

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