Screening of Fresh, Boiled and Pasteurized Milk for Aflatoxin-M<sub>1</sub> Contamination and Antibiotic Residues in Pakistan

Muhammad, Muhammad Rizwan, Shafqatullah and Nafees Bacha

Centre for Biotechnology and Microbiology, University of Swat, Pakistan
Centre of Biotechnology and Microbiology (COBAM), University of Peshawar, Pakistan
Mycotoxin Laboratory Pakistan Council of Scientific and Industrial Research (PCSIR), Peshawar, Pakistan

Abstract: In the present study Aflatoxin M<sub>1</sub> (AFM<sub>1</sub>) and various Antibiotic Residue (AbR) including oxytetracyclin, streptomycin, gentamicin, neomycin and penicillin were comparatively evaluated through Thin-Layer Chromatographic (TLC) technique in various types of milk samples i.e. fresh, boiled and pasteurized milk samples. AFM<sub>1</sub> contamination was recorded in 20% of the total fresh milk and 15% of the boiled milk. It was a point of concern that most of the contaminated samples were having AFM<sub>1</sub> level higher than permissible limit (0.05 µg/L) reported by European Union. However on the other side 5.71% of the samples (fresh, boiled and pasteurized milk) were found contaminated with antibiotic oxytetracycline, 4.28% with gentamicin and 2.85% with penicillin. In contrast streptomycin and neomycin residues were never detected in any of the selected samples. We further observed that all the pasteurized milk samples were free of both AFM<sub>1</sub> and AbR. Hence at this stage, we suggest that the pasteurized milk is free of health threats.

Key words: Milk - Aflatoxin M<sub>1</sub> (AFM<sub>1</sub>) - Antibiotic Residues (AbR)

INTRODUCTION

Pakistan is the fourth largest milk producing country in the world, where consumption of milk is higher than thirty seven thousands tons per year [1]. The livestock sector is important for the development of rural economy because more than 30 million individuals are dependent upon it [2]. Fresh milk has a nutritive value for health. Milk consist of proteins, saturated fats, vitamin C and calcium etc., therefore it is advised for the new born babies, where the secondary food is difficult to digest [3]. Beside this there are some problems associated with milk due to the presence of aflatoxins, pesticides and antibiotics residues.

Aflatoxins are the toxin produced by fungi in various forms including B1, G1, B2, G2 and M1. Looking into the toxicity level, several toxicologist have classified AFM<sub>1</sub> is the most toxic in importance for human. Due to carcinogenic effects of AFB<sub>1</sub>, International Agency of Research on Cancer classified it as a group-1 human carcinogen [4]. In human’s liver and other types of mammals, Aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) is converted into AFM<sub>1</sub> [5, 6]. The presence of AFM<sub>1</sub> can be detected in milk and its products within 12-24 h after the first exposure of AFB<sub>1</sub> [7]. During the process of sterilization, pasteurization and various dairy products preparation the AFM<sub>1</sub> is stable [6, 7]. AFM<sub>1</sub> is produced when animals feed on AFB<sub>1</sub> contaminated food, the gastrointestinal tract rapidly absorb it and convert it into metabolite AFM<sub>1</sub>, which appears in the blood of animal within 15 minutes and then secreted in milk through mammary glands [8, 9]. It has been demonstrated that, up to 6% of ingested AFB<sub>1</sub> is secreted in milk in the form of AFM<sub>1</sub> [10]. AFM<sub>1</sub> is considered to be carcinogenic and hepatotoxic for humans and various other species [11]. It has been estimated that due to contaminated foods, throughout the world in progressing countries, more than 500 million people have risk of chronic exposure to aflatoxins [12]. Due to aflatoxins the hepatocellular carcinoma is the primary disease (liver cancer, or HCC). According to WHO, globally hepatocellular carcinoma is the leading cause of cancer death [13]. Due to hepatocellular carcinoma each year 550,000-600,000 new cases occur, out
of which in Sub Saharan Africa and East Asia eighty three percent of deaths occur [14]. Since milk and its derivatives are consumed daily and, moreover, that they are of primary importance in the diet of children, most countries have set up maximum permissible levels of AFB in feed [15] and for AFM, in milk, which vary from the 0.05 µg/kg established by the European Union (EU), to the 0.5 µg/kg established by United States Food and Drug Administration (FDA) [16, 17]. Based on acceptable level, the intake of AFM, from milk has been computed between 0.0001 µg to 0.012 µg/ per day per person in Africa and Far East respectively. (Middle East: 0.0007 µg/person per day, Latin America: 0.0035 µg/person per day and Europe: 0.0068 µg/person per day) [18].

Another problem with consumable milk is the presence of antibiotic residue. Antibiotics are broadly used for the treatment of different diseases, e.g. bacterial infections, especially mastitis. Sometimes these antibiotics appear in milk as residues that could result in allergic reaction in humans, as well as increase bacterial resistance, result in many health problems [19, 20]. For the first time in late 1940s, shortly after the development of antibiotics, antibiotics were used for the treatment of infection in veterinary medicine [21]. For the treatment of bovine mastitis, antibiotics are used widely and its improper application may cause contamination of milk at farm level. Nowadays amino glycoside (streptomycin, neomycin, etc), tetracycline (oxytetracycline, etc) and beta lactam antibiotics (penicillin G, etc), antibiotics are widely use for antimicrobial purposes, e.g. for treatment of mastitis in dairy cows and consequently are most commonly type of residues found in milk [22]. ß-lactams, which had been approved for the treatment of mastitis by the food and drug administration, belongs to primary class of antibiotics. It is considered that the beta lactam group of antibiotics probably may cause 95% of milk antibiotic contamination and its residues may cause hygienic as well as industrial problems [23]. It is important to regulate antibiotic residues in milk and to fix the residues limits to minimum. Therefore, National monitoring programs are present in different countries like Turkey, while their primary purpose is to regulate and control the antibiotics residues in milk [24, 25]. Quantity of antibiotic residues in milk higher than maximum residues limits (MRLs) are illegal.

In the present work Thin Layer Chromatography (TLC) technique is used for detection of antibiotic residue and determination of AFM in various types of milk samples. This technique is a simple, cheap, easy, quite sensitive and specific method for the screening of contaminated milk [26, 27].

MATERIALS AND METHODS

Collection of Samples: The samples were collected from different dairy shops and dairy farms. A total of 140 milk samples including 80 fresh, 40 boiled and 20 pasteurized milk samples were examined. These samples were collected in sterilized plastic bags and refrigerated till inspected.

Extraction Procedure: The extraction procedure of AFM, from milk was followed by Horwitz [28]. As 50 mL milk sample was treated with 10 mL of 40% sodium chloride (NaCl) solution. The mixture was mixed with 120 mL extraction solvent chloroform in separated funnel and mixed well. Two layer of solvent was formed. The chloroform portion having AFM, was collected from passing it through anhydrous sodium sulphate to remove the water content from the layer. While antibiotic residues were extracted by the reported method of Tyczkowska et al. [29] in which 1 mL of each sample is subjected for analysis, 1 mL sample were mixed with extraction solvent mixture [acetonitrile, methanol, deionizer water (40:20:20)] and centrifuged for 10 minutes at 3000 rpm, to remove proteins. Supernatant was used for detection. The extracts evaporated at 45°C under gentle stream of nitrogen to dryness. The residues were mixed with 100 µL of spotting solvent (benzene 9 mL + acetonitril 1mL) for AFM, and with 1 mL methanol for antibiotic residues.

Spotting and Detection: Spotting of standard and samples on TLC plate were carried out by the help of analytic syringes on analytic TLC autospottor, each samples and standard spots loaded on Merc TLC plate with three different concentrations. The plates were developed and visualized under UV light of 365 nm for AFM, and 254 nm for antibiotic residues. The detection was carried out by comparing of sample spot with standard spot of same Reference front (Rf) value and was quantified by the following formula [28, 29].

Effective weight (E.W) = original volume or weight of test portion x filtrate volume/120

For calculation the concentration of AFM, in µg/kg I use the formula:

$$\mu g/kg = \frac{S \times Y \times V}{X \times W}$$

where S = µLAFM, standard equal to test solution spot; Y = concentration of AFM, standard µg/mL; V= µL of
Antibiotic Residues Screening: Another problem which also persists in milk samples is the occurrence of antibiotic residues which is broadly used to treat diseases or to prevent them. Sometimes these antibiotics produce residues in milk, due to which it provide allergic reaction as well as bacterial resistance in humans [33]. So, in this study we focused on the detection of those major antibiotic residues which are mostly used in the targeted areas i.e. oxytetracyclin, gentamicin, penicillin, streptomycin and neomycin.

The results revealed that out of 140 milk samples, a total of 18 (12.84%) contained antibiotic residues, out of them oxytetracyclin was 8 (5.71%), gentamicin was 6 (4.28%), penicillin was 4 (2.85%), while streptomycin and neomycin was not found in any analyzed samples (Table 1, Fig. 1). A similar study carried out by five Veterinary Control and Research Institutes in different cities of Turkey, that analyze 3084 milk samples and 377 (12%) out of these samples had detectable level of antibiotic residues [34].

The Contamination of raw milk was higher than boiled milk samples that were 14 (17.5%) and 4 (10%) consecutively, while the pasteurized milk samples were free from antibiotic residues contamination. The reasons for the low contamination of boiled milk and free of contamination of pasteurized milk could be that; these substances are tending to destroyed in thermal processing [35]. Oxytetracyclin was detected in 6 (7.5%) out of 80 raw milk, 2 (5%) out of 40 boiled milk. gentamicin was 6 (7.5%) out of 80 raw milk. penicillin was 2 (2.5%) out of 80 raw milk, 2 (5%) out of 40 boiled milk samples. Ceyhan and Bozkurt analyzed 200 milk samples (100 raw milk, 50 pasteurized milk and 50 UHT sterilized milk samples) collected around Ankara region, 11 (5.5%) of them were reported as penicillin positive [36].

### Table 1: Aflatoxin-M₁ concentrations in fresh milk, boiled milk and pasteurized milk determined by TLC

<table>
<thead>
<tr>
<th>Samples</th>
<th>No of samples</th>
<th>AFM₁ Containing sample N (%)</th>
<th>Concentration (µg/kg) AFM₁</th>
<th>Sample above 0.05 µg/kg AFM₁ N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh milk</td>
<td>80</td>
<td>16 (20)</td>
<td>0.43 1.50 0.90 0.32</td>
<td>16 (20)</td>
</tr>
<tr>
<td>Boiled milk</td>
<td>40</td>
<td>6 (15)</td>
<td>0.40 0.75 0.55 0.16</td>
<td>6 (15)</td>
</tr>
<tr>
<td>Pasteurize milk</td>
<td>20</td>
<td>0 (0)</td>
<td>UDL UDL 0.00 0.00</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Total</td>
<td>140</td>
<td>22 (15.71)</td>
<td>UDL 1.50 0.80 1.13</td>
<td>22 (15.71)</td>
</tr>
</tbody>
</table>

Min: Minimum; Max: Maximum; SD: Standard deviation; UDL: Under the detection limit.

### Table 2: Detection of antibiotic residues in fresh milk, boiled milk and pasteurized milk by TLC

<table>
<thead>
<tr>
<th>Samples</th>
<th>No of samples</th>
<th>Penicillin N (%)</th>
<th>Streptomycin N (%)</th>
<th>Gentamicin N (%)</th>
<th>Neomycin N (%)</th>
<th>Oxytetracycline N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh milk</td>
<td>80</td>
<td>2 (2.5)</td>
<td>0 (0)</td>
<td>6 (7.5)</td>
<td>0 (0)</td>
<td>6 (7.5)</td>
</tr>
<tr>
<td>Boiled milk</td>
<td>40</td>
<td>2 (5)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>2 (5)</td>
</tr>
<tr>
<td>Pasteurized milk</td>
<td>20</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Total</td>
<td>140</td>
<td>4 (2.85)</td>
<td>0 (0)</td>
<td>6 (4.28)</td>
<td>0 (0)</td>
<td>8 (5.71)</td>
</tr>
</tbody>
</table>
CONCLUSIONS

Milk is the main source of nutrition for humans especially for children, but its contamination with aflatoxins, antibiotics and pesticide residues would be assessed. Therefore we used TLC to screen contamination of AFM, and antibiotic residues in milk (raw, boiled and pasteurized). Our findings conclude that in total 15.71% of the milk samples were contaminated with AFM, while 12.84% of the samples were contaminated with various antibiotic residues. As in Pakistan majority of the population are relying on fresh, non pasteurized and un-packed milk, hence the government should adopt TLC method to screen such contaminations in milk and to prevent health complications due to these contaminants.

ACKNOWLEDGMENT

We are thankful to Arshad Husain (Head of Mycotoxin Laboratory, Pakistan Council of Scientific and Industrial Research Peshawar, Pakistan) for support and facilitation of our research work.

REFERENCES


