Introduction

Antibiotics are secondary metabolites of microorganisms which in low concentration can prevent the growth or kill the bacteria by affecting some metabolic or structural elements that are crucial for their survival (Stolker, 2005). It was the late 1940s shortly after their development when the first antimicrobial used in veterinary medicine for the treatment of infections (Mitchell et al., 1998). Antibiotics are used in food-producing animals mainly for three purposes: 1) to treat ill animals 2) to prevent infections in healthy animals (prophylactic uses) and 3) as a growth promoter to increase body weight through boosting feed efficiency. In animal application of antibiotics for long duration in less than the therapeutic concentrations (1-10 mg/kg of feed) improve the efficiency of digestion and absorption by changing in the composition of microbial flora in the digestive system and subsequently cause an increase of animal growth rates. Responsible use of antibiotic is important to ensure food safety and public health. However inappropriate, unnecessary, and overdose of antibiotics in food-producing animals has remarkably overcast its benefit through the accumulation of drug residue and the development of resistant bacteria within the animal body. Residues of drugs are either the parent compound or their metabolite that can be accumulated, deposit or store within various cells, tissues and organs of the body and subsequently may present different consumable food products of animal origin like milk, meat, egg and skin (Chowdhury et al., 2015**)**. These residues might excrete through urine and feces lead to the contamination of the environment. Milk is considered as an ideal food for its high nutritional value and is consumed by the people of all levels- from newborns to the elderly. So, the quality of milk is a subject of public health concern throughout the world. Milk and its products provided for human consumption must be safe and free from any microbiological, physical or chemical contaminants (Kaya et al., 2010). Veterinary drug residues in milk might occur in many ways like failure of maintain the recommended withdrawal time/periods, long time use and incorrect dosage (Kurwijila et al., 2006). These residues in consumable food is a serious threat for public health especially when occurs above the maximum residue limit (MRL) that triggers allergic reactions, vomiting, diarrhea, kidney failure and anemia etc. Besides, exposure to a low dose of

antibiotics for long duration might alter the nature of gut microflora results in the enhancement of many diseases and moreover, it has the potential of growth of resistant strains of bacteria in the human body. In summary the public concern over the presence of antibiotic residues in animal originated food occurs in two ways; firstly, it produces direct toxicity to humans and secondly, it facilitates the development of antibiotic-resistant bacteria that leads to the failure of antibiotic therapy and threaten the human life. It also creates problem for the commercial dairy industry as literatures say these chemical residues does not significantly reduces by the conventional heat treatment used frequently for manufacturing pasteurized milk (72^0C). So industrial technologies are considered ineffective to eliminate these residues. In addition, the process of making the fermented milk products like cheese, dahi and yogurt are also affected by the presence of antibiotic residues in raw materials, as these can partially or fully inhibit the growth of lactic acid-producing bacteria (starter cultures), which is mandatory for the quality and structural characteristics of these milk products and causing serious economic problems for the dairy industry (Fonseca et al., 2009). Presence of antibiotic residue in milk above the maximum level (MRL) is recognized as illegal worldwide by various regulatory authorities (Aning et al., 2007). The Codex Alimentarius Commission recommends that when dairy cows are treated with antibiotics one should follow the recommended withdrawal period specified for each veterinary drug and their milk should be discarded within this period no matter how many days or even weeks (Sattar et al., 2014)

Different analytical methods have been developed to determine the presence of antibiotic residues in milk. All these tests can be divided in two main groups-screening test and confirmatory test. Screening methods are qualitative and usually provide semi-quantitative results also. Through these methods a drug or a family of drugs can be detected at the level of interest. Screening methods possess the characteristic of low rate of false-positive result, high throughput, ease of use, short analysis time, good selectivity and low cost. Screening methods could be classified in two broad types according to the reaction taking place i.e. microbiological assays and immunoassays (Cháfer-Pericás et al., 2010). Microbiological tests are able to detect antibiotics or any metabolite having antibacterial activity. These methods are based on the specific

reaction between a bacterium and the antibiotic present in the sample. These are highly reliable, simple and cost effective. Immunoassays are semi-quantitative methods based on the specific reaction between antibody and antigen. These have been classified as enzyme-linked immunosorbent assay (ELISA), fluoro-immunoassay (FIA) and time-resolved fluoro-immunoassay (TRFIA).

Confirmatory methods are time-consuming, expensive, and require complex laboratory settings and trained personnel for exhausting procedures of sample-preparation based on solid-phase extraction (SPE) and multi-step clean-up (Moreno-Bondi et al., 2009). These are mostly based on Liquid Chromatography (LC) and to detect analyze concentrations it is coupled with different detection mode like LC with mass spectrometry (MS) and LC with UV (Benito-Peña et al., 2009). Other analytical methods commonly used by the investigators include High Performance Liquid Chromatography (HPLC) and Capillary electrophoresis (CE) (Gracia, 2009). In analytical chemistry, HPLC is regarded as one of the most robust tools **(**Jayalakshmi et al., 2017). HPLC contains characteristics like a variety of mobile phases, extensive library of column packing's and variation in modes of operations, the use of HPLC increasing day by day for the detection of residue (Jank et al., 2017)

Now-a-days in Bangladesh, food safety issues are gaining considerable attention to the government and consumers are also much concerned. In this context, this research work was undertaken to detect and determine the concentration or level of antibiotic residues in milk of local and commercial farms in Chattogram city and Patiya Upazila of Chattogram, Bangladesh. Moreover, the perception of farmers regarding antibiotic residue and resistance was evaluated. This study will provide information on the chemical safety and quality of milk in the study area and create baseline data for further investigation on milk safety issues. Understanding the quality and safety of milk sold to the market has ultimate benefits to the consumers and dairy industries where the milk safely processed to other products. This study will help the regulatory body by providing information on the use of veterinary antibiotics in treating and preventing various cattle diseases and the knowledge and practice of dairy farmers.

Objectives

The overall objective of this study is to access the dairy farmers perceptions regarding AMU and AMR and determine the level of antibiotic residue present in both raw and processed cows' milk produced and marketed in the Chattogram city and Patiya Upazila of Chattogram, Bangladesh. Besides, few specific objectives were as follows

i) Access the general knowledge, attitudes and practices of dairy farmers regarding AMR and AMU

ii) Qualitative screening of antibiotic residues in raw and processed milk available in the study area.

iii) Quantification of the concentration of antibiotic residue levels in the positive raw and processed milk samples.

Review of Literature

Relevant literature on antimicrobial uses, drug withdrawal period, prevalence and concentration of residues, diagnostic methods, treatment effects, consequences and public health importance have thoroughly been reviewed in this chapter. The main purpose of this chapter is to provide up-to-date scientific information based on past studies and accordingly identify gaps and justify the present MPH thesis research on antibiotic residues in milk and its public health significance. The review findings of relevant published and non-published articles have been presented under the following headings as below.

Antimicrobials and their use in dairy cattle

Antimicrobial agents are found in different groups which are available for treatment of infected livestock and also for prevention of infection as well as a growth promoter. The most common groups includes tetracyclines, beta-lactams, sulphonamides, aminoglycosides, macrolides, and chloramphenicol (McGrane, 2000; Movassagh and Karami, 2010; Pecou and Diserens, 2011). These antibiotics may be used alone or in combination when treating dairy cattle. Antimicrobials are administered to animal through various routes like injections, orally in feed or water, topically on the skin and by intramammary and intrauterine infusions (Mitchell et al., 1998). Babapour (2012) stated that due to cost effective availability antibiotics have always been extensively used in the dairy, livestock, poultry, aquaculture and honey production sectors in various countries around the globe. Antibiotics are also used prophylactically to prevent the occurrence and spread of infections in intensive production systems such as cattle, pigs and poultry. The prophylaxis application of antibiotic can be to both individual animal and to groups of animals; the application of antibiotic to groups of animal at the time when only single animal of the group present symptoms of the disease, but it is expected that most of the group will become affected, is referred to as metaphylaxis with such treatment regimes, the antibiotics

are commonly applied via feed or water; and also antibiotics are used in animal production as growth promoters to improve feed utilization and production (Katakweba et al., 2012; Grane, 2000; Kurwijilaet al., 2006). In cattle, among the various indications, penicillin G has been parenterally administered for the treatment of mastitis, arthritis and respiratory infections (Ranheim et al., 2002) and 1st generation cephalosporins used for the treatment of mastitis (Homish 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).

Antimicrobials residue

Antimicrobial residue is the trace amount of an antimicrobial or its breakdown product(s) which remains in or on an agricultural product (livestock, cereal grains, fishes etc.) during the time of consumption following treatment with that antimicrobial (Botsoglou and Fletouris, 2001; Goodman, 2001; Brunton, 2011). Residue of veterinary drugs means all pharmacologically active substances, whether active ingredients or degraded products and their metabolites which remain in foodstuffs of animal to which these veterinary medicinal products have been administered. Usually every living being is receiving antibiotics in direct or indirect ways. Antibiotics are used not only for treatment purpose but also for prevention as well as a growth promoter. In livestock, intramuscular, subcutaneous and intravenous routes are followed for medication. Theoretically, all of these routes may lead to residues appearing in foods of animal origin such as milk, meat and eggs (Johnston, 1998). Besides, antimicrobials are poorly adsorbed in the gut of the animals and the majority is excreted unchanged in feces and urine. The excretion rate of chlortetracycline, sulfamethazine and tyolsin via feces and urine are 75, 90, and 50–100%, respectively (Kim et al., 2011).

Present status of antimicrobial residue 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 3.7 µg/l to 6340 µg/l and amoxicillin in 5.7% samples at concentrations ranging from 8.5 µg/l to 53 µg/l and

cefaprin in 3.8% samples at the concentrations of 5.7 µg/l and 6.4 µg/l. Amatya (2010) found 14% of raw milk samples contained Amoxicillin and 16% contained penicillin. Amoxicillin and penicillin were the most common residue found in milk samples. Khaskheli et al., (2008) showed that of all samples 36.5% were contaminated by beta- lactam antibiotic residues in cow raw milk in Pakistan. Ardic and Durmaz (2006) reported 21.3% of beta-lactam antibiotic residues in unpacked milk consumed in Sanliurfa region, Turkey. Elizabeta et al., (2011) measured range of concentrations (µg/kg) as 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 quinolones and 41.9 for tetracyclines. Kaya and Filazi (2010) found the minimum detectable concentrations for penicillin G, oxytetracycline, gentamicin, streptomycin and neomycin, as µg/1 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 150.4 µg/l for oxytetracycline 33.5 µg/l for penicillin G and 7688.4 µg/l for neomycin among raw samples. According to the total number of samples analyzed, the percentages of contamination with antibiotics were detected as 1.25%. Syit (2011) studied 400 milk samples by Delvotest SP assay and HPLC. 8.5% were found positive with antimicrobial residues. The mean residue level of oxytetracycline was 142.0 µg/l and penicillin G was 4.78 µg/l. The concentration of oxytetracycline was found above WTO/FAO/CAC established residue limit of 100 µg/l. The result suggested that oxytetracycline and penicillin G were imprudently used in dairy farms. Abbasi et al., (2011) suggested that the mean value of total tetracycline residues in 114 samples were 97.6ng/g and that of pasteurized, sterilized and raw milk samples were 87.1 ng/g, 112.0 ng/g and 154.0 ng/g respectively. Twenty five percent of all the 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 (l00 ng/g).

Status of antimicrobial residue 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 the prevalence 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) found 21% of 1109 milk samples were contaminated with antimicrobial residues in Kenya. Aning et al., (2007) carried out a study to understand the extent to which antimicrobial drugs may be translocated into milk and the related risk of exposure by consumers using Charm aim-96 antimicrobial inhibition assay screening kit. Among total screened milk samples 35.5% (140/394) were contaminated with one or more of the antimicrobials. 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, level of contamination ranged from 16.6% (9/54) for wholesalers or milk assemblers to 54.2% (13/24) for milk processors. There were no significant differences found in prevalence of drug residues in milk collected from different types of traders between and within locations.

The causes of antimicrobial residues in milk

A number of factors may be responsible for persistence of drugs residues in food of animal origin. The main reason reported is failure to observe withdrawal times (Shitandi, 2004). Fecal recycling, where the drug excreted in feces of treated animals contaminates the feed of untreated animals, can be the cause of residues of certain antimicrobial groups (McCaughey et al., 1990). The presence of residues may result from failure to observe the mandatory withdrawal periods, illegal or extra-label use of drugs and incorrect dosage, non-existence of restrictive legislation or their inadequate enforcement, poor records of treatment failures, lack of advice of withdrawal period especially in the developing countries (Ivona and Mate, 2002). Drug residues can also occur in calves fed milk and/or colostrums 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). Residues of antibiotics in milk most often originate from poor livestock diseases management, poor milk handling and unhygienic condition at farm-level, but do not rule out market-level practices which introduce antimicrobials to milk (Kurwijila et al., 2009; Shitandi, 2004). However,

unconfirmed reports suggest that some unscrupulous milk market agents may add antibiotics, among other chemicals, to lengthen the shelf life of milk (Kurwijila et al., 2006).

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. Following antibiotic administration, withdrawal times are specified after which time the animal or animal products are fit for human consumption (Katakweba et al., 2012; Kurwijila et al., 2006 and Shitandi et al., 2004). 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 the earliest time at which it may be slaughtered for food (Treves-Brown, 2013). 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.

Table 2.1: Withdrawal periods of different antimicrobials used in dairy cows

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

Source: (Mumtaz et al., 2000)

Maximum residue limits (MRLs)

It is the levels of drug legally permitted and recognized as acceptable in a food, resulting from the correct use of a veterinary drug, which should occur in food. MRLs are based on the type and amount of residue considered to be without any toxicological hazard for human health as expressed by the acceptable daily intake and

an additional safety factor. MRLs give an indication of food safety and provide trading standards (FAO/WHO-CAC, 2012). According to Council Regulation 2377/90 (Passantino, 2008) 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. 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 (Passantino, 2008). The European Union has established Maximum Residue Limits (MRLs) for several classes of antibiotics in animal products, such as milk and edible tissues, with the aim of minimizing risk to human health. In milk, the MRL ranges are between 4 and 30 g/kg for penicillins, 20 and 100 g/kg for cephalosporins, and 30 and 100 g/kg for quinolones.

Table 2.2: Maximum Residue Limits (MRLs) of different antimicrobials in milk

Antimicrobials	**MRL (µg/l)**
Tetracycline	100
Oxytetracycline	100
Amoxycillin	4
Cloxacillin	30
Gentamycin	200
Streptomycin	200
Benzyl Penicillin /procaine	4

Source:FAO/WHO-Codex Alimentarius Commission: Maximum Residues Limits (MRL) for Veterinary Drugs in Foods- CAC/MRL 2-2012

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 (Passantino, 2008). The Acceptable daily intake is 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. Due to the lack of toxicological data available, no ADI is established for chloramphenicol and, in consequence, no MRL could be attributed (JECFA, 2002).

Table 2.3: Acceptable Daily Intake (ADI) of different antimicrobials residue in milk

Antibiotics	ADI (mg/kg bw/day)
Amoxycillin	0.2
Gentamicin	0.05
Oxytetracycline	0.03
Ceftiofur sodium	0.03
Streptomycin (and dihydrostreptomycin)	0.05

Source: (OCS, 2013)

Harmful effects of antimicrobial residues

Antimicrobial residues in foods of animal origin may cause problems from various aspects. The presence of antibiotic residues in animal products such as milk, meat and eggs can present hazards for the public health, industry and environment (Nonga et al., 2009; and Katakweba et al., 2012). In addition to toxicity, effects on intestinal microbiota and the immune system also are important problems. 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). 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). The presence of antibiotic residue in milk also causes inhibition of starter cultures in production of cultured milk products such as yogurt and also manufacture of cheese (Movassagh and Karami, 2010; Kaya and Filazi, 2010).

Public health importance of antimicrobial residues in milk

Administration of drugs to food-producing animals has significant effects on humans who consume food from these livestock. The antibiotic residues when taken above the maximum residue limit (MRL) can result in potential health effects to the human being (Goffova et al., 2012). Imprudent use of antimicrobials in animals may unnecessarily result in increased human morbidity, increased human mortality, reduced efficacy of related antibiotics used for human medicine, increased health care costs, increased potential for carriage and dissemination of pathogens within human populations and facilitated emergence of resistant human pathogens (Gaurav, 2014). The effects include the occurrence of resistant strains of bacteria in humans, toxicity effects of the drug, allergic reactions (hypersensitivity reactions) in sensitized persons. 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. 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). In addition to toxicity, other important effects include intestinal dysbiosis (Goffová et al., 2012) and the impairment of immune system (Perrin-Guyomard et al., 2001). Also some drugs or their metabolites possess carcinogenic potential e.g. meat preserved with sodium nitrate and contains sulphamethazine residues, may develop a triazine complex that has a considerable carcinogenic potential. Prolonged ingestion of tetracycline in food has detrimental effects on teeth and bones in growing children. Some reports showed that drug residues destroy useful microflora of gastrointestinal tract, especially in children leading to enteritis problems (Goffová et al., 2012). A recent study has shown that altering the gut micro flora early in life can have lifelong effects, and may contribute to the development of obesity from a high-fat diet, or the development of other diseases (Cox et al., 2014).

Risk assessment for antibiotic residue consumed by food

Few research trials have evaluated the risk for antibiotic residue contamination of milk during early lactation following pre-partum intra-mammary antibiotic treatment in heifers. Vragovic et al., (2011) assessed the quantitative risk of streptomycin and tetracycline. The median value for streptomycin in milk and meat was 11.50 and 38.00 µg/kg, respectively (milk: average: 15.57 µg/kg; range from 0 to 73.82 µg/kg; meat: average 44.14 µg/kg; range from 0 to 278.35 µg/kg). The median value for tetracycline in milk and meat was 1.50 µg/kg (milk: average 1.5 µg/kg; range, from 0 to 4.26 µg/kg; meat: average 1.62 µg/kg; range from 0 to 5.35 µg/kg). Based on the median value it was concluded that the estimated daily intake of streptomycin and tetracycline through milk and meat in Croatia was low (streptomycin: 7.33 µg/person/day; tetracycline: 0.52 µg/person/day), and the risk was assessed as negligible.

Lopez de Souza et al., (2009) presented an environmental risk assessment (ERA) for the most used intravenous antibiotics in the Intensive Care Unit (ICU) of a hospital in Curitiba (Brazil). The amount of antibiotics used in the ICU was evaluated during a period of 18 months (June 2006 to November 2007), in order to calculate the Predicted Environmental Concentration (PEC1). The Predicted No-Effect Concentration (PNEC) of pharmaceuticals was also considered to assess the environmental risk by calculating the PEC/PNEC ratios. All PECs were $\geqslant$1 ng L^{-1}. The worst-case PEC estimations (PEC1 and PEC2) were observed for sodic ceftriaxone, sodic cefazolin, meropenem, ampicillin, cefepime and sodic piperacillin

Antibiotic residues and emergence of antibiotic resistance

Alali et al., (2009) conducted a longitudinal ecological study to examine the relationship between the prevalence of antibiotic-resistant (AR) commensal *Escherichia coli* isolates from both monthly human wastewater and composite swine fecal samples. Human and swine *E. coli* isolates (n = 2469 human and 2310 swine, respectively) were tested for antimicrobial susceptibility using a commercial broth micro dilution system. The relative odds of ciprofloxacin resistance were significantly increased for ciprofloxacin use in non-swine workers (OR = 5.5) as compared to the referent (non-use). The relative odds of tetracycline resistance were increased significantly for chlortetracycline use in medicated feed for the upper tertile of MMD

category (OR = 2.9) as compared to the referent category (no use) across all swine production groups. Cho et al., (2012) compared the antibiotic resistance of *Escherichia coli* isolates from faecal samples of workers who often used antibiotics. A total of 163 *E coli* strains were analyzed by agar disc diffusion to determine their susceptibility patterns to 16 antimicrobial agents. Most of the tested isolates showed high antimicrobial resistance to ampicillin and tetracycline. The isolates showed higher resistance to cephalothin than other antibiotics among the cephems. Among the aminoglycosides, the resistance to gentamicin and tobramycin occurred at higher frequencies compared with resistance to amikacin and netilmicin. The data indicated that faecal *E. coli* isolates of livestock workers showed higher antibiotic resistances than non-livestock workers (restaurant workers), especially cephalothin, gentamicin, and tobramycin ($p < 0.05$). Ji et al., (2012) quantified eight antibiotic resistance genes (ARGs), 7 heavy metals, and 6 antibiotics in manures and soils collected from multiple feedlots in Shanghai. The results revealed the presence of chloramphenicol, sulfonamides and tetracyclines at concentration ranges of 3.27–17.85, 5.85–33.37 and 4.54–24.66 mg/kg respectively. Overall, sulfonamide ARGs were more abundant than tetracycline ARGs. Except for sul II gene, only a weak positive correlation was found between ARGs and their corresponding antibiotics. On the contrary, significant positive correlations ($p < 0.05$) were found between some ARGs and typical heavy metals. For example, sul A and sul III were strongly correlated with levels of Cu, Zn and Hg. Gao et al., (2012) conducted a study in which total concentrations of tetracycline and sulfonamide antibiotics in final effluent were detected at 652.6 and 261.1 µg/L respectively, and in treated sludge, concentrations were at 1150.0 and 76.0 g/kg dry weights (DW), respectively. The gene abundances of tet O and tet W normalized to that of 16S rRNA genes indicated an apparent decrease as compared to sul I genes, which remained stable along each treatment stage. No significance ($R2 = 0.15$, $p > 0.05$) was found between tet genes (tet O and tet W) with concentration of tetracyclines identified in wastewater, while a significant correlation ($R2 = 0.97$, $p < 0.05$) was observed for sulI gene and total concentration of sulfonamides. Roug et al., (2013) conducted a study with an aim to screen cattle, sheep, goat, chicken, rabbit and horse feces from a livestock fair in California for the potentially zoonotic pathogens *Escherichia coli* O157:H7, Salmonella, Campylobacter, Vibrio, Cryptosporidium and Giardia spp., as well as determining the level of antimicrobial resistance in *E. coli* and Salmonella spp. The prevalence of antimicrobial resistance as well as multi-drug

resistance patterns were highest for *E. coli* and Salmonella spp. cultured from pigs and chickens were generally widespread but at lower levels for other animal groups and included resistance to ampicillin and streptomycin, two antimicrobial drugs of importance for human medicine. Novo et al., (2013) conducted a study in which raw and treated waste water composite samples were collected from an urban treatment plant over 14 sampling dates. Samples were characterized for the i) Occurrence of tetracyclines, penicillin, sulfonamides, quinolones, triclosan, arsenic, cadmium, lead, chromium and mercury; ii) Antibiotic resistance percentages for tetracycline, sulfamethoxazole, ciprofloxacin and amoxicillin and iii) 16S rRNA gene-DGGE patterns. Antibiotic resistance percentages presented different trends of variation in heterotrophs/enterobacteria and in enterococci, varied over time and after wastewater treatment. Antibiotic resistance was positively correlated with the occurrence of tetracycline residues and high temperature.

Effect of heat treatment on antibiotic residues

Zorraquino et al., (2008) conducted a study to analyze the effect of different heat treatments (40^0C for 10 min, 60^0C for 30 min, 83^0C for 10 min, 120^0C for 20 min and 140^0C for 10 sec) on milk samples fortified with three concentrations of nine betalactam antibiotics. The method used was a bioassay based on the inhibition of *Geobacillus stearothermophilus* var. calidolactis. The results showed that heating milk samples at 40^0C for 10 min hardly produced any heat inactivation at all, while the treatment at 83^0C for 10 min caused a 20% loss in penicillin G, 27% in cephalexin and 35% in cefuroxime. Of the three dairy industry, heat treatments studied in this work, low pasteurization (60^0C for 30 min) and treatment at 140^0C for 10 sec only caused a small loss of antimicrobial activity, whereas classic sterilization (120^0C for 20 min) showed a high level of heat inactivation of over 65% for penicillins and 90% for cephalosporins. Roca et al., (2011) conducted a study to calculate the kinetic parameters for the degradation of beta-lactam antibiotics in milk and to develop prediction models to estimate the concentration losses of these compounds in conventional dairy heat treatments. Increasing the temperature from 60^0C to 100^oC decreased the half-life of amoxicillin (372 to 50 min), ampicillin (741 to 26 min), cloxacillin (367 to 46 min), and penicillin G (382 to 43 min). These increases in temperature caused further degradation in cephalosporins, which was accompanied by a decrease in half-life times to reach very low values; for instance, 4, 5 and 6 min for

cefoperazone, cephurexime and cephapirin, respectively. Heat treatments at high temperatures and long times (e.g., 120°C for 20 min) led to a further degradation of beta-lactam antibiotics with percentages close to 100% for cefoperazone and cefuroxime. In contrast, when milk was subjected to heat treatments at lower temperatures and times (e.g., 72°C for 15 sec), the degradation of beta- lactam in milk did not exceed 1% for the 10 antibiotics tested.

Table 2.4: Heat stability of antimicrobials after autoclaving at 121°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)

Hsieh et al., (2011) conducted a study aimed to evaluate the heat stability of 14 veterinary antibiotics under a short term heating scenario by characterization of their structural degradation and their relationship to resultant changes in antimicrobial activity. Mutagenicity was also examined in four representative antibiotics after 15 min heat treatments at two temperatures (100°C and 121°C). Differential heat stabilities of antibiotics between drug classes, between temperature levels and among the same class of drugs were discovered. Heat treatment resulted in the reduction of the main peak and the production of new peaks in certain antibiotics, contributing to minimum inhibitory concentration increases of 2 to 1024 fold. Ranking of heat stability by antibiotic classes at 121^0C was highest for sulfonamides, followed by lincomycin, colistin, tetracyclines and beta-lactams while at 100°C sulfonamides equaled lincomycin and was greater than colistin but variability was observed within different tetracyclines and beta-lactams. Furthermore, the markedly variable heat stabilities within the classes of tetracyclines and beta-lactam antibiotics highlighted the fact that heat stability within these two classes can be very different despite their

structural similarity; hence, it is not appropriate to predict heat stability simply by antibiotic class. Mutagenicity (Ames) tests on heated chlortetracycline (CTC) resulted in 2 to 6 fold revertant changes in *Salmonella typhimurium* TA98 and TA100.

Different methods for determination of antibiotic residues in milk

There are several methods for analysis of antibiotics in various biological and pharmaceutical matrices and these consist of screening methods and chromatographic techniques for detection of qualitative and quantitative levels of antibiotic residues Alkan et al., (2007). The screening is performed by microbiological, enzymatic and immunological methods and they are based on the susceptibility of bacteria to different antibiotics Syit (2011). Chromatography methods include Thin-Layer Chromatography (TLC), High Performance Liquid Chromatography (HPLC) and Gas Chromatography (GC). There are also various chemical methods used like Radioimmunoassay and Electrophoresis (Ramirez et al., 2003). Efficient control of residues requires good screening tests, which must be less expensive, less time consuming than the more specific quantitative or confirmatory method, easy to perform, allow simultaneous analysis of large numbers of samples and give rapid results Cháfer-Pericás et al., (2010). The microbiological assay is easy, fast, simple and cheapest method (Muriuki et al., 2001; Abbasi et al., 2011). They may detect the presence of an antibiotic residue or a class of antibiotics and usually allow high sample throughput Alkan et al., (2007). Screening methods have the capability for a high samples throughput and are used to sift large numbers of samples for suspect or potential non-compliant results. They are specifically designed to avoid false compliant results (Okerman et al., 2004), thus, the number of so- called false-negative results of a screening test should be as low as possible, while a few false-positive results can be accepted as long as all positive results of the screening test are confirmed with chromatographic method. Microbiological assays for detection of antibiotic residues utilizes bacteria such as *Bacillus stearothermophilus* and *Bacillus subtilis* because of their high sensitivity to detect a wide range of antibiotics commonly used in animal disorders (Syit, 2011). With agar diffusion methods like the four-plate test, two different microorganisms *B. subtilis* and *Micrococcus luteus* are used as indicator microorganism.

The Enzyme Linked Immunosorbent Assay (ELISA) is a rapid test that can be used to detect the presence of specific antimicrobials in tissues (Mc Glinchey et al., 2008; Wang et al., 2006). The assay is performed by bringing cloned antibodies, either monoclonal or polyclonal, into contact with the analyte and adding an amount of radio-enzyme or fluorescent-labelled analyte, which competes with the non-labelled analyte for the available binding sites. The amount of labelled analyte bound is then determined directly or after the addition of a suitable substrate that is trans- formed into a selectively detectable product using an ELISA reader (Aerts et al., 1995). ELISA methods using both monoclonal and polyclonal antibodies have been used to screen milk, meat and eggs for chloramphenicol and sulphachlorpyridazine at low levels (Aerts et al., 1995; Spinks et al., 2006). Instead of the immunoassays and microbiological tests used previously, Thin Layer Chromatography and Ultra High Performance Liquid Chromatography are used commonly nowadays to detect antimicrobial residues in tissues of food producing animals (Cetinkaya et al., 2012; Tajick and Shohreh, 2006). Thin Layer Chromatography (TLC) uses a solid-stationary polar phase and a liquid- mobile phase. It involves spotting the sample to be analyzed near one end of the adsorbent solid phase placed in a covered developing jar containing a shallow layer of solvent. The solvent rises by capillary action up through the adsorbent and differential partitioning occurs between the components of the sample mixture dissolved in the solvent. The plate is removed from the developing chamber, dried, and the separated components of the sample are visualized straightforward or using ultra-violet (UV) lamp. It is used to support the identity of a compound in a mixture when the retardation factor (Rf = distance moved by analyte / distance moved by solvent front) of a test compound is compared with the Rf of a known compound; preferably both run on the same TLC plate (Wellesley Education, 2009). Grzelak et al., (2009) developed a simple thin-layer chromatography screening method for the determination of two cephalosporins (cefacetrile and cefuroxime) in milk. Only two developments of TLC plates with concentrating zones were required: pre-development with hexane, as a clean-up procedure to remove lipids from milk samples, and a proper development with methanol-toluene-ethyl acetate-98% formic acid (5:20:65:10) phase. The recoveries of both antibiotics in milk were calculated over five days from the preparation of the samples. The best results, obtained on the second day of the experiment, were 97.66% for cefacetrile and 86.13% for cefuroxime

High performance liquid chromatography technique

High performance liquid chromatography (HPLC) is a chromatographic technique used to quantify the concentration of antibiotics residues in different biological, pharmaceutical and food matrices. It has got different detection modes such as spectrometry, fluorescence, mass spectrometry, particle beam (PB), fast atom bombardment (FAB), atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) (Pena et al., 2007). Antibiotics residues such as penicillin G and oxytetracycline can be successfully detected and quantified in various biological matrices using HPLC in the reverse phase mode, with different detection modes, such as spectrometry, fluorescence and mass spectrometry (Abbasi et al., 2011; Bedada et al., 2012; Muriuki et al., 2001). HPLC usage is increasingly being used in the field of residue analysis. The variety of mobile phases, the extensive library of column packing and the variation in modes of operations are the reasons for this method to be in demand. In residue analysis of edible animal products, the samples often have much higher concentrations of endogenous interfering components but a very low content of residues. It is necessary to assess variety of producers for isolations, derivatization and quantitation of the compound of interest since the nature and concentration of these components can vary wider (Nollet, 1992). Sample deproteinization is the first step in animal originated food residue analysis. Mineral or organic acids like hydrochloric or trichloroacetic acid and/or water miscible organic solvents such as acetonitrile, acetone or methanol, which precipitate the proteins and allow their removal by centrifugation, are used frequently. Sample deproteinization helps releasing protein- bound residues besides protecting the HPLC column from irreversible contamination (Pena et al., 2007). In most conditions analyte extraction into a solvent is the second step where extraction efficiency is determined by the polarity of the extracting solvent, the pH of the sample/solvent system and the sample-to-solvent volume ratio. Extract cleanup process is usually involved as the third step in sample preparation. The easiest procedure is a simple liquid-liquid partitioning between two immiscible solvents, where the analyte is selectively partitioned in one of the two phases (Nollet, 1992). HPLC analysis of antimicrobial residues is mainly performed in either reverse phase mode or in the ion exchange mode. The efficiencies in the ion exchange mode are determined to be lower than those obtained by normal-or reverse-phase HPLC. In most conditions analyte extraction into a solvent is the second step where extraction efficiency is determined

by the polarity of the extracting solvent, the pH of the sample/solvent system and the sample-to-solvent volume ratio. Extract cleanup process is usually involved as the third step in sample preparation. The easiest procedure is a simple liquid-liquid partitioning between two immiscible solvents, where the analyte is selectively partitioned in one of the two phases (Nollet, 1992). HPLC analysis of antimicrobial residues is mainly performed in either reverse phase mode or in the ion exchange mode. The efficiencies in the ion exchange mode are determined to be lower than those obtained by normal-or reverse-phase HPLC. Usually excessive tailing due to the in-homogeneity of the absorbent surface is obtained. Many parameters can influence both the resolution of the compounds and column efficiency in reverse-phase HPLC. In order to obtain best results a combination of the appropriate stationary/mobile phase system and the mode of elution (isocratic or gradient) must be determined. Alkyl-bonded (C8, C18) stationary phases are used with mobile phases such as methanol or acetonitrile. The content of the organic modifier in the mobile phase is a function of both the polarity of the analyte and the type of column packing (Nollet, 1992). For residue analysis fluorescence detection has been proved to be valuable tool where interferences from food components must be reduced or eliminated. Fluorescent derivatives of many non-fluorescing solutes emerging from the chromatographic column can be prepared using specific fluorescence-labeling reactions. Comparing retention times is the key for identification of eluted compounds with reference compounds processed in an identical manner. Sometimes retention times are not enough by itself since a retention time can be observed for more than one compound or several components can be eluted at same retention time and chromatograph may show only one peak. Repeating the sample analysis on a different packing material can contribute to more satisfying results (Nollet, 1992). Furusawa (2000) developed a simple and rapid HPLC method for determination of residual penicillin G (benzyl penicillin, PCG) in milk. The sample preparation was performed by stirring with ethanol and reacting with 5 M 1, 2, 4-triazole mercury (II) chloride solution at 65 C for 10 min followed by an ultra-centrifugation step.

Control of antimicrobial residues in milk

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. Veterinary drugs are monitored for Maximum Residue Limits compliance. The directive establishes the groups of substances to be controlled for each food commodity. European Commission Decision 97/747/EC (Passantino, 2008) 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. In addition to the control programme, the load of antimicrobial residues would be reduced by different ways. More than 90% of excreted antimicrobial residues in livestock manures are degraded when proper composting was done and this is an important way to reduce the environmental loads of antimicrobial residues (Kim et al., 2011). To protect the public health against possible health risks caused by drug residues hazards, regulations regarding veterinary use of drugs including withholding periods after antibiotics therapy and tolerance levels have been formulated (WHO/FAO-CAC, 2012) and are strictly adhered in developed countries (Donoghue 2003; Lee et al., 2001)

It is expected that there might be a high prevalence of antimicrobial residues in milk of Bangladesh since very recently some regulations were placed at field level and, stakeholders are not yet used to the regulations. Moreover, antimicrobials are used in wide scale by veterinarians and producers to treat disease and improve animal production. Improper use of antimicrobials and absence of knowledge or avoiding the withdrawal periods might be the driving factor for the presence of antimicrobial residue in milk in Bangladesh perspective. Sometimes the farmers use antimicrobials in animals without consulting any veterinarian. All these factors might lead to higher concentration of antimicrobial residue in milk

Materials and Methods

The present study was conducted with the purpose to identify antibiotic residues in milk and access the perception of dairy farmers towards antibiotic residue in Chattogram, Bangladesh. This chapter includes a brief description of study area, various materials used and methodologies adopted to conduct this study.

Description of study area

This study was carried out at Chattogram city and Patiya Upazila of Chattogram, Bangladesh. Chattogram has a total area of 168.07 square kilometers. Due to great demand of milk for the city dwellers, a developed dairy farming and market is quite huge here. Patiya Upazila is considered as a major milk pocket nearby this city. In this study, commercial dairy farms were selected for collecting milk samples from Chattogram metropolitan area (CMA) and Patiya Upazila under Chattogram district.

Study period

The study was conducted during the period of January to June 2019.

Study design

A cross-sectional study was conducted to achieve the study objectives

Reference farms and populations

A list of commercial dairy farms in Chattogram metropolitan area (CMA) and Patiya Upazila, which had at least three dairy cows, was collected from the Department of Livestock Services. All the cows in selected farms of CMA and Patiya Upazila were the reference population. Processed milk samples of different brands were collected from different markets of CMA.

Target population

All the lactating cows in commercial farms of CMA and Patiya Upazila were the target population. The sample size was estimated using Epi-Tool software http://epitools.ausvet.com.au/.

Pretesting of data collection tool

Pretesting of questionnaires was done to test the clarity, sequence of questions and

estimate the duration of each questionnaire. A total of five respondents were interviewed and findings were used to improve the questionnaires. The revised version of the questionnaire was then translated into —Bengali, the mother tongue of the people

Data collection

A well structured and pre-prepared questionnaire was developed for data collection from commercial dairy farms to identify risk factors for the presence of antimicrobial residues in milk (Appendix-A). Sizes of the farms, farmer status, 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 farm attendant. 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.

Knowledge, attitudes and handling practices in usage of veterinary drugs

A simple random sampling technique was employed in which the dairy farmers willing to participate in the study were interviewed at the farm level. Semi-structured questionnaire was prepared to capture information on diseases status, most common antibiotics used, frequency of using veterinary drugs, common type of farming system practiced, knowledge and awareness of withdrawal period/time, means of access to veterinary drugs, the reasons for using antibiotics, awareness on the effects of antibiotic residues in human health, storage practices and condition for drugs.

Sampling

A total 300 milk samples were collected based on purposive sampling which included 200 raw milk samples and 100 processed packet milk samples. Raw milk samples possessed 150 individual cow milk and 50 pooled milk samples brought from different farms under CMA and Patiya Upazila. Whereas, 100 Processed packet samples of different brands purchased from various market places of CMA only.

Sample collection

To collect milk samples, the dairy farms from different zones of Chattogram city and Patiya Upazila were visited. Individual samples were collected from few purposively

selected cows of every dairy farm. Whereas, pooled samples were collected from the milk tank after milking the herd. About 20 ml raw milk was collected in a falcon tube from each animal. All samples were immediately carried to clinical pathology laboratory of CVASU through cool box containing ice cubes. The samples were stored in deep freezer at -20^0 C and were analyzed within 48 hours of collection.

Selection of antimicrobials

Five commonly used antimicrobials-Amoxicillin, Gentamicine, Ceftriaxone, Oxytetracycline and Streptomycin were selected for the present study and standards were prepared for comparison with the extracted samples. All the antibiotic standards were procured from Sigma Aldrich (Fluka and Vetranal), Co, USA and the purities ranged from 98-99 %. The standard operating procedures were followed for obtaining, labeling, storing and handling of antibiotic standards. Reference standards were initially stored in deep freezer under dry storage conditions and were brought to room temperature prior to weighing.

Experimental set-up

As per the mandate of this study, experimental work was accomplished in Clinical Pathology Laboratory and Poultry Research and Training Center (PRTC) of Chattogram Veterinary and Animal Sciences University (CVASU).

Analytical tests

All milk samples were tested by the TLC method for determining the presence of any residue against the five commonly used antibiotics of dairy farm in the study area - Gentamicin, Amoxicillin, Ceftriaxone, oxytetracycline and streptomycin. Then only two antimicrobials viz amoxicillin and oxytetracycline positive samples were examined through HPLC machine to measure the quantity of specific residue.

Thin Layer Chromatography (TLC)

Sample preparation: About 1 ml of milk was added with 1 ml of acetonitrile-methanol-deionized water at a ratio of 40:20:20 in a centrifuge tube. After mixing properly, the mixture was centrifuged at 3000 rpm at about 10 minutes. Then the supernatant was collected for performing TLC (Tyczkowska et al., 1989)

Preparation of standard:

Reference standards were initially stored in deep freezer under dry storage conditions and were brought to room temperature prior to weighing. Then reference standard working solutions were prepared for different antibiotics in a volumetric glass flask. Standards were prepared by dissolving 0.1 gm of standard in 2 ml of methanol. The stock solutions were diluted further by using same solvent to make working standard solution with different concentrations ranging from 0.02-5 ppm. The working standards solutions were stored in deep freezer at -18°C. Particular attention was given to compound toxicity and likelihood of exposure to the compound during handling.

Thin Layer Chromatography (TLC) technique:

Thin Layer Chromatography was run on silica plates (20 cm^2). Plates were cut into 8 equal parts. Then, a line was drawn with pencil 2 cm up from the bottom of the plate. For preparation of the mobile phase, 25 ml of acetone was combined with 25 ml of methanol (in a 1:1 ratio). A 2 µL of sample was spotted on the plate at the 2 cm line. The spot was then allowed to dry before placing it in the TLC chamber containing the mobile phase. Precautions were taken to make sure that the mobile phase fell below the 2 cm line on the TLC plate before running the TLC plate. After the TLC plates were placed in the chamber, the mobile phase was allowed to run until it was approximately 1 cm away from the end of the plate (approximately 45 minutes). Then, the plate was removed and allowed to dry. After drying, the plate was placed in a UV chamber (254 nm) to expose any compounds present in the sample. Bands from chemical compounds that showed up under UV light were marked. The Retention factor (Rf) value of each sample band was measured and compared to the Rf values of the prepared standards. The Rf value was calculated by measuring the distance traveled by the sample, and dividing it by the distance traveled by the solvent (Sattar et al., 2014). The distance that each spot had traveled from the start line was measured in 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:

RF = Distance moved by substances ÷Distance moved by solvent

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

High Performance Liquid Chromatography (HPLC)

In the present study, a confirmatory technique which is able to detect residues of different antibiotics at sub MRL levels has been developed using HPLC-DAD (High Performance Liquid Chromatography- Diode Array Detector). The developed method was validated for specificity, precision, recovery and linearity. The targeted antibiotics in the study included only oxytetracycline and amoxicillin. The extracted samples were centrifuged for 15 minutes at 3000rpm in eppendorf tube followed by filtration using 0.2nm MFS filters. The final extracted samples were set to run in the HPLC system. Determination of amoxicillin residues was 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).

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). Acitonitrile and other solvents were supplied by (Philipsburg, NJ, USA). Deionized water obtained from Mille- Q Plus analytical deionization system (Bedford, MA, USA).

Preparation of standard and test solutions

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

Test solution: Extract antibiotic solutions for thin layer chromatography were filtered through 0.2 M FS syringe filters (0.2 mm 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 PH 5.0.

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

Preparation of buffer solution: Dilute sodium hydroxide was added to 250 ml of 0.2 M potassium dihydrogen phosphate R up to pH 5.0 and diluted to 1000 ml

with waster R.

UHPLC Procedure: The Chromatographic procedure was carried out by the following ways:

1. A stainless Colum C 18 (2 mum) P/N 891 -5002, 2 mm ID* 100 mmL No.22G2C- 001 was used for chromatography.
2. Mobile phase was run at a flow rate of 0.2 ml/min
3. Spectrometer detector was set at 254 nm to measure the wave length.
4. Injection volume: 20 µl.

Determination of oxytetracycline residue

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

Preparation of standard and test solutions

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

Test solution: Extracted antibiotic solutions for thin layer chromatography were filtered through 0.2 M FS syringe filters (0.2 mm Advanced MFD. Inc., Japan).

Preparation of mobile phase:

Mobile phase A: It was a mixture of distilled water containing H2SO4 at PH 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 PH 5.0. Preparation of buffer solution Dilute sodium hydroxide was added to 250 ml of 0.2 M Potassium dihydrogen phosphate R up to P11 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 C l8 (2 mum) P/N 891-5002, 2 mm ID*10O mmL No.22G2C-001 was used for chromatography.
2. Mobile phase was run at a flow rate of 1.5 ml/min.
3. Spectrometer detector was set at 360 nm to measure the wave length.
4. Injection volume: 20 µl

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

Data analysis

Raw questionnaire and sample data exported to STATA-13 (Stata Corp, 4905, Lakeway Drive, College station, Texas 77845, USA). Prevalence of different antimicrobials in milk were calculated using TLC positive number of samples divided by the total number of samples tested and the results were expressed as percentage with 95% confidence interval (CI). Univariable significance test was conducted to observe the effect of different variables on the common perception regarding antimicrobial residue and resistance

Estimation of Hazard Quotient and risk assessment

Risk assessment based on the estimated and acceptable daily intake was performed to evaluate hazards associated with milk consumption and its public health significance. The mean level of antibiotic concentrations in raw milk was calculated. The data of the mean detected concentrations for the analyzed residues and average daily consumption based on 60 kg body weight were taken into account. Then, Estimated

Daily Intake of antibiotics was calculated. Risk analysis was done by using suitable models like Hazard Quotient. Numerically the hazard of antibiotic residues was assessed by calculating the Hazard Quotient as below.

$$\text{Hazard Quotient} = \frac{EDI}{ADI}$$

And, the estimated daily intake (EDI) was calculated by the following equation as given by (Juan et al 2010):

$$\text{EDI} = \frac{(\text{Mean of mg antibiotic per kg of food}) \times (\text{Daily Intake of food})}{\text{Adult body weight (60 kg)}}$$

Estimation of consumption for milk in Bangladesh was carried out based on surveys of the Department of Livestock Services. The per capita availability of milk in Bangladesh was estimated to be 165.07 ml/day (DLS 2018-19).

Ethical Consideration

Research permit was obtained from the ethical committee, Chattogram Veterinary and Animal Sciences University and verbal consent was obtained from each of the farm owner prior to commencement of interviews and sampling. Participation in the study was on voluntary basis. All the information collected from the participants and the laboratory results obtained by the analysis of milk samples were kept under the custody of the researcher as confidential.

Results

Study on perception of farmers of AMR and AMU

A questionnaire survey was conducted on 101 dairy farmers to understand their perception regarding AMR and AMU.

Table 4.1: Description of the study population

Variable	Category	Frequency	Percent (95% CI)
Area	Chattogram city	88	87.13 (79-93)
	Patiya	13	12.87 (7-21)
Farm size	0-25	25	25 (16.9-34.7)
	26-50	36	36 (26.6-46.2)
	50-190	39	39 (29.4-49.3)
Education of farmer	Primary to HSC	57	57.58 (47.2-67.5)
	Graduation and post-graduation	42	42.42 (32.5-52.8)
Advice on treatment	His/herself	5	4.95 (1.6-11.2)
	Local drug seller	3	2.97 (0.6-8.4)
	Another farmer	6	5.94 (2.2-12.5)
	Vet	87	86.14 (77.8-92.2)
Purpose of antibiotic use	Both (prevention & treatment)	18	17.82 (10.9-26.7)
	Treatment	83	82.18 (73.3-89.1)
Storage of antibiotic	Store room	90	89.11 (81.3-94.4)
	Shed	11	10.89 (5.6-18.7)

All types of farms- small, medium and large were visited through this study. Results showed that 42% of farm owner had a graduate degree which indicates more and more educated young people are involving with dairy farm industry in recent times. Most of the farmers of the study area follow the advice of veterinarians for the treatment of sick animal; however, few farmers depend on their own choice or on opinions of drug sellers. Farmers mainly store the drugs in a separate room which is

used for the workers accommodation and as a store room also and 11% farmers keep the drugs in the cattle shed (Table 4.1).

Table 4.2: Descriptive statistics of farmers perception regarding AMU and AMR

Questions	Category	Frequency	Percent (95% CI)
Ever heard about antibiotic?	Yes	101	100 (96.4-100)
	No	0	0 (00-3.6)
What is antibiotic?	Act against bacteria	91	90.10 (82.5-95.1)
	Act against virus	4	3.96 (1.1-9.8)
	Don't know	6	5.94 (2.2-12.5)
Heard about antimicrobial resistance?	Yes	88	87.13 (79-93)
	No	13	12.87 (7-21)
What is antimicrobial resistance?	Don't know	81	80.20 (71.1-87.5)
	Poor response to treatment	9	8.91 (4.2-16.2)
	Treatment failure	11	10.89 (5.6-18.7)
Heard about antibiotic residue?	Yes	89	88.12 (80.2-93.7)
	No	12	11.88 (6.3-19.8)
Do you keep record of drugs	Always	31	30.69 (211.9-40.7)
	Most frequently	1	0.99 (0.0-5.4)
	Sometimes	62	61.39 (51.2-70.9)
	Rarely	5	4.95 (1.6-11.2)
	Never	2	1.98 (0.2-7)
Use of antibiotic in last 1 year	Yes	101	100 (96.4-100)
	No	0	0 (00-3.6)
Do you add AB in homemade feed	Yes	0	0 (00-3.6)
	No	101	100 (96.4-100)
Prescriber mentioned the withdrawal period?	Yes	0	0 (00-3.6)
	No	101	100 (96.4-100)
Number of AB used at a time	Single	93	92.08 (85-96.5)
	Multiple	8	7.92 (3.5-15)
Course completed last time?	Yes	93	92.08 (85-96.5)
	No	8	7.92 (3.5-15)

Do you follow withdrawal period?	Yes	1	0.99 (0.0-5.4)
	No	100	99.09 (94.6-100)
Follow the prescription of vet?	Always	27	26.73 (18.4-36.5)
	Sometimes	74	73.27 (63.5-81.6)

Both farm owner and worker were found familiar with the term antibiotic residue and antimicrobial resistance (AMR) although they have little idea about what actually they are. Around 80% farmers didn't know about the effects of AMR on the treatment of their animal. Most of the farmers recognized the term antibiotic residue but only 30% of them always maintain the record of the drugs used in their farm. Almost all farmers acknowledged that their prescribers don't mention the withdrawal period in the prescription and 99% farmers don't follow the withdrawal period of the drugs after administration (Table 4.2).

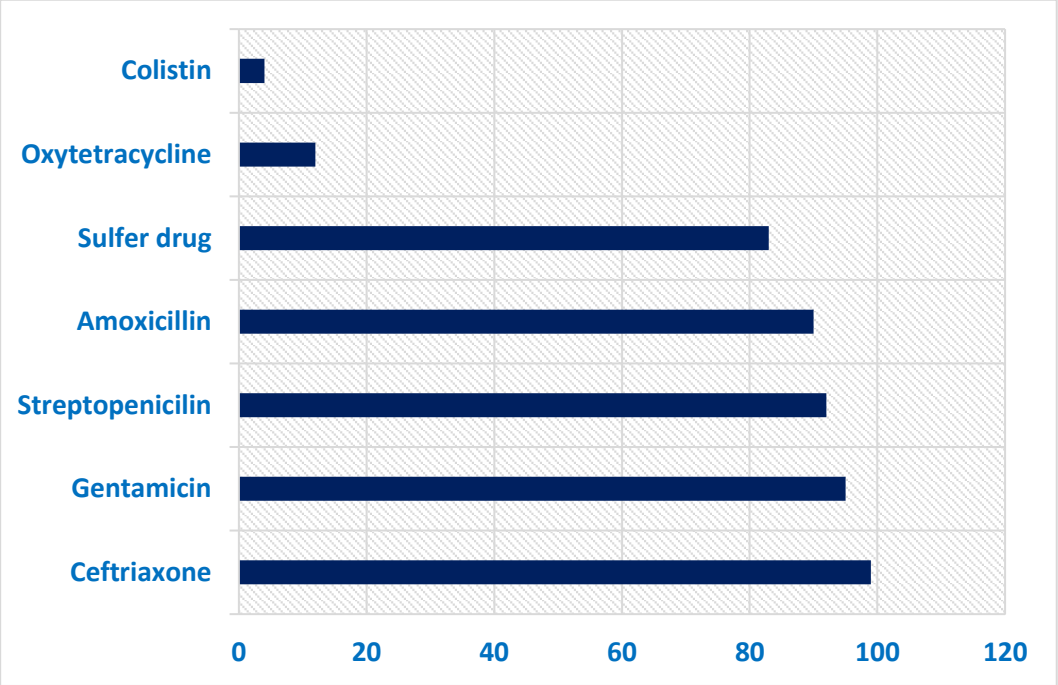

Fig 1: Different types of most commonly used antibiotics in dairy farms

The dairy cattle are mainly affected by mastitis, joint pain, diarrhea and the farmers used different types of antibiotics when required. In the study area, the most commonly used antibiotics were ceftriaxone, gentamicin, streptopenicilin, amoxicillin. Different types of sulfur drugs were also used in a good proportion. Oxytetracycline is still a common drug of dairy farming. Colistin isn't commonly

used as a single antibiotic but in combination with other antibiotics in dairy cows (Figure 1).

Table 4.3: Association of educational status of the farmer with the perception of AMR and AMU

Questions	Category	Primary to HSC (% shown in bracket)	Graduate and post-graduate (% shown in bracket)	P value
Ever heard about antibiotic?	Yes	50 (87.72)	39 (92.86)	0.68
	No	3 (5)	1 (2)	
What is antibiotic?	Act against bacteria	4 (7)	2 (5)	0.49
	Act against virus	49 (85.96)	38 (90.48)	
	Don't know	8 (14)	4 (9)	
Heard about antimicrobial resistance?	Yes	50 (87.72)	39 (92.86)	0.68
	No	3 (5)	1 (2)	
What is antimicrobial resistance?	Don't know	51 (89)	28 (67)	0.01
	Poor response to treatment	3 (5)	6 (14)	
	Treatment failure	3 (5)	8 (19)	
Heard about antibiotic residue?	Yes	50 (89)	38 (91)	0.66
	No	7 (12)	4 (10)	
Do you keep record of drugs	Always	17 (30)	14 (33)	0.56
	Most frequently	0	1 (2)	
	Sometimes	35 (61)	25 (60)	
	Rarely	3 (5)	2 (5)	
	Never	2 (4)	0	
Purpose of antibiotic use	Both (prevention & treatment)	12 (21)	6 (14)	0.38

	Treatment	45 (79)	36 (86)	
Use of antibiotic recommended by	Drug seller	6 (11)	2 (5)	0.32
	Another farmer	3 (5)	5 (12)	
	Own	8 (14)	3 (7)	
	Vet	40 (70)	32 (76)	
Number of AB used at a time	Single	51 (89)	40 (95)	0.29
	Multiple	6 (11)	2 (5)	
Course completed last time?	Yes	51 (89)	40 (95)	0.29
	No	6 (11)	2 (5)	
Do you follow withdrawal period?	Yes	0	1 (2)	0.24
	No	57 (100)	41 (98)	
Follow the prescription of vet?	Always	16 (28)	10 (24)	0.63
	Sometimes	41 (72)	32 (76)	

Univariable significance test was conducted to observe if educational status of the farmer had any effect on the common perception regarding antimicrobial residue and resistance. Except one, none of the association showed significant relationship. It was observed that more farmers (89%) having primary to HSC level education had no idea about 'what is antimicrobial resistance' compared to when the farmers had graduate or post graduate degree (67%) (P value 0.01). On the other hand, a smaller number of farmers depend on vet's opinion when they are less educated compared to more educated farmers (Table 4.3) however the association was not found significant.

Screening of milk samples for presence of antibiotic residues

In total 300 milk samples were screened for 5 different varieties of antibiotics named amoxicillin, oxytetracycline, streptomycin, ceftriaxon and gentamicin. The individual milk samples were collected from individual animals during milking at the dairy farms while the pooled milk samples were obtained from both the farms and the market level. The processed milk samples of different brands were purchased solely from different markets of Chattogram Metropolitan Area (CMA).

Prevalence of antimicrobial residues in milk

Among the milk samples, the prevalence of antimicrobial residues irrespective of antimicrobial types was highest (6%) in the individual milk samples than 4% found in pooled milk samples. The prevalence of antimicrobial residue was observed 0% in samples of processed packet milk collected from different markets. Results for TLC positive specific antibiotic residue in individual, pooled and processed milk samples is shown in (Table 4.4 and 4.5).

Table 4.4: Overall prevalence of antimicrobial residues in milk (descriptive analysis)

Categories of milk	No of samples tested	No of positive samples	% of positive samples (95% CI)
Pooled samples	50	2	4 (0.5-13.7)
Individual samples	150	9	6 (2.8-11.1)
Processed samples	100	0	0 (0.0-3.6)
Total	**300**	**11**	**3.6 (1.8-6.5)**

Table 4.5: Prevalence of different types of antimicrobial residues in different milk samples

Sample	Amoxicillin	Oxytetracycline	Streptomycin	Gentamicin	Ceftriaxone
Individual (n= 150)	3 (2%)	2 (1.33%)	2 (1.33%)	1 (0.66%)	1 (0.66%)
Pooled (n= 50)	0 (0%)	0 (0%)	0 (0%)	2 (4%)	0 (0%)
Processed (n= 100)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)

Table 4.6: Comparative prevalence of antimicrobial residues in two study areas

Location	sample	Total sample	positive	% of positive (95% CI)
Chattogram city	Individual	97	7	7.22 (3-14.3)
	Pooled	40	2	5 (0.6-16.9)
Patiya Upazila	Individual	53	2	3.77 (0.5-13)
	Pooled	10	0	0 (0.0-30.8)

In this study the samples were collected from 2 regions- Chattogram city and Patiya Upazila. The percentages of positive samples in individual milk were almost double in farms of Chattogram city (7.22%) compared to Patiya Upazila (3.77%). Two pooled sample were found positive in Chattogram city but none were in Patiya Upazila (Table: 4.6).

Different types of processed milk available in the markets of Chattogram city were tested against the studied antibiotics in this study. Processed milk samples included pasteurized, UHT, Mango, Chocolate and Strawberry milk. None of the processed milk samples were detected positive for antibiotic residue in the TLC method.

Method validation for oxytetracycline

The present study used a highly sensitive, accurate and reproducible HPLC method for the determination of oxytetracycline in milk samples (Fig. 2 & 3). Sample preparation was simpler and the recovery was better. The standard concentration was 520 µg/l with recovery time 6.087 minutes and the peak area was 38953708

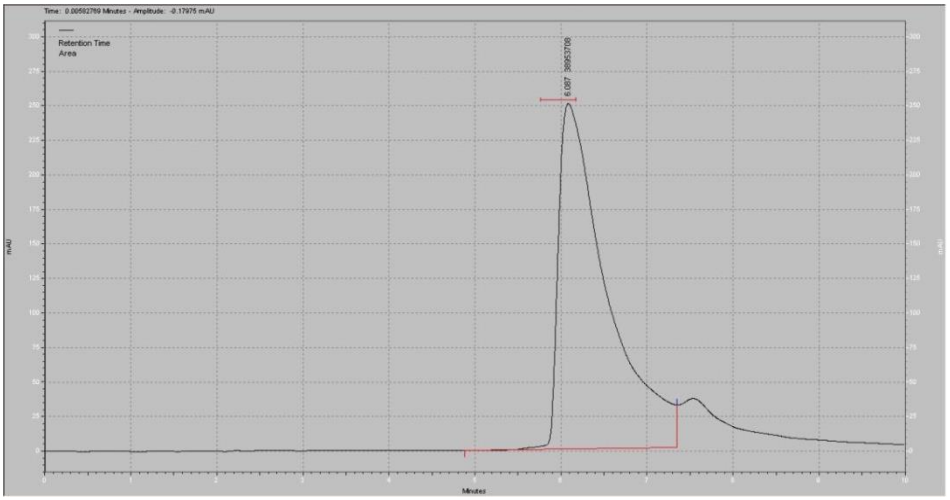

Fig. 2 (a) Chromatogram of oxytetracycline standard (520 µg/l)

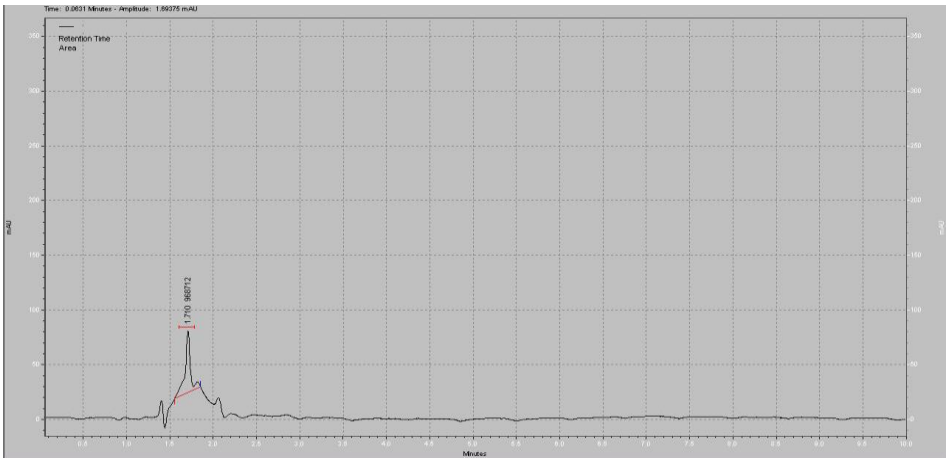

Fig. 2 (b) Chromatogram of blank milk sample

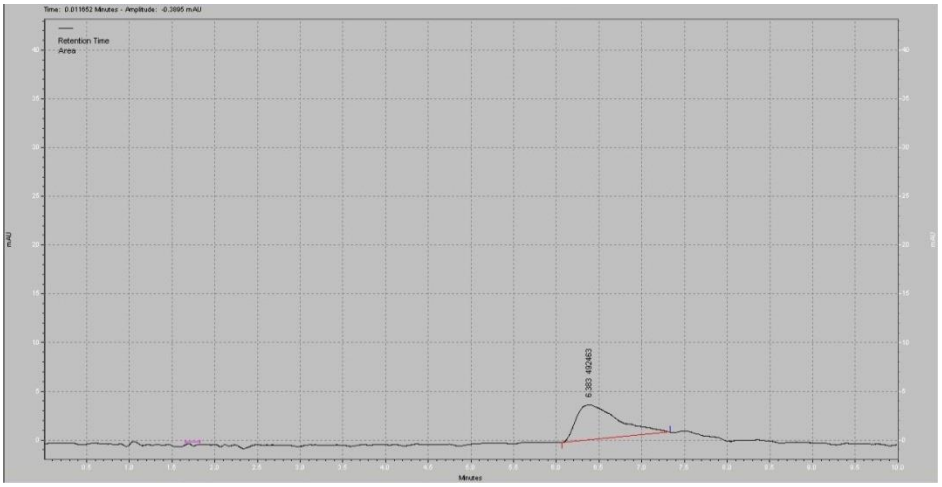

Fig. 2 (c) Chromatogram of oxytetracycline positive sample (conc. 6.57 µg/l)

Fig. 2: HPLC chromatograms for standardization of oxytetracycline (a,b,c)

The concentration of oxytetracycline residue in two positive individual milk samples were 116 and 6.57 µg/l, where one sample (116 µg/l) exceeded the maximum residue levels (MRLs) set by the European Union (EU) and the Codex Alimentarius Commission (CAC).

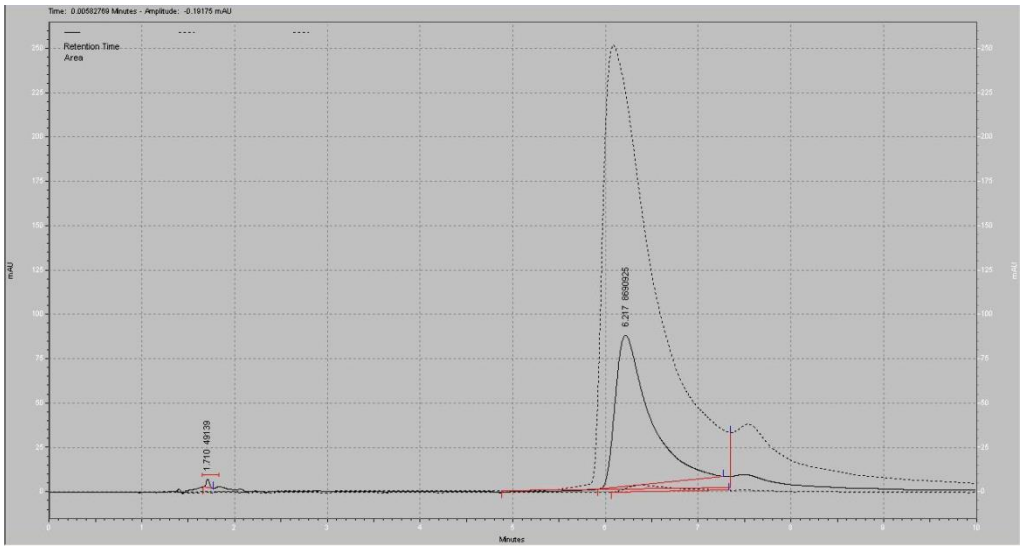

Fig 3: Chromatogram of oxytetracycline positive sample (conc. 116 µg/l) with standard

Method validation for amoxicillin

Similar to oxytetracycline determination the present study used a highly sensitive, accurate and reproducible HPLC method for the determination of Amoxicillin residue in milk samples (Fig. 4 & 5). The standard concentration was 200 µg/l with recovery time 3.7 minutes and the peak area was 2143200

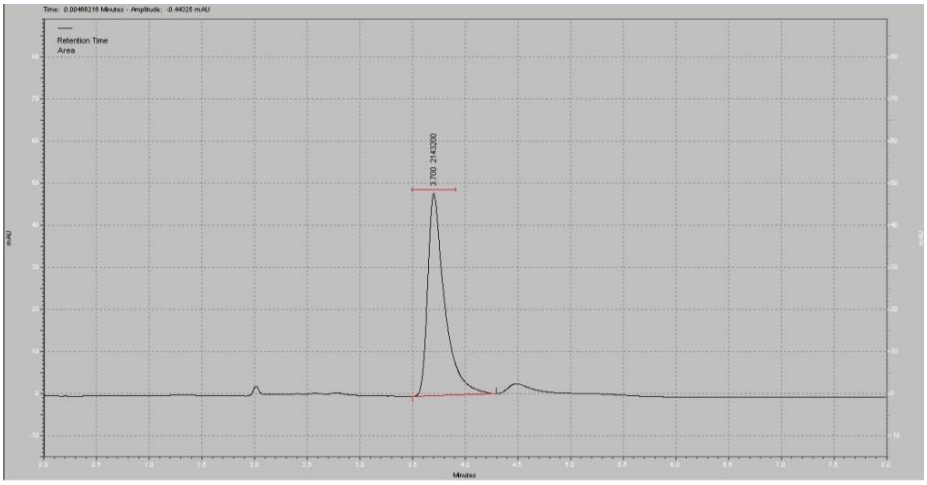

Fig 4 (a): Chromatogram of amoxicillin Standard (concentration 200 µg/l)

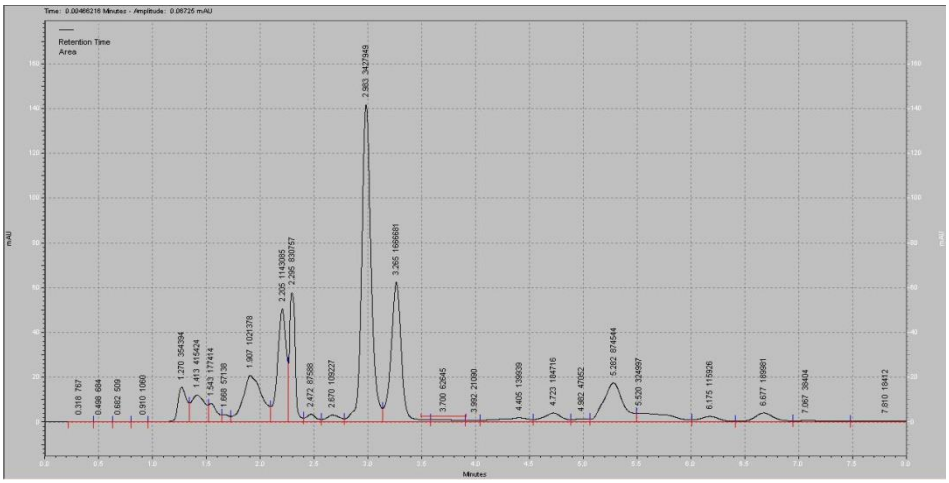

Fig 4 (b): Chromatogram of amoxicillin negative sample

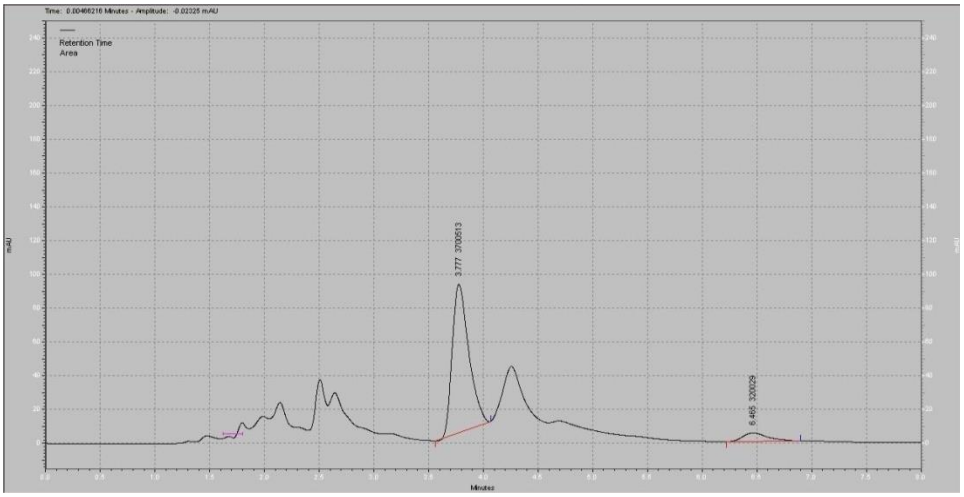

Fig 4 (c): Chromatogram of amoxicilin positive sample (conc. 345 µg/l)

Fig. 4: HPLC chromatograms for standardization of Amoxicillin (a,b,c)

The maximum concentration of amoxicillin in all positive milk samples were 345 µg/l and minimum were 5.85µg/l. All of the 3 positive samples exceeded the maximum residue levels (MRLs) set by the European Union (EU) and the Codex Alimentarius Commission (CAC).

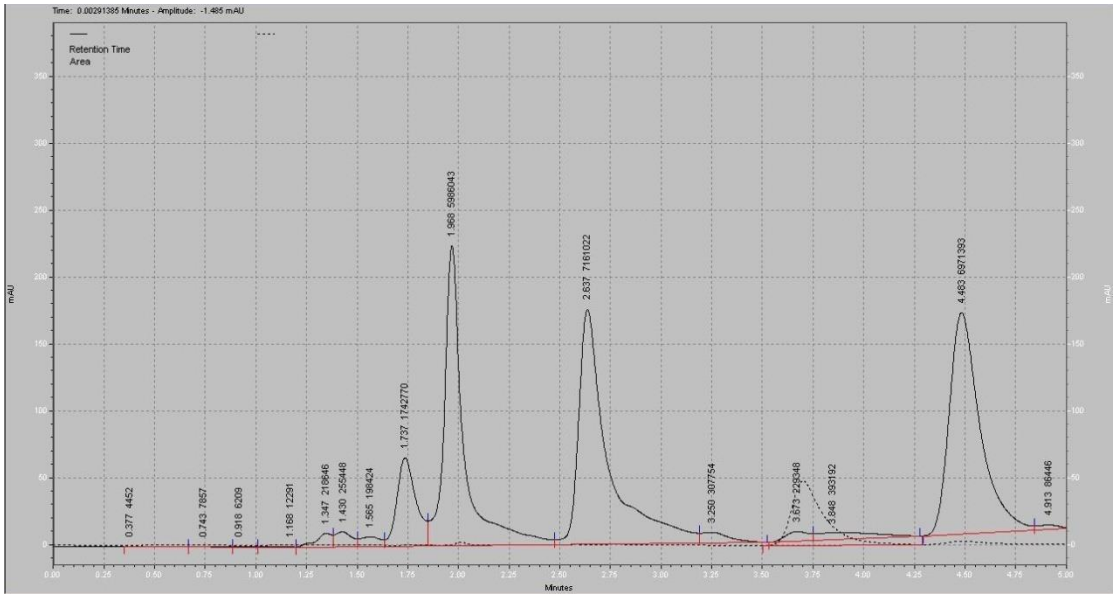

Fig 5: Amoxicillin positive Sample (conc. 21.4 µg/l) with standard

Concentrations of antimicrobial residues in raw milk

The mean concentrations of amoxicillin and oxytetracycline in raw milk were 124 µg/l, and 61.29 µg/l. Amoxicillin residue was in the highest concentrations in raw milk under study as stated in Table 4.7.

Table 4.7: Concentration of antimicrobial residues in individual milk samples

Antimicrobials	No of positive samples	Maximum conc. (µg/l)	Minimum conc. (µg/l)	Mean conc. (µg/l)
Oxytetracycline	2	116	6.57	61.29
Amoxicillin	3	345	5.85	124

Risk analysis in humans for intake of milk having residues

In the present study, the mean level of residues of oxytetracycline and amoxicillin in individual raw milk samples were found to be 61.29 and 124 µg/kg. Based on the mean values of antibiotic residues, the Hazard Quotient was evaluated for oxytetracycline and amoxicillin in individual raw milk samples having values 0.0056, and 0.0017 (Table 4.6).

Table 4.6: Estimation of risk assessment based on Hazard Quotient for raw milk (mean concentration)

Antibiotic	EDI (µg/kg/day)	ADI (µg/kg/day)	Hazard Quotient	Reference
Oxytetracycline	0.168	30	0.0056	OCS, 2013
Amoxicillin	0.341	200	0.0017	OCS, 2013

Discussion

Most studies on antibiotic resistance and residue in Bangladesh in animal originated food used qualitative methods, focusing only on residue detection or isolation of resistant bacteria. To our knowledge, this is the study that has targeted quantitative determination of antibiotic residue in raw and processed milk samples and approached the subject by exploring the knowledge levels as well as the attitudes and practices of livestock owners regarding antibiotic residue and resistance development. The present study observed a significant relationship between the knowledge of AMR and the educational status of the farmer. The result of the TLC analysis revealed a higher prevalence of the antimicrobial residue in individual milk samples than the pooled samples. The average concentrations of amoxicillin and OTC residue in raw milk samples were calculated 124 µg/l and 61.29 µg/l.

This study evidenced that in general farmers were aware of the term antibiotic residue and antibiotic resistance but the knowledge on the concept of AMR has a significant relation with the educational status of the farmer. Eltayb et al., (2012) also observed a significant relationship between awareness on antibiotic resistance and the level of education. Our study revealed that a remarkable percentage of farmers considered the use of antibiotics for both treatment and prevention purposes as like as the findings of (Eltayb et al., 2012). Aminoglycoside, cephalosporin, beta-lactams, oxytetracycline and sulphur drugs were the most commonly used antibiotic groups in this study area which coincide with the findings of (Syit, 2011) who found beta-lactams and OTC were imprudently used in commercial dairy farms for treatment purpose. A similar finding was also stated by (Brogden et al., 2003) where they found beta-lactams, tetracyclines, aminoglycosides, quinolones, macrolides and sulfonamides are used at commercial farm levels of dairy cows for preventive and treatment purposes in Bangladesh. Although the majority of the farmers in the study reported using antibiotics recommended by veterinarians in cows, it revealed that only a small proportion (26.73%) of them always properly follow the given prescription. Previous studies (Jones et al., 2015) stated 14% of farmers admitted to modifying the advised dosage. Very frequently the manipulation of prescription of the veterinarian occurred by dealer, drug seller or directly by the farmer. Almost all respondents of this study were not maintaining the withdrawal period as they recognize their prescribers didn't

mention it in their advice. This result is concordant to the findings reported from Nigeria (Adesokan et al., 2009) where 91.67% of the livestock owners were never advised by their veterinarians to follow the antibiotic withdrawal periods. So, it suffices to say that the poor veterinary profession also contributed to the poor knowledge and practices of these livestock owners on antibiotic use and resistance development. Therefore, it might be suggested that to reduce the prevalence of antibiotic residue, policy-makers should target these prescriber groups and institutions with the aim of proper guidance in responsible medicine use.

However, farmers are often a neglected group in most developing countries in the drive to combat antibiotic residues in animals. We revealed from this study that they might play a major role in the growing concern for antibiotic residue and resistance development in Bangladesh. Besides, public health extension education of livestock owners therefore, remains a vital tool in ensuring proper use of antibiotics in food animals as well as preventing development of resistance in both animals and humans. In addition, the authors recommend the enforcement of veterinary professional's ethics as well as controlled sales of veterinary drugs in order to safeguard antibiotic effectiveness in both animals and humans now and in the near future.

The present study estimated a higher prevalence of the antimicrobial residues in the individual milk samples compared to pooled samples. It could be due to the fact that pooled samples were mixed with the milk of other healthy cows of the farm so the concentrations of the residues were diluted at an undetected level. But in the individual samples, milk came from the diseased cows contained a high level of antibiotic residue which was easily detected by the methods used in this study. We detected oxytetracycline, amoxicillin, streptomycin, ceftriaxone and gentamicin residue in individual milk samples but in pooled samples, only gentamicin residue was identified. It might be due to the frequent use of gentamicin for treating mastitis in dairy cows in the study areas. Among the processed milk samples none of the collected samples were recognized as positive for the antibiotic residue. The finding is supported by (Fonesca et al., 2009; Movassagh and Karami, 2011) who found that market milk had a lower percentage of antimicrobial residues and it might be because that market milk was collected from different areas, standardized and pasteurized (heat-treated) simultaneously. The variation might be resulted due to application of different range of temperatures during pasteurization of milk in different plants and

also regional variation in terms of sickness of cow and use of antimicrobials. According to the present study the individual raw milk samples from dairy farms were found antimicrobial residues positive with amoxicillin, OTC, ceftriaxone, gentamicin and streptomycin in 2%, 1.33%, 0.66%, 0.66% and 1.33% respectively. The current percentages were lower than the findings of (Aydin et al., 1989; Khaskheli et al., 2008; Ardic and Durmaz, 2006), because of the effect of other factors like study area and period, sample designs, sample distribution, sample size, seasons etc.

In this study, the average concentration of amoxicillin residue in individual raw milk was 124 µg/l which was several times higher than the acceptable Maximum Residue Limit (MRL) of amoxicillin residue (4 µg/l) in milk (Passantino, 2008). The finding was higher than those of (Ghidini et al., 2002) who observed up to 53.7 µg/l amoxicillin residues in raw milk. Besides, the average level of OTC residue in individual raw milk samples was found 61.29 µg/l which was lower than the accepted MRL (l00 µg/1) prescribed by Passantino (2008). This concentration of OTC residue was also lower than the previous results of (Elizabeta et al., 2011; Kaya and Filazi, 2010; Syit, 2011) which were 149.4 µg/l, 150µg/l and 142 µg/l, respectively. The lower concentrations of OTC residue in milk of present study might be due to the decreased use of this antimicrobial in present time.

In this study the risk (Hazard Quotient) in humans for intake of milk having residues was calculated. However, no local literature was found available for comparing the calculated result. This study found the estimated daily intakes (EDI) was lower than the acceptable daily intake (ADI) for both amoxicillin and oxytetracycline residue similarly, Elizabeta et al., (2011) calculated the estimated daily intakes (EDI) for the average daily consumption of 200 ml of milk for an adult in Macedonia for the examined antimicrobials and obtained levels 2 to 100 times lower than the values of the acceptable daily intakes fixed by the World Health Organization. In a recent study conducted by (Vragovic et al., 2012) was reported the estimated dietary exposure based on the data on average consumption of milk and the estimated concentration of amoxicillin, ampicillin, benzylpenicillin, cloxacillin, cephapirin, cefazolin, cefoperazone and ceftiofur were not exceeding the relevant toxicological reference value (acceptable daily intake). Vragovic et al., (2011) also reported that the median value for streptomycin in milk was 11.50 µg/kg and the median value for tetracycline in milk was 1.50 µg/kg. Based on the median value, the

estimated daily intake of streptomycin and tetracycline through milk in Croatia was estimated to be low (streptomycin: 7.33 µg/person/day; tetracycline: 0.52 µg/person/day) and the risk assessed was negligible. Likewise, our result also indicated that toxicological risk associated with the consumption of analyzed milk could not be considered as a public health issue with regards to these veterinary drugs.

The study was conducted for six months only which was a very short period to reveal the comprehensive status of antimicrobial residue in milk and farmers' general perceptions. Present study has considered two antimicrobials for residue quantification because the time and resources were limited. It would be much better if we use all the antimicrobials used in treating dairy cows for screening and quantification of residues. To the best of our knowledge, it was the first report in Bangladesh of risk analysis in humans for the intake of milk having residue**s** and therefore no local literature was found to compare and discuss with the present scenario. The results of the risk analysis could be used as baseline data for future researches on this fact.

Conclusions

Results of data analysis showed the educational status of the farmers has a significant relationship with the knowledge of AMR. Respondents of this study were not habituated to maintaining the withdrawal period of any antimicrobials and they claim their prescribers for ignoring it during their advice. TLC based detection of antibiotic residues revealed the individual milk positive for oxytetracycline, amoxicillin, gentamicin, streptomycin and ceftriaxone however, in pooled milk sample only found the residue of gentamicin. No antibiotic residue was detected in processed packet milk samples. In individual sample, the mean residue level of amoxicillin was found 124 µg/l while the mean residue level of oxytetracycline was found 61.3 µg/l. Risk assessment was done which indicated that there was no risk associated with the consumption of milk of the study area. Thus, it can be concluded that sometime the raw milk of Chattogram may contain antibiotic residues above the MRL value but due to less per capita milk consumption, it doesn't consider as harmful for our health. Finally, the results of this study will contribute in creating awareness regarding milk quality and public health security to policymakers, scientists, veterinarians, farmers and consumers.

Recommendations

- Educating the farmers about the detrimental effect of antimicrobial residue for their better understanding and realization and encourage them to maintain the withdrawal period.

- Ensure the availability of veterinary services to the farmers to prevent the unnecessary use of antimicrobials during different stages of production.

- Regularly arrange the professional development modules for field veterinarians to train them with a proper guideline for the prudent use of antibiotic and antibiotic stewardship.

- Develop a fast screening method to test the milk for antibiotic residue before marketing.

- Establish the national policy and standard for proper maintaining the quality of milk

- Strong rules and regulations should be made to monitor the market of veterinary drugs. Specially antibiotics

- A countrywide more comprehensive study should be taken at the earliest basis to uncover the total scenario of this highly concerned issue.

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Appendix A:

Questionnaire survey form regarding antibiotic usage pattern and its residual knowledge of Farmer's

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A. General information (সাধারণ জ্ঞাতব্য)

1. Name of the farm (খামারের নাম) :.....

2. Name of the owner/caretaker (মালিক/তত্তাবধায়কের নাম):.....

3. Address of the farm (ঠিকানা):.....

Phone (ফোন):.....E-mail (ই-মেইল):.....

4. Type of farm (খামারের ধরন):.....

5. Total population (মোট সংখ্যা):.....

6. Age (Birds/Animals) (বয়স): Single Age Group (একক) ☐ Multiple Age Group (বহু) ☐

7. How many sheds in the farm (সেডের সংখ্যা):.....

8. Educational status of farm owner/caretaker (শিক্ষাগত যোগ্যতা) :

Illiterate (অশিক্ষিত) ☐	Primary (PSC) (প্রাথমিক/পিএসসি) ☐	Junior Secondary (JSC) (জুনিয়র মাধ্যমিক/জেএসসি) ☐
Secondary (SSC) (মাধ্যমিক/এসএসসি) ☐	HSC/Diploma (উচ্চমাধ্যমিক/ডিপ্লোমা) ☐	Graduate (স্নাতক) ☐

B. Use of Antibiotics in livestock and poultry (অ্যান্টিবায়োটিকের ব্যবহার)

1. From whom you usually get suggestions or treatment for your sick animals? (কার কাছ থেকে অসুস্থ্য প্রাণীর চিকিৎসা সমন্ধে মতামত পাওয়া যায়?)

a) Veterinary practitioner (ভেটেরিনারিয়ান) ☐

b) Veterinary paraprofessional (প্যারা ভেটেরিনারিয়ান) ☐

c) Local drug seller (দোকানদার) ☐

d) Family/Friend (পরিবার/বন্ধু) ☐

e) Other Farmers (অন্য খামারি) ☐

f) Do not go any where (কেউ না) ☐

g) Other's (অন্যান্য)**…….**

2. Have you ever heard about antibiotic? (এন্টিবায়োটিক সম্পর্কে কোন জ্ঞান?)

a) Yes (হ্যাঁ) ☐ b) No (না) ☐

3. What is antibiotic? (এন্টিবায়োটিক কি?)

Act against bacteria (ব্যাকটেরিয়ার বিরুদ্ধে কাজ করে) ☐

Act against virus (ভাইরাসের বিরুদ্ধে কাজ করে) ☐

Others (specify) (অন্যান্য).....

4. Have you ever heard about antimicrobial resistance? (এন্টি বায়োটিক প্রতিরোধী কথা জানা)

a) Yes (হ্যাঁ) ☐ b) No (না) ☐

5. What do you know about antibiotic resistance? (এন্টি বায়োটিক প্রতিরোধী সম্পর্কে কোন জ্ঞান?)

a) It causes treatment failure (চিকিৎসায় ব্যর্থতা) ☐

b) It causes poor response to treatment (চিকিৎসায় ধীর গতি) ☐

c) Do not know (জানি না) ☐

d) Others (অন্যান্য).....

6. Have you ever heard about antibiotic residue? (এন্টিবায়োটিক অবশিষ্ট সম্পর্কে কোন জ্ঞান)?

a) Yes (হ্যাঁ) ☐ b) No (না) ☐

7. Do you keep record of using any drug? (এন্টিবায়োটিক ব্যবহারের রেকর্ড রাখা)

a) Always (সর্বদা) ☐

b) Most Frequently (প্রায় সময়ই) ☐

c) Sometimes (কোন কোন সময়) ☐

d) Rarely (কদাচিত) ☐

e) Never (কখনো না) ☐

f) Do not know (জানি না) ☐

8. Any record of antibiotics used within past one year? (১ বছরের অ্যান্টিবায়োটিক ব্যবহারের কোন রেকর্ড আছে কিনা?)

a) Have (আছে) ☐ b) Don't have (নাই) ☐

9. Do you add antibiotics during self-feed processing (প্রস্তুতকৃত খাবারে এন্টি বায়োটিক মেশানো)

a) Yes (হ্যাঁ) ☐ b) No (না) ☐

10. Purpose of antibiotic use (এন্টিবায়োটিক ব্যবহারের উদ্দেশ্য)

a) Treatment (প্রতিরোধক) ☐ b) Prevenpion (প্রতিষেধক) ☐

c) Both (a+b) (উভয়) ☐ c) Growth promotor (বৃদ্ধি তরান্বিত) ☐

11. Regular use of antibiotics to prevent any specific disease (নিয়মিত এন্টিবায়োটিক ব্যবহার)

a) Yes (হ্যাঁ) ☐ b) No (না) ☐

12. Which are the specific diseases for livestock? (নির্দিষ্ট রোগ সমূহ)

a) Mastitls (দুধ জ্বর) ☐ b) Fever (জ্বর) ☐ c) Diarrhoea (ডায়রিয়া) ☐

d) Foot and mouth disease (ক্ষুরা রোগ) ☐ e) PPR (পিপিআর) ☐

f) Others (অন্যান্য) .....

13. Do any of your prescriber mentioned about withdrawal period?

(প্রেসক্রিপশনকারী এন্টিবোয়টিকের উইথড্রয়াল পিরিয়ড সমন্ধে লিখে কি না)

a) Yes (হ্যাঁ) ☐ b) No (না) ☐

14. Use of antibiotics recommended by (এন্টিবায়োটিক ব্যবহারের পরামর্শ)

Veterinarian (ভেটেরিনারিয়ান) ☐

Other farmers (অন্য খামারি) ☐

Shopkeeper (দোকানদার) ☐

Representative of pharmaceutical company (ঔষধ কোম্পানির প্রতিনিধি) ☐

15. Please mention five common antibiotics you frequently use in livestock

(৫টি কমন অ্যান্টিবায়োটিক এর নাম লিখুন যেটা প্রায় প্রাণীতে ব্যবহার করেন)

a) Penicillin b) Tetracycline c) Doxycycline d) Oxytetrecycline
e) Streptomycin f) Gentamycin g) Cephalexine h) Ceftriaxone
i) Ciprofloxacin j) Enrofloxacin k) Levofloxacin L) Sulphar drug
M) Amoxycillin N) Cefixime O) Lincomycin p) Azithromycin
q) Amikacin r) Others (অন্যান).....

16. Number of antibiotics use at a time (একই সময় অ্যান্টিবায়োটিক ব্যবহারের সংখ্যা)

a) Single (একক ভাবে) ☐ b) Combined/Multiple (একাধিক) ☐

c) Do not know (জানি না) ☐

18. Frequency of use of antibiotics in livestock/poultry (ব্যবহারের ফ্রিকোয়েন্সি)

a) Daily (দৈনিক) ☐ b) Weekly (সাপ্তাহিক) ☐

c) Monthly (মাসিক) ☐ d) When needed (যখন প্রযোজন) ☐

19. Route of administration of antibiotics for livestock/poultry (খাওয়ানোর পদ্ধতি)

a) Feed (খাদ্য) ☐ b) Water (পানি) ☐ c) Feed & Water (খাদ্য ও পানি) ☐

d) Injection (ইনজেকশন) ☐ e) Others (অন্যান্য) ☐

20. Antibiotic course completed last time in livestock/poultry (এন্টিবায়োটিক কোর্স সম্পন্ন)

a) Yes (হ্যাঁ) ☐ b) No (না) ☐

21. Withdrawal period follows (উইথ ড্রয়াল সময়ের অনুসরণ)

a) Yes (হ্যাঁ) ☐ b) No (না) ☐

22 Storage of drug (ঔষধ সংগ্রহ স্থল)

a) Store room (স্টোর রুম) ☐ b) Refrigerator (ফ্রিজ) ☐

c) Poultry shed (পোল্ট্রিসেড) ☐ d) Others (অন্যান্য) ☐

23. Any idea about self-life/Expiry date of Antibiotics? (অ্যান্টিবায়োটিকের তারিখ সম্পর্কে ধারণা)

a) Yes (হ্যাঁ) ☐ b) No (না) ☐

24. Antibiotics used by yourself or without taking prescription from any veterinarian?
(অ্যান্টিবায়োটিক ব্যবহার করেন নিজে বা ভেটেরিনারিয়ান-এর প্রেসক্রিপশন না নিয়ে?)

a) Yes (হ্যাঁ) ☐ b) No (না) ☐

25. Following the prescription of veterinarian during purchasing of exact prescribed antibiotic (সঠিক এন্টিবায়োটিক কেনার ক্ষেত্রে ভেটেরিনারিয়ান এর প্রেসক্রিপশন অনুসরন)

a) Always (সব সময়) ☐

b) Sometimes influenced by dealer of shopkeeper (ডিলার বা দোকানদার কর্তৃক পরিবর্তন) ☐

Brief bio-data of the Author

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