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Chapter 2

A Decade of Aflatoxin M_1 Surveillance in Milk and Dairy Products in Developing Countries (2001-2011): A Review

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Additional information is available at the end of the chapter

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1. Introduction

Aflatoxin M_1 is the 4-hydroxylated metabolite of aflatoxin B_1 that can be found in animal and human breast milk and dairy products [1]. Aflatoxin M_1 presence in milk is considered as a potential risk for human health because of its carcinogenicity potential and thus a need of regular monitoring in milk and dairy products. Unpredicted climatic and environmental variations as well as poor economic and agriculture practices could easily influence the increase of AFM_1 in milk and dairy products. Aflatoxins are particularly known to be mainly produced in food and feed materials by *Aspergillus flavus* and *A. parasiticus* and at low level by *A. tamarii* and *A. nomius* as well as other emerging fungal *spp.* including *A. ochraceoroseus, A. rambellii, Emericella astellata* and *E. venezuelensis* [2, 3]). *Aspergillus flavus* and *A. parasiticus* (AF producers) mainly contaminate cereals (maize) and nuts (peanuts) and their by-products including animal feeds [4-6]. *Aspergillus* contamination is regarded as a storage problem [7] and may also contaminate plant on the field [4], especially during drought stress and low soil moisture content [3]. Aflatoxin M_1 (AFM_1) was initially classified by the International Agency for Research on Cancer (IARC) as a group 2B agent carcinogenic to humans [1] due to lack of data. However, following further investigations that demonstrated in vivo the genotoxicity and cytotoxicity of AFM_1 [8], the toxin has since been classified as a group 1 human carcinogen [9]. The importance of AFM_1 can be evaluated after considering the quantity of milk and milk products consumed daily; moreover they are of primary importance in infants' diet around the world [10].

The aim of this book chapter therefore was to review the incidence of AFM_1 in milk, dairy products as well as in human breast milk and in addition it was to look into advances of

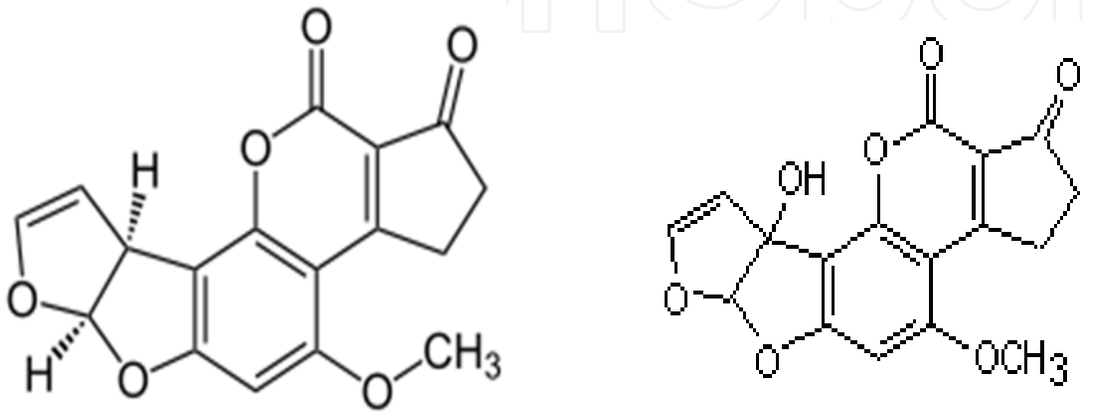
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techniques applied for extraction and detection of AFM_1 globally and developing countries in particular during the last decade (2001-2011) and to evaluate extraction and detection methods improvement.

2. Chemistry and metabolism of aflatoxin M_1

Aflatoxin M_1 (Figure 1) is the 4-hydroxy derivative of aflatoxin B_1 (Figure 1) which has a relative molecular mass of 328 Da and has the molecular formula $C_{17}H_{12}O_7$ [28].



Aflatoxin B1 Aflatoxin M1

Figure 1. Molecular structures of aflatoxin B1 and M1 (AFM_1)

[28] reviewed extensively on the biochemistry and metabolism of aflatoxins and AFM_1 in particular and it was shown that the metabolism of aflatoxin B_1 was more carcinogenic as compared to AFM_1. It has also been shown that human do not form as much aflatoxin B_1 8, 9-epoxide as rats, but suggested that human do not have glutathione-*S*-transferase (GST) isozymes which have high specific activity towards this epoxide. Significant individual differences in aflatoxin B_1 metabolism and binding suggest the presence of genetic and/or environmental factors that may result in large differences in susceptibility. In a study of the metabolism of aflatoxins M_1 and B_1 in vitro in human liver microsomes, they had a very limited capacity to catalyse epoxidation of aflatoxin M_1 [28]. It is important to mention that the metabolism pathway of AFs is of importance in the determination of toxicity degree [12]. Aflatoxin is metabolized by cytochrome p450 group of enzymes in the liver and converted into different metabolic products like aflatoxicol, aflatoxin Q1, aflatoxin P1, and AFM_1, depending on the genetic predisposition of the species [13]. Along with the above another metabolite called aflatoxin 8, 9 epoxide is also formed. The amount of this metabolite decides the species susceptibility as this can induce mutations by intercalating in to DNA, by forming adduct with guanine moiety in the DNA [14]. The ingested aflatoxin B_1 (AFB_1) through contaminated food and feed by mammals is metabolised to aflatoxin M_1 and excreted mainly into milk and dairy products. Aflatoxin M_1 is also detectable in urine, blood, and internal organs in addition to milk [15]. Aflatoxin B_1 is excreted into milk of lactating dairy cows primarily in the form of aflatoxin M_1 (AFM_1) with residues approximately equal to 1-3% of the dietary concentration [16]. The appearance of AFM1 in

milk have been determined to be within 15 minutes to an hour after consumption and returns to baseline levels within two to three days after removal from the diet [17, 11]. Studies of AFM_1 metabolism have shown that the rate between the amount of AFB_1 ingested by cows and the quantity excreted in milk is usually 0.2 to 4% [11, 18]. Studies have shown that it takes 3-6 days of constant daily ingestion of aflatoxin B_1 before steady-state excretion of AFM_1 in milk can be achieved, whereas AFM_1 becomes undetectable 2-4 days after withdrawal of animals from the contaminated diet [28]. Tolerable limits worldwide including South Africa on AFs vary between 10-20 ppb for AFB_1 and 0.05 ppb for AFM_1 in Europe and South Africa and 0.5 ppb in the United States (US) [19]. Studies done have also demonstrated that concentrations of 20 ppb of AFB_1 in the total mixed ration dry matter of lactating dairy cattle could result in AFM_1 levels in milk below the FDA set up limit of 0.5ppb. European Union and several other countries including South Africa have however, presently set up acceptable level of AFM_1 in milk and milk products at 0.05 ppb [11, 19]. Assumed safe feeding levels may result in milk concentrations above the limited level because absolute concentrations of mycotoxins in the feed are difficult to determine, concentrations may not be uniform throughout a lot of feed and concentrations can change over time [19]. Unfortunately most developing countries and mainly African ones have not yet set tolerable limits on food and feed contamination levels of AFs as well as AFM_1 in milk. This is mostly due to lack of data and information availability in these countries.

3. Stability of aflatoxin M1

Several studies have been done regarding AFs and particularly AFM_1 stability in milk and dairy sub products. Yousef et al [20] extensively reviewed information on the stability of AFM_1. Studies have shown that there was no significant changes of AFM_1 concentration after heat processing (Pasteurisation or boiling) or Ultra-high temperature processing (UHT) technique [21, 11, 22]. The stability measurements on powder milk showed no significant trends for both short- and long-term stability studies [23]. In addition, studies done on AFM_1 concentration changes in cheese showed no significant change of concentration even after 3 months of storage [24]. However, Khoury et al. [25] investigated the binding ability of AFM_1 by Lactic acid bacteria (LAB) such as *Lactobacillus bulgaricus* and *Streptococcus thermophilus* and found that they were effective in reducing the extent of free AFM_1 content in liquid culture medium and during yogurt processing. Therefore, this is a first study showing the capacity which can be played by LAB in AFM_1 removal and could be used as a biological agent for AFM_1 reduction. It is important to mention that the stability of AFM_1 during processing and storage makes it dangerous.

4. Aflatoxins and Aflatoxin M1 contamination

Most of mycotoxins poisoning problems occur in developing countries and particularly in sub-Saharan African region where maize and groundnuts are the staple foods [28]. It is estimated that about 250.000 hepatocellular carcinoma related deaths occur annually in Africa and around the world [28]. Acute aflatoxicosis has been reported in countries such as: Taiwan, Uganda, Kenya and Thailand [29, 30]. Ciegler et al.[31] reported that between 1974

and 1975, there was a disease outbreak affecting humans, killing about 106 among the 397 registered cases caused by the consumption of badly moulded corn contaminated with aflatoxin (between 6.5 and 15.6ppm). In July 2004, over 100 people died in Kenya due to acute aflatoxicosis after eating maize contaminated with aflatoxins [32]. Human aflatoxicosis (acute hepatitis) in Kenya is problematic among 20 hospitalized, 20% mortality was associated with the consumption of maize highly contaminated with AF [33], while during 2004, 125 deaths were recorded from a total of 317 reported cases in an outbreak of acute aflatoxicosis associated with Reye's syndrome [34]. Although such mortalities are viewed as a direct consequence of mycotoxin poisoning, Sharma et al. [35] indicated that these mortalities might have resulted from a predisposition to infectious diseases resulting from immunosuppressive effects probably caused by mycotoxins. In Malaysia, exposure to aflatoxins contaminated foodstuff was strongly implicated in the death of 13 children in 1988 [36]. Contamination occurs through exposure to contaminated AFM_1 milk, milk products such as cheese, yoghurt because as said earlier, AFM_1 is mostly excreted in milk of lactating animals and women exposed to diets previously contaminated with aflatoxin B1 and B2 [26, 27]. Aflatoxin M1 can also be found in the organs, e.g., kidney, liver, and excreta of animals exposed to AFB_1 [11]. Aflatoxin B1 is excreted into milk of lactating dairy cows primarily in the form of AFM_1 with residues approximately equal to 1-3% of the dietary concentration [16]. It has been shown experimentally to present high hepatotoxic and mutagenic risk [11].

5. Health implications of AFM_1

Aflatoxins are classified as mutagen and carcinogen [37] and their exposure and exact effects on human and animals are difficult to determine with precisions due to lack of experimental data, in addition due to co-occurrence with other mycotoxins in food and feed. Aflatoxin M1 has been demonstrated to be cytotoxic on human hepatocytes in vitro and its acute toxicity in several species is similar to that of aflatoxin B1 and liver cancer has been related to dietary intake of aflatoxins [11, 38]. Aflatoxin M1 exhibits a high level of genotoxic activity and certainly represents a health risk because of its possible accumulation and linkage to DNA [39, 38]. Moreover, AFB1 contamination at higher levels has also been correlated with reduced birth weight and jaundice in neonates [40]. The capacity of biotransformation of carcinogens in infants is generally slower than that of adults, this result in a longer circulation time of the chemicals [41].

6. Update on aflatoxin M1 extraction, detection and quantification methods

Several methods of extraction and detection have been used or developed for detection of AFM_1 in milk dairy products during the past decade. It is however important that to consider the type of matrix (fresh, stored, pasteurised milk, liquid or powder milk, cheese) as this can affect the final results [42]. In addition, most of commercial kits or rapid tests are designed for specific matrix. This makes the extraction of mycotoxins and AFM_1 from different matrices a challenge and costly. The detection of AFM_1 in milk or milk products

remains a challenge because of the very low concentrations. Therefore there is a need of sensitive methods for extraction and detection. Among screening methods, the enzyme-linked immunosorbent assay (ELISA) has been the most used as a screening method for AFM_1 [43]. The method is invariably based on the competition enzyme-linked immunosorbent assay (competition ELISA). The ELISA microtiter plate is coated with a bound antibody against AFM_1 and the detecting reagent is a covalent complex of this mycotoxin and an enzyme, usually horseradish peroxidase or alkaline phosphatase. The reagent is mixed with a sample of the mycotoxin extract and the mixture is placed in the well. In the control well (absence of mycotoxin in the sample), the mycotoxin-enzyme conjugate can saturate the bound antibody, and addition of a chromogenic substrate results in the development of colour. In the test well, free mycotoxin molecules in the extract compete with the conjugate on the bound antibody [44]. The higher the concentration of mycotoxin, the less the conjugate can react with the bound antibody, leading to fainter colour development [44]. The competition ELISA approaches that of the LC-MS method [44]. However, ELISA has been noticed to be not fully reliable due to cross-reaction interferences, especially at concentrations lower than 0.05 μg/L [44]. Magliulo et al. [45] reported a more specific chemiluminescent assay reaching 0.001μg/L. Another new screening assay using a headspace sensor array obtains results comparable to those of ELISA [119]. While Goryacheva et al. [128] have developed Immunoaffinity pre-concentration combined with on-column visual detection for rapid screening of AFM_1 in milk. This method showed a 2% of false negative results. Kanungo et al. [46] also developed an ultra-sensitive sandwich for the detection of AFM_1 in milk. The assay involved the immobilization of rat monoclonal antibody of AFM_1 in 384 microtiter plate to capture AFM_1 antigen. The miniaturised assay (10μL) enabled ultra-trace analysis of AFM_1 in milk with much improved lower limit of detection at 0.005pg/mL [46]. Sensitive magnetic nanoparticles (MNPs) based ELISA has been also developed and coupled with micro plate ELISA for analysis in milk. The hybrid-assay, by coupling the 1 antibodies (Ab) immobilized MNPs column with microwell plate assay enabled simultaneous measurement of low (0.5 pg/mL) and high AFM_1 contamination (200 pg/mL). The MNPs-ELISA advantages were that it had a small column size, high capture efficiency and lower cost over other reported materials [46].

In addition, commercial portable devices such as Ridascreen ELISA kits (R-Biopharm Germany); ROSA aflatoxin M1 SL, Charm (Charm Sciences Inc. 2009); Lateral Flow Immunoassay (LFIA) or Gold-Colloid Based Immunoassay [47] have since been developed to speed up the detection of AFM_1. Typical lateral flow immunochromatography (LFIA) strip is composed of a loading pad where a sample of the extract is applied; a zone containing coloured particles (e.g., latex, gold) coated with a mouse monoclonal anti-mycotoxin antibody; a zone of nitrocellulose membrane that allows the migration of the particles together with the mycotoxin sample; a test line that contains immobilized mycotoxin; a positive control line that contains a secondary anti-mouse antibody, and an absorbent pad [44]. In principle the LFIA is that AFM_1 extract is applied and migrates along the strip and once the conjugate zone is reached, the mycotoxin binds the anti mycotoxin–particle complex. Free and mycotoxin-containing particles now migrate to the test line. The

immobilized mycotoxin captures only the free particles that form a visible coloured line, whereas mycotoxin-containing particles continue to migrate [44].

The presence of a mycotoxin in the sample at higher concentration than the cut-off point of the strip (saturation of the particles with mycotoxin) will fail to bind to the test line, and vice versa. Thus, the intensity of the colour in the test line is inversely proportional to the concentration of the mycotoxin. Upon reaching the positive control line, both free and mycotoxin-containing particles can bind the anti-mouse antibody, thus forming a strongly coloured line regardless of the presence or absence of mycotoxin. The sensitivity of LTF is very high, and is comparable to those of sophisticated methodologies such as LC-MS-MS and surface plasmon resonance (SPR) (see below). The use of fluorescent reagents can bring the LOD to 50–200 ppt, as has been shown with other toxins [49]. The highest sensitivity in the detection of a mycotoxin by LTF was 5 ppb of AFB2 in pig feed, using a commercial immunaffinity column for the purification and concentration of the extract [44].

It is important to mention that the use different of devices available on the market depend on the objectives intended to be achieved by the analysis. It may be screening, confirmatory or both. The most reported methods conventionally used for AFM_1 extraction and detection are liquid-liquid extraction, silica gel, SPE cartridges (C18) cartridges [50] or immunoaffinity column (IAC), [57]. The inconvenience of this method is excessive use of chlorinated solvent in liquid-liquid extraction [21]. recently multifunctional clean-up columns (MFC) have been successfully applied to the clean-up of aflatoxins B_1, B_2, G_1, and G_2 when analysed by LC-FLD. MFC entrap matrix materials of cereal extracts but let the analytes pass through. This is simple, quick, and more stable than IAC [54] will probably be used for AFM_1 in the future as there have been no reports on this. Reports have shown that the detection and quantification of AFM_1 can be done by separation using thin-layer chromatography (TLC) or high-performance liquid chromatography (HPLC) coupled with fluorescence detector [6, 55-57). The inconvenience of the TLC method is that it is challenging in the determination of mycotoxin concentrations with exactitude. In order to enhance AFM_1 fluorescence, pre or post derivatization with trifluoroacetic acid with detection limits (LOD) (0.01- 0.3 μg/L) or post column derivatization with pyridinium hydrobromide perbromide and lowered the detection limit to 0.001 μg/L on HPLC [50]. The inconvenience of these methods is that they are time consuming as samples are run singularly to ensure the validity of results. In addition, the derivatizing agents (tirfluoroacetic and pyridinium hydrobromide) are corrosive and therefore dangerous for the technician and have corrosive effect on the HPLC column which reduces its longevity.

There are reports on the use of mass spectrophotometer (MS) for AFM_1 detection and quantification [51, 52]. The advantage of this is the low sensitivity. Plattner et al. [52] and Kokkonen [53] also reported on the use of tandem MS (MS/ MS) for AFM_1 detection in dairy products. However, by their sample preparation procedures these two methods did not eliminate matrix effects and must use matrix standards for calibration. Blank matrixes free of the analytes are not easy to obtain, and their storage time is short. As a result, there would be considerable practical benefit from further work on sample preparation and methods for separation prior to MS. Patel et al. [21] reported on AFM_1 detection 1 in milk

and milk powder using an LC-MS/MS method and this contrasted the clean-up efficiencies of IAC and MFC. Dutton et al. [57] reported the use of IAC clean-up and HPLC coupled with a fluorometer detector coupled with a Coring cell (Kobra cell) for AFM_1 detection. The advantage of this method is the avoidance the derivatization procedure which is labour cost and health risky for the technician and corrosive for the column. It is important also to mention that data presented in this study cannot be compared because of the use of different extraction and analytical methods. The use of different analytical methods (TLC, HPLC, LC-MS, ELISA) also affected reported concentrations of aflatoxin M1 in milk and therefore may affect intake estimates; therefore there is a need for trained people to give correct estimations of intake or AFM_1 in milk for better evaluation of exposure and anticipation of health problems.

In addition to herein mentioned techniques, Stark [44] in his review on other simple techniques such as the identification of mycotoxins based on molecular techniques for AFs and other mycotoxins detections from fungal strains are being developed and experimented [44]. Bhatnagar et al. [58] mentioned also that the biosynthesis and regulations of AFs involved at least 25 genes. Shapira et al. [59]; Chen et al. [60] used Primers pertaining to sequences of afl-2, aflD, aflM and aflP, (apa-2, nor-2, ver-2, omt-2, respectively) to detect and identify aflatoxigenic strains of *A. flavus* and *A. parasiticus* among isolated colonies or in DNA extracts from food and feedstuff. Differential medium (ADM) as diagnostic medium is another method that enables the identification and enumeration of aflatoxigenic *Aspergillus* [61]. Non-expensive reagents, an autoclave and a simple 365 nm UV lamp [62] allowed the identification of aflatoxigenic fungi. This method can be used by any mycologist in developing countries where the acquisition of expensive equipment or the availability highly trained personnel remains a concern. In this method common media such as czapek, sabouraud dextrose or yeast extract sucrose (YES) can support the growth of *Aspergillus* [44]. Addition of methyl-b-cyclodextrin(Wacker, Munich) [63] or of a combination of methyl-b-cyclodextrin plus bile salts (0.6% Na-deoxycholate) [64] enhances the natural fluorescence of aflatoxins, allowing detection of aflatoxigenic colonies after 3 days [63] or 36 h [64] of incubation.

Rojas-Dura´n et al. [64] also noted that the detection of AFs by studying the fluorescence of fungal colonies remained a challenge because non-aflatoxigenic strains of *Aspergilli,* such as *A. parasiticus* and *A. niger* also fluoresce under UV and erroneous interpretation due to the presence of AFs could happen. Further diagnostic tests done at room temperature and which revealed a phosphorescence of AFs that lasts 0.5 s after switching off the UV light were needed to confirm the presence of AFs in AF-producer aspergilli [64] –aflatoxigenic. In addition to fluorescence and phosphorescence observed during the exposure of AFs to UV light, AFB1 and AFB2 are activated by 365 nm UV light, resulting in AFB2–8,9-oxide [44]. It was also observed that AFB2–8,9-oxide bound to DNA at the N7 position of guanine residues yields 8,9-dihydro-2-(N7-guanyl)-3-hydroxyaflatoxin B2 [44]. It is therefore important to mention that the structure of the AF-DNA photo adduct [65] is identical to the AF-DNA adduct that is formed in vivo from ingested AFB2 by cytochrome P450, mainly in the liver [66]. The formation of AF-DNA adducts is considered as the initiating step of AF-induced carcinogenesis while covalent DNA- and protein adducts are also responsible for

acute toxicity [44]. Munkvold et al. [68] exposed AFs contaminated kernel corns to 365 nm UV light and the observation of an intense blue-green fluorescence confirmed the presence of aflatoxin-containing kernel. In this method the presence of more than four fluorescent kernels in a 5-pound sample of corn (approximately 6,000 kernels) was indicative of AFs contamination of at least 20 pp [68}

The use of Polymerase Chain Reaction (PCR) for the detection of mycotoxigenic fungi has been applied recently to prevent contamination of crops by mycotoxigenic fungi [44]. Bhatnagaret al. [58] demonstrated also that at least 25 genes were involved in the biosynthesis of AFs and its regulation. To detect and identify aflatoxigenic strains of *A. flavus* and *A. parasiticus* among isolated colonies, or in DNA extracts from foodstuff and feedstuff primers pertaining to sequences of afl-2, aflD, aflM and aflP, (apa-2, nor-2, ver-2, omt-2, respectively) have been used[59; 60]. In this method, the amplification of genes involved in AF biosynthesis is done using the DNA of *Aspergilli* as template. The identification of AFs biosynthetic genes is confirmed by sequencing of the amplified fragments of the DNA [44]. It is however important to mention that the presence of the genes reflects only AFs productions potential by *Aspergilli* [44]. Several environmental factors such as temperature, humidity, composition of the growth medium, growth phase and age of the culture can influence AFs production. A recent application of reverse transcription polymerase chain reaction (RT–PCR) for the characterization of aflatoxigenic *Aspergilli*, relies on the presence of mRNAs pertaining to AF biosynthesis genes [44]. The RT–PCR has been also used for the confirmation of the presence of aflatoxigenic fungus and of AF biosynthetic enzymes [44]. Multiplex RT–PCR containing 4–5 primer pairs of various combinations of aflD, aflO, aflP, aflQ, aflR and aflS (aflJ) were used to detect toxigenic fungi [69]. The genes, their enzyme products have their functions in the AF biosynthetic pathway while non-aflatoxigenic strains lack one or some AF biosynthetic genes [59) and mRNA products [69].

Other methods used for mycotoxin detection and not yet applied for AFM_1 and will be the future in AFM_1 detection and quantification include Molecular Imprinting (MI) in which a pseudo receptor is formed after polymerization of the surrounding molecules and washout of the mycotoxin. The selective binding of other molecules of the same mycotoxin to the imprinted polymer relies on immuno-competition reactions which are enhanced as compared to binding to a non-imprinted polymer [70]. It is important to mention that frequent production of several mycotoxins by a single fungus or in the same sample [6], and the contamination of crops with several toxigenic fungi are some of the reasons for the development of the arrays [44]. The Arrays biosensors have been developed to perform the simultaneous assays of more than one mycotoxin [71]. Electronic Nose sensors have been developed to identify volatile biomarker compounds such nitrosamines emitted from grains. These volatile biomarkers are dependent also on the types of mycotoxin in the contaminated grains [44]. Solid State SsDNA Odor Sensors with ssDNA 22-mers of ssDNA containing a fluorescent chromophore and dried onto a solid support can interact non-covalently with volatiles resulting in fluorescence increase have been developed. Volatile precursors of AFB_2, ochratoxin A and DON [72] could be used to identify toxigenic fungi by such odour sensors.

Although all the above mentioned methods of evaluating AFs contamination and identification in corn or food are applicable, there is an absolute necessity that the identification and determination of AFs levels with portable kits, and by confirmation of their identity in the laboratory be carried out.

7. Review of a decade survey of AFM_1 in dairy milk

Due to AFs and AFM_1 health hazard potential, it is therefore important to monitor and regulate the level of AFM_1 in milk and milk products through control of animal feed quality for consumer's safety purposes [19]. Aflatoxin M_1 is a metabolite of AFB_1 that can occur in milk and milk products from animals consuming feed contaminated with AFB_1 [73]. Data from this review (Table 1) revealed that not much is done in regard to AFM_1 surveys in many developing countries and particularly in African countries. The list of countries presented in Table 1 is not exhaustive however does represent the trend in AFM_1 survey in developing countries. Numerous epidemiological studies have shown in some areas a correlation between high aflatoxin exposure and high incidence of hepatho carcinoma in several countries in Africa [74]. Data in Table 1 show that only few African countries apart from South Africa [57], Egypt [75] and Morocco [76] and Nigeria [77] were involved in the survey for AFM_1. However the following Middle East, Asian and Latin American countries are implementing controls of the toxin in both dairy and human milk Iran [78] and Kuwait [79] and Pakistan [80-83] India, Argentina [84] and Brazil [18, 85-87]

Data obtained clearly show that ELISA immunoassay technique has been the most used analytical method in the past decade to measure AFM_1. This has also been confirmed in a survey done in Italy on AFM_1 in dairy products by [88] The use of ELISA immunoassay can be justified by the fact that it is affordable, simple and easy, to use and does not need expensive equipment such as liquid chromatography. However, several researchers mostly from developed countries combined Imunoaffinity column and Liquid Chromatography for specificity and confirmation of results [18, 48, and 57].

Survey in developing countries showed high levels of AFM_1 contamination (> 0.05 μg/L) in many milk samples analysed as compared to data obtained from developed countries such as France [89], Italy [90], Portugal and Spain [91] in which the results were mostly low or with very few samples above the European Commission regulation of 0.05 μg/L. The explanation to this might be that, developed countries have imposed strict control on the quality of feed provided to animals which reduces chances for aflatoxin contaminations. Such regulations are not yet implemented or being implemented in developing countries. In addition, climatic conditions mostly tropical; hot and humid conditions favourable for aflatoxin producing fungi contamination in cereals [3] recorded in most developing countries could be the pivotal reason for AFM_1 contamination in milk. In addition, differences noticed between data shown in Table 1 could be also explained by the use of different extraction, and analysis (ELISA, TLC, HPLC, and LC-MS) techniques as said before which could affect significantly the level of mycotoxin detection.

Country	Sample size	Methods of detection	Positive (%)	ranges (μg/L)	References
Argentina	77	ELISA	18(23)	0.012-0.014	[84]
Brazil	107	IAC/HPLC	79 (73.8)	0.010-0.5	[39]
	36	IAC/HPLC	25 (69.4%)	0.001-0.2	[87]
	79	RP-18/HPLC	58(73.4)	0.015-0.5	[85]
	27	IAC/TLC/HPLC	16(59.3)	0.01-0.53	[86]
Croatia	61	ELISA	2(1.6)	0.011-0.058	[106]
Egypt	175	ELISA	86(49)	0.01-0.250	[75]
Libya	49	ELISA	35(71)	0.03–3.13	[114]
Italy	161	ELISA	128(78)	0.015–0.280	[22]
Iran	111	ELISA	85(77)	0.002-0.725	[93]
	126	ELISA	(80)	<0.05	[80]
India	225	ELISA	151(67.1)	0.059-0.515	[78]
Syria	87	ELISA	76(86)	0.028-1.064	[107]
	167	ELISA	81.4	0.007-0.47	[117]
Pakistan	225	ELISA	151(67.1)	0.0056-0.523	[78]
	74	ELISA	70(95)	0.020–0.690	[80]
	168	ELISA	168(100)	0.01–0.70	[81]
	232	ELISA	76 (32.7)	0.002-0.794	[82]
Thailand	40	IAC/ HPLC	15(37.5)	0.008-0.036	[83]
	240	IAC/ HPLC	240(100)	0.014–0.197	[108]
	123	ELISA	103(84)	0.003-0.5	[109]
Turkey	129	IAC/ HPLC	75(58.1)	0.025-0.543	[110]
	90	ELISA	63(63)	0.054-0.065	[111]
Portugal	598	IAC/HPLC	394 (65.8)	0.005-0.08	[91]
South Africa	90	ELISA/IAC/HPLC	85(94.5)	0.02-1.50	[57]
Lebanon	64	ELISA	26(40.62)	0.005-0.05[+]	[114
Morocco	54	IAC/HPLC	48(88.8)	0.001-0.117	[76]
Nigeria	101	AOAC/TLC	6(5.9)	0.2-0.40	[77]
Indonesia	113	ELISA	65(57.5)	0.005-0.025	[112]
Kuwait	309	ELISA	176(56.9)	0.004-0.083	[79]
Slovenia	60	ELISA	4(10)	0.051-0.223	[116]
South Korea	100	IAC/HPLC	48(48)	0.05–0.10	[48]
Sudan	44	AOAC/HPLC	42(95.45)	0.22-6.90	[113]

[+] Some samples were above 0.05μg/L

Table 1. Review of aflatoxin M1 survey in dairy products from different countries between 2001 and 2011

The investigation of AFM_1 contamination in milk according to climatic season variations (winter and summer) showed clear effect of seasons on the occurrence and concentration of AFM_1 in milk. Most of the studies showed that higher levels of AFM_1 are obtained in winter milks as compared to summer samples [22; 57, 92]. The reason being that during the

summer, animals are fed on pasture, grass, weeds and green fodder while during winter, due to shortage or unavailability of fresh green feed, animals are more on concentrate feeding based on corn, wheat, and cotton seeds which could harbour mycotoxins than the fresh fodder [92]. Moreover, green fodder and hay preserved as silage under inadequate storage conditions which is ideal for toxigenic fungi such as *Aspergillus* contamination and aflatoxins production may occurred under favourable conditions [5, 93-95]). There is also evidence that milk yield is lower in winter, which means that AFM_1 and other components become more concentrated [92].

A comparison study was also carried out between milk samples obtained from rural subsistence and commercial farm in South Africa [96]. Analysed samples revealed the incidence of contamination with AFM_1 of 86.0% in rural subsistence farms samples while in samples from commercial farms, the incidence of contamination was of 100%. The lower frequency of contamination of AFM_1 in rural milk samples, as compared to those from commercial farms was explained by the fact that in subsistence farming animals were not fed with commercial feed on daily basis but mainly with leftovers from harvest season found on poor pastures with very little or no supplementation [6] while in commercial farms animals were fed on concentrates and silage which were also contaminated with toxigenic fungi and aflatoxins. This explaining the presence of AFM_1 in all milk samples analysed [4, 57]. Hence, the low intake of AFs in rural subsistence farm animals as compared to commercial farm animals exposed on feed.

8. Update on Aflatoxin M1 in human breast milk

Human breast milk has nutritional and immunological beneficial components for children and may contain trace amounts of a wide range of contaminants including AFM_1 following maternal dietary exposures [97]. Maternal consumption of aflatoxin contaminated food such as grain products, milk and milk products, legumes, meat, fish, corn oil, cottonseed oil, dried fruits, and nuts during breastfeeding can result in the accumulation of aflatoxins and their metabolites in breast milk [20]. Approximately 95% of AFB1 metabolite is excreted in milk as AFM_1in breast milk [98]. Studies on possible effects of infant exposure to AFs and AFM_1 have revealed growth retardation in human children [98] and foetal growth retardation in some animals exposed to aflatoxins prenatally [98]. In addition, AFs have been detected in blood of pregnant women [100]. A review done by Weidenborner [101] (Table 2) revealed the presence of AFM_1 in breast milk of lactating women in several countries with contamination levels varying according to countries and type of food exposed to. In addition to this, other studies confirmed the presence of AFM_1 in milk of lactating women (Table 2) at varying concentrations with some samples being above accepted limit in the USA and Europe (25ppb) or Australia and Switzerland (10 ppb). Methods used in studies to determine AFM_1 contamination in human breast milk have been mainly ELISA and HPLC as observed in studies done with dairy milk. Similar to the situation of dairy milk, few developing countries and in particular African countries have done investigations regarding AFM_1 contamination in human breast milk. Such investigations would be indicative of human exposure to AFs through food.

Country	Sample size	Positive (%)	ranges (μg/L)	References
Sierra Leone	113	25(23)	0.003-336	[101]
	113	35 (30.9)	0.2-99	"
UAE	140	129(73.8)	≤0.0034	[118]
	64	10(6.4)	0.3-13	
	445	443(99.5)	0.002-3	[101]
Australia	73	13 (69.4%)	0.028-1.031	"
Thailand	11	5(73.4)	0.039-1.736	"
Egypt	10	2(20)	0.5-5	"
	120	66(55)	0.2-2.09	"
	388	138(35.6)	5.6-5.13	"
	443	245(55.3)	0.0042-0.889	[126]
	150	98(65.3)	0.01-0.05	[101]
Gambia	5	5(100)	≤0.0014	[127]
Sudan	99	13(78)	0.005-0.064	[101]
	94	51(54.3)	0.401 - 0.525	"
Sudan/Kenya/	800	12(77)	0.005-1.379	[121]
Ghana	231	1(80)	0.194	[101]
Italy	82	4(4.8)	0.007-0.140	[22]
	75	75(100)	0.006-0.229	[123]
Turkey	61	8 (13.1)	0.0051-0.0069	[122]
	63	63(70)	0.054-0.65	[111]
Iran	160	157(98)	0.0003-0.0267	[20]
	2022	(9)	0.0069	[125]
	80	(1.3)	0.0069	[120]
	132	13(6)	0.007-0.018	[124]

Table 2. Summary of aflatoxin M1 survey in women's breast milk from different countries

9. Worldwide Aflatoxins control and regulations

Tolerable limits worldwide including South Africa for AFs vary between 10-20ppb [19]. Studies done have demonstrated that a concentration of 20 ppb of AFB_1 in the total mixed ration dry matter of lactating dairy cattle will result in AFM_1 levels in milk below the FDA set up limit of 0.5ppb [19]. European Union and several other countries including South Africa have however, presently set up acceptable level of AFM_1 in milk and milk products at 0.05 ppb. It is estimated that safe feed and food is the one in which detection levels may result in mycotoxin concentrations in milk being above limited level because absolute concentrations of mycotoxins in these feed and food are difficult to determine and concentrations may not be uniform throughout a lot of feed and concentrations can change over time [19]. The application of these regulations requires the use of sophisticated, expensive scientific equipment, and highly trained professional personnel commonly not

found in developing countries where contamination of cereals by AFs producing fungi are mainly found and observed. To reduce crops contamination by these AFs producing fungi, the trend is the experimentation of molecular enabling detection of their mycotoxins producing potential [44]. In addition, recently researchers are proposing the use of strains of lactic acid bacteria to effectively remove AFB_1 and AFM_1 from contaminated liquid media and milk (102, 103]. In vitro binding ability of AFM_1 by *Lactobacillus bulgaricus* and *Streptococcus thermophilus* was investigated in PBS liquid medium and during yogurt making by Ayoub et al. [104].and obtained positive results with reduction of AFM_1.

Binders or sequestering agents added to feed have been another approach to reduce toxicity of mycotoxins by reducing reactivity of bound mycotoxins and reducing their intestinal absorption. Substances used as mycotoxin binders include indigestible adsorbent materials such as silicates, activated carbons, complex carbohydrates and others. Whitlow [67] extensively reviewed information on mycotoxins binders. The addition of mycotoxin binders to contaminated diets has been considered the most promising dietary approach to reduce effects of mycotoxins [105]. The use of binders offers an approach to salvaging feeds with low levels of mycotoxins and to protecting animals from the background levels of mycotoxins that, although low in concentration, routinely occur and may cause chronic disease problems and losses in performance [67]. Researchers have also noticed that there is no binder product that meets all the desirable characteristics, however, the potential currently exists for practical judicial use of mycotoxins binders for reducing mycotoxin exposure to animals [67]. Aflatoxin and some other mycotoxins which have chemical structure similar to aflatoxin such as sterigmatocystin, can bind to silicate. Silicates vary and bind to mycotoxins depending on the structure of mycotoxins. Chemically modification of silicates can increase binding to mycotoxins such as deoxynivalenol and zearalenone. Activated carbon (charcoal) has produced variable binding results most probably because of differences in physical properties of the test product [105]. Aflatoxin binding by activated charcoal has been variable, but mostly positive [67]. Complex indigestible carbohydrate polymers derived from [44] yeast cell walls are shown effective in binding aflatoxin and restoring performance to animals consuming multiple mycotoxins (generally *Fusarium* produced). Bacterial cell walls also have potential to bind mycotoxins, but limited research has been conducted. Inorganic polymers such as cholestyramine and polyvinylpyrrolidone also have binding potential [67].

There is an excellent potential for binders to help manage the mycotoxin problem. Various materials can bind mycotoxins in feed and thus reduce toxic exposure to consuming animals. No product currently meets all the characteristics for a desirable binder. Mycotoxin control measures may require many approaches [67]. Animals may also be supplemented with antioxidants and other beneficial substances. In addition, the enforcement of legislation against mycotoxins and public enlightenment on hazards and control of mycotoxins should be emphasized by government in developing countries to ensure the control of mycotoxins contamination in food and milk.

10. Conclusion

Aflatoxin M1 remains a mycotoxin that is yet to be investigated in most of developing countries including Africa. As long as conditions favourable for aflatoxin contamination in food and animal feed are present, AFM_1 in milk and milk products will continue to be an issue that needs constant monitoring because of the serious effects it could cause on human health, particularly children. Developing countries compared to developed nations need to develop and implement regulations and control systems that would regulate AFM_1 in milk and its products thus ensuring food quality and safety. The coming decade will definitively focus on development and application of new, quick and low cost technology for aflatoxin detection. This would be key to developing strategies that would improve prevention, promotion awareness with regards to fungi and aflatoxins contamination.

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