ANTIMICROBIAL RESIDUES IN MILK AND SELECTED MILK PRODUCTS OF CHITTAGONG, BANGLADESH

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Roll No. 113/01

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A thesis submitted in the partial fulfillment of the requirements for the degree of Master of Science in Dairy Science

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Dedicated

To My

Beloved Family

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Md. Saiful Bari
June 2014

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This is to certify that we have examined the above Master's thesis and have found that is complete and satisfactory in all respects, and that all revisions required by the thesis examination committee have been made.

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Table of contents

List of Tables

List of Figures

List of abbreviations

Abbreviation	Elaboration
AR	Antimicrobial residue
%	Percentage
^{o}C	Degree centigrade
ADI	Acceptable Daily Intake
β-lactam	Beta-lactam
BBS	Bangladesh Bureau of Statistics
CI	Confidence Interval
DLS	Department of Livestock Service
EC	European Commission
EEC	European Economic Community
FAO	Food and Agriculture Organization
FID	Farm Identification Number
Gm	Gram
Km	Kilometer
MIC	Minimum Inhibitory Concentration
MRL	Maximum Residue Limit
μg	Microgram
Ml	Milliliter
OTC	Oxytetracycline
TLC	Thin Layer Chromatography
UHPLC	Ultra High Performance Liquid Chromatography
VRC	Veterinary Residue Committee
WHO	World Health Organization

Abstract

Antimicrobials are imprudently used in dairy cows for treating different infectious diseases and their indiscriminate use followed by unawareness about withdrawal periods leave antimicrobial residues in milk and milk products. A cross sectional study was conducted to determine antimicrobial residue in milk and selected milk products of Chittagong area and effect of heat on residue during the period of November 2013 to May 2014. For this, 280 milk samples from commercial and household dairy farms were collected from Chittagong Metropolitan Area and Patiya upazila of Chittagong district. Forty milk samples were collected from different milk distributing points of the city and also 50 samples from the available brands milk in Bangladesh. A total of 144 milk products sample including dahi, rasogolla and powder milk were collected from different brand's showroom and grocery shops. A pretested questionnaire was also implemented for identifying the associated risk factors of prevalence of antimicrobial residue during sample collection from commercial farms. Both the milk and products samples were screened for antimicrobial residue by Thin Layer Chromatography and then confirmed by using Ultra High Performance Liquid Chromatography. The oxytetracycline and gentamicin residues were found in milk at higher percentages in commercial dairy farms. Categories of farm, cow illness, treatment given and antimicrobials used have significant ($p \leq 0.05$) relationship on prevalence of antimicrobial residue in milk (18%). Five percent of the dairy products had antimicrobial residue including 4.2% dahi and 2.1% powder milk samples. But no antimicrobial residue was in rasogolla. The effect of heat on prevalence of antimicrobial residue in milk was insignificant ($p > 0.05$). The concentrations of amoxicillin (339.9µg/l) and oxytetracycline (195µg/l) residue were significantly ($p \leq 0.01$) reduced by heat treatment. The amoxicillin and oxytetracycline residues were higher than the Maximum Residue Limit in milk and the ciprofloxacin residue in dahi (0.6µg/kg) was within the Maximum Residue Limit. However, most of the antimicrobials in milk were beyond the acceptable Maximum Residue Limit and assumed to causing serious public health threat. This work will contribute to understanding the levels of antimicrobial residue in milk and selected milk products as well as in taking measures regarding public health concerns.

Keywords: *Antimicrobials, heating, residues, milk and milk products, Chittagong*

Chapter-1: Introduction

Antimicrobials are the substances produced by living microorganism or their products, identical synthetic or similar semi-synthetic products that inhibit the growth of or destroy microorganisms (Soanes and Stevension, 2005). Antimicrobials are widely being used in commercial dairy farms in South and South-East Asian countries including Bangladesh with the aim of preventive and therapeutic measures. Approximately 80% of all food-producing animals receive antimicrobials for part or most of their lives (Lee et al., 2001). Globally, most commonly used antimicrobials belonging to β-lactams, tetracyclines, aminoglycosides, quinolones, macrolides and sulfonamides are used to prevent infection and promote rapid growth of farm stock (Brogden et al., 2003).

Antimicrobial residues are small amount of antimicrobials or their active metabolites which remain in milk after treating the cows (CAC, 1998). Mastitis is the most prevalent disease in cattle which usually requires antimicrobial treatment (Suhren, 2002; Mohsenzadeh et al., 2008). Thus antimicrobials are indiscriminately used in lactating cows and withdrawal periods are not being maintained in Bangladesh. Ultimately dairy cows are leaving Antimicrobial residues in milk during and after medication period. Drug residues in milk cause a potential health hazard for the consumers like cancers, allergic reactions, interference in the intestinal flora and resistant bacteria in the general populations, thereby rendering antibiotic treatment ineffective (Dewdney et al., 1991; Companyo et al., 2009; Donoghue, 2003). Consumers want to be confident that their food is free of contamination by herbicides, pesticides, drugs or antibiotics etc.

Varieties of milk products are being produced from milk in dairy enterprises and households everyday throughout the world including Bangladesh. Milk products have been contaminated with Antimicrobial residues as derived from milk (Helio et al., 2007). Among the available milk products dahi, rasogolla and powder milk are very popular in this country.

Problems associated with Antimicrobial residues in milk products include the risk of allergic reactions after consumption by penicillin-sensitized persons, increased resistance of pathogens towards antibiotics and inhibition of bacterial starter cultures

used in production of dairy products. The concerns arise mainly from the possibility that antibiotic-resistant bacteria may be transferred from animals to humans, through contact, water, manure or contaminated milk products (CAC, 1998). Antimicrobial residues may persist in milk and milk products at unacceptable levels and consumers can be easily exposed to them. The presence of residues may result from failure to observe the mandatory withdrawal periods, illegal or extra-label use of drugs and incorrect dosage levels. Unauthorized antibiotic use may result in residues of these substances in milk and tissues (Ivona and Mate, 2002).

Determination of antimicrobial residues in milk and milk products has been investigated across the world. The reported prevalence estimates of antimicrobial residues in raw milk were 5.7% amoxicillin and 3.8% cefaprin (Ghidini et al., 2002), 14% amoxicillin and 16% penicillin (Amatya, 2010), 21.3% beta lactams in Turkey (Ardic and Durmaz, 2006) and 36% beta lactams in Pakistan (Khaskheli et al., 2008). The reported prevalence estimates antimicrobial residues in market milk as 2.7% beta lactams in Iran (Movassagh and Karami, 2011), 4% in Brazil (Fonesca et al., 2009), 10.8% in Trinidad (Adesiyun and Webb, 1997), 21% in Kenya (Shitandi, 2001) and 35.5% (Aning et al., 2007). The concentration levels of antimicrobial residues in raw milk were reported to be 8.5µg/l to 53.7µg/l for amoxicillin and 5.7µg/l to 6.4µg/l for cefaprin (Ghidini et al., 2002), 150.4µg/l for oxytetracycline, 33.5µg/l for penicillin G and 7688µg/l for neomycin whereas in market milk antimicrobial residues concentration reported 87.1µg/l for beta lactams (Abbasi et al., 2011). In case of milk products, 8.3% powder milk containing 0.4µg/g dicloxacillin residue was reported by Helio et al., (2007).

The people of developing countries like Bangladesh have been consuming milk after boiling. Some antibiotics such as amoxicillin, oxytetracycline and ceftriaxone are heat labile, whereas gentamicin, sulphadimidine and ciprofloxacin are heat stable (EC, 2001). Hence, boiling has effect on concentration of antimicrobial residues in milk. The prudent use of antimicrobials in food animals is a collaborative effort involving veterinarians, industry or commodity groups and government to preserve antimicrobial efficacy and to reduce the risk of antimicrobial residues entering the food chain (Lohren et al., 2008). However, these efforts are not practicing at all in Bangladesh. Veterinarians or livestock professionals in this country usually do not suggest farmers properly to follow drug withdrawal period for food producing

animals, therefore farmers lack knowledge about persistence of drug residues in milk and milk products which can easily affect human and become drug resistant against human pathogens (Apata, 2009) and cause related problems such as allergic reactions, cancers etc. (Companyo et al., 2009).

It is of utmost importance to know the level of antimicrobial residues in milk and associated risk factors to take necessary actions for minimizing the prevalence of antimicrobial residues in milk and milk products with a view to reduce public health hazards. A study is therefore required to assess the association between drugs used in dairy cows, cow sickness, and treatment given with the status of antimicrobial residues in dairy farms. Although drugs are widely being used in dairy production in Bangladesh, to the best of my knowledge there is no published scientific reports on level of antimicrobial residues in milk and effect of heat treatment on the level of residue except few unpublished data (Chawdhury, 2012) available with considering limited antibiotics in only raw milk. However, the prevalence and level of antimicrobial residues concentration in milk products has still remained unexplored. Hence, the present study was conducted to determine the antimicrobial residues in milk and the selected milk products of Chittagong, Bangladesh and assess the heat effect on residues.

The specific objectives of the present study were enlisted as follows:

- To estimate the prevalence and determine the level of concentrations of antimicrobial residues in milk and milk products (dahi, rasogolla and powder milk) of Chittagong
- To identify the risk factors associated with the prevalence of antimicrobial residues in milk
- To assess the effect of heat treatment on concentrations of antimicrobial residues in milk

Chapter-2: Review of literature

Pertinent literatures on drug uses in commercial dairy farms and drug resistance as well as residues in dairy based food products and possible public health risk(s) are reviewed in this chapter. The main purpose of this chapter is to provide up-to-date information concerning the research work which is addressed here. Important information related to the present study is presented below under the following headings and sub-headings.

2.1. Antimicrobials residue

Antibiotic residue is the small amount of an antibiotic or its break down product(s) that remain in or an agricultural product (livestock, cereal grains, fishes etc.) following treatment with that antibiotic (Bremmer and Johnston, 1996). Residue of veterinary medicinal products means all pharmacologically active substances, whether active principles recipients or degradation products and their metabolites which remain in foodstuffs obtained from animals to which the veterinary medicinal products have been administered. Every living being is receiving antibiotic in direct or indirect ways. Antibiotics are used not only for treatment purpose, but also for prevention as well as growth promoter. In livestock, intramuscular, subcutaneous and intravenous routes are followed for medication (Bremmer and Johnston, 1996).

2.2. Antimicrobials and their use for production animals

Antimicrobials are administered to animals by injections, orally in feed or water, topically on the skin and by intramammary and intrauterine infusions (Mitchell 1998). Theoretically, all of these routes may lead to residues appearing in foods of et al., animal origin such as milk, meat and eggs (Johnston, 1998). Among the various indications, parenterally administered penicillin G has been used for the treatment of mastitis, arthritis and respiratory infections (Ranheim et al., 2002) and 1st generation cephalosporins for the treatment of mastitis (Hornish and Kotarski, 2002). Oxytetracycline is used for the treatment of respiratory and gastrointestinal infections, fluoroquinolones for the treatment of infections of the respiratory, gastrointestinal, and urinary tracts and macrolides to treat respiratory and enteric infections (Draisci et al., 2001). Aminoglycosides are used mainly in the treatment of infections caused by aerobic, gram-negative bacteria, Sulphonamides and trimethoprim are used for the

treatment of respiratory and alimentary tract infections (Boison et al., 1996).

2.3. The origin of antimicrobial residues in milk

The most likely cause of drug residues is the failure to observe withdrawal times (Paige, 1994), improper maintenance of treatment records or failure to identify treated animals (Sundlof, 1989). Fecal recycling, where the drug excreted in faeces of treated animals contaminates the feed of untreated animals, can be the cause of residues of certain antimicrobial groups (McCaughey et al., 1990). Drug residues can also occur as a result of improper use of a licensed product or through the illegal use of an unlicensed substance. Extra label dosages and use of drugs which have not been approved for the species in question may lead to drug residues (Higgins et al., 1999). Residues can also occur in calves fed milk and/or colostrum from cows receiving antimicrobials. Disease may affect the pharmacokinetics of the drugs, metabolism, or the presence of infection and/or inflammation may cause the drug to accumulate in affected tissues Subcutaneous and intramuscular administrations increase the potential for residues at the injection sites (Berends et al., 2001).

2.4. Harmful effects of antimicrobial residues

Antimicrobial residues in foods of animal origin may cause problems for several reasons. In addition to toxic effects, effects on intestinal microbiota and the immune system are important (Perrin-Guyomard et al., 2001). Microbiological endpoints are considered more valid and sensitive in the safety evaluation of antimicrobial residues in production animals than standard toxicological endpoints (Boisseau, 1993). Four microbiological endpoints have been identified that could be of public health concern: modification of the metabolic activity of microbiota, changes in bacterial populations, selection of resistant bacteria, and perturbation of the barrier effect (Perrin-Guyomard et al., 2001).

Drug hypersensitivity is defined as an immune-mediated response to a drug agent in a sensitized patient, and drug allergy is restricted to a reaction mediated by IgE (Riedl and Casillas, 2003). The principal types of disorder are: Type I: anaphylactic shock, asthma and angioneurotic edema; type II: hemolytic anaemia and agranulocytosis; type III: serum sickness and allergic vasculitis, and type IV: allergic dermatitis (Riedl and Casillas, 2003). Notwithstanding their non-toxic nature, ß-lactams appear to be responsible for most of the reported human allergic reactions to antimicrobials (WHO, 1991). Aminoglycosides, sulphonamides and tetracyclines may also cause allergic

reactions (Paige et al., 1997).

Hazards of chloramphenicol observed in association with clinical use in humans include dose-related, reversible suppression of the bone marrow, gray baby syndrome, which is a circulatory collapse in children less than 30 days on high doses, and irreversible, idiosyncratic, non-dose related aplastic anemia (Waltner-Toews and McEwen, 1994). Toxic and allergic reactions in humans and animals caused by tetracyclines have only been observed at therapeutic doses (Berends et al., 2001). Residual antibiotics can induce cancers and other non cancer hazardous effects on the body (Movassagh and Karami, 2011).

2.5. Acceptable daily intake of Antimicrobial residues

The Acceptable Daily Intake (ADI) is an estimate of the residue, expressed on a body weight basis which can be ingested daily over a lifetime without any appreciable health risk (EC, 2001). The Acceptable daily intake was calculated by dividing this by a suitable safety factor, usually 100, which assumes that humans are 10 times more sensitive than animals and that within the human population there is a 10-fold range of sensitivity (Woodward, 1998). In the EU, the classical toxicology tests required include single dose toxicity, repeated dose toxicity, tolerance in the target species, reproductive toxicity, mutagenicity and carcinogenicity. Studies on other effects include immunotoxicity, microbiological properties of residues, observations in humans, and neurotoxicity (EC, 2001).

According to Council Regulation 2377/90 (EEC, 1990) maximum residue limit means the maximum concentration of residue resulting from the use of a veterinary medicinal product which may be legally permitted or recognized as Development of microbiological methods for the detection and identification of antimicrobial residues in milk. Possible persistence of residues in organs or at the injection site is also considered (EC, 2001). Once the process of safety evaluation is complete and Maximum Residue Limits (MRL) have been derived for a particular substance, consideration is given to the likely level of residue which may be expected to remain after the use of the substance in accordance with good veterinary practice, and to the availability of analytical detection methods suitable for use for routine monitoring purposes. The maximum residue limits may be further reduced to take account of these factors (EC, 2001).

Table 2.1: Acceptable limits of antimicrobial residues in milk and milk products

Antimicrobials	Minimum (µg/kg)	Maximum (µg/kg)
Amoxicillin	4	40
Tetracycline	15	100
Oxytetracycline	15	100
Chlortetracycline	15	100
Sulphonamides	25	100
Trimethoprim	8	50
Erythromycin	12	40
Quinolones	47	147

(Source: EC, 2001)

2.6. Withdrawal period

To ensure that drug residues have declined to a safe concentration following the use of drugs in animals, a specified period of drug withdrawal must be observed prior to providing any products for human consumption. It is the time which passes between the last dose given to the animal and the time when the concentration of residues in the tissues: muscle, liver, kidney, skin/fat or products milk, eggs, honey is lower than or equal to the Maximum Residue Limits (Jackson, 1980). The CVMP recommends the use of a statistical method in the assessment of a withdrawal period (CVMP, 1995) whenever possible, and particularly for products containing new chemical entities. A withdrawal period is determined at the time when the upper one-sided tolerance limit with a given confidence is below the Maximum Residue Limits. For old chemical entities data are often insufficient to assess the withdrawal time by a statistical method. A simpler method consists of declaring the withdrawal time as the time in which the residues in all tissues of all observed animals have fallen below the respective MRLs (Concordet and Toutain, 1997). However, establishment of accurate pre-slaughter withdrawal times is hardly possible with irritative drug formulations which are administered intramuscularly or subcutaneously (Nouws *et al*., 1990).

Table 2.2: Wthdrawal periods of different antimicrobials in dairy cows

Antimicorbials	Withdrawal periods (Days)
Amoxicillin	5
Oxytetracycline	7
Ciprofloxacin	6
Trimethoprim	10
Sulphaquinoxaline	10
Sulphadimethoxine	5

(Source: Mumtaz et al., 2000)

2.7. Treatment effects on antimicrobial residues

Heat stabilities of different classes of antimicrobials were examined by using autoclave and temperature 121^{o}**C** for 15 minutes heating under pressure (Furusawa and Hanabusa, 2002). The study revealed the following heat stability of different classes of antimicrobials. The antimicrobials residue were found to reduce in percentages by heat treatment in milk as 7% to 5% for ciprofloxacin, 13% to 12% for amoxicillin and 23% to 21% for oxytetracycline by15 minutes boiling (Chawdhury, 2012).

Table 2.3: Heat stability of antimicrobials after autoclaving at 121^{o}C for 15 minutes

Stable	Partially stable	Labile
Ciprofloxacin	Nitrofurantoin	Amoxicillin
Gentamicin	Polymixin B	Cefixime
Trimethoprim	Amoxicillin	Doxicycline
Sulfamethoxazole	Penicillin G	Ceftriaxone
Clindamycin	Rifampicin	Erythromycin
Nalidixic acid	Ampicillin	Tetracycline

(Source: Furusawa and Hanabusa, 2002)

2.8. Control of antimicrobial residues in Milk

Veterinary drugs are monitored for Maximum Residue Limits compliance. The directive establishes the groups of substances to be controlled for each food commodity. Commission Decision 97/747/EC (EC, 2001) provides further rules for certain animal products: milk, eggs, honey, rabbit and game meat. In the USA, the National Residue Program conducts two types of residue testing programs. Under the monitoring programme, a statistically based selection of random samples from normal animal population is collected. The surveillance program focuses on obtaining samples from animals suspected to contain drug residues in their tissues (Dey et al., 2003). In Finland, the national residue control programme is carried out in accordance with both national and EU legislation. The samples are taken from both live animals and foodstuffs of animal origin. In addition to the control programme, antimicrobial residues in meat are tested in meat inspection at slaughterhouses (MAF, 2001). In 2003 a total of 4422 suspected kidney samples were tested with microbiological methods in meat inspection, and 5241 samples according to the national residue control programme (NAF, 2004). Antimicrobials are indiscriminately used in lactating cows and withdrawal periods are not being maintained in Bangladesh. Ultimately dairy cows are leaving Antimicrobial residues in milk during and after medication period.

2.9. Detection and identification of antimicrobial residue

A screening method is the first-hand analysis of the sample to establish the presence or absence of residues (Aerts et al., 1995). It should be a low-cost and high-sample throughput method, optimized to prevent false-negative results and to have an acceptable number of false-positive results. In order to prevent false-negative results, it should be positive for all samples that contain residues at MRL levels; preferably at 50% of the MRL (Korsrud et al., 1998). Microbiological methods are suitable for large scale screening because of their convenience and broad spectrum characteristics (Haasnoot et al., 1999). In the search for rapid methods for determining the interaction of Development of microbiological methods for the detection and identification of antimicrobial residues in meat antimicrobial agents and organisms, intermediate and end products of bacterial metabolism, as well as the interaction of the organism with various energy sources have been examined (Amsterdam, 1996). Microbiological tests are unspecific, indicating only the presence of an inhibiting agent. Therefore, a

post-screening test such as Thin Layer Chromatography (TLC) is needed for the preliminary characterisation of the residue (Aureli et al., 1996; Ferrini et al., 1997).

2.10. Methods for determination of antimicrobial residues in milk and milk products

Commonly used procedures for the detection of veterinary drug residues include High Performance Liquid Chromatography (HPLC), gas chromatography (GC), thin layer chromatography (TLC) and mass spectrometry (McCracken et al., 2000). Chemical methods usually proceed with a preliminary extraction in order to isolate the drugs of interest from the biological matrix. The main objectives of sample treatment are removal of macromolecules and other matrix constituents that may either adversely affect the chromatographic system or interfere with the detection, and enrichment of the analytes in order to achieve the required low limits of detection (Aerts et al., 1995). Liquid chromatography (LC) has emerged as the method of choice for determination of antimicrobials which are rather polar, non-volatile, and sometimes heat sensitive (Kennedy et al., 1998). With the automated sequential trace enrichment of dialysates sample pretreatment is restricted to homogenization and dilution of the samples; clean-up is by on-line dialysis and on-line solid-phase extraction (Zurhelle et al., 2000).

2.11. Public health importance of antimicrobial residues in milk and milk products

Administration of drugs to food-producing animals requires not only consideration of effects on livestock and poultry but also effects on humans who consume food from these livestock. In short after food-producing animals have been exposed to drugs in order to cure or prevent disease or to promote growth, the effects of the residues of such treatments may have on humans should be known. These residues consist of parent compound or compounds derived from the parent drugs (or both) including metabolites and residues bound to macromolecules (Weber, 1982). Concern has been expressed about possible harmful effects on humans through the use of drugs, as follows: (1) increased microbial drug resistance, (2) drug residues in food, (3) allergic reactions and sensitization to antimicrobials, and (4) drug toxicity (Black, 1984; Dewdney et al., 1991; Companyo et al., 2009). Antimicrobial residues in foods of animal origin may cause problems for several reasons. In addition to toxicity, effects

on intestinal microbiota and the immune system are important (Perrin-Guyomard et al., 2001).

2.12. Antimicrobial residues in milk and milk products

2.12.1. Antimicrobial residues in raw milk

Ghidini et al., (2002) investigated 53 bovine raw milk samples and found penicillin G in 49.1% samples at concentrations ranging from 3.7µg/l to 6340µg/l and, amoxicillin in 5.7% samples at concentrations ranging from 8.5µg/l to 53µg/l and cefaprin in 3.8% samples at the concentrations of 5.7µg/land 6.4µg/l. Amatya, (2010) found 14 % of raw milk samples contained Amoxicillin and 16 % contained Penicillin. Amoxicillin and Penicillin was the most common residue found in milk sample. Khaskheli et al., (2008) showed that of all samples 36.5% were contaminated by beta-lactam antibiotic residues in cow raw milk in Pakistan. In a study by Ceyhan and Bozkurt, (1987) from a total 200 milk samples collected from Ankara region, 5.5% was positive for antimicrobial residues. Ardic and Durmaz (2006) reported 21.3% of beta-lactam antibiotic residues in unpacked milk consumed in Sanliurfa region, Turkey. Aydin et al., (1989) in 204 raw milk samples, 44% was positive for antimicrobial residues in Turkey. Kang'ethe et al., (2005) showed 16% incidence of antibiotic residues in milk in Kenya. Rybinska et al., (1995) studied on antibiotic residues in milk in Poland and found 13-22% samples were positive for antimicrobial residues. Elizabeta et al., (2011) studied and measured range of concentrations (in µg/kg) was 13.5-147.9 for sulfonamides, 0.6-22.0 for quinolones and 17.4-149.1 for tetracyclines, with calculated mean values (in µg/ kg) 24.7 for sulfonamides, 12.6 for qinolones and 41.9 for tetracyclines.

Kaya and Filazi, (2010) found the minimum detectable concentrations for penicillin G, oxytetracycline, gentamicin, streptomycin and neomycin, as µg/l were 4, 100, 200, 100 and 1000, respectively and recovery rate were 75.6%, 79.7%, 80.9%, 84.7% and 73.5%, respectively. The concentrations found among pasteurized samples were 150.4µg/l oxytetracycline and 33.5µg/l penicillin G and 7688.4µg/l of neomycin among raw samples. According to the total number of samples analysed, the percentages of contamination with antibiotics was detected as 1.25%. Syit, 2008 studied 400 milk samples by Delvotest SP assay and HPLC. 8.5% were found positive with antimicrobial residues. The mean residue level of oxytetracycline 142.0µg/l and

penicillin G was 4.78µg/l. The concentration of oxytetracycline was found above WTO/FAO/CAC established residue limit of 100µg/l. The result suggested that oxytetracycline and penicillin G were imprudently used in dairy farms. Abbasi et al., (2011) suggested that the mean of total TCs residues in 114 samples were 97.6ng/g and that of pasteurized, sterilized and raw milk samples were 87.1ng/g, 112.0ng/g and 154.0ng/g respectively. Twenty five percent of the all samples, and 24.4%, 30% and 28.6% of the pasteurized, sterilized and raw milk samples, respectively had higher tetracycline residues than the recommended maximum levels (100ng/g).

2.12.2. Antimicrobial residues in market milk

Movassagh and Karami, (2011) found 2.7% samples were positive for beta-lactam antibiotic residues in pasteurized milk in the northwest region of Iran. Fonseca et al., (2009) studied on the incidence of antimicrobial residues in Brazilian UHT milk and got 4% samples indicated probable presence of antibiotic residues. Adesiyun et al., (1997) studied the prevalence of antimicrobial residues in preprocessed and processed cow milk in Trinidad, and showed that 10.8% samples were positive. Shitandi (2001) showed 21% of 1109 milk samples were positive for antimicrobial residues in Kenya.

Aning et al., (2007) carried out a study to determine the extent to which antimicrobial drugs may be translocated into milk and the associated risk of exposure by consumers by using Charm aim-96 antimicrobial inhibition assay screening kit. Overall, 35.5% (140/394) of the milk samples collected were contaminated with one or more of the antimicrobial drugs screened. This translates into an average risk of exposure every third time a consumer drinks locally produced milk. There was no significant difference in contamination levels between season and area of sampling. Among market agents, contamination levels ranged from 16.6% (9/54) for wholesalers or milk assemblers to 54.2% (13/24) for milk processors. There were no significant differences in prevalence of drug residues in milk from different types of traders between and within locations.

2.12.3. Antimicrobial residues in milk products

Helio et al., (2007) stated that, the twelve powder milk samples were analyzed by HPLC method and dicloxacillin was found at the concentration of 0.4µg/l in one

brand of powder milk i.e. 8.3% samples of one brand was found antibiotic residue positive.

From the above discussion, it is clearly apparent that the determination of antimicrobial residues in milk and milk products in Bangladesh is justifiable. It is also crucial to identify the factors associated with the levels of antimicrobial residues in milk to prevent the occurrence of antimicrobial resistance and possible public health threat. Whether the boiled milk is free of antimicrobial residues or not is still now confused. Scientific works on determination of antimicrobial drugs' residue in milk products are also limited in Bangladesh as well as world aspect. Therefore, a study on determination of antimicrobial residues in milk and milk products in Bangladesh and effect of heat treatment on residues is of utmost important to minimize the future public health threat.

Chapter-3: Materials and Methods

3.1. Study area

Chittagong has a total area of 168.07 square kilometres (64.89 sq mi). The city is known for its vast hilly terrain that stretches throughout the entire district and eventually into India. The city is located at 22°22′0″N 91°48′0″E on the banks of the Karnaphuli River. Patiya is located at 22.30°N 91.98°E. It has 70218 units of house hold and total area 316.47 km. Chittagong Metropolitan Area (CMA) and Patiya upazila of Chittagong district were purposively selected for the study. Commercial dairy farms were selected for collecting milk samples in CMA. On the other hand, household dairy farms were selected for milk sample collection from Patiya.

The market milk and milk products samples were collected from available grocery shops and sweetmeat shops (brand's showroom), respectively of CMA.

3.2. Study period

The study was conducted during the period of November 2013 to May 2014.

3.3. Study design

A cross-sectional study was conducted to determine the prevalence and associated risk factors of antimicrobial residues in milk and selected milk products in Chittagong, Bangladesh.

3.4. Reference farms and population

A complete list of dairy farms in CMA who had at least three dairy cows was developed. All the cows in selected farms of CMA (Annex-1) and the entire selected household cows (farmers having less than three cows) in Patiya upazila were the reference population.

3.5. Target population

All the lactating cows in commercial farms of CMA and all the household lactating cows in Patiya upazila were the target population.

3.6. Data collection

A pre set questionnaire was developed in relation to the objectives of the study for

data collection from commercial dairy farms to identify risk factors for antimicrobial residues in milk (Annex-2). Sizes of the farms, disease prevalence, treatment history, antimicrobial use and dose, route of administration, withdrawal periods etc. were considered as distinguished variables. Data were collected by face to face interview of the farm owners and sometimes the animal rearer. Before interviewing the objectives of the study were clearly defined to the respondents. Sample collection and data collection were done simultaneously during the study period.

The commercial dairy farms under study in CMA were categorized into A, B and C based on the number of cows (milch cow and dry cow) present in the farms such as Category A farms having 3 to 25 lactating and dry cows, category B farms containing 26 to 50 cows and category C farms having more than 50 cows (DLS, 2012).

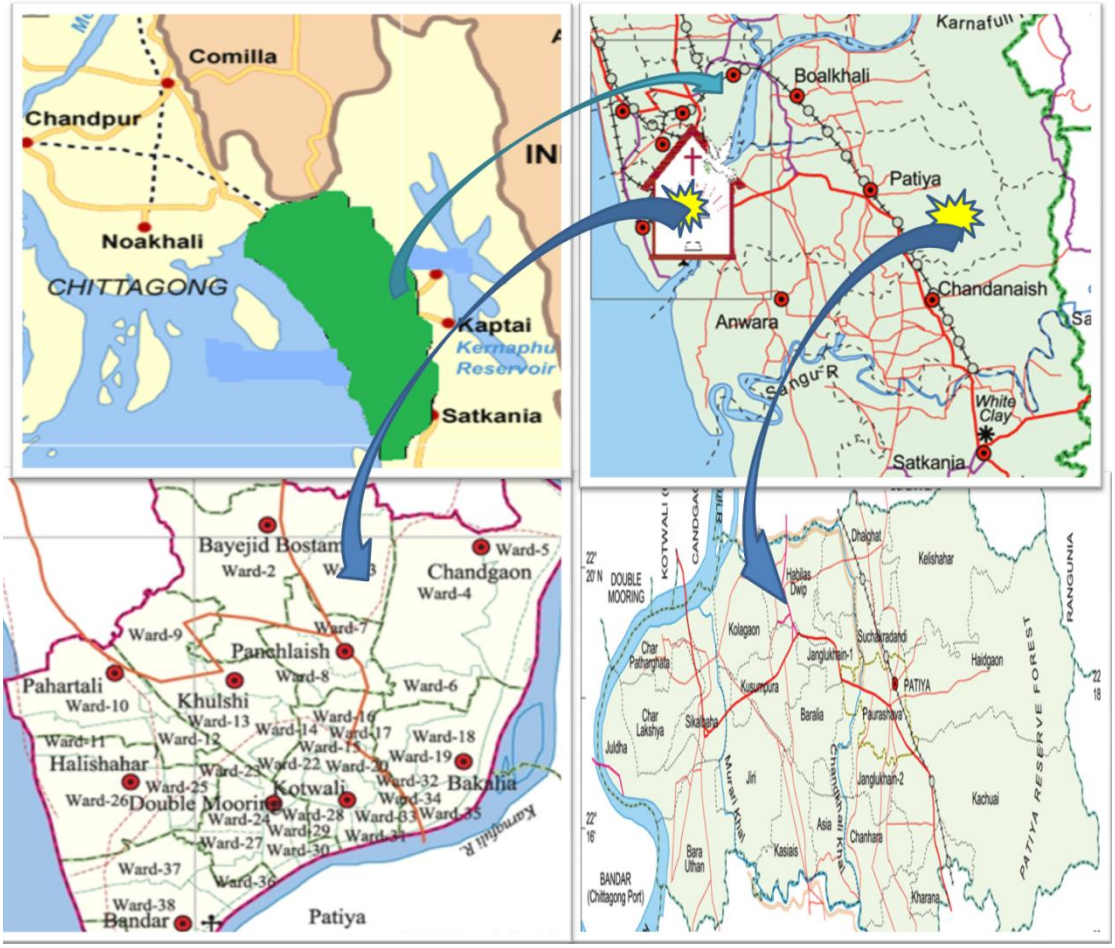

Figure: 3.1. Maps of Chittagong, Bangladesh indicating the study area

3.7. Selection of farms and sample collection

3.7.1. Milk samples

The following sampling strategy was adopted for the collection of milk samples from CMA. A total of 50 commercial dairy farms were selected randomly and 1 pooled sample was collected from each farms. Accordingly, 50 pooled samples per farm were collected. About 3-5 samples from randomly selected individual cow of the 50 commercial farms were also collected. Following this, a total of 180 individual cow milk samples were collected. A total of 50 households were randomly selected in Sikalbaha of Patiya upazila for milk sample collection from household dairy farms. Household dairy farms were selected based on the criteria of having 1-2 dairy cows. Thus, we got 50 samples from household dairy farms. Each milk sample (approximately 10 ml) was given unique identification number. Then the samples were transported to the laboratory using ice box and stored at -20°C until further analysis.

A total of 40 samples, 10 samples from each distributing points (Sholasohor, Janalir hat, Karnafuli bridge and Potenga) were collected. Every sample was collected from each point in two days interval. A total of 50 market milk samples were collected from Milk vita, Arong, Pran, Farm fresh and RD brands (10 samples from each brand). Every sample was collected from each brand at weekly interval.

3.7.2. Milk product samples

A total of 48 samples, 12 samples from each brand of rasogolla, were collected from Food plaza, Modhubon, Banoful and Fulkoli brands. Every sample was collected from each brand at seven days interval. A total of 48 dahi samples, 12 samples from each brand, were collected from Food plaza, Modhubon, Banoful and Fulkoli brands. Every sample was collected from each brand at seven days interval. A total of 48 powder milk samples, 12 samples from each brand were collected from Milk vita, Dano, Marks and Fresh brands. Every sample was collected from each brand at weekly interval.

3.8. Heat treatment on milk samples

To determine the effect of heat treatment the positive milk samples by TLC were heated at 100^{o}C for 15 minutes and 30 minutes in hot air oven.

3.9. Extraction of milk and milk products samples for Thin Layer Chromatography

3.9.1. Preparation of milk sample

Both raw and boiled milk samples were used. The antibiotic residue positive milk samples were boiled for 15 minutes and 30 minutes at 100^{o}**C** and then again extracted for TLC. All categories of milk samples were extracted for TLC. A mixture of acetonitrile-methanol and Deionized water at a ratio of 40:20:20 was made. In order to precipitate the protein in milk, 1ml of mixture was added to 1ml of milk sample in sterile falcon tube and mixed properly. This mixture was then centrifuged at 3000 rpm for 20 minutes and the supernatant was collected in eppendorf tube for TLC (Tyczkowska et al., 1989).

3.9.2. Preparation of milk product sample

Five gram of rosogolla sample was weighed by using electronic balance. The sample was then well macerated after adding 45 ml of distilled water. Then 1 ml of macerated fluid was taken in a falcon tube. Previously prepared 1 ml of reagent mixture containing Acetonitrile, methanol and deionized water at the ratio of 40 : 20 : 20, respectively was added to it. The reagent with sample mixture in the falcon tube was then well mixed and centrifuged at 3000 rpm for 20 minutes. The supernatant was collected in an eppendorf tube. The supernatant was used for chromatogram in TLC.

Five gram of dahi sample was weighed by using electronic balance. The sample was then well mixed after adding 45 ml of distilled water. Then 1 ml of mixed fluid was taken in a falcon tube. Previously prepared 1 ml of reagent mixture containing Acetonitrile, methanol and deionized water at the ratio of 40 : 20 : 20, respectively was added to it. The reagent with sample mixture in the falcon tube was then well mixed and centrifuged at 3000 rpm for 20 minutes. The supernatant was collected in an eppendorf tube. The supernatant was used for chromatogram in TLC.

Five gram of powder milk sample was weighed by using electronic balance. The sample was then dissolved by adding 38 ml of distilled water followed by heating at 45^{o}**C** for 5 minutes (according to the standard procedure of reconstitution). Then 1 ml of reconstituted milk was taken in a falcon tube. Previously prepared 1 ml of reagent mixture containing Acetonitrile, methanol and deionized water at the ratio of 40 : 20 : 20, respectively was added to it. The reagent with sample mixture in the falcon tube

was then well mixed and centrifuged at 3000 rpm for 20 minutes. The supernatant was collected in an eppendorf tube. The supernatant was used for chromatogram in TLC.

3.10. Thin layer chromatography

TLC procedure for qualitative evaluations of antimicrobial residues was done as described by Popelka et al., (2005). TLC detects type-specific antimicrobial residues. The detail procedure is given in Annex-3.

3.11. Determination of antimicrobial residues in milk and milk products by Ultra High Performance Liquid Chromatography (UHPLC)

Determination of amoxicillin and ciprofloxacin residues were done by using the methods of Wang et al., (2009) and the oxytetracycline residue was quantified by using the method established by Senyuva et al., (2000). In both cases the extracted samples for TLC were used. The samples were again centrifuged for 15 minutes at 3000rpm containing in eppendorf tube followed by filtration by using 0.2µm MFS filters. The finally extracted samples were set to run in the UHPLC system. The detail procedures of UHPLC are given in Annex-4.

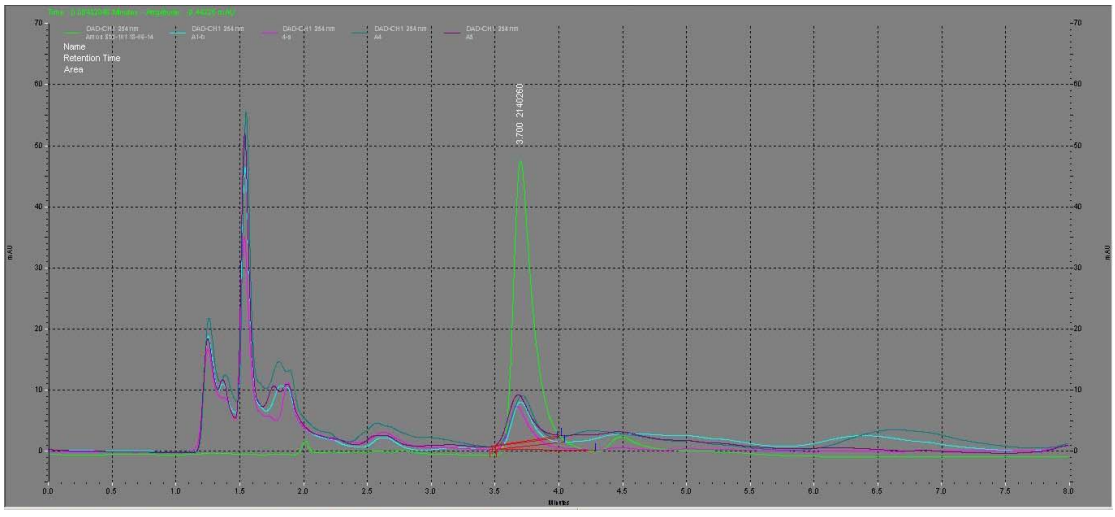

Figure 3.2: Graphical representation of amoxicillin residue by UHPLC

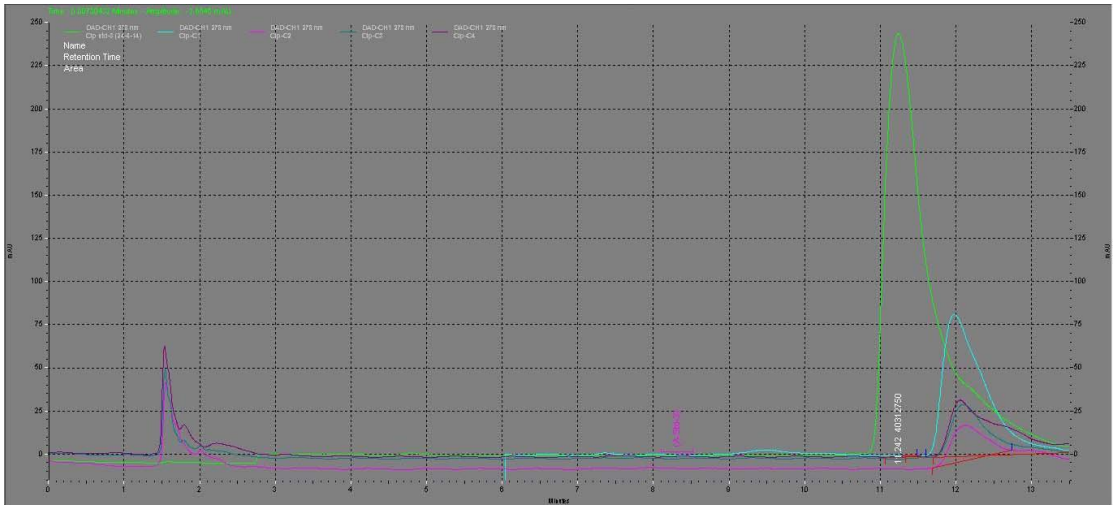

Figure 3.3: Graphical representation of ciprofloxacin residue by UHPLC

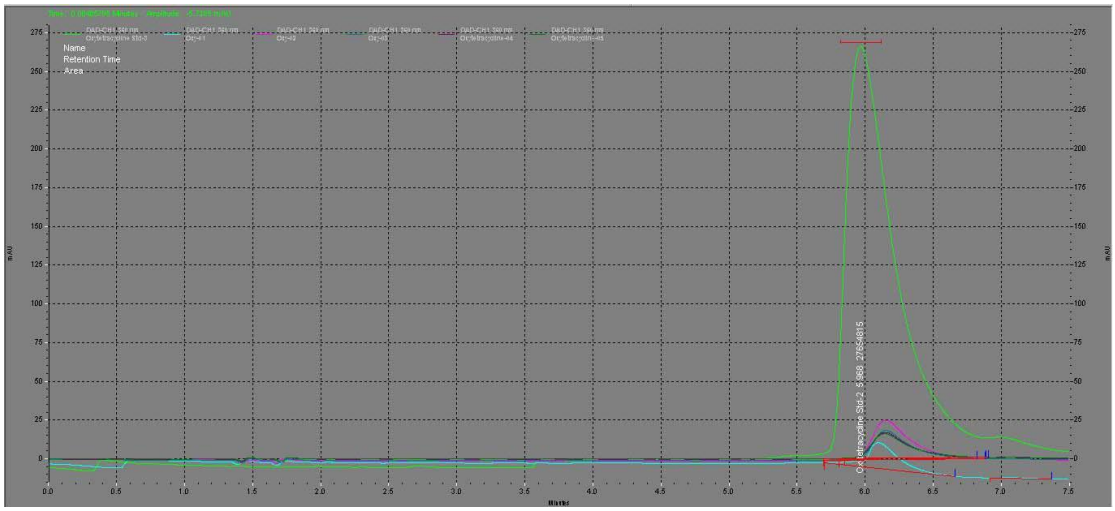

Figure 3.4: Graphical representation of oxytetracycline residue by UHPLC

3.12. Statistical analysis

The data obtained from field and laboratory was entered into spread sheets of the MS Excel-2007 program. Data were checked and sorted in the Excel program before exporting to STATA-11 (STATA Corp, USA). Descriptive analysis was performed using percentages (%) for every farm related variables. Chi-square test was done for association of risk factors (category of farms, illness of cow, treatment given, antibiotic use, withdrawal period etc.) with prevalence of antimicrobials residue. Chi-square test was also performed to correlate the effect of heat on prevalence of antimicrobials residue in milk. Finally one-way ANOVA with Bonferroni test was ueed to assess the effect of heat on level of antimicrobials concentrations in milk. The level of significance was set ≤ 0.05.

Chapter-4: Results

4.1. Prevalence of antimicrobial residues in milk

Among the milk samples, the prevalence of antimicrobial residues irrespective of antimicrobial types was the highest (18%) in the pooled samples from commercial farm and the lowest (4%) in market milk samples. The prevalence of antimicrobial residue was 9.4% in case of individual cow from commercial farm, 6% in household and 5% in distributing point samples (**Table 4.1**).

Table 4.1: Overall prevalence of antimicrobial residues in milk (descriptive analysis)

Category of milk samples	No. of samples tested	No. of positive samples	% of positive samples
Commercial farm (pooled)	50	9	18%
Commercial farm (individual)	180	17	9.4%
Household	50	3	6%
Distributing point	40	2	5%
Market milk	50	2	4%

4.1.1. Prevalence of antimicrobial residues in commercial farm and household raw milk

In commercial farms (pooled samples), the prevalence of antimicrobial residues for amoxicillin, oxytetracycline, gentamicin, ceftriaxone and sulphadimidine were 4%, 6%, 4%, 2% and 2%, respectively (**Table 4.1.1)**. The prevalence of antimicrobial residues in individual samples of commercial dairy farms for amoxicillin, oxytetracycline, gentamicin, ceftriaxone and sulphadimidine were 1.11%, 3.33%, 2.78%, 1.67% and 0.56%, respectively. In this study the prevalence of antimicrobial residues in household milk samples were oxytetracycline 4% and gentamicin 2% as documented in **Table 4.1.1**.

Table 4.1.1: Prevalence of antimicrobial residues in commercial farm and household cow milk

Source	N	Prevalence of antimicrobial residues %(N)					
		Amoxicilln	Oxytetracycline	Gentamicin	Ciprofloxacin	Ceftriaxone	Sulphadimidine
Pooled (Farm)	50	4 (2)	6 (3)	4 (2)	0 (0)	2 (1)	2 (1)
Individual cow (Farm)	180	1.1 (2)	3.3 (6)	2.8 (5)	0 (0)	1.7 (3)	0.6 (1)
Household	50	0 (0)	4 (2)	2(1)	0 (0)	0 (0)	0 (0)

Table 4.1.2: Prevalence of antimicrobial residues in milk from distributing point

Sources	N	Prevalence of antimicrobial residues %(N)					
		Amoxycillin	Oxytetracycline	Gentamicin	Ciprofloxacin	Ceftriaxone	Sulphadimidine
Sholasohor	10	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Karnafuli Bridge	10	0 (0)	10 (1)	0 (0)	0 (0)	0 (0)	0 (0)
Jan Alir hat	10	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Potenga	10	0 (0)	0 (0)	0 (0)	10 (1)	0 (0)	0 (0)

4.1.2. Prevalence of antimicrobial residues in raw milk from distributing points

Among the milk samples of different distributing point, Karnafuli bridge's milk samples were 10% oxytetracycline and Potenga's were 10% ciprofloxacin residue positive. The samples from other points were investigated as free from any types of antimicrobial residues (**Table 4.1.2)**.

4.1.3. Prevalence of antimicrobial residues in market milk

In case of market milk samples, 10 % of Aarong's milk for amoxicillin residue and 10 % of Farm fresh's milk for OTC residue were investigated as positive. Other samples of remaining brands understudy were negative regarding the presence of selected antimicrobials (**Table 4.1.3**).

4.2. Association of risk factors with prevalence of antimicrobial residues in raw milk

The prevalence of antimicrobial residues was significantly (p = 0.03) higher in category C farms (60%) than category B (9.1%). The farms having sick cows had the significantly (p = 0.01) higher prevalence of antimicrobial residues (32%) than the farms having no diseased cows (4%). The dairy farms having the history of ongoing treatment had significantly (p = 0.01) higher prevalence of antimicrobial residues (33.3%) in milk and the farms having no such history had the lower prevalence of antimicrobial residues (3.8%) as presented in **Table 4.2**.

The dairy farms treated with antimicrobials had significantly (p = 0.00) higher (50%) prevalence of antimicrobial residues in milk (50%) than the farms without treatment history (10%) (**Table 4.2**).

Table 4.1.3: Prevalence of antimicrobial residues in market milk

Brands	N	Prevalence of antimicrobial residues %(N)					
		Amoxicillin	Oxytetracycline	Gentamicin	Ciprofloxacin	Ceftriaxone	Sulphadimidine
Milk vita	10	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Aarong	10	10 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Pran	10	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Farm fresh	10	0 (0)	10 (1)	0 (0)	0 (0)	0 (0)	0 (0)
RD	10	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

Table: 4.2: Association of risk factors with prevalence of antimicrobial residues in raw milk (Chi-square test, univariate analysis)

Variable	Category	Antimicrobial residues N (%)		*p*
		Positive	**Negative**	
Category of farm	3 – 25 cows (A)	5 (14.7)	29 (85.3)	0.033
	26 – 50 cows (B)	1 (9.1)	10 (90.9)	
	≥ 51 cows (C)	3 (60.0)	2 (40.0)	
Cow illness	Yes	8 (32.0)	17 (68.0)	0.010
	No	1 (4.0)	24 (96.0)	
Treatment given	Yes	8 (33.3)	16 (66.7)	0.007
	No	1 (3.9)	25 (96.2)	
Antibiotic use	Yes	5 (50.0)	5 (50.0)	0.003
	No	4 (10.0)	36 (90.0)	

N = 50

4.3. Effect of heat treatment on antimicrobial residues in raw milk

Among the pooled milk samples obtained from commercial dairy farms, the oxytetracycline residue of raw milk (N = 3) reduced from 6% to 4% by 15 minutes boiling and 2% by 30 minutes boiling. The gentamicin positive samples (N = 2) unchanged at 15 minutes boiling but reduced at 30 minutes boiling from 4% in raw to 2% in 30 minutes boiled samples. The other positive antimicrobial residue samples (N = 4) remained unchanged at even 30 minutes boiling. In case of individual cow milk samples the amoxicillin (N = 2) and oxytetracycline (N = 6) remained unchanged at 15 minutes boiling but in 30 minutes boiling the percentages of positive samples were reduced from 1.1% to 0.6% and 3.3% to 2.8%, respectively. The other antimicrobial residues positive samples (N = 9) of the same source were remained unchanged in heat treatment (**Table 4.3**).

Table 4.3: Effect of heat on prevalence of antimicrobial residues in raw milk

Sources of sample	Antimicrobials	Raw milk %(N)	After boiling %(N)	
			15 minutes	30 minutes
Commercial farm (Pooled sample)	Amoxicillin	4 (2)	4 (2)	4 (2)
	Oxytetracycline	6 (3)	4 (2)	2 (1)
	Gentamicin	4 (2)	4 (2)	2 (1)
	Ceftriaxone	2 (1)	2 (1)	2 (1)
	Sulphadimidine	2 (1)	2 (1)	2 (1)
Individual cow sample (Farm)	Amoxicillin	1.1 (2)	1.1 (2)	0.6 (1)
	Oxytetracycline	3.3 (6)	3.3 (6)	2.8 (5)
	Gentamicin	2.8 (5)	2.8 (5)	2.8 (5)
	Ceftriaxone	1.7 (3)	1.7 (3)	1.7 (3)
	Sulphadimidine	0.6 (1)	0.6 (1)	0.6 (1)
Household	Oxytetracycline	4 (2)	4 (2)	2 (1)
	Gentamicin	2 (1)	2 (1)	2 (1)
Distributing point	Oxytetracycline	2.5 (1)	2.5 (1)	0 (0)
	Ciprofloxacin	2.5 (1)	2.5 (1)	2.5 (1)

Among the household milk samples, the oxytetracycline positive samples (N =2) remained unchanged at 15 minutes boiling but reduced in percentage at 30 minutes boiling from 4% to 2% positive. The other positive antimicrobial residues samples (N = 1) remained unchanged at even 30 minutes boiling **Table 4.3**. The oxytetracycline residue positive samples (N = 1) from distributing points remained unchanged at 15 minutes boiling but became changed at 30 minutes boiling. The other positive antimicrobial residues samples (N = 1) remained unchanged at even 30 minutes boiling (**Table 4.3**).

4.3.1. Comparison of effect of heat on presence of antimicrobial residues in raw milk

About 18% pooled milk samples from commercial farm were positive for antimicrobial residues. After boiling for 15 minutes and 30 minutes, antimicrobial residues reduced to 16% and 12%, respectively ($p = 0.70$) as presented in **Table 4.3.1**.

The raw individual milk samples from commercial dairy farms were 9.4% antimicrobial residues positive. After boiling for 15 minutes and 30 minutes the antimicrobial residues positive percentages reduced to 8.9% and 7.8%, respectively. Among raw milk samples, 15 minutes boiled and 30 minutes boiled samples, the antimicrobial residues percentages varied insignificantly ($p = 0.85$). The antimicrobial residues percentage was same (6%) both in raw milk and 15 minutes boiled samples from household dairy cows. But in 30 minutes boiling the prevalence of antimicrobial residues reduced to 4%. There was insignificant ($p = 0.88$) differences in the antimicrobial residues positive percentages of raw milk, 15 minutes boiled and 30 minutes boiled household cow milk samples. In case of samples from distributing point, the raw milk and 15 minutes boiled milk had 5% antimicrobial residues, whereas the 30 minutes boiled samples had only 2.5%. There was no significant ($p = 0.81$) differences in antimicrobial residues positive percentages among the different categories of milk samples from distributing point as documented in **Table 4.3.1**.

Table: 4.3.1: Comparison of effect of heat on antimicrobial residues in milk (Chi-square test, a univariate analysis)

Sources	Category	Antimicrobial residues		*p*
		% Negative	% Positive	
Commercial farm (pooled sample)	Raw milk	82	18	0.698
	15 min boiled	84	16	
	30 min boiled	88	12	
Commercial farm (individual sample)	Raw milk	90.6	9.4	0.849
	15 min boiled	91.1	8.9	
	30 min boiled	92.2	7.8	
Household	Raw milk	94	6	0.876
	15 min boiled	94	6	
	30 min boiled	96	4	
Distributing point	Raw milk	95	5	0.812
	15 min boiled	95	5	
	30 min boiled	97.5	2.5	

4.4. Concentrations of antimicrobial residues in raw milk

The concentrations of amoxicillin, oxytetracycline and ciprofloxacin in raw milk were 339.9±13.2µg/l, 195.0±35.6µg/l and 9.2µg/l, respectively. Amoxicillin residue was in the highest and ciprofloxacin in the lowest concentrations in raw milk under study as stated in **Table 4.4**.

Table: 4.4: Concentrations of antimicrobial residues in raw milk

Antimicrobials	N	Mean±SD (µg/l)	Minimum (µg/l)	Maximum (µg/l)	Threshold value (µg/l)
Amoxicillin	4	339.9±13.2	323.6	355.8	40
Oxytetracycline	12	195.0±35.6	137.8	258.5	100
*Ciprofloxacin	1	9.2	-	-	147

*N=1, not subjected to any statistical analysis

4.5. Effect of heat on concentrations of antimicrobial residues in raw milk

The highest concentrations (339.9±13.2µg/l) of amoxicillin residue in raw milk reduced to 257.7±18.4µg/l by 15 minutes of boiling and 119.5±11.7µg/l by 30 minutes of boiling (**Table 4.5**).

Table: 4.5: Effect of heat on concentrations of antimicrobial residues in raw milk (One way ANOVA)

Antimicrobials	Treatment	N	Residue (µg/l) Mean ±SE	*p*
Amoxicillin	Raw milk	4	339.9±13.2	0.001
	15 min boiled	4	257.7±18.4	
	30 min boiled	4	119.5±11.7	
Oxytetracycline	Raw milk	12	195.0±10.3	0.000
	15 min boiled	12	100.2±14.4	
	30 min boiled	12	27.8±10.5	
*Ciprofloxacin	Raw milk	1	9.2	-
	15 min boiled	1	0.1	
	30 min boiled	1	0.01	

*N=1, not subjected to any statistical analysis

There was highly significant (p = 0.00) differences in amoxicillin residue concentration in raw milk, 15 minutes boiled and 30 minutes boiled milk samples.

The oxytetracycline residue in raw milk was 195.0±10.3µg/l. The concentration of oxytetracycline reduced in 15 minutes boiled (100.2±14.4µg/l) and 30 minutes boiled milk samples (27.8±10.5µg/l). The oxytetracycline residue concentration varied significantly (p = 0.00) among the raw milk, 15 minutes boiled and 30 minutes boiled milk samples. The ciprofloxacin residue in raw milk, 15 minutes boiled milk and 30 minutes boiled milk were 9.2µg/l, 0.1µg/l and 0.01µg/l, respectively. There was wide variation in the concentration of ciprofloxacin residue in three categories of milk samples **Table 4.5**.

4.5.1. Comparison of amount of antimicrobial residues in milk by heat treatment

In case of amoxicillin residue, the effect of heat on concentration was not varied significantly (p = 0.16) between raw milk and 15 minutes boiled milk. There was highly significant (p = 0.00) differences between raw milk and 30 minutes boiled milk and significant (p = 0.02) between 15 minutes boiled and 30 minutes boiled milk. For oxytetracycline residue, the effect of heat was highly significant (p = 0.00) between raw milk and 15 minutes boiled milk, raw milk and 30 minutes boiled milk as well as 15 minutes boiled and 30 minutes boiled milk (**Table 4.5.1).**

Table: 4.5.1: Comparison of concentrations of antimicrobial residues in milk by heat treatment (One way ANOVA, Bonferroni test)

Antimicrobials	Comparison	*p*
Amoxicillin	Raw milk and 15 min boiled milk	0.164
	Raw milk and 30 min boiled milk	0.001
	15 min boiled milk and 30 min boiled milk	0.015
Oxytetracycline	Raw milk and 15 min boiled milk	0.000
	Raw milk and 30 min boiled milk	0.000
	15 min boiled milk and 30 min boiled milk	0.000

4.6. Prevalence of antimicrobial residues in selected milk products

The highest percentages (4.2%) of antimicrobial residues were in dahi among the studied milk products. The rasogolla were free from antimicrobial residues. About

2.1% samples of powder milk were detected as antimicrobial residues positive (**Table 4.6**).

Table 4.6: Overall prevalence of antimicrobial residues in selected milk products

Milk products	N	Antimicrobials positive	Prevalence
Dahi	48	2	4.2%
Rasogolla	48	0	0%
Powder milk	48	1	2.1%

4.6.1. Prevalence of antimicrobial residues in dahi and rasogolla manufactured by different sweetmeat producers

Overall the dahi were 4.2% positive for antimicrobial residues. About 8.3% of Modhubon brand dahi for ciprofloxacin and 8.3% of Banoful brand dahi samples for gentamicin were investigated by TLC as positive. The dahi of other sources were free from residue of antimicrobials. The rasogolla were residue negative of any type of antimicrobials understudy (**Table 4.7.1)**.

4.6.2. Prevalence of antimicrobial residues in powder milk

As investigated in the study the powder milk samples of marks brand had 10% antimicrobial residues positive for sulphadimidine. Other brands were free from antimicrobial residues for any antimicrobials selected in the study (**Table 4.6.2**).

4.7. Concentrations of antimicrobial residues in market milk and selected milk products

Among the market milk and milk products sample, the amoxicillin, oxytetracycline and ciprofloxacin residues were 132.9µg/l, 78.3µg/l and 0.6µg/kg, respectively (**Table. 4.7**).

Table 4.6.1: Prevalence of antimicrobial residues in dahi and rasogolla

Brands	Sample (N)	Prevalence of antimicrobial residues %(N)					
		Amoxicilln	Oxytetracycline	Gentamicin	Ciprofloxacin	Ceftriaxone	Sulphadimidine
Food plaza	Dahi (12)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Rasogolla (12)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Modhubon	Dahi (12)	0 (0)	0 (0)	0 (0)	8.3 (1)	0 (0)	0 (0)
	Rasogolla (12)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Banoful	Dahi (12)	0 (0)	0 (0)	8.3 (1)	0 (0)	0 (0)	0 (0)
	Rasogolla (12)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Fulkoli	Dahi (12)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Rasogolla (12)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

Table 4.6.2: Prevalence of antimicrobial residues in powder milk

Brands	N	Prevalence of antimicrobial residues %(N)					
		Amoxicilln	Oxytetracycline	Gentamicin	Ciprofloxacin	Ceftriaxone	Sulphadimidine
Milk vita	12	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Dano	12	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Marks	12	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	8.3 (1)
Fresh	12	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

Table: 4.7: Concentrations of antimicrobial residues in market milk and selected milk products

Sample	Category	Antimicrobials	N	Residue	Threshold value
Market milk	Aarong	Amoxicillin	1	132.9µg/l	40µg/l
	Farm fresh	Oxytetracycline	1	78.3µg/l	100µg/l
Dahi	Modhubon	Ciprofloxacin	1	0.6µg/kg	147g/kg

Chapter-5: Discussion

The present study investigated the antimicrobial residues in milk and selected milk products of Chittagong, Bangladesh and effect of heat on residue.

The present study suggested the prevalence of the antimicrobial residues was more in the pooled samples from commercial dairy farms than individual samples. It could be due to the pooled samples were the mixture of milks from all the individual cows and the individual samples were not all subjected to sampling. There were only oxytetracycline (OTC) and gentamicin residue in household milk. It might be due to the frequent use of OTC and gentamicin for treating cows in rural areas. The beta lactams and OTC were imprudently used in commercial dairy farms for treatment purpose which coincide with the findings of Syit, (2008). The prevalence of antimicrobial residues in milk samples from distributing points were lower compared to other sources of milk. It can be caused due to the dilution of milk from different farm in same can. Among the milk samples, the market milk had the lower percentage of antimicrobial residues. The finding was also supported by Fonesca et al., (2009); Movassagh and Karami, (2011). It is because that market milk was collected from different areas, standardized and pasteurized (heat treated) simultaneously.

According to the present study the milk samples from commercial farms were found antimicrobial residues positive with amoxicillin, OTC, ceftriaxone, gentamicin and sulphadimidine. The same finding is stated by Brogden et al., (2003). They found β-lactams, tetracyclines, aminoglycosides, quinolones, macrolides and sulfonamides are used at commercial farm levels of dairy cows for preventive and treatment purpose in Bangladesh. The prevalence of antimicrobial residues in commercial dairy farms milk was 18% which accords with Rybinska et al., (1995) who studied on antimicrobial residues in milk in Poland. The present findings show higher values in comparison to that of some previous findings (Amatya, 2010; Ceyhan and Bozkurt, 1987; Kang'ethe et al., 2005). It might be due to the regional variation and the differences in intensity of use of antimicrobial drugs in dairy cows. On the other hand, the current findings were lower than the findings of Aydin et al., (1989); Khaskheli et al., (2008); Ardic and Durmaz, (2006). It can be possible due to the other factors like sample designs, sample distribution, sample size, seasons etc.

The results of the current study revealed that the prevalence of antimicrobial residues was positively correlated with size of the farms. There was found significant variation in the prevalence of antimicrobial residues among the small, medium and large dairy farms. To the best of my knowledge, I reported the factors for the first time and no available studies were found regarding the association of size of the farms and prevalence of antimicrobial residues during the study. But the author thinks it is assumed to be due to the higher frequency of using antimicrobials in large dairy farms.

The present study suggested that the milk of farms having sick cows had the highest prevalence of antimicrobial residues than farms having no sick cows. There was significant difference in the prevalence of antimicrobial residues in milk between the farms having sick cows and without sick cows. The dairy farms having the history of continuing treatment had higher prevalence of antimicrobial residues than the others having no such history. The antimicrobial residues prevalence in the milk of farms having cows with treatment was significantly higher than other farms. There was found significant differences in antimicrobial residues between farms where antimicrobials treatment was going on and the farms with no case of antimicrobials treatment continued at present. As far I know, this is the first time study to discuss the risk factors with prevalence of antimicrobial residues in milk. But the author thinks when the cow sickness occurs, frequencies of treatment using antimicrobial drugs are also higher, ultimately the prevalence of antimicrobial residues might be higher.

In the present study, about 4% of the market milk samples contained antimicrobial residues. The current result agreed with the findings of Fonseca et al., (2009) who studied the incidence of antimicrobial drugs in Brazilian Ultra High Temperature (UHT) milk. Movassagh and Karami, (2011) found a bit lower prevalence of antimicrobial residues in market milk in the northest region of Iran. The present finding was lower than the results of Adesiyun and Webb (1997); Shitandi, (2001); Aning et al., (2007). The variation might be resulted due to application of different range of temperature during pasteurization of milk in different plants and also regional variation in terms of sickness of cow and use of antimicrobials.

The current study suggested that OTC was heat labile as the positive percentages of OTC was reduced at 15 minutes boiled and 30 minutes boiled samples. The finding is

agreed by Javadi et al., (2009). The gentamicin residue was partially heat stable. This result is also supported by Javadi et al., (2009). The amoxicillin residue was also partially heat labile in the present study and ciprofloxacin and sulphadimidine were completely heat stable which accords with Furusawa and Hanabusa, (2002). Gentamicin residue was partially heat labile where ceftriaxone was heat stable in the current study. These results are not agreed by Javadi et al., (2009). This might be due to improper temperature effect and differences in temperature time combination between the studies. The present study suggested that the effect of heat on positive antimicrobial residue percentages was insignificant. It is possible, because only amoxicillin and OTC were heat labile among the antimicrobials selected for the study (Javadi et al., 2009).

In this study the average concentrations of amoxicillin residue in raw milk was 339.9±13.2µg/l which was several times higher than the acceptable Maximum Residue Limit (MRL) of amoxicillin residue (40µg/l) in livestock products (EC, 2001). The finding was higher than those of Ghidini et al., (2002) who investigated up to 53.7µg/l amoxicillin residue in raw milk. The OTC residue in raw milk was 195.0±10.3µg/l which was about two times higher than the acceptable MRL (100µg/l) prescribed by EC, (2001). The OTC residue concentrations in the present study was also slightly higher than the previous findings of Elizabeta et al., (2011); Kaya and Filazi, (2010); Syit, (2008) which were 149.4µg/l, 150µg/l and 142µg/l, respectively. The differences in concentrations of OTC residue in milk can be possible due to higher doses of antimicrobials used during treatment. The ciprofloxacin residue in raw milk was higher than the findings of Elizabeta et al., (2011). But the finding was within the acceptable MRL (147µg/l) as suggested by (EC, 2001).

The present study suggested that the differences in concentrations of amoxicillin residue in raw milk and boiled milk samples were highly significant. The effect of heat for 15 minutes on concentrations of antimicrobial residues was insignificant in the present study. The boiling for 30 minutes was found to cause significant reduction in concentrations of antimicrobial residues in raw milk. As similar results Javadi et al., (2009) also reported amoxicillin residue as heat labile. The OTC residue was found to be reduced at highly significant level from raw milk to boiled milk samples. There were highly significant differences in concentrations between raw milk and 15 minutes boiled milk samples as well as raw milk and 30 minutes boiled milk samples.

Javadi et al., (2009) also showed same result regarding heat lability of OTC. The ciprofloxacin residue was found to be reduced in milk after 15 minutes and 30 minutes boiling. The current finding was disagreed with the results of Javadi et al., (2009) who stated that ciprofloxacin residue is heat stable under autoclaving temperature and conditions. This might be occurred due to the long time heating of milk in the present study.

The current study revealed that the amoxicillin residue in market milk sample was 132.9µg/l that was several times higher than the acceptable MRL as suggested by EC, (2001). The concentration of amoxicillin residue in market milk was lower than the raw milk samples. It might be due to the effect of heat during pasteurization. To the best of my knowledge, there is no literature available on amoxicillin concentrations in market milk. So, no comparisons can be made with other studies. The concentration of OTC residue in market milk was 78.3µg/l. The result of OTC concentrations in the current study was within the acceptable MRL as reported by EC, (2001). The finding of present study also coincide with the study of Abbasi et al., (2011) who found 87.3µg/l OTC residue in pasteurized milk.

In the present study, among the selected milk products the Rasogolla samples were antimicrobial residues negative. On the other hand dahi and powder milk samples were antimicrobial residues positive as 4.2% and 2.1%, respectively (**Table 4.7**). The Powder milk samples from Marks brand were 8.3% antimicrobial residues positive. This finding is supported by Helio et al., (2007). Dahi and rasogolla have lower prevalence of antimicrobial residues than milk. In the current study, I reported for the first time the prevalence of antimicrobial residues in dahi and rasogolla. And no comparisons could be made with other studies due to unavailable data on this topic. As far the author's knowledge it might be due to most of the milk products are processed by heating.

In this study the ciprofloxacin residue in market dahi (0.6µg/kg) was within the acceptable MRL (EC, 2001). The residue concentration in dahi was lower than that in milk. As far I know no data exists on this topic in the literature as I measured the antimicrobial residues in milk products for the first time; no comparisons could be made with other studies. The reason for such a difference is currently unclear, but it

could be explained by the fact that longer heat treatment in milk followed by coagulation of milk is done for dahi preparation.

The study was conducted for seven months only which is a very short period to reveal the exact scenario of antimicrobial residue in milk and milk products. I have considered only 3 antimicrobials for residue quantification because the resources were not available. It could be better to use all the antimicrobials used in treating dairy cows for screening and quantification of antimicrobial residues in milk and milk products. There was also another factor that, it is the first time study for analysis of risk factors related to antimicrobial residues in milk and no available data were found to compare and discuss with the present scenario. The determination of antimicrobial residue in milk products is also the first time study. So comparison of values with other study was not possible.

Chapter-6: Conclusions

The present study includes the investigation of levels of antimicrobial residues in milk and selected milk products in Chittagong, Bangladesh and effect of heat on residues.

The overall prevalence of antimicrobial residues was 18% in milk samples of commercial farms. The oxytetracycline (OTC) and gentamicin were mostly used in commercial dairy farms. Overall 5% of the dairy products were having antimicrobial residues. About 4.2% of the dahi samples were investigated as antimicrobial residues positive in the study. The effect of boiling was found to reduce the prevalence of antimicrobial residues in milk for amoxicillin, gentamicin, ciprofloxacin and OTC.

The effect of heat on prevalence of antimicrobial residues in raw milk was insignificant. The concentrations of amoxicillin and OTC residue in raw milk samples were 339.9±13.2µg/l and 195.0±10.3µg/l, respectively. The concentrations of amoxicillin and OTC residue in milk both before and after boiling were higher than the acceptable Maximum Residue Limit (MRL). The effect of heat on concentrations of amoxicillin and OTC residue was highly significant. The amoxicillin and OTC residue concentrations in market milk were 132.9µg/l and 78.3µg/l, respectively. The amoxicillin residue was higher than the MRL and the OTC residue was within the MRL. The ciprofloxacin residue in dahi (0.6µg/l) was also within the MRL.

Most of the antimicrobial concentrations were above the MRL. These may cause hypersensitivity, antibiotic resistance as well as cancers in humans. This necessitates that all effort including awareness creation, observance of withdrawal period, effective surveillance, monitoring and control on the use of veterinary drugs to prevent drug residues in animal derived products be employed. Moreover, the proper authority should pay depth attention on maintaining withdrawal periods of antimicrobials in farm level. Finally, the present study will contribute in understanding the level of antimicrobial residues in milk and milk products. In addition, the dairy scientists, veterinarians, farmers, might be beneficial from the present findings. Therefore, it can be concluded that the present results will contribute in awareness building regarding public health to a great extent.

Chapter-7: Recommendations

This study on the investigation of levels of antimicrobial residues in milk and milk products in Chittagong, Bangladesh and effect of heat on residue suggests the following recommendations:

- Indiscriminate or excessive use of drugs should be restricted through education and motivation of dairy farmers and practicing veterinarians.
- Veterinarians are advised to be more rigorous when prescribing veterinary medicinal products and to become aware of rules for the prudent use of antimicrobials.
- Owners should respect the prescribed withdrawal periods of drugs. It is also necessary to organize seminars on the risk of the excess use of antimicrobial substances in food animals for public health.
- Drug withdrawal periods should strictly be maintained in the dairy farms to produce safe milk and milk products for human consumption.
- Regulatory authorities should ensure proper withdrawal period before milking the animals and definite supervisions are necessary on application of these drugs.
- This study covered only few areas of Chittagong district for investigation of drug uses and antimicrobial residues. Therefore, a comprehensive study is required to determine the level of antimicrobial residues in milk all over Bangladesh.

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Annex-1: List and contact address of sampled commercial dairy farms of Chittagong Metropolitan Area

Serial No.	Farm name	Address	No. of animals
1	Faisal Dairy	Nasirabad	66
2	Bhuian Dairy	Kotoali	54
3	Hossain Dairy	Nasirabad	34
4	Zakir hossain Dairy	Khulshi	21
5	Amin Dairy	Pahartali	32
6	Nurul Dairy	Potenga	54
7	Well Dairy	Chalkbazar	59
8	Altaf Dairy	Kumira	37
9	Wajedia dairy	Aturar dipo	26
10	Alam dairy	Panchlaish	81
11	Amin Dairy	Patenga	25
12	Jarip Dairy	Nasirabad	39
13	Molla Dairy	Potenga	199
14	Samiya dairy	Bahaddarhat	105
15	kamini dairy	Kotoali	86
16	Hamid dairy	Kotoali	100
17	Eliyas dairy	Agrabad	11
18	Moti dairy	Oxygen	50
19	Liza dairy	Kalamia bazaar	112
20	Hossain dairy	Kattoli	51
21	Jane Alam dairy	Jalalabad	71
22	Monoara dairy	Chandgao	23
23	Poly dairy	Ambagan	24
24	Raj dairy	Panchlaish	24
25	Zarif dairy	Chaktai	84
26	Shadiq dairy	Karnafuli	15
27	Rasel dairy	Faillatali	14
28	Rahnania dairy	Halisahar	34
29	Haque vandari dairy	Baizid	14
30	Khaja dairy	Kalamia bazaar	19
31	Upa dairy	Baizid	14

32	Hossain dairy	Chaktai	34
33	Sumon dairy	Pahartali	9
34	Bhuiya dairy	Potenga	44
35	Bijay ghosh dairy	Chalkbazar	20
36	Dula mia dairy	Kumira	3
37	Sofia dairy	Aturar dipo	32
38	Jesmin dairy	Panchlaish	30
39	Ajit ghosh dairy	Patenga	13
40	Nirmal ghosh dairy	Nasirabad	6
41	Janata dairy	Potenga	55
42	Monoara dairy	Bahaddarhat	15
43	Kartic dairy	Kotoali	16
44	Chisrhia dairy	Kotoali	36
45	Shofiq dairy	Agrabad	14
46	Eva dairy	Oxygen	79
47	Khokon ghosh dairy	Kalamia bazaar	6
48	Jagat ghosh dairy	Kattoli	10
49	Indrajit dairy	Jalalabad	16
50	J N dairy	Pahartali	27

Annex-2: Questionnaire

Title: Antimicrobial residues in milk and selected milk products of Chittagong, Bangladesh

Department of Dairy and Poultry Science,
Faculty of Veterinary Medicine
Chittagong Veterinary and Animal Sciences University, Bangladesh

SECTION - A

1. Farm name, Address with Mobile no:

   ……
   ……
2. Educational Qualification: Illiterate/Primary- Secondary/Higher Secondary/ Graduate to higher
3. Total income of farmer (monthly): ……
4. Income from farm (monthly): ……..
5. Main profession of the farmer: …….
6. Farm composition: a) Milch cow: …b) Dry cow: …c) Preg:..d) Bull: …e) Calf:…
7. Average milk production/cow/day: ……..
8. Average feed (concentrate) given/cow: ……
9. Price of milk/liter: …….
10. Selling of milk: dairy plant/household/market/ ghosh /others
11. Is any vaccination done or not? Yes/ No
12. If yes, name of vaccine with date, route and dose

    1.
    2.
    3.
    4.

SECTION-B

13. Is any cow ill? 1) Yes 2) No
14. If yes, what's the problem: …….
15. Is any treatment given? a) Yes b) No

16. If yes, what kind of drug is used (with route and dose)? ……

17. How many days the cow is ill? ……

18. Was any cow ill in the last month? Yes/ No

19. If yes, what's the problem: …….

20. Was any treatment given? a) Yes b) No

21. If yes, what kind of drug was used (with route and dose)?

22. Was the milk selling done during treatment? Yes / No

Signature of respondents Signature of interviewer

Annex-3: Procedure of Thin Layer Chromatography

Reagents for Thin Layer Chromatography

De-ionized water, distilled water, Acetonitrile, Methanol, Deionized water, and Acetones were required for Thin Layer Chromatography (TLC).

Silica plates for sample running

TLC plate with 0.25mm thickness (MERCK, Germany), was activated at 120^{o}**C** for two hours before use.

Preparation of solvent system

In order to perform Thin layer Chromatography along with stationary phase or adsorbent, a mobile phase or solvent preparation was done as directed by Thangadu et al., (2002). Here, a volume of 50ml of methanol and 50ml acetone were mixed properly and used as mobile phase.

Preparation of standard solution

Stock solution of pure amoxicillin, OTC and ciprofloxacin were prepared by dissolving exactly 0.5ml of each in 10ml of methanol. Working solutions were prepared as required dilution. These solutions were stored in well-closed vessels and direct light was avoided (Thangadu et al., 2002).

Preparation of developing chamber (TLC tank)

A glass made beaker with a watch glass on the top was used as developing chamber.

Pointing on TLC plate:

For pointing on TLC plate first precoated TLC plates were cut according to the shape of TLC Tank with scissors. Then the following steps were performed.

1. Firstly a line was drawn with the help of pencil on TLC plate and scaled. This line was sufficiently high up (0.5cm) the plate so that when it was placed in the solvent the spots made on the TLC plate, this was remaining high on the level of the solvent.
2. Then pointing of standard solution was done with capillary tube on this line. Proper care was taken to ensure that the spots were kept as small as possible. The spots were never greater than 2-3mm in diameter.
3. After drying the spot of standard solution points the spot of sample solution by

capillary tube about 2 cm distance from previous spot. After 2 spots had been put and dried up, the plates then were placed in the TLC tank and were allowed for running.

Development of Chromatogram

1. Five milliliter solvent (Methanol and Acetone mixture) was placed in the developing chamber. The solvent level has to be below the starting line of the TLC plate, otherwise the spots were dissolved away.
2. The lower edge of the plate was then dipped in the solvent. The solvent (elute) travels up the matrix by capillarity, moving the components of the sample at various rates because of their different degrees of interaction with the matrix (stationary phase) and solubility in the developing solvent. The solvent was allowed to travel the solvent up the plate until 1 cm below from the top.
3. The plates were taken out and marked the solvent front immediately. The solvent was not allowed to run over the edge of the plate. Then, the plates were dried to evaporate the solvent completely (Thangadu et al., 2002).

Examination of Chromatogram under UV detector

In the TLC, the chromatogram was examined under ultra-violet lamp at 256nm for spots i.e., spot that fluorescence. The outline of the spot was marked with a series of dots using a sharp pencil. The color of each fluorescent spot was recorded on a separate paper. Then again visualizing agent ferric chloride was sprayed on the paper and dried with hot air oven at temperature of 105^{o}C.Then again the plates were examined under ultraviolet lamp for further fluorescent spots and color was noted on the paper (Thangadu et al., 2002).

Determination of Retardation factor (RF) value

The distance that each spot had traveled from the start line was measured (cm). This was taken from the center of the spot to the last point of the traveling of that spot. Also the distance of the solvent was measured from the start line. Then calculation of RF values was done using the following equation:

$$\mathrm{RF} = \frac{\text{Distance moved by substances}}{\text{Distance moved by solvent}}$$

Results of all RF values were recorded on a paper of tabular form.

Interpretation of result:

The Chromatogram of the standard solution and sample were compared based on following criteria.

I. Same color under UV light.
II. Same color with the spray reagent.
III. Same RF value as those of the reference sample.

If the color of the standard and sample spot was same in Ultraviolet (UV) ray after traveling or after using ferric chloride or the Refractive value of standard and sample solution was same, the sample was positive to that standard antibiotic.

Annex-4: Procedure of Ultra High Performance Liquid Chromatography (UHPLC)

Determination of amoxicillin residue

Chemicals

All chemicals and reagents used were of UHPLC grade or analytical grade. Amoxicillin trihydrate, sodium hydroxide and potassium dihydrogen phosphate were obtained from Sigma (St. Louis, MO, USA). Actonitrile and other solvents were supplied by J.T Baker (Philipsburg, NJ, USA). Deionized water obtained from Mille-Q Plus analytical deionization system (Bedford, MA, USA).

Preparation of standard and test solutions

Standard solution

30mg of Amoxicillin trihydrate CRS was dissolved in mobile phase A and diluted to 50ml with mobile phase A.

Test solution

Extract antibiotic solutions for thin layer chromatography were filtered through 0.2MFS syringe filters (0.2mm Advanced MFD, Inc., Japan).

Preparation of mobile phase

Mobile phase A: It was a mixture of 1 volume of acetonitrile R and 99 volumes of buffer solution P^H 5.0.

Mobile phase B: It was a mixture of 20 volume of acetonitrile R and 80 volumes of buffer solution P^H 5.0.

Preparation of buffer solution

Dilute sodium hydroxide was added to 250ml of 0.2M potassium dihydrogen phosphate R up to pH 5.0 and diluted to 1000ml with waster R.

UHPLC Procedure

The Chromatographic procedure was carried out by the following ways:

1. A stainless Colum C18 (2µm) P/N 891-5002, 2mm ID×10 0mmL No.22G2C-001 was used for chromatography.
2. Mobile phase was run at a flow rate of 0.2ml/min.

3. Spectrometer detector was set at 254nm to measure the wave length.
4. Injection volume: 20µl.

Ciprofloxacin:

Chemicals:

All Chemicals and reagents used were of HPLC grade or analytical grade. Ciprofloxacin hydrochloride, acetonitrile (Sigma Aldrich), phosphoric acid (Sigma Aldrich) and deionized water.

Preparation of mobile phase:

Mobile phase A: 10 volume of acetonitrile

Mobile phase B: 90 volume of 0.1% H_3PO_4

A spectrophotometer detector was set at 254nm to measure the wavelength.

Oven Temperature : 40^oC

Injection Volume : 10µl

Standard Solutions:

30 mg of ciprofloxacin HCl was dissolved in mixed Mobile phase A and B and diluted to 50ml with the same mobile phase.

Test solution:

Extracted antibiotic solutions for thin layer chromatography were filtered through 0.2 MFS syringe filters (0.2-m, Advance MFD, Inc. Japan)

The chromatographic procedure was carried out by the following ways:

1. A stainless steel column C18 (2µm), 2 mm ID × 100mmL was used for chromatography.
2. Mobile phase was run at a flow rate of 1ml/min

Oxytetracycline

Chemicals

All chemicals and reagents used were of UHPLC grade or analytical grade. Oxytetracycline hydrochloride, methanol and sodium hydroxide were obtained from Sigma (St. Louis, MO, USA). Actonitrile and other solvents were supplied by J.T

Baker (Philipsburg, NJ, USA). Deionized water obtained from Mille-Q Plus analytical deionization system (Bedford, MA, USA).

Preparation of standard and test solutions

Standard solution

30mg of Oxytetracycline hydrochloride was dissolved in mobile phase A and diluted to 50ml with mobile phase A.

Test solution

Extracted antibiotic solutions for thin layer chromatography were filtered through 0.2MFS syringe filters (0.2mm Advanced MFD, Inc., Japan).

Preparation of mobile phase

Mobile phase A: It was a mixture of distilled water containing H_2SO_4 at P^H 2.1 and acetonitrile at the ratio of 85 : 15.

Mobile phase B: It was a mixture of 20 volume of acetonitrile and 80 volumes of buffer solution P^H 5.0.

Preparation of buffer solution

Dilute sodium hydroxide was added to 250ml of 0.2 M Potassium dihydrogen phosphate R up to P^H 5.0 and diluted to 1000 ml with distilled water.

UHPLC Procedure

The Chromatographic procedure was carried out by the following ways:

1. A stainless steel column C18 (2µm) P/N 891-5002, 2mm ID×10 0mmL No.22G2C-001 was used for chromatography.
2. Mobile phase was run at a flow rate of 1.5ml/min.
3. Spectrometer detector was set at 360 nm to measure the wave length.
4. Injection volume: 20µl.

Assay validation

The column was equilibrated with a mobile phase with ratio A: B of 98:8. After that standard solution was injected. And the assay was validated until the resolution between the 2 principal peaks was used for quantification. The calibration curves were used to calculate the amoxicillin concentration of the quality control samples

and known samples. The spiked samples were processed and analyzed with the developed procedure. Therefore, the extraction recovery was obtained by comparing the observed peak area obtained from the processed standard samples to direct injection of standard aqueous solution prepared at concentrations with represented 100% recovery.

Annex-5: Status of commercial dairy farm in Chittagong metropolitan area

1. Status of commercial dairy farms in CMA

The literacy level of the dairy farmers were the highest at primary (36%) followed by secondary to higher secondary (32%), graduate or post graduate (28%) and least of them were illiterate (4%) in the present study (**Table 4.1**). The main profession of the dairy farm owners was farming (56%). Some of the dairy farmers were business man (36%) and least of them (8%) were engaged with others profession. There were category A farms having 3 to 25 lactating and dry cows found in the highest number (64%) whereas category C farms (≥ 51 cows) were present in the lowest number (12%). Moderate numbers of farm (24%) having 26 to 50 cows were categorized as category B farms.

The maximum percentage of farmers (48%) sold milk to the surrounding households followed by ghosh (32%), market (16%) and others place (4%). Most of the farmers (88%) continued routine vaccination to their cows whereas only few of them (12%) didn't continue vaccination. About 50% of the farms had diseased cows (**Table 4.1**). Among the farms 48% having the history of ongoing treatment and 52 % had no such therapeutic history in the time of sampling. On the other hand there were only 20% of farms had the history of Antimicrobials use during sampling (**Table 4.1)**. The survey also revealed that about 60 % of the farmers sold milk from cows during medication.

Table 1: Status of commercial dairy farms in CMA (descriptive analysis)

Variable	Category	Percentage (%)	SE	95% CI
Farmer's education	Illiterate	4	0.04	- 0.04 – 0.12
	Primary	36	0.10	0.16 – 0.56
	SSC to HSC	32	0.09	0.12 – 0.52
	≥ Graduate	28	0.09	0.09 – 0.47
Farmer's profession	Farming	56	0.10	0.35 – 0.77
	Business	36	0.10	0.16 – 0.56
	Others	8	0.05	- 0.03 – 0.19
Category of farm	3 – 25 cows (A)	64	0.10	0.44 – 0.84
	26 – 50 cows (B)	24	0.09	0.06 – 0.42
	≥ 51 cows (C)	12	0.07	- 017 – 0.26
Selling place of milk	Household	48	0.10	0.27 – 0.69
	Market	16	0.07	0.01 – 0.31
	Ghosh	32	0.09	0.12 – 0.52
	Others	4	0.04	-0.04 – 0.12
Vaccination	Yes	88	0.07	0.07 – 1.02
	No	12	0.07	- 0.02 – 0.26
Illness of cow	Yes	50	0.07	0.36 – 0.64
	No	50	0.07	0.36 – 0.64
Treatment given	Yes	48	0.07	0.34 – 0.62
	No	52	0.07	0.38 – 0.66
Antibiotic use	Yes	20	0.06	0.09 – 0.32
	No	80	0.06	0.69 – 0.92
Milk selling during treatment	Yes	60	0.10	0.39 – 0.81
	No	40	0.10	0.19 – 0.61

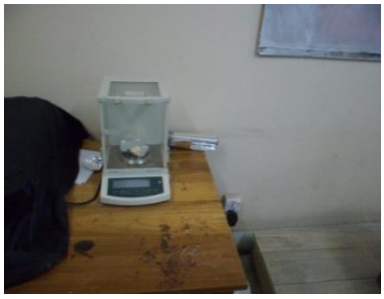
The current study evidenced that most of the dairy farmers were educated up to primary level. This finding coincided with the results of Uddin et al., (2012) who found about 65% dairy farmers were educated up to primary level. Of the dairy farms at CMA, maximum belongs to category A farms (having 3-25 cows). This finding is agreed by Bari et al., (2014) who stated most of the dairy farms in Chittagong are category A farms. Half of the farms were found having at least one cow sick and treatment was continued. The same findings also reported by Uddin et al., (2009) who found almost half of the dairy farms having at least one sick cow. Most of the farmers were found selling milk from the sick animals during treatment and leaving antimicrobials residue in milk; and hence in human bodies. The present finding coincide the findings of Ivona and Mate, (2002) who found drugs are indiscriminately used at farm level and withdrawal periods are not maintained. Thus, the residual antimicrobials may cause antimicrobial resistance, hypersensitivity reactions and even cancers in human bodies (Movassagh and Karami, 2011).

Annex-6: Picture gallery

Thin Layer Chromatography (TLC)

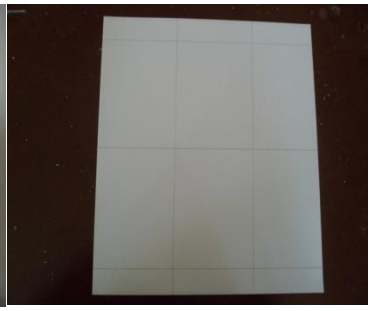
Adding reagent

Heating

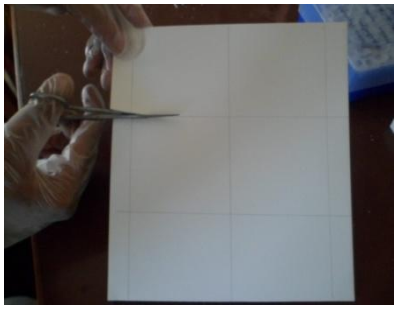


Weighing

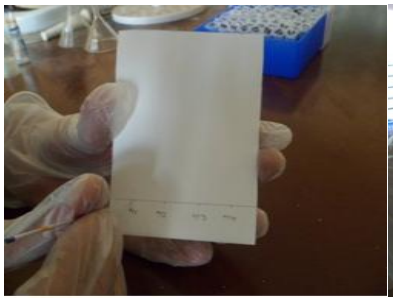
Centrifuging



TLC plate



Cutting of plate

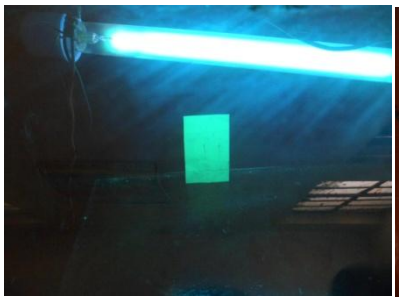


Pointing on TLC plate

Running

Drying

Placing of plate at UV chamber samples



Detection

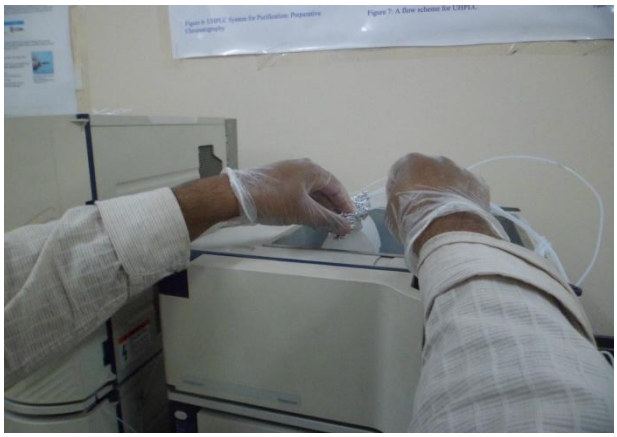
Positive

Ultra High Performance Liquid Chromatography (UHPLC)

UHPLC system

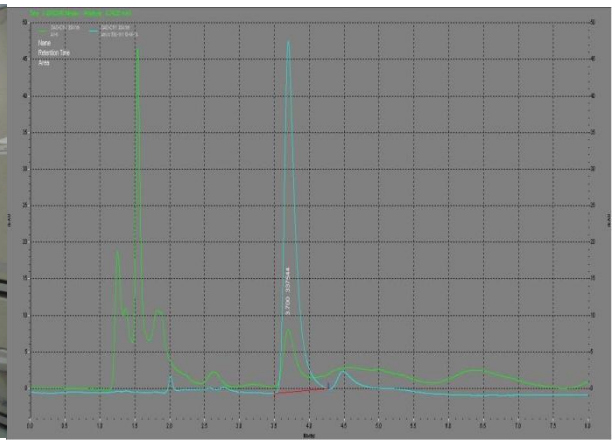
0.2 µm filter

Filtering



Setting of mobile phase

Placing of samples



Peak of sample

Brief Biography

Md. Saiful Bari passed the Secondary School Certificate Examination in 2002 followed by Higher Secondary Certificate Examination in 2004. He obtained his Doctor of Veterinary Medicine Degree in 2010 (held in 2012) from Chittagong Veterinary and Animal Sciences University (CVASU), Bangladesh. Now, he is a Candidate for the degree of MS in Dairy Science under the Department of Dairy and Poultry Science, Faculty of Veterinary Medicine, CVASU. He has been working as a lecturer in the Department of Dairy and Poultry Science, Faculty of Veterinary Medicine, CVASU since January 2013. He published ten scientific articles in national and international peer- reviewed journals. He has immense interest to work in UHPLC based determination of residual drugs and hazardous chemicals in food affecting public health.