



# Occurrence, Public Health Implications and Detection of Antibacterial Drug Residues in Cow Milk

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## Abstract

In India, although antibiotics have been used in large quantities for some decades, until recently the existence of these drugs in the milk and meat has received a little concern. It is only in recent years that a more extensive investigation of antibiotic substances has been attempted in order to permit an assessment of the public health risks pose by them. Antibiotic usage in livestock production as therapeutics, prophylactics and as growth promoter has become vital to the growing dairy industry, since the prolonged or inappropriate usage of such antibiotics may lead to residues appearing in milk, which pose the risk of human health hazards and also interfere with the processing of the milk and milk products. The administration of antibiotics against bacterial infection is a significant driving force for selection of resistant strains of bacteria, which can spread from animal to human population and complicate the therapeutic management of such infections. Despite the numerous investigations performed, there is still a lack of understanding and knowledge about antibiotic residues in the milk and milk products. In this review we addressed the present state of knowledge concerning the occurrence, fate, public health hazards and the methods used to detect of antibiotic residues in milk

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## Introduction

The increasing human population and changing standard of living across the globe demand for the more food and other resources. The practical solution for this was

to adopt the practices to improve the agriculture production and industrial output. Several measures have been initiated by the Indian government to increase the productivity of livestock through various schemes and policies including Delhi Milk Scheme (DMS), Key Village Scheme (KVS), Integrated Cattle Development Project (ICDP) and some other programmes were launched to improve breeding, feed and fodder availability and effective disease control which has resulted in increasing the milk production significantly and at present, India stands first in the milk production (Kumar *et al.*, 2011). The average growth in the milk production is about 5 % per annum with sustained growth in the availability of milk and milk products for the growing population. Dairy sector has become an important secondary source of income for millions of rural families and has assumed the most important role in providing employment and income generating opportunities. Milk is an excellent source of nutrients and considered as a nature's perfect food (and as almost complete food) since the antiquity (Enb *et al.*, 2009). Milk contains the optimal balance of proteins, fats, carbohydrates, vitamins and minerals providing a range of benefits for growth, immunity and development for the calves and also to human.

Milk is, however a potential ready source for disease agents and other biological and chemical contaminants. Animal products and byproducts can potentially be contaminated with thousands of chemicals which are used in day today life for the various purposes. The most promising residues in animal derived products include pesticides, antibacterial drugs, hormonal growth promoters, industrial chemicals and heavy metals (Unnikrishnan *et al.*, 2005; Khaniki, 2007). Antibiotics are the most widely used veterinary drugs for therapeutic and prophylactic purposes and also as growth promoter in dairy animals which may appear in milk as residues for a certain time period (Wassenaar, 2005). Food Safety and Standards Act, 2006 defines veterinary drug residues as “*the parent compounds or their metabolites or both in any edible portion of any animal product and include residues of associated impurities of the veterinary drugs concerned*” (FSSA, 2006). The residues can also be defined as the substances having a pharmacological action, their metabolites and other substances transmitted to animal products that are likely to be harmful to human health (Serratos *et al.*, 2006). The antibacterial residues are sometimes called as “bacterial inhibitory substances” because of the microbiological basis of the screening tests frequently used to detect them and specific bacterial organism inhibited by them (McEwen *et al.*, 1992). The presence of residues may be the result of failure to monitor the withdrawal periods, illegal or off-label use of drugs and incorrect dosage levels or dosing schedule. Unauthorized antibiotic use and lack of good veterinary practices may result in residues of these substances in milk and tissues (Ivona and Mate, 2002; Paturkar *et al.*, 2005; Gaare *et al.*, 2012). The detailed understandings behind occurrence of residue in milk are explained in the later in the text.

Safe levels of residues in milk and other animal products result from the participation of all activities involved in the food chain ‘from stable to table’ (Serratos *et al.*, 2006). Milk producers, dairy cooperatives, personnel involved in processing, marketing and the veterinarians are directly or indirectly responsible to provide the safe milk for the society. Veterinary drugs are extensively used to promote the animal health, control and treat the infection and to step up the production (Botsoglou and Fletouris,

2001). Presence of any drug or antibiotic residue in milk and meat is considered as illegitimate and also lead to economic losses to dairy industry. A consumer demands the food which is free from chemical residues. Many countries have imposed regulatory limits (maximum residue limit/MRL) on the level of various drugs used for treatment of dairy animals. MRL could be defined as maximum concentration of residues in a product (milk, meat, egg) considered by the regulatory authorities as without sanitary hazard for the consumer and without effect on the manufacturing processes (Paturkar *et al.*, 2005).

### **Antibacterials used in dairy animals**

Several antibiotics have been isolated from various sources and being used to control disease pathogens. These agents are not only used for treating the infectious diseases, but also used as feed additives such as chemotherapeutic agents (antibiotics, anthelmintics) and growth promoters (Serratos *et al.*, 2006; Babapour *et al.*, 2012). Antibiotics can be grouped by either their chemical structure or mechanism of action. The most commonly used antibacterials in veterinary medicine include  $\beta$ -lactams, tetracyclines, aminoglycosides, macrolides, quinolones and sulfonamides (Mitchell *et al.*, 1998; Unnikrishnan *et al.*, 2005). These antibacterials may be used singly or at times in combination when treating dairy cattle. Antibacterials are administered by various routes to the animals. The most common routes are parenteral, topical, oral (sometimes through water and feed), intra-mammary and intra-uterine. All of these routes may result to residues in milk either parent drug or its metabolites (Hubbert *et al.*, 1996). Among the above mention antibacterials oxytetracycline, chloramphenicol and streptomycin are commonly excreted through milk by virtue of their pharmacokinetic properties (Zahid Hosen *et al.*, 2010).

Mastitis is the most prevalent and economically important widespread disease in cattle which requires intensive antimicrobial treatment (Mohsenzadeh and Bahrainpour, 2008). Treatment of mastitis with antibacterials is of greatest regulatory concern because of possibilities of antibacterial residues in milk (Sandholm *et al.*, 2009). Mastitis is treated with antibiotics delivered directly into the udder and sometimes injecting those by parenteral routes. Treated cows are required to be excluded from the milk supply for a specific time period to ensure that antibiotic residues are not excreted in their milk. Antibiotic residues enter the milk supply when treated cows are returned to the milking herd before the withholding period or when a cow retains antibiotic residues in her system for an extraordinary length of time. Therefore milk from treated cows must not be marketed until the recommended withholding period has elapsed (Khaniki, 2007). Observance with recommended withholding time helps minimizing the risk of antibiotic residues to occur in milk which is the exclusive responsibility of dairy farmer.

Research has quite extensively examined that, the reasons for contamination of bulk tank milk is bad management practices involving delivery of milk from treated cows during the withholding period, insufficient information flow between milking personnel, failures during milking, treatment failures or milking of dry cows after antibiotic treatment at drying off (Doherr *et al.*, 2007). Other major reasons for occurrence of drug residues in milk are incorrect milking order of cows and insufficient cleaning of milking

cluster or milking installation. Few cases of prolonged occurrences of residues in milk are related to veterinary error and insufficient cleaning of milk contact surfaces after milking of treated cows. The milking interval is reported to have more or less relation to residues excretion in milk. Henschelchen and Walser (1983) reported significantly shorter excretion periods for procain-penicillin G and oxytetracycline when cows were milked with 2 hour intervals after treatment. Different milking intervals in cows milked with automatic milking systems may influence the withdrawal period and excretion of antibiotics in milk of treated cows (Knappstein *et al.*, 2003).

In general, the health of cow and udder also has profound effect on excretion of antibiotics in milk. The fibrosis of udder tissue in chronic mastitis leading to poor distribution and absorption of penicillin cause higher concentrations and longer retention of penicillin in milk of the affected quarters compared to healthy quarters (Edwards, 1964). Intravenous infusion of high dosages of ceftiofur in cows with experimentally induced *Escherichia coli* mastitis resulted in significantly longer excretion of ceftiofur in milk compared to healthy cows (Erskine *et al.*, 1995). In a study Cagnardi and colleagues (2010) evaluated the effect of udder health on persistence of cefoperazone in the systemic circulation and mammary tissue after intra-mammary administration. They reported that, in the cows with subclinical mastitis the retention time of cefoperazone is more than that of healthy cow. The transfer of antibiotics from treated quarters to non-treated quarters and subsequent excretion in milk has been described for a number of antibiotics (Aureli *et al.*, 1990). In India the off label use of antibiotics mainly dosages deviating from recommendations of the drug manufacturer fall under the main reason for occurrence of antibiotic residues in milk after the end of the withholding period in cows. Due to off-label use of veterinary drugs or noncompliance withdrawal periods, much higher residue levels might appear in the milk and milk products (Nisha, 2008) (The term off-label is employed whenever a drug is used in a manner other than which it is licensed for). Off label use might include giving the drug to a different species or by different route or against the infection that it isn't licensed or recommended.

As prophylactics and growth promoters, the antibacterials are used at lower concentrations than those used for treatment. Because prevention of disease transmission and enhancement of growth and feed efficiency are critical in modern animal husbandry practices, there has been widespread incorporation of antibiotics into animal feeds in many countries including India (Khachatourians, 1998). The prolonged use at low concentration encourages the production of antibiotic resistant strains of bacteria (Simonsen *et al.*, 1998; Lin *et al.*, 2013). One example is the emergence of fluoroquinolone resistant *Campylobacter*, one of several bacterial species that cause severe food poisoning in humans (Cheng *et al.*, 2012).

### **Beta-lactam antibiotics**

It is interesting to note that the  $\beta$ -lactam antibiotics, including the penicillins, cephalosporins, carbapenems and others, make up the largest share of antibiotics used in most countries (Kummerer, 2009).  $\beta$ -lactam antibiotics are broad spectrum antibiotics interfering with cell wall synthesis, used generally to treat Gram positive and Gram

negative bacterial infections (Droumev, 1983; Sun *et al.*, 2013).  $\beta$ -lactam antibiotics include penicillins, cephalosporins, carbapenems and monobactam group of antibiotics. Among the beta-lactam antibiotics, penicillins and cephalosporins forms the major category used in veterinary medicine and are frequently used for the treatment of animals all over the globe.

Penicillins are the most commonly applied antibiotics for the treatment of bovine mastitis (Haapapuro *et al.*, 1997) which frequently results in their residues in milk. The residues of these antibiotics in milk cause problems in dairy industries and human health hazards (Ghidini *et al.*, 2002). Penicillins are not inactivated at pasteurization temperature or on drying and may cause allergic reaction manifested by skin rashes in very sensitive individuals at very low concentration of 0.03 IU/ml (Bjorland *et al.*, 1998) to 0.01 IU/ml (Waltner-Toews and McEwen, 1994) in milk. Cross reactivity is observed between penicillins and cephalosporins for development of allergic reactions. Approximately 4 % of patients with a history of penicillin allergy experience an anaphylaxis reaction to a cephalosporin (Kelkar and Li, 2001) and patients with a history of a penicillin related allergic event have a increased risk of a reaction when given a sulfonamide or a cephalosporin (Apter *et al.*, 2006). The carbapenems and aztreonam shows merely cross reactivity with penicillin and other beta lactams (Romano *et al.*, 2007). Beta lactam antibiotics are sometimes associated with neurotoxicity manifested by hallucinations, twitching, and seizures (Snively and Hodges, 1984). Pre-existing brain lesions, renal dysfunction and hyponatremia can trigger the neurotoxic symptoms even at lower concentration of these antibiotics (Granowitz *et al.*, 2008).

### **Tetracyclines**

The tetracyclines are broad-spectrum antibacterials active against *Mycoplasma*, *Chlamydomphila* and *Rickettsia* in addition to bacteria. Tetracyclines are bacteriostatic and acquired resistance is now widespread among bacteria (Fuoco, 2012). Tetracyclines may be administered parenterally, orally through feed or water or by intra-mammary infusion. The widely used oxytetracycline and the less often used tetracycline and chlortetracycline have similar properties. Fraction of tetracyclines excreted in bile gets reabsorbed through entero-hepatic circulation, and may persist in the body for a long time after administration (Chambers, 2006). The rate of metabolism of tetracyclines in cows has been estimated to 25-75 % and a significant percentage of the administered tetracyclines are excreted in bovine milk (Abbasi *et al.*, 2011). If these antibiotics administered improperly or if the withdrawal period for the treated cows has not been passed, the parent drug and their metabolites may end up in milk and may cause harmful effects to consumers (Fritz and Zuo, 2007). Demeclocycline, doxycycline and other tetracyclines to a lesser extent associated with mild to severe photosensitivity reactions upon exposure to sunlight in the individuals which are previously treated with these antibiotics. Photo-onycholysis and pigmentation of the nails may develop with or without accompanying photosensitivity (Chambers, 2006). Photo-onycholysis is a phototoxic reaction, which is a drug-induced separation of the nail from the nail bed when exposed to ultraviolet radiation. Tetracyclines can cross the placenta and enter the fetal circulation and amniotic fluid. Relative to the maternal circulation, tetracycline concentrations in umbilical cord

plasma and amniotic fluid are 60 % and 20 % respectively. Relatively high concentrations of these drugs also are found in breast milk. Children receiving tetracyclines for long or short duration may develop permanent brown discoloration of the teeth (Navratilova *et al.*, 2009). The larger the amount received relative to body weight, the more intense the enamel discoloration. Exposure of pregnant women with tetracyclines may produce discoloration of the teeth in her children.

### **Aminoglycosides**

Aminoglycoside were the first antibiotics discovered by systematic screening of natural product sources for antibacterial activity (Hermann, 2007). The laboratory of Waksman reported the discovery and isolation of the streptomycin from soil bacteria *Streptomyces griseus* in 1944, which was the first antibiotics used effectively against *Mycobacterium tuberculosis* (Schatz and Waksman, 1944). Aminoglycosides are usually used synergistically with  $\beta$ -lactams for the treatment of serious infections due to Gram positive and Gram negative bacteria. They act by binding to the A site of the 30 S small ribosomal subunit, inhibiting translation process in protein synthesis (Hermann, 2005). The antibiotic, gentamicin in this class is used mainly to treat uterine infections in cattle, particularly dairy animals. However, one of its side effects is its tendency to accumulate and persist in bovine kidney tissue for several months (Elezov *et al.*, 1984). Semi-synthetic derivatives of natural products like amikacin, netilmicin and tobramycin have been the most fruitful source of new clinically useful antibiotics (Von Nussbaum *et al.*, 2006). In humans, the nephrotoxicity of aminoglycosides is associated with small portions of the drug being accumulated in the renal cortex and leading to reversible renal impairment (Mingeot-Leclercq and Tulkens, 1999; Granowitz *et al.*, 2008). Considering the antibiotics induced nephrotoxicity, the aminoglycosides and amphotericin are the prototypical classes associated with acute renal failure; however, other agents including sulfonamides, beta-lactams and acyclovir, have been reported to cause renal impairment in variable severity. In addition to nephrotoxicity, aminoglycosides can cause irreversible ototoxicity that occurs both in a dose-dependent and idiosyncratic manner (Fischel-Ghodsian, 2005). Some animal study data demonstrated the probable role of reactive oxygen species to induce specific ototoxicity (Bates, 2003).

### **Sulphonamides**

Sulphonamides are among the oldest groups of antibacterials widely used in the treatment of bacterial and coccidial diseases of dairy cattle and as growth promoter in swine. They have a wide spectrum of bacteriostatic action, effective against both Gram positive and Gram negative organisms. These types of antibiotics dissolve quickly, easily distribute in all tissues and body fluids, including the cerebrospinal fluid and fetal circulation. About 90 % of these antibiotics bind to plasma proteins, and their maximum concentration seen 3-6 hours after the administration. They are metabolized primarily in the liver and excreted in the urine by glomerular filtration. The primary mechanism of adverse effects produced by these antibacterials in humans is associated with the impairment of thyroid-hypothalamus-pituitary axis and should be assessed by measuring parameters of thyroid and pituitary function (JECFA, 1990). Sulfonamides are also

known to produce sensitization and are one of the most common classes of antibacterial that produce allergic reactions at therapeutic dose (Golembiewski, 2002), but there have been no cases of human allergies that involved exposure to residues in animal foods (Paige *et al.*, 1999).

### **Chloramphenicol**

Chloramphenicol is a broad spectrum antibiotic, isolated from bacterium *Streptomyces venezulae* in the year 1947. It is clinically used to treat chronic infection of respiratory tract, bacterial meningoenzephalitis, brain abscesses and intraocular infections and is active against a variety of pathogens including bacteria, *Spirochaetes* and *Rickettsiae* (Shukla *et al.*, 2011). Chloramphenicol and its metabolites may appear in milk after parenteral administration, however after oral administration to cattle it is not excreted in milk (De Corte-Baeten and Debackere, 1976). Chloramphenicol has been associated in a non-dose related manner with aplastic anemia (Shukla *et al.*, 2011) and bone marrow suppression (Ambeker *et al.*, 2000) in a small proportion of human to whom the drug was exposed. Some of the individuals who survive the bone marrow depression may develop leukaemia, which creates concerns about possible carcinogenicity (Doody *et al.*, 1996). Although, most countries have banned the use of chloramphenicol in food and dairy animals, nevertheless traces of it have been detected in shrimp and other aquaculture products (Shukla *et al.*, 2011).

### **Other antibacterials**

Nitrofurans are the broad spectrum synthetic antibacterials, primarily used to treat Gram positive bacterial infections. In veterinary medicine these are used for topical application on infected burns and skin infections e.g. nitrofurazone (Vasheghani *et al.*, 2008) for the oral treatment of cholera e.g. furazolidone (Roychowdhury *et al.*, 2008), bacterial diarrhoea and giardiasis (Petri, 2005); and to treat urinary tract infections e.g. nitrofurantoin (Guay, 2008). These antibacterials are sometime associated with adverse reactions involving hemorrhagic diathesis, anemia, nausea and vomition in livestock and cardiotoxicity in birds. Animal studies demonstrated the mutagenic (Ahmed *et al.*, 2008) and neurotoxic effects of long term exposure to nitrofurans. The possible mechanism linked to the mutagenic effect of furazolidone and other nitrofurans involves the irreversible damage to the DNA of epithelial cells as well as endocrine dysfunction occurred prevalently when cells are exposed to furazolidone (De Angelis *et al.*, 1999). Based on animal study data, nitrofurans and some anti-parasitic drugs, such as dimetridazole, also raise some concern of carcinogenicity (Waltner-Toews and McEwen, 1994).

Another synthetic class, fluoroquinolones are broad spectrum antibacterials primarily active against Gram negative pathogens. These are effective for the therapy of serious infections, e.g. septicemia, gastroenteritis and respiratory diseases and also used for the treatment of infections of the urinary tract and soft tissues (Nizamlioglu and Aydın, 2012). They are effective in the therapy of mycoplasma infections and infections caused by atypical bacteria (Navratilova *et al.*, 2011). In veterinary medicine, they are

useful especially in the therapy for gastrointestinal and respiratory tract infections (Botsoglou and Fletouris, 2001), enrofloxacin being the most widely used fluoroquinolone in veterinary medicine (Monica *et al.*, 2011). Fluoroquinolone preparations are also used for the prevention and treatment of mastitis in lactating cows and for dry cow therapy (Gruet *et al.*, 2001).

A few antibacterials are also known to cause functional changes in the rhythm and rate of cardiac contraction. Macrolides, some quinolones, azoles, pentamidine and quinine can prolong the Q-T interval of the cardiac cycle and cause ventricular arrhythmia. Individual receiving oral erythromycin is found to have increased risk of sudden death probably due to Q-T interval prolongation (Ray *et al.*, 2004). On the other hand erythromycin, azithromycin and other macrolides can cause dose dependant and serum concentration dependent ototoxicity associated with bilateral hearing loss or labyrinthine dysfunction (Swanson, 1992; Coulston and Balaratnam, 2005; Hajjioannou *et al.*, 2011) and sometimes may results in permanent hearing loss or vertigo (Ress and Gross, 2000).

### **Public health hazards imposed by antibacterial residues**

The prevalence of food borne illness is increasing worldwide and it has a major public health impact (Nguz, 2007). Food borne illnesses are caused by eating or drinking beverages and other food articles contaminated with bacteria, parasites, viruses or chemical contaminants usually as a result of food mishandling and mismanagement practices. In India insufficient data is available regarding the food borne hazards to human and environment. In China the estimated incidents of food borne hazards was 200-400 thousand people annually (Xie and Yongda, 2002). Centers for Disease Control and Prevention (CDC) estimated that food borne illnesses affect 48 million people each year in United States (CDC, 2011).

Most of the antibacterials currently used in the control and treatment of farm animal diseases are relatively non toxic even at higher concentration, but there are few antibiotics which pose a significant threat to public health when present in sufficiently high concentrations in food (McEwen and McNab, 1997). Antibiotic residues in milk are of great public health concern since milk is being widely consumed by infants, youngster and adults throughout the globe (Khaniki, 2007). Considering the issue of public health hazards, milk and milk products contaminated with antibiotics and other chemical contaminants beyond a given residue levels, are considered unfit for human consumption (Hillerton *et al.*, 1999; Goffova *et al.*, 2012). Occurrences of veterinary drug residues pose the broad range of health consequences in the consumers. The residues of antibacterials may present pharmacological, toxicological, microbiological and immunopathological health risks for humans (Drackova *et al.*, 2009). In rare situations, the pathogens against which the antibiotics are being used have less public health hazards than those posed by the improper use of antibiotics. To enlist one example, mastitis pathogens in milk pose a lower threat to public health if milk is pasteurized. On the other hand, the careless antibiotic therapy to eliminate mastitis pathogens becomes a public health concern due to their residues in milk [84] (Hameed, 2006).



Antibacterial causes broad range of health effects, to summarize they can cause development anomalies e.g. bone marrow aplasia (chloramphenicol) and can alter the normal gastrointestinal microflora resulting in GI disturbances (intestinal dysbiosis) and development of resistant strains of bacteria. Therefore, the use of antibacterials may result in emergence of antibiotic resistant strains of pathogens, complicating the treatment for both human and animal diseases (Dewdney *et al.*, 1991; Goffova *et al.*, 2012). In addition some of the antibacterial may act as carcinogens and pro-carcinogens (Oxytetracycline and furazolidone). Carcinogenic and genotoxic effects in consumers could be possible at very low exposure levels, especially if the chemical in question is ingested regularly over a long period of time. Oxytetracycline can react with nitrite and the combination thereof called as nitrosamine is a potential carcinogen (Mitchell *et al.*, 1998). In the year 1995 European Union (EU) prohibited the use of nitrofurans for the treatment of bacterial diseases in livestock production, due to concerns about the carcinogenicity of their residues in edible tissue (Vass *et al.*, 2008). In subsequent years Australia, USA, Philippines, Thailand and Brazil also prohibited the use of nitrofurans in food animals (Khong *et al.*, 2004).

These hazards can be categorized in to two types as direct-short term hazards and indirect-long term hazards, according to duration of exposure to residues and the time onset of health effects (Muhammad *et al.*, 2009). The direct health hazards includes the health effects caused due to excretion of drug in milk, as an example the beta-lactam group of antibiotics regardless of their low concentration in milk causes allergic hypersensitive reaction in sensitized individual immediately after consumption (Paige *et al.*, 1997; Sierra *et al.*, 2009). Another example includes the aplastic anemia caused by chloramphenicol not related to level of exposure (Rich, 1950; Granowitz *et al.*, 2008).

Several antibiotics are potent antigens or act as a haptens and occupational exposure on a daily basis can lead to allergic reactions. Most of the reported allergic reactions are related to  $\beta$ -lactam antibiotic residues in milk or meat and the allergic reaction has been associated with exposure to antibiotic residues in foods. Many of the cases refer to people previously treated with antibiotics and hypersensitized to a degree that subsequent oral exposure evoked a response. A hypersensitivity reaction to a drug is either IgE-mediated or Non-IgE-mediated reactions. IgE-mediated reactions occur shortly after drug exposure. Instances of IgE-mediated hypersensitivity reactions include urticaria, anaphylaxis, bronchospasm and angioedema. Non IgE-mediated reactions include hemolytic anemia, thrombocytopenia, acute interstitial nephritis, serum sickness, vasculitis, erythema multiforme, Stevens-Johnson syndrome and toxic epidermal necrolysis (Granowitz *et al.*, 2008).

Indirect and long term hazards are the effects caused by long term exposure of an individual to residues and include carcinogenicity, teratogenicity and reproductive effects. Long term exposure to diethyl stilbesterol can cause vaginal clear cell adenocarcinoma and benign structural abnormalities (Offman and Longacre, 2012). The long term exposure to antibiotic residues in milk may result in alteration of the drug resistance of intestinal microflora (Ram *et al.*, 2000). The use of the antibiotic avoparcin as a growth promoter in food animals resulted in the development and amplification of

vancomycin resistant enterococci. Subsequent colonization in human intestine of these resistant strains causes the clinical disease that would be difficult to treat (Tzavaras *et al.*, 2012). Even if the resistant strains bacteria are not human pathogens, they may still be dangerous because they can transfer their antibiotic resistance genes to other pathogenic bacteria (Hayek, 2013). Antibiotic resistant strains of bacteria, including *Salmonella*, *E. coli* and *Campylobacter* spp., have been isolated from farm animals in many countries (Aarestrup *et al.*, 1997; Brichta-Harhay *et al.*, 2011).

Antibacterial agents like tetracyclines, nitrofurans and sulfonamides are used as feed additives in cattle feed which may excrete in milk and sometimes associated with toxicological effects in human (DeVries, 1997). Carcinogenic and mutagenic effects were demonstrated on animals due to nitrofurans used at high and lengthened doses (De Angelis *et al.*, 1999; Gutierrez *et al.*, 2011). These antibacterials are now prohibited in most countries for veterinary use in animal production. The other substances considered as potentially carcinogenic are antibiotic additives: quinoxaline, carbadox and olaquinox (FAO/WHO, 1995). In long term animal studies, amoxicillin (Abou-Tarboush, 1994), chloramphenicol (Fritz and Hess, 1971), doxycycline (Schaefer *et al.*, 1996), gentamicin (Nishio *et al.*, 1987) and rifampin (Holdiness, 1987), have shown effects on reproduction performance in parents and developmental toxicity in their offsprings. This data hold functional insight on possible adverse effects of these antibacterials in pregnant and lactating women and to their young ones. Recently in a study, Etminan and coworkers (2012) postulated the risk of retinal detachment in individuals upon continued exposure to fluoroquinolones. Chloramphenicol is also associated with optic neuropathy (Wong *et al.*, 2013) and brain abscess (Wiest *et al.*, 2012) with varied intensities and clinical manifestations.

Apart from the health hazards, antimicrobial residues in milk are responsible for interference with starter culture activity and hence disrupt the manufacture process of milk products (Katla *et al.*, 2001). In the fermented dairy products manufacturing plants, such as cheeses and yogurts, the presence of antimicrobial agents can lead to the partial or total inhibition of the lactic bacterial growth. Antibiotic residues can also interfere with the methylene blue test, intended to estimate the total microbial load in milk. The time taken for reduction of the dye will be increased, hence causing under estimation of the microbial load. All of these concerns may result in major economic losses to the dairy industry.

### **Antimicrobial residues and regulatory issues in India**

Milk offered for sale is a specific food item that requires strict control and enforcement by all legal regulations and relevant authorities that must allow its marketing at home country and abroad only through special and official permission.

In regard to dairy animals in developed countries, to avoid risks related to drug residues in milk, in many countries maximum residue limits (MRLs) have been established for each antimicrobial agent by law, below which it is considered that the drug may safely be used without harming the consumer. In the European Union, the

Table.1. Maximum Residue Limits (MRL) for commonly used veterinary drugs in cow milk (Codex Alimentarius Commission 2012)

Sr. No.	Veterinary drug	Class of drug	MRL ( $\mu\text{g/l}$ )
1	Albendazole	Benzimidazole anthelmintic	100
2	Amoxicillin	Penicillin antibiotic	4
3	Benzyl-penicillin	Penicillin antibiotic	4
4	Ceftiofur	Cephalosporin antibiotic	100
5	Chlortetracycline	Tetracycline antibiotic	100
6	Oxytetracycline	Tetracycline antibiotic	100
7	Tetracycline	Tetracycline antibiotic	100
8	Colistin	Polymyxin antibiotic	50
9	Dihydrostreptomycin	Aminoglycoside antibiotic	200
10	Streptomycin	Aminoglycoside antibiotic	200
11	Diminazene	Aromatic diamidine trypanocide	150
12	Doramectin	Avermectin anthelmintic agent	15
13	Eprinomectin	Avermectin anthelmintic agent	20
14	Febantel	Phenylguanidine anthelmintic agent	100
15	Fenbendazole	Benzimidazole anthelmintic agent	100
16	Oxfendazole	Benzimidazole anthelmintic agent	100
17	Gentamicin	Aminoglycoside antibiotic	200
18	Imidocarb	Carbanalide antiprotozoal agent	50
19	Isometamidium	Trypanocide	100
20	Ivermectin	Avermectin anthelmintic agent	10
21	Lincomycin	Lincosamides antimicrobial	150
22	Monensin	Polyether ionophores antimicrobial	2
23	Neomycin	Aminoglycoside	1500
24	Pirlimycin	Lincosamide antibiotic	100
25	Spectinomycin	Aminocyclitol antibiotic	200
26	Spiramycin	Macrolide antimicrobial	200
27	Sulfadimidine	Sulfonamide antimicrobial	25
28	Thiabendazole	Benzimidazole anthelmintic agent	100
29	Tylosin	Macrolide antimicrobial	100
30	Procaine benzyl-penicillin	Penicillin antibiotic	4

MRLs in food of animal origin are established by the Codex Alimentarius Commission (Codex Alimentarius Commission, 2012). These MRLs for commonly used veterinary drugs as listed in the table 1 would be followed by the countries wherein the regulatory limits are not established by the concern authorities. In India the manufacturing of antibiotics and other drugs for human and veterinary use are regulated by Central Drug Standard Control Organization (CDSCO) under Drugs and Cosmetics Act of 1940 and the rules therein. However, there is intrinsically no regulation especially for use of antibiotics in animals for treatment and as growth promoter. Multiple regulations for food have been ordained at different points of time to supplement each other. This incremental approach has lead to incoherence and inconsistency in the food sector regulatory scenario.

The Food Safety and Standards Authority of India (FSSAI) under the Ministry of Health and Family Welfare is the main authority for establishing the scientific standards for articles of food including milk and milk products and to regulate their manufacture, storage, distribution, sale and import, to ensure availability of safe and wholesome food for human consumption and for matters connected therewith as per the rules specified by Food Safety and Standard Act, 2006 (FSSA, 2006). The Food Safety and Standards Act, 2006 is a new legislative act which replaced the Milk and Milk Products Order, 1992 on August 5, 2011. The FSSA integrates eight different existing food laws, and is a major transformation that ensures to bring paradigm shift in the food regulatory scenario in India. The FSSAI and State Food Authorities are responsible for implementation and enforcement of the FSSA. The Section 21(1) of FSSA, 2006 specifies that the “food should not contain any insecticides or pesticides residues, veterinary drugs residues, antibiotic residues, solvent residues, pharmacological active substances and micro-biological contaminants in excess of limits prescribed under the regulation” (FSSA, 2006). FSSAI have recognized few laboratories from different regions of country for complete analysis of the food samples for presence of as per the Food Safety and Standards (Food Products Standards and Food Additives-Part-I & II) Regulations, 2011 including the chemical contaminants, mycotoxins and veterinary drug residues.

### **Methods for detection of antibacterial residues in milk**

To assure consumer’s safety and high quality dairy products intended for export, raw milk is to regularly analyze for the presence of antibiotic residues. Detecting violative levels of antimicrobial residues in milk through the use of residue screening and other qualitative tests can help prevent contaminated milk from entering the human food chain. Although milk is not regulated at the individual cow basis, use of antibiotic screening tests for individual cow milk is associated with reduced risk of bulk milk residue incidence (McEwen *et al.*, 1991). Different methods and assays for the detection of residues of antimicrobials, mostly in cow milk, have been developed and validated, whereas few studies have been carried out so far for detection of residues in sheep and goat milk (Wang *et al.*, 2006; Comunian *et al.*, 2010).

To detect antibiotic residues, different kinds of methods were developed. These methods can be classified in two main groups as of screening methods and

chromatographic methods to detect as many antibiotics as possible at very low concentration. The screening tests are generally performed by microbiological (Nouws *et al.*, 1999; Babapour *et al.*, 2012), enzymatic and immunological methods (Strasser *et al.*, 2003). The working principle of screening methods is based on the variable susceptibility of bacteria to different antibiotics. The antibiotic residue detection assays that are currently available use different methods and test microorganisms (Mitchell *et al.*, 1998). Microbiological assays for the detection of antibiotic residues utilize bacteria such as *Bacillus stearothermophilus* because of its high sensitivity to the majority of antibiotics. The first test for establishing antimicrobial agent residues in milk (microbial inhibitor test) was developed as early as 1952 (Mitchell *et al.*, 1998). The developments of tests for detection of antibiotic residues were initiated to establish the inhibitor agent levels in milk, since the presence of these agents could cause the inhibition of the starter cultures used in dairy industry (Navratilova, 2008). These methods are relatively cheap, simple to carry out and capable of detecting a wide variety of antimicrobial agents. A drawback which limits their use is a long incubation period. Therefore, rapid assays for antibiotic agent detection in milk have been developed which enable obtaining the results in short duration. The rapid tests are simple to perform, sensitive and specific. The rapid assay developed include Penzyme test which was developed in 1980's. Later on, in 1988, Charm II test for detecting 7 types of antimicrobial agents was introduced to the market, followed by other rapid assays, e.g. the LacTec test (1991), SNAP test (1994), Beta Star test, Charm Safe Level test (Mitchell *et al.*, 1998) and Charm MRL-3 (Reybroeck *et al.*, 2011; Fejzic *et al.*, 2014).

### **Microbiological inhibition assays**

In microbial growth inhibition test, standard culture of the test microorganism in a liquid or solid medium is used; e.g. *Geobacillus stearothermophilus* var. *calidolactis*, *Bacillus subtilis*, *Bacillus megaterium*, *Sarcina lutea*, *Escherichia coli*, *Bacillus cereus* or *Streptococcus thermophilus* (Heeschen, 1993). Milk sample to be analysed is applied on the agar surface and the plates are incubated for diffusion of the sample into the medium, and if the sample contains inhibitor agents, inhibition of growth occurs of the tested microorganism. The positive test is indicated either by formation of a clear zone of inhibition around the disc or a change in the colour of medium (Reybroeck, 2014). At present, many commercially produced microbial inhibitor tests are applied along with selective rapid tests for milk sample screening in primary dairy industry for rapid and precise detection of residues (Kozarova *et al.*, 2009). The advantage of these methods is that they have a wide detection spectrum, simple to carry out, reliability and they are cheap and can be used for the screening of a large number of samples. Microbial inhibitor tests detect a wider range of antimicrobial substances, including  $\beta$ -lactam antibiotics, and give a result within 3 hours or less.

### **Receptor/ Protein binding assays**

For detection of the  $\beta$ -lactam antibiotics residues in bulk or individual cow milk samples, antibiotic specific receptor proteins or penicillin-binding proteins (PBP) were successfully used in some methods and commercially available tests (Biacore

analysis, Penzym test, Beta Star test, SNAP test, Charm Safe Level test and DELVO-X-Press test and others) (Navratilova, 2008). Receptor binding assays are also a common class of antibiotic residue screening tests. This type of assay involves a receptor protein conjugated to an enzyme. The conjugate will bind to free  $\beta$ -lactam antibiotics that may be present in the milk sample (Massova and Mobashery, 1998). One of the most common receptor binding assays available commercially in market is the Beta star test. Beta Star test is a receptor assay for rapid detection of the beta-lactam antibiotics penicillin, ampicillin, amoxicillin, cloxacillin and cephapirin. This test is validated for use with raw, commingled cow's milk (Movassagh and Karami, 2011).

Table.2. FSSAI recommended analytical methods for detection and quantitation of antibiotic residues:

Sr. No.	Antibacterials	Analytical method
1	Beta-lactam antibiotics	<i>Bacillus stearotherophilus</i> qualitative disc Method-II
2	Chloramphenicol	HPLC-MSMS method
3	Nitrofurans metabolites	HPLC-MSMS method
4	Tetracyclines	HPLC-UV/ MSMS method
5	Sulphonamides	HPLC-MSMS method
6	Quinolones	HPLC-MSMS method
7	Nitromidazoles	HPLC-MSMS

### Immunoassays

Immunoassays are quantitative or semi-quantitative methods characterized by their high specificity, sensitivity, simplicity and cost-effectiveness, which make them particularly useful in routine work. They are based on the specific reaction between antibody and antigen. Nonisotopic immunoassays such as ELISA (Enzyme Linked Immunosorbent Assay), FPIA (Fluorescence Polarisation Immunoassay), PCIA (Particle-Concentration Immunoassay), PCFIA (Particle-Concentration Fluorescence Immunoassay), and monoclonal-based immunoassays play an important role in antibiotics screening immunoassay (Roeder and Roeder, 2000; Gaurav *et al.*, 2014). These methods can also be applied for a preliminary identification of classes of antibiotics (Sternesjo and Johnsson, 1998). Recently, Jiang and colleague (2013) described a dual-colorimetric ELISA for the simultaneous detection of 13 fluoroquinolones and 22 sulfonamides with the detection limit of 2.4 and 5.8 ng/ml, respectively. Further, ELISA is easy to perform, portable and reported to be very sensitive and can be economical when many samples need to be analyzed.

Monoclonal antibodies (Mab) also have been used to detect antibiotic residues in milk. Dietrich and colleagues (1998) described an assay to detect ampicillin residues using monoclonal antibodies raised against ampicillin-keyhole limpet hemocyanin conjugate coupled by a glutaraldehyde in mice. Sensitivity and specificity of these Mabs were tested with a direct competitive enzyme immunoassay, in which an ampicillin-horseradish peroxidase conjugate prepared by a carbodiimide method served as the labelled antigen.

Although the microbiological inhibition assay have been encourage because of their simplicity, these methods lack specificity and allow for only semi-quantitative measurements of residues detected and sometimes may produce false positives results (Kurittu *et al.*, 2000; Abbasi *et al.*, 2011). Therefore, for quantitative measurements and to detect the specific antibiotic and their metabolites, chromatographic techniques, such as HPLC (high performance liquid chromatography), GC (gas chromatography) and capillary electrophoresis (CE), have been developed to substitute microbiological assays (Chen and Gu, 1995; Posyniak *et al.*, 2005; Petkovska *et al.*, 2006; Kantiani *et al.*, 2009; Kukusamude *et al.*, 2010; Adetunji and Olaoye, 2012; Tona and Olusola, 2014). Also, to avoid the condemnation of bulk milk quantity due to exceeding tolerance levels of residues, it is required to analyze the sample with highly selective and sufficiently sensitive analytical methods. The main drawbacks of chromatographic methods are: they are expensive, non-portable, and therefore non-suitable to be use at farm level and require expertise to operate. Hence, these methods are used only as confirmatory test and to support the results of rapid screening tests. For the successful implementation of national regulation and surveillance monitoring for antibiotic residues FSSAI recommend various analytical and screening tests for detection of these residues in milk and other food products (Table.2).

### **Possible strategies for prevention of antibacterial residues in Indian scenario**

Preventing drug contamination of milk is the responsibility of every farm. Drug residues can be avoided by a well planned drug use program. The sale of contaminated milk will cause the responsible party to be subjected to severe penalties, including suspension of permits and monetary loss. Milk with drugs can adulterate a whole truckload or holding tank of milk.

1. Establishment of pharmacokinetics and withholding time for antibacterial used in dairy animal to describe metabolism and distribution of drugs in different tissue and milk. Withholding periods after treatment of cows with veterinary drugs should be valued. The pharmacokinetics of a drug is also dependent on the vehicle used in a certain drug formulation. Therefore the withholding time is valid for the specific drug. Different withholding periods may be appropriate for two drugs containing the same antibiotic.
2. One practical approach to cut down the residues in milk would involve good hygiene and good management practices at farm and the milk processing units. Modifying and implementing the good management practices is also very

important for preventing the spread of disease among livestock which could reduce the need of antibacterial use.

3. Evaluation and use of alternative to antibiotic growth promoter e.g. probiotic microorganisms, immune modulators, organic acids (acidifiers) and other feed supplements.
4. Although, Directorate General of Health Services under Ministry of Health and Family Welfare has set out the policy for use of antimicrobials to combat antimicrobial resistance in human and animal in India, there is no regulation or policy regulating the use of antibacterials in animals for treatment or as growth promoter. Establishing the use policy for antibacterial in animals will help for monitoring and surveillance of the usage of these drugs.
5. Pharmacovigilance programme would be developed for veterinary pharmaceuticals concerning the safety of veterinary medicines used for the treatment, prevention or diagnosis of disease in animals.
6. Establishment of pharmacovigilance working group and an effective reporting system involving veterinarians, immunologists, pharmacologists, toxicologists and ecotoxicologists is an important prerequisite for the risk assessment of antibacterial drug residues for human and environment.
7. Maintaining treatment records of cows in order to determine appropriate withholding periods. This will also help to treat dry cow with long acting substances so the withholding period can be adjusted if the dry period is shorter than expected.
8. Recommendations of the drug manufacturer regarding dosage, route of administration, treatment intervals and storage condition of antimicrobials should be followed intimately because any deviation may contribute to extended withholding periods.
9. Development and validation of rapid screening tests for detection of antimicrobial residues in milk at individual cow basis to make sure that milk of individual cows is free of inhibitors after the end of the withholding period.

Establishing regulatory standards and good management practices that reduce the risk of antibiotic residues in milk supply are essential components of human food safety. Within the last decade an increasing number of investigations covering antibiotic input, occurrence, fate and effects have been published, but there is still a lack of regulations and guidelines regarding use of antibiotics in veterinary practice in India. The issue of antibiotic residues in food chain warrants the further policies and guidelines to address the possible risk to public health and environment.

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