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Screening of Fresh, Boiled and Pasteurized Milk for Aflatoxin-M_1 Contamination and Antibiotic Residues in Pakistan

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Abstract: In the present study Aflatoxin M_1 (AFM_1) 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_1 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_1 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_1 and AbR. Hence at this stage, we suggest that the pasteurized milk is free of health threats.

Key words: Milk · Aflatoxin M_1 (AFM_1) · 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_1 is the most toxic in importance for human. Due to carcinogenic effects of AFB_1, 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_1 (AFB_1) is converted into AFM_1 [5, 6]. The presence of AFM_1 can be detected in milk and its products within 12-24 h after the first exposure of AFB_1 [7]. During the process of sterilization, pasteurization and various dairy products preparation the AFM_1 is stable [6, 7]. AFM_1 is produced when animals feed on AFB_1 contaminated food, the gastrointestinal tract rapidly absorb it and convert it into metabolite AFM_1, 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_1 is secreted in milk in the form of AFM_1 [10]. AFM_1 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

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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_1 in feed [15] and for AFM_1 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_1 from milk has been computed between 0.0001µg/ to 0.012µg/ 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]. â-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_1 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_1 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_1 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_1 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 Merck TLC plate with three different concentrations. The plates were developed and visualized under UV light of 365 nm for AFM_1 and 254 nm for antibiotic residues. The detection was carried out by comparing of sample spot with standard spot of same Reference front (R_f) value and was quantified by the following formula [28, 29].

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

For calculation the concentration of AFM_1 in µg/kg I use the formula:

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

where S = µLAFM_1 standard equal to test solution spot; Y = concentration of AFM_1 standard µg/mL; V= µL of final dilution of test solution; X=µL test solution spotted giving fluorescent intensity equal to S (M_1 standard); W= g test portion (effective weight) used in analysis.

RESULTS AND DISCUSSION

Aflatoxin-M_1 Screening: For screening of AFM_1 and antibiotic residues, a total of 140 milk samples were collected from different regions of Pakistan. These included 80 raw (fresh), 40 boiled and 20 pasteurized (tetra packed) milk samples. From the analysis we found that 22 (15.71%) of the total 140 milk samples were contaminated with AFM_1. The AFM $_1$ contaminated samples include 16 (20%) out of 80 raw milk, 6 (15%) out of 40 boiled milk and 0 (0%) out of 20 pasteurized milk (Table 1, Fig. 1). From the quantification of AFM_1 the detection limits were between 0.43 ppb and 1.50 ppb in raw milk, whereas, in boiled milk samples the AFM_1 contamination comprised between 0.40 ppb and 0.75ppb. In addition it was found that on the basis of AFM_1 analysis the pasteurized milk samples were free from AFM_1 contamination. The quantity of detection in contaminated milk samples was higher than the permissible limits reported by European Union i.e. 0.05 µg/kg (i.e. 0.05 ppb). In October 2003 a study was carried out for 20 days in Lombary (northern Italy), in which 2,061 farms and 808 dairy factories milk samples were analyzed for AFM_1 and found that 33% of them was above 0.05 µg/kg (i0.05 ppb) [30]. Bakirci found AFM_1 in 87.77% of milk samples in Turkey, 35 (38.89%) of the positive samples were found higher than the maximum limits [31]. While in our study 22 (15.71%) of the detected milk sample was higher than the maximum limits. Galvano *et al*. reported that the level of AFM_1 in milk is significantly affected by seasonal changes, developmental level of countries and geographical region, etc. [32].

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 oxytetrcyclin 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 2, Fig. 2). 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 ree 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-M_1 concentrations in fresh milk, boiled milk and pasteurized milk determined by TLC

Samples	No of samples	AFM1 Containing sample N (%)	Concentration (µg/kg) AFM1 Min	Max	Mean	SD	Sample above 0.05 (µg/kg) AFM1 N (%)
Fresh milk	80	16 (20)	0.43	1.50	0.90	0.32	16 (20)
Boiled milk	40	6 (15)	0.40	0.75	0.55	0.16	6 (15)
Pasteurize milk	20	0 (0)	UDL	UDL	0.00	0.00	0 (0)
Total	140	22 (15.71)	UDL	1.50	0.80	1.13	22 (15.71)

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

Sampless	No of samples	Penicillin N (%)	Streptomycin N (%)	Gentamicin N (%)	Neomycin N (%)	Oxytetracycline N (%)
Fresh milk	80	2 (2.5)	0 (0)	6 (7.5)	0 (0)	6 (7.5)
Boiled milk	40	2 (5)	0 (0)	0 (0)	0 (0)	2 (5)
Pasteurized milk	20	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Total	140	4 (2.85)	0 (0)	6 (4.28)	0 (0)	8 (5.71)

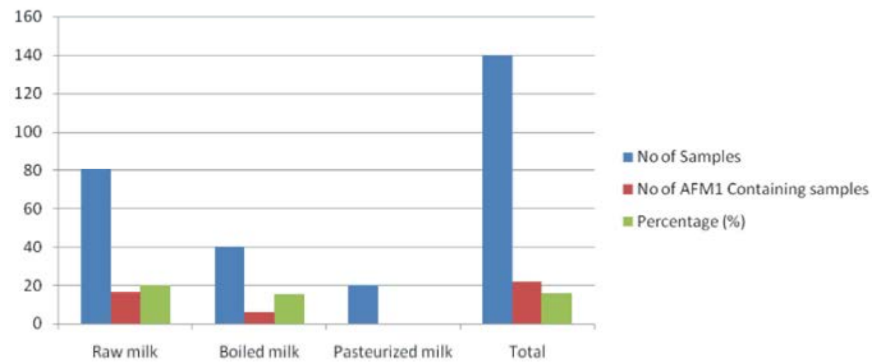

Fig. 1: Aflatoxin-M_1 concentrations in raw milk, boiled milk and pasteurized milk determined by TLC

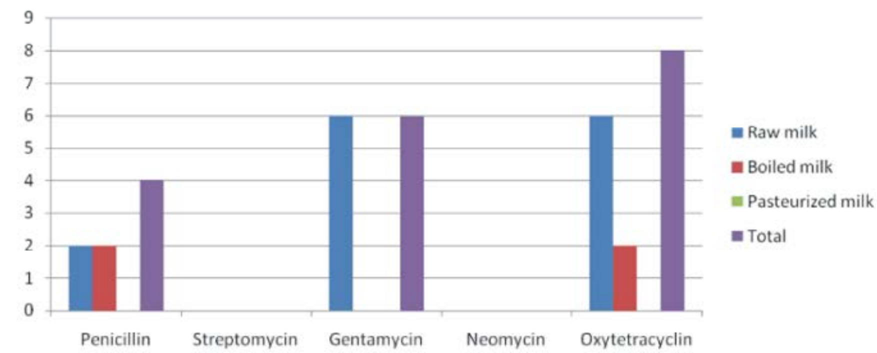

Fig. 2: Detection of antibiotic residues in raw milk, boiled milk and pasteurized milk by TLC

For analysis of AFM_1 and antibiotic residues TLC method was found to be simple, cheap, easier, quite sensitive and specific, which can be applied easily and adapted to laboratory condition.

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_1 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_1, 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.

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