Determination of Antibiotics Residues in Milk Samples Collected in the Different Sites of Kathmandu, Nepal

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ABSTRACT

Background: Antibiotics are commonly used in animal husbandry for medicinal, prophylactic and growth promotion purposes.

Method: The prevalence of three groups of antibiotics; namely Gentamycin, Streptomycin and sulfonamides was studied in fresh milk available in five different study areas; namely Budhanilkantha, Dharmasthali, Tokha, Nepal and Gaushala from Kathmandu Valley of Nepal. The milk samples (n=100) were collected from local dairy collection centers of Kathmandu Valley. Qualitative and semi-quantitative analysis with rapid screening kits revealed that 6% (n=6) samples were positive for antibiotic residues in the fresh milk for gentamycin, streptomycin and sulfonamide groups. Total 5% milk samples contained unidentified antibiotic residues. Sulfonamides residues were found to be highest 3% (n=3) followed by gentamycin 2% (n=2) and streptomycin 1% (n=1) with no any milk sample with multiple residues. Total 2 samples were positive in Dharmasthali, 2 in Gaushala, 1 in Nepal and 1 in Tokha and no positive samples from Budhanilkantha area. There was no statistical significant difference between study areas and commonly used antibiotics residues in milk samples (P>0.05).

Conclusion: The antibiotic residues were found above the maximum residue limits that presented serious threat to consumer health and raised a serious concern regarding the implementation and monitoring of international regulations.

Key words: Antibiotics, Gentamycin, Milk, Streptomycin, Sulphonamides.

INTRODUCTION

Milk is an opaque white fluid rich in fat and protein, secreted by female mammals for the nourishment of their young ones. As an agricultural product, milk is extracted from non-human mammals during or soon after pregnancy (Pehrsson et al., 2003). Nepal is the fifth largest producer of buffalo milk around world. In our domestic context, milk production from cattle and buffalo are 5,57,669 metric tons and 11,67,154 metric tons respectively (De Vries and Kaylegian, 2018).

Antibiotics, which are commonly used in animal husbandry for medicinal, prophylactic and growth promotion purposes can be grouped either by chemical structure or by their mechanism of action. The most commonly used antibiotic in veterinary medicine includes Beta-lactams, Tetracyclines, Aminoglycosides, Macrolides, Quinolones and Sulfonamides (Mitchell et al., 1998; Unnikrishnan et al., 2005). These antibiotic may be used singly or at times in combination when treating dairy cattle. Oxytetracycline, Chloramphenicol and Streptomycin are commonly excreted through milk by virtue of their pharmacokinetic properties (Zahid Hosen et al., 2010).

Beta-lactam antibiotics are broad spectrum antibiotics interfering with cell wall synthesis, used generally to treat both Gram +ve and Gram -ve bacterial infections (Droumev, 1983). Among the Beta-lactam antibiotics, Penicillin’s and Cephalosporin’s forms the major category used in veterinary medicine and are frequently used for the treatment of animals all over the globe. Penicillin’s 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 Carbapenems and Aztreonam show 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).

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 Nepal, 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...
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United States (CDC, 2011). Long-term antibiotic residue intake through food consumption may pose some specific adverse health effects such as gastrointestinal and liver implications. Antibiotic residues in milk as well as other animal-originated foods are regulated by safety legislation and standard agencies in developed countries. Safe limits have been established by international organizations such as the Food and Agriculture Organization (FAO), World Health Organization (WHO), Codex Alimentarius (CA) and Scientific Committee of Food of European Union (EU).

The withdrawal period is the interval between the last doses of a medicine given to the animal and the time when the level of residues in the tissues (muscle, liver and kidney, skin/fat) or products (milk, eggs, honey) falls below the Maximum Residue Limit (MRL). The specified period of drug withdrawal must be observed prior to providing any food from animal products to the market for human consumption (Jackson, 1980). In context of Nepal, as there is no strong veterinary drug regulation, the country has adopted European Union standards for drug withdrawal period. Till date, very less research on the milk residues and their impact on human health have been specified in Nepal. Therefore, the present study aims to lessen this research gap.

**MATERIALS AND METHODS**

The study was carried out during the month of April to May 2017 in Kathmandu district (Fig 1). This area is among the important pocket areas for milk production. Since the site of study is not much far from the major cities and being linked with road network, it has got easy access to Veterinary services and Artificial Insemination (A.I) facility. Except few organized farms most of the house hold rare cattle in traditional shed. The samples were collected from Five sites of Kathmandu district (Budhanilkantha, Dharmsatelli, Tokha, Nepalitar and Gaushala). This study was conducted in different sites of Kathmandu district at Bagmati zone, central development region province number 3. Kathmandu district is popular for annual milk production (75,739 metric ton) of study area, 11 samples were found to be positive with 6 known (Sulfonamide, Streptomycin, Gentamycin) and five unknown milk residues (Fig 2).

A total of 100 raw milk samples (5-10 ml) were collected in sterile McCartney bottle from the people selling milk at local dairy collection centers. Each sample was labeled clearly representing animal identification, date and place of collection.

Samples were transported in cool box along with ice pack and transported to the laboratory of VSDAO, Budhanilkantha. All the samples were stored under refrigeration until further processing was carried out.

The processing of the sample was done according to the protocol of the rapid residue test kit provided by the manufacturer. Before performing the analysis, the milk samples were heated in water bath at 82±2°C for 2 minutes to destroy heat-labile natural inhibitors and microorganisms contaminated in raw milk.

3 drops (0.1ml) of milk samples were added into the prepared tubes of RR test kit (Rodejanarug pharmaceutical limited partnership, Thailand). 3 drops (0.1ml) of UHT fresh milk was added into another prepared tube for negative control. All the tubes were incubated for 2 hours 30 mins-2 hours 45 min. in water bath and the temperature of water bath was maintained at 64±2°C, keeping the medium in the tube under water level, until the color of medium in negative control tube changes completely from purple to yellow. The color change of medium in sample tubes were observed. Positive sample were further screened for: Tetracycline, Sulfonamides, Streptomycin, Gentamycin, Penicillin and Fluoroquinolones Separate laboratory protocol was prescribed for each device in product guide provided by the manufacturer (The N.K. Biotech Residue Rapid Test Device, China and Quicking Biotech Residue Rapid test device, China).

The collected data were coded and entered into Microsoft Excel spread sheet. Data were statistically analyzed using Pearson’s Chi-square test with Yates’ continuity correction, performed by “R version 3.3.1” software packages (R Core Team, 2020). Percentage was used to calculate prevalence. Data were statistically analyzed using Chi-square. In all cases 95 % confidence interval (CI) and p<0.05 was considered for statistically significant difference.

**RESULTS AND DISCUSSION**

**Overall presence of milk residues**

Among 100 milk samples collected from different 5 locations of study area, 11 samples were found to be positive with 6 known (Sulfonamide, Streptomycin, Gentamycin) and five unknown milk residues (Fig 2).

Out of six identified commonly used antibiotics, most frequently identified was Sulfonamide (n=3) (3%) followed by Gentamycin (n=2) (2%) and Streptomycin (n=1) (1%). The unidentified milk residues were determined as (n=5) (5%) (Table 1). The proportionate findings of antibiotics was 6% and unidentified milk residues was 5% (Fig 3).

Antibiotics are vital drugs considered as the ultimate strategy to treat animal infections. Their effectiveness is however, threatened by extensive and inappropriate use of these, not only in cattle but also in human medicine. In veterinary practice, antibiotics are utilized at therapeutic levels primarily to treat diseases and to prevent infections (Aning et al., 2007). They are also used at sub-therapeutic levels to increase feed efficiency, promote growth and prevent diseases (Mosalagae et al., 2011). The frequent use of antibiotics may result in antibiotic residues that can be found at different concentration levels in products of animal origin, such as milk or meat. Presence of drugs or antibiotics residues in food above the maximum level recognized worldwide by various public authorities is illegal (Aning et al., 2007).

The most commonly used antibacterial in Veterinary Medicine includes Beta-lactams, Tetracycline, Aminoglycosides, Macrolides, Quinolones and Sulfonamides (Mitchell et al., 1998; Unnikrishnan et al., 2005). On basis of these antibiotics; Penicillin, Tetracycline, Gentamycine, Streptomycin,
Sulfonamides, Fluoroquinolone were selected as major antibiotics for the study. According to proportionate study of use of those antibiotics, 18.18% Gentamycin, 27.27% Sulfonamides and 9.09% Streptomycin was observed and 45.45% was observed as unidentified positive samples. In this study, out of 100 sample, total prevalence of 11% (11sample) of were found positive. Out of the total positive samples, 2% (n=2) were found to contain Gentamycin residue, 3% (n=3) were found to contain Sulfonamides and 1% (n=1) were found to contain Streptomycin. Quantification of antibiotics in positive case could not be carried out. This study was supported by a study of Tetracycline, Chloramphenicol and Streptomycin residues in milk samples sold at different places of Kathmandu valley, out of 140 milk samples from three different sources (organized dairies, cottage dairies and individual farmers). Enzyme Linked Immunosorbent Assay (ELISA) detected 27% milk samples positive for Tetracycline, 28% for Chloramphenicol and 2% for Streptomycin (Sharma et al., 2008). Since no ELISA was done, the difference in result might have been varied due to difference of sensitivity of kits used. Proportion has been found different because of difference in subject (antibiotics group) of study.

Similar studies were conducted by VSDAO (2013) out of a total of 150 samples tested, 17.3% (n=26) samples were found to be positive for antimicrobial residue. Out of 26 positive samples, Sulfonamide residue was detected in 8 (5.3%) of the samples and Penicillin residue in 18 (12%) of the samples. The result was also found higher than the previous research of Kathmandu valley as reported by (Baynes et al., 1999) and (Sedhain, 2008). Variation in result might have been observed because this research was performed in winter where as in this case it was mid rainy season. Lesser amount of residue in positive sample may be due to lesser use of antibiotics during early winter season (time when study was conducted) when disease occurrence is comparatively lower. According to (Yamaki et al., 2004), the seasonal factor also affects the prevalence of antibiotic residue, because in his study the highest percentages of “positive plus doubtful” results were observed in late summer and early autumn.
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Table 2: Determination of antibiotic residues in milk sample in different areas.

| Area           | No. of samples collected | Commonly used antibiotics | Not Identified Antibiotics | Frequency | Negative Samples | P-value |
|----------------|--------------------------|---------------------------|----------------------------|-----------|------------------|---------|
| Budhanil-kantha| 20                       | 0                         | 0                          | 1         | 19               | 0.7997  |
| Dharmasthali   | 20                       | 1                         | 1                          | 0         | 1                | 17      |
| Nepalat        | 20                       | 0                         | 1                          | 0         | 2                | 17      |
| Gaushala       | 20                       | 1                         | 1                          | 0         | 0                | 18      |
| Tokha          | 20                       | 0                         | 0                          | 1         | 1                | 18      |
| Total          | 100                      | 2                         | 3                          | 1         | 5                | 89      |

The study clearly shows that there was no statistical significant difference between study areas and commonly used antibiotics residues in milk samples. ($\chi^2 = 2.79$, $P > 0.05$).

According to the results of the technical report prepared for milk hygiene by World Health Organization and Joint Expert Committee on Food Additives (JECFA), the rate of contamination of milk and dairy products with antimicrobial additives in developed countries such as USA, Australia, UK and Scotland was 7-10% until 1969. After that year the rate of contamination of the same products decreased to 0.5% in USA, 2.1% in Australia, 1.5% in UK and 3.4% in Scotland due to the precautions taken after the given date. In that study, 3084 milk samples were analyzed using interest method for the presence of Penicillin, Tetracycline and Chloramphenicol in which 377 of the samples (12%) were found as positive. In our study, the overall prevalence of 11.32% is much higher than these developed nations. The variation might have occurred mainly due to not following guideline of antibiotic used and farmers are unaware of hazards. Lack of implication of rules and regulation.

Out of 100 milk samples, 20 samples were collected from each study area. Among them, two milk samples were positive in Dharmasthali, two in Gaushala, one in Nepalat, one in Tokha and no any sample with commonly used positive antibiotics residue was found in milk samples of Budhanilkantha area (Table 2).

Antibiotic residues in milk sample in different areas

Location wise distribution of identified milk residue was more in Dharmasthali and Gaushala (n=2) (33.33%) followed by Nepalat and Tokha (n=1) (16.67%) and Balambu (37.50%). Budhanilkantha study area did not show any identified antibiotics residue. Similarly, distribution of unidentified milk residue was more in Nepalat (n=2) (40%) and less in Budhanilkantha, Dharmasthali and Tokha (n=1) (20%) (Table 2). Gaushala study area did not show any unidentified antibiotics residue. This study was supported by the study in Turkey by Ceyhan and Bozkurt (2009), from a total of 200 milk samples collected around Ankara, 11 of them were reported as Penicillin positive (5.5%). However, in our context it is more than 13%. The variation might be due to lower in sample size or the sensitivity of the kits used. One survey that was administered in 381 herds in Washington State, reported that 23% of dairy farmers used one or more unapproved or prohibited uses of antimicrobial, of which the most common compounds cited were Gentamicin, Neomycin and Florenicol (Raymond et al., 2006). In our context, we do not have strong regulatory mechanism to guide the use of antibiotics nor are the records maintained about antibiotics used in field level. There is no mechanism of strict recording of entry of drugs in nation and its consumption pattern so even prohibited medicines are readily procurable in our market.

In similar study by (Koyu, 2010) in Ankara-Turkey, a total of 240 milk samples between April 2003 and March 2004 were analyzed for penicillin G, Oxytetracycline, Gentamicin, Streptomycin and Neomycin by using TLC (Thin
Layer Chromatography) Bioautographic method. According to the total number of samples analysed, the ratio of contamination with antibiotics was detected as 1.25%. The variation in result might have been observed due to variation in procedure and since EU is strict about public health concern policy level control might be responsible for low contamination. According to a study carried out in Turkey, by (Kaya and Filazi, 2010) from all analyzed samples only 1.25% were found to have been contaminated with beta lactam residues. The variation in result might be due to variation in stage of development. This is one of the major challenges for most of the developing countries to be able to consume milk without any type of contamination.

CONCLUSION

The study has indicated a clear risk involved in the consumption of dairy milk with antibiotics residue. Therefore, it is vital that all inspection should be strongly controlled and records should be monitored on the treatment of animals as well as the proper use of antibiotics. This study has clearly indicated the presence of antibiotic residue in the marketed milk of Kathmandu. Use of antibiotics should be made only under the supervision of veterinarian; Food Act and Feed Act has to be strictly implemented, regarding antibiotics residue in food animals; training should be provided to dairy worker regarding antibiotic Act and its implementation; Gentamycin is only approved in human to use but result showed that it was used in animals, so use of Gentamycin should be strictly prohibited, regular investigation of residue has to be carried out by dairy industries, focus has to be given for consumption of pasteurized milk.

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