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Investigating Diagnostic Accuracy of Strip Kit (Rapid Test) for Detecting Aflatoxin M1 in Milk Based on the Concentration Obtained by ELISA and HPLC

Hanieh Amiri; MSc [1], Naser Morgan Azghadi; PhD [2], Azadeh Saki; PhD [3], Zeinab Rezaei; PhD [4], Ali Asghar Najafpoor; PhD *[5], Mahsa Khakpour; PhD [6]& Mansooreh Soltani; MSc[7]

[1] Student Research Committee, Department of Environmental Health Engineering, School of Health, Mashhad University of Medical Sciences, Mashhad, Iran; [2] Veterinary Head Office of Khorasan-Razavi Province, Iran Veterinary Organization, Mashhad, Iran; [3] Department of Biostatistics, School of Health, Mashhad University of Medical Sciences, Mashhad, Iran; [4] Center of Cheshme Noshan khorasan (Alis), University of Applied Science and Technology, Chanaran, Iran; [5] Social Determinants of Health Research Centre, Mashhad University of Medical Sciences, Mashhad, Iran; [6] Department of Food Hygiene and Aquaculture, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran; [7] Food Laboratory Qom University Medical Sciences, Qom, Iran.

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Corresponding author:
najafpooraa@mums.ac.ir
Social Determinants of Health Research Centre, Mashhad University of Medical Sciences, Mashhad, Iran.

Postal code:9178845334
Tel: +98 51 38552610

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ABSTRACT

Background: The use of easy, fast, and accurate methods can be an important help in evaluating milk quality. The purpose of this study was to investigate the accuracy of diagnostic strip kits and compare it with ELISA and HPLC as a reference diagnostic method, which as the fastest diagnostic tool can play an important role in providing results. **Methods:** According to the catalog provided by the manufacturer, the samples were analyzed by rapid diagnostic kit and ELISA kit. Finally, the positive samples were checked by HPLC. Validation was done by comparing the results obtained from three methods as well as the values provided by the manufacturer. The limit of detection (LOD) was set at 100 ppt (ng/kg) and 50 ppt for Strip Kit and ELISA, respectively, and the limit of quantification (LOQ) was 5 ppt for confirmatory HPLC. **Results:** A total of 68 samples were selected to measure aflatoxin M1 (AFM1). Based on the results, 10 samples (14.7%) were positive by ELISA and 7 samples (10.29%) were detected to be positive by strip kit, of which only 3 (4.4%) samples were positive by reference method (HPLC). Moreover, among the strip kit test results, no sample was found as false-negative compared to HPLC, and the results of spike solution test with different concentrations also confirmed the results. The sensitivity and specificity of strip kit were calculated 70% and 100%, respectively. **Conclusion:** The strip kit can be used as a cheap, fast, and with acceptable accuracy method based on HPLC results for on-site detection of AFM1 in milk with saving time and money while guaranteeing high analytical precision and accuracy.

Introduction

Among many factors causing food spoilage, fungal toxins are very important. Among these toxins, aflatoxins are an important group of fungal toxins (mycotoxins) that are found in agricultural products due to the growth of some species of Aspergillus, especially *Aspergillus flavus*, and *Aspergillus parasiticus* (Atabati *et al.*, 2020, Unusan, 2006, Zinedine *et al.*, 2007).

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Aflatoxins are known to be hepatotoxic, mutagenic, teratogenic, immunosuppressive, and neoplastic (Farhadi *et al.*, 2022, Mwanza *et al.*, 2015). The most common and toxic aflatoxins are B1 and B2 species, which are found in moldy food, grains, and fodder more than other types of aflatoxins. After entering the mammalian body, these mycotoxins are hydroxylated in the liver and converted to M1 and M2 metabolites, which are excreted as the predominant aflatoxin through milk and urine (Nguyen *et al.*, 2020, Salmani *et al.*, 2016). Milk is the most important food source for the human body, especially for children and infants. The relationship between animal feed and milk health and safety is very close and may be the main way for aflatoxins to enter the human body (Khosravi-Darani *et al.*, 2019, Nguyen *et al.*, 2020). Considering that pasteurization, sterilization, and processing of milk do not have much effect on the stability and reduction of aflatoxin M1 (AFM1) toxicity, this toxin endangers the health of consumers through the consumption of various dairy products (Fallah, 2010, Sani *et al.*, 2010, Shamloo Aghakhani *et al.*, 2012); therefore, the European Union has set the permissible amount of AFM1 in milk at 50 ng/l, which in Iran, the maximum permissible M1 in dairy products is 100 ng/l (Fallah, 2010, Karim *et al.*, 1998). Preventing the entry of B1 as a precursor of AFM1 into dairy feed seems to be a good solution to control it, but this control method currently seems very difficult and impractical. The most appropriate and practical solution is the measurement of the AFM1 values in milk and its products to prevent the distribution and consumption of dairy products contaminated with values higher than the allowable level of this toxin in the community. For this reason, a wide range of methods are now available for detecting AFM1 in milk and dairy products; however, achieving key analytical functions, such as sensitivity, accuracy, and reliability, and suitability to apply regulatory limits in the low range (ng/kg) is still quite challenging (Karim *et al.*, 1998, Sadighara *et al.*, 2023). The main method based on the immunological system is ELISA (Li *et al.*, 2009), but other methods are also based on electrochemical and optical principles such as chromatography (Manabe *et al.*, 1978, Yousefi *et al.*, 2022) and spectroscopy (Jaiswal *et al.*, 2018). In the meantime, high-performance liquid chromatography (HPLC) using fluorescent detectors are widely used to measure AFM1 (Liu *et al.*, 2016, Rezaei *et al.*, 2021, Shamloo *et al.*, 2015). However, there are significant drawbacks to this method, such as high cost, complexity of management, and sample preparation (Alimohammadi *et al.*, 2014, Mahdavi *et al.*, 2012, Rezaei *et al.*, 2022). Considering that simpler and more practical methods are needed to routinely monitor milk and its products, various enzyme-linked immunosorbent assays (ELISAs) have been developed for measuring AFM1. Strong analytical methods are needed to identify mycotoxins and to select the most appropriate method, the target molecule, chemical characteristics, complex matrix, test timing, and detection limits should be considered (Bellio *et al.*, 2016).

Recently, competitive direct ELISA (cdELISA) has become more common compared to competitive indirect ELISA (ciELISA), which is a time-consuming diagnostic method. However, ELISA method in terms of rapid and on-site detection have significant limitations such as long incubation time, multiple washing steps, and the complexity of the tools needed to complete their process (Anfossi *et al.*, 2008, Magliulo *et al.*, 2005). Therefore, the use of rapid test has received a lot of attention in the field of analysis in recent years. Among rapid methods, immunochromatographic methods, in which the basis of analyte detection is the reaction between antibody and antigen, can be mentioned. This method has received a lot of attention due to the lack of laboratory facilities, low costs, and easy operation; an immunostrip is used in rapid screening to detect molecular weight toxins. These diagnostic kits include reddish-gold nanoparticles that combine with antibodies and provide a visual detection signal through color change (Zhao *et al.*, 2016). However, although antibodies are highly specific and sensitive compounds with chemical

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functional groups similar to mycotoxins, as interfering factors, they can interfere with the detection process with antibodies (Salari *et al.*, 2020, Zheng *et al.*, 2005). This effect likely makes errors in rapid test results through diagnostic kits in measuring AFM1 and show their amount more or less than its actual value; therefore, it seems necessary to check the accuracy of the results of this method. Various studies have been conducted to measure AFM1 levels using various diagnostic methods such as HPLC with fluorescence or mass spectrometer (Solfrizzo *et al.*, 2011), ELISAs (Radoi *et al.*, 2008, Salari *et al.*, 2018), and colloidal gold immunoassays (Zhang *et al.*, 2013). According to previous studies and the reports presented in this field, there is no report on comparison of HPLC, ELISA, and Strip Kits methods in measuring the amount of AFM1 in milk for validating the Strip Kits rapid test. In 2012, Zhang *et al*. validated the construction of an immunochromatographic test at the level of China's PPT 500 limit by ELISA method, and in 27 collected milk samples, there was a 100% correlation between the results of the rapid test and the ELISA method (Zhang *et al.*, 2012). In another study conducted by Alberto Bellio *et al*. in 2016 in Italy entitled "Aflatoxin M1 in cow's milk: method validation for milk samples in northern Italy", a total of 1668 milk samples were analyzed and 36 milk samples by ELISA method were positive (2.2%), which was subsequently confirmed by HPLC (Bellio *et al.*, 2016). The use of ELISA and HPLC tests in series and consecutively allows the analysis of a large volume of samples. Therefore, it saves time and money, and at the same time guarantees high analytical accuracy. In the present study, ELISA was validated as a qualitative (screening) approach and HPLC as a quantitative (confirmatory) approach. By analyzing a number of milk samples, it was tried to find whether there is a difference in the determination of the number of AFM1-contaminated samples. Validation of the method was done by comparing the results obtained from three techniques and the values declared by the manufacturer. Therefore, the aim of this study was to compare and evaluate the efficiency of different analytical methods for measuring AFM1 in milk and to validate the rapid test strip method based on the results of HPLC and ELISA.

Materials and Methods

Materials and equipment

Rapid test kits for measuring AFM1 were provided by Rojan Azma Production Research Company, Tehran, Iran. Also, ELISA test kits were purchased from TECNA, Italy. Methanol, chloroform, and standard solution with a concentration of 10 ppb (laboratory grade) were prepared by Merck, Germany. In all stages of the experiment, double distilled water was used to dilute and prepare the required solutions during the process. In this study, HPLC (model 1260, made by US company AGILENT) with immunoaffinity column, ELISA Washer (BioTek® ELx 50), and ELISA Reader (®BioTek ELx808, USA) were used.

Validation of ELISA

ELISA performance and efficiency were evaluated. Analysis of specificity showed that the β error was ď5%, confirming that the test is able to discriminate the analyte. The different incubation temperatures had no significant effect on assay performance, indicating that the test is sufficiently rugged. Finally, sensitivity was 1.00 (95% confidence interval CI 0.91–1.00) (Bellio *et al.*, 2016).

Validation of HPLC

The HPLC method was in good agreement with the criteria stated in standard. The method was linear in the range of 0.75–25 ppb (pg/µl), corresponding to 0.006–0.2 ppb in matrix, indicating no interference by the food matrix and acceptable specificity. The tests to check repeatability and recovery were considered satisfactory according to internal requirements and parameters (Bellio *et al.*, 2016).

Collection and storage of samples

Sixty-eight samples of pasteurized milk were randomly collected from stores in the city and 20 ml of samples was transferred to test tubes and stored in a freezer at -20 °C. Then, 24 hours before

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the test, the samples were transferred from the freezer to the refrigerator to gradually melt at 2-8 °C.

Preparing Spike Samples of AFM1 in Milk

Stock solution of AFM1 with a concentration of 1 µg/ml was prepared from the standard of aflatoxin powder in which the solvent used was acetonitrile. In order to ensure that the concentration of the prepared solution is 1 ppm, its concentration was measured by spectrophotometry. Concentration was obtained according to the following formula:

$$C = (MW .1000. Amax) / \varepsilon \quad (1)$$

Where C represents the concentration, A is the absorption intensity read by the spectrophotometer, MW is the molecular weight, and ε is the solvent coefficient used for dilution. Then, the following stoichiometric equation was used to prepare different concentrations of spike solution:

$$C_1V_1=C_2V_2 \quad (2)$$

Where C and V represent the concentration and volume of stoke and spike solutions, respectively. For example, to make a concentration of 100 ppt, 0.25 ml of 10 ppb standard solution was taken and was brought to a volume of 25 ml using milk to prepare the spike solution of 100 ppt. In the same way, all concentrations of spike solution were calculated.

Kit Strip

Two drops of the thoroughly stirred milk sample were added to the micro-tube, and the lid was then closed and shaken gently occasionally for 5 minutes. Then, the contents of micro-tube were added to the kit (well S) and after 5 minutes, the presence of only one clear colored strip in region "C" indicates a definite positive result, which means that the level of AFM1 is more than 100 ppt in the sample. If a very faint colored strip appears in the T region, the result will be considered positive (it may be around 100 ppt). The presence of two clear colored strips (C and T) indicates a negative result and shows that the concentration of AFM1 does not exceed 100 ppt in the sample. If the strip is not visible in area C, the result will be considered invalid (**Figure 1**).

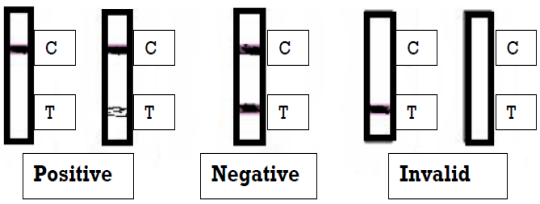

Figure1. Interpretation of strip kits results.

Determination of AFM1 in milk by ELISA method

The amount of 2 cc of milk stored at 2-8 °C was transferred to a glass tube. The desired sample was centrifuged for 10 minutes at 3000 rpm, then the fat phase was separated and 1 cc of the lower phase was transferred to a new tube, and 1 ml of distilled water was added to it and stirred. The required number of micro-plate wells (the number of standards in this kit is 7) was placed in the well holder; the wells were coated with AFM1-specific antibodies. In the next step, 100 µl of each standard solution and samples were added separately to the wells and mixed gently, and the samples were covered to prevent evaporation and kept at room temperature for 45 minutes. The material was then emptied and washed 5 times, 100 µl of enzyme conjugated solution was added to micro-wells and coated, giving 15 minutes to complete the process. After these steps, the wells were emptied again and rinsed for 5 times, and 100 µl of chromogen solution was added to each micro-wells and stirred gently, and the samples were covered for 15 minutes. In the last step, 50 µl of stopping solution was added to each of micro-wells and shaken, and the amount of light absorption at 450 nm was read by ELISA reader and the amount of aflatoxin was calculated with the help of software provided by the manufacturer of the kit.

Determination of AFM1 by HPLC

Sixty milliliter of sample was centrifuged and its fat was separated. The immunoaffinity column was then brought to room temperature and 10 ml of Phosphate Buffer Saline solution was poured into the tank attached to the column and allowed to pass through the column at a rate of 1 to 2 drops per second without external pressure. Then, 20 ml

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of skim milk was poured into the graduated cylinder and then passed through the immunoaffinity column. The graded cylinder containing the sample was washed twice with 10 ml of water, and each time, washing water was passed through the column. In the next step, 2500 µl of acetonitrile was passed through a column at a rate of 2-3 ml/min and collected in a vial, and then mixed with vortex. The vial contents were dried in a laboratory water bath at 40-50 °C and 1 ml of the mobile phase (70% water: 30% acetonitrile) was added to the vial and mixed for one minute by the vortex. The vial contents were mixed again with the vortex for 1 minute, and then, the column was washed with 20 ml of PBS. Finally, the standard AFM1 calibration solutions were injected into the injection device. After that, a suitable volume of sample was injected into the device and the resulting peaks were compared with standard peaks in terms of retention time. Contamination was determined and its amount was calculated using the calibration curve.

Data analysis

Kolmogorov-Smirnov test was used to test the normality hypothesis, which indicates abnormality (P-value<0.001); therefore, non-parametric tests were used to compare the data. On the other hand, in order to compare ELISA data with HPLC (difference in results), Wilcoxon non-parametric test was used, based on which a significant difference between the results was obtained (P<0.007).

Results

In this study, 68 samples of pasteurized milk were analyzed. Considering the limit of detection (LOD) of 5 ppt and the limit of quantification (LOQ) of 50 ppt for AFM1, it can be concluded that the proposed methods are very suitable for low concentrations of AFM1. Samples were tested for initial testing by AFM1 rapid test kit in three repetitions. The results showed 7 positive samples out of 68 samples in this method. Then, all 68 positive and negative milk samples (samples above 100 ppt and below) tested by the rapid detection kit were analyzed by ELISA kit in three repetitions, and finally, samples above the allowable limit in the ELISA method (100 ppt) for validation of the results, were injected into the HPLC machine after extraction steps. In comparison, the ELISA method showed 10 positive samples and 58 negative samples. The results are shown in **Table 1**.

Table1. Comparison of results and concentrations higher than AFM1 standard in milk by STRIP test, ELISA, and HPLC.

Sample	HPLC (ppt)	ELISA (ppt)	STRIP test
1	117	191	+
2	110	202	+
3	13	225	+
4	8	343	+
5	18	134	+
6	61	104	+
7	737	<500	+
8	15	<500	-
9	4	264	-
10	5.5	116	-

As clarified by the results, the contamination frequency of samples with AFM1, among 68 milk samples, was 10.29% (7 samples) for strip kit, 14.7% (10 samples) for ELISA method, and 4.4% (3 samples) for the HPLC method. Out of 7 positive samples reported by rapid test kits and 10 samples reported by ELISA test, only 3 samples were confirmed by HPLC analysis. On the other hand, the results of examining the normality of ELISA data (**Figure 2**) showed that due to the lack of bell-shaped data, the data graphs do not have a normal distribution

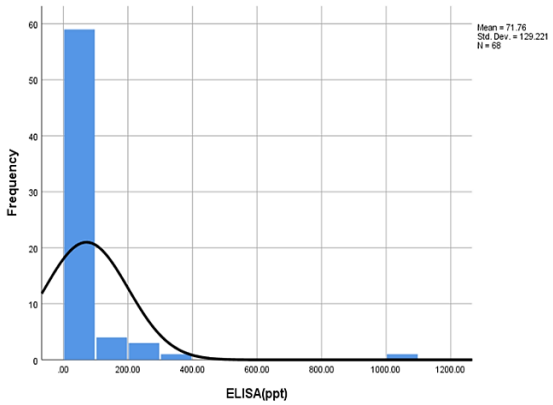

Figure 2. Investigating the normality of data obtained from ELISA method.

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The validation of strip Kit and ELISA (**Figure 3** and **Table 2**) showed that the area under the curve is more than 0.8, which indicates the high predictive accuracy of this test.

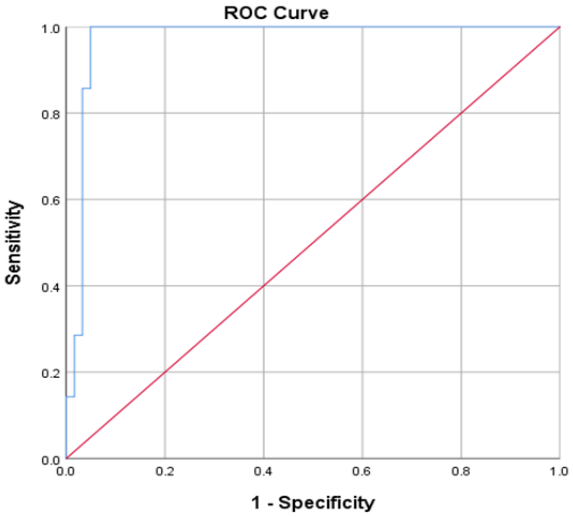

Figure 3. Determining the STRIP TEST vs. ELISA

Table 2. Information obtained from the ROC curve

Area	SE	Asymptotic Sig	Asymptotic 95% confidence interval	
			Lower Bound	Upper Bound
0.972	.019	<0.001	.934	1.000

According to the results presented in **Table 1**, if values obtained from HPLC test are higher than the standard (100 ppt), the strip kit will show100% compliance, but at values below the standard, this compliance will be 43%. Furthermore, if values obtained from the ELISA test are higher than 100 ppt, the strip kit will show 70% compliance, but at values less than 100 ppt, 100% compatibility will be observed between ELISA test and the strip kit. The sensitivity and specificity of strip kit in this study, compared to the ELISA method, as shown in **Table 3** were calculated to be 70% and 100%, respectively

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Table 3. Determination of sensitivity, specificity, and false negative of STRIP test based on ELISA test results.

STRIP test	ELISA	
	Positive n(%)	Negative n(%)
Positive	7 (70)	0(0.0)
Negative	3(30)	58(100)
Total	10	58

The number of false negatives was equal to 3 cases (30%). On the other hand, these values had a sensitivity of 100% and a specificity of 43% compared to the HPLC method (**Table 4**), and the results indicate that there is no false negative in this case.

Table 4. Determination of sensitivity, specificity, and false negative of STRIP test based on HPLC test results.

STRIP test	HPLC	
	Positive n(%)	Negative n(%)
Positive	3 (100)	4(57)
Negative	0 (0.0)	3 (43)
Total	3	7

Although the ELISA method is effective in detecting positive AFM1 cases, it is more likely to report false positives than strip kit and HPLC methods. However, in the evaluation of strip kit by reference method (comparison with HPLC results), it was found that strip kit with higher accuracy and recording less false positives, compared to ELISA method, can be used as a suitable alternative to measuring AFM1. Furthermore, after checking the

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accuracy of the strip kit method, which was performed by HPLC and ELISA diagnostic methods, in order to ensure more, the measurement of AFM1 in diluted spike solutions was re-evaluated. The results are shown in **Table 5**. Considering the cut-off point of 100 ppt, the kits have sufficient accuracy and sensitivity to measure AFM1.

Discussion

Food safety has always been the concern of consumers. Today, consumers tend to use healthy food without contamination, which requires more and comprehensive monitoring of the amount of residual toxins and pollutants in food (Abdi-Moghadam *et al.*, 2023, Marzban *et al.*, 2017). One of the easy ways to achieve this goal is to use quick and inexpensive diagnostic methods to identify the residual toxins in food (Salmani Nodoushan *et al.*, 2015). The use of various kits for rapid detection and quantitative measurement of AFM1 in industries related to milk and its products has become very widespread. These kits and diagnostic methods should be validated to ensure their scientific and practical performance for monitoring the quality of milk and its contamination with AFM1, especially in developing countries (Pecorelli *et al.*, 2020). This study is the first study conducted to evaluating the operational accuracy of strip kits in comparison with ELISA and HPLC methods and health risk assessment for milk and dairy consumers according to AFM1 standards in Iran. Since milk and dairy products play a very important role in human diet and health, in parallel with the increasing consumption of these products, the assessment of the presence of AFM1 in these products using high-precision methods and as fast as possible is increasing (Başkaya *et al.*, 2006). In a study conducted by Reza Noorian *et al*. in Qazvin in 2015 with the title of "determining the level of AFM1 in raw milk samples produced in Qazvin province by ELISA and HPLC ", 170 raw and pasteurized milk samples were collected and the level of contamination was measured using the ELISA method. Samples higher than 0.5 ng/ml were analyzed by high performance liquid chromatography method for the final confirmation that reference and confirmation methods are similar to the present study method. The results of the study showed that all the samples were contaminated with AFM1, of which 33.52% of the samples were contaminated above the permissible limit of the Iranian standard (Norian *et al.*, 2015). Asim Mohammad Zakaria et al. in 2018 in Egypt, conducted a study on rapid detection of AFM1 residues in market milk and the effect of probiotics on its remaining concentration. They found that among 90 milk samples, 37 samples (49%) were positive milk. Thirty seven positive milk samples were analyzed by HPLC to determine the level of AFM1. Similar to the present study, positive samples of rapid method were confirmed by standard and reference methods (Zakaria *et al.*, 2019). In a similar study conducted by Jing-Jhih Wang *et al*. in Taiwan entitled "Sensitivity of Direct Competitive ELISA and Gold Nanoparticle Immunochromatography Strip for Detection of Aflatoxin M1 in Milk", the detection limit of the strip was 1 ng/ml for AFM1 in milk samples (Wang *et al.*, 2011). In addition, the entire analysis was performed in 10 minutes. Detailed examination of 15 samples by ELISA method showed that 6 samples were slightly infected with AFM1, which was consistent with the present study. In addition, all samples were negative due to the level of contamination lower than the detection limit of the immunochromatographic strip. According to the results of this study, the important point is that direct competitive ELISA and immunochromatographic strip methods have high sensitivity in the rapid detection of AFM1 in milk and milk products (Wang *et al.*, 2011). Shim *et al.* conducted a similar study entitled “The development of an immunochromatographic test strip for rapid detection of aflatoxin B1 in grain and feed samples”. In this study, a total of 172 grain and feed samples were collected and analyzed by a rapid test kit and HPLC. The results of rapid test showed good agreement with HPLC results. These results showed that rapid test kit has the potential as a rapid and cost-effective screening

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tool for the determination of AFB1 in real samples and can be applied in the preliminary screening of mycotoxins in food and agricultural products within 15 minutes (Shim *et al.*, 2007). Reybroeck *et al*. validated a Lateral Flow Test to detect AFM1 at a concentration of 50 ng/l. This test was accepted by the Belgian Federal Agency for Food Safety. The cut-off level of the rapid test was based on the AFM1 limit regulated in the European Union; but in the current study, the cut-off level is specifically equal to Iran's permissible limit (0.1 ppb) (Reybroeck *et al.*, 2014). In most of similar studies and references, the rapid STRIP test methods have been confirmed with only one reference method, which, like the present study, require confirmation of the samples with a concentration higher than the standard by the rapid method. Most rapid screening methods for detecting mycotoxins rely on antibodies (immunological assays) and vary depending on how antibodies are used in the method. In the present study, we used basic techniques of ELISA and strip kit and compared their results with HPLC as a reference method for measuring AFM1 and evaluated their accuracy. Based on the results, it was observed that the strip kit method, with no false-negative results compared to the reference method (HPLC), can be used as a fast and high-accuracy method for on-site monitoring of AFM1 in milk. Furthermore, the ELISA test results showed significant false positives compared to the reference method, which can be attributed to the nature of milk. Milk protein and fat may affect results in a variety of ways (for example, due to the possibility of non-heterogeneity of milk, sampling from the upper part of milk storage container, which has more fat, can lead to higher error rates), and each component of milk can interact specifically with the immuno-reagents used, resulting in this false positive (Anfossi *et al.*, 2011). Also, the ELISA method may not be completely reliable due to the interaction of the reaction, especially at concentrations below 50 ng/l (Stark, 2009), which results in high prevalence of contamination in studies using this method, and it is better to use confirmatory methods such as HPLC in addition to the ELISA test. The use of strip kit has many advantages, including saving time and expert manpower, easy analysis of results, short time, and no need for advanced electronic devices and instruments. In addition, the detector used in strip kit is gold nanoparticles, while in the case of the ELISA method an enzyme marker is typically used, which this factor makes the stability and storage time of strip kit significantly longer than ELISA kits (Liu *et al.*, 2016). Furthermore, strip kit analytical protocols are very simple and hassle-free, and calibration is possible automatically by loading the QR code (Lattanzio *et al.*, 2012, Plotan *et al.*, 2016). The results of this study are in line with the findings of other studies that have been previously done, confirming the accuracy of the current study results (Wang *et al.*, 2011, Zakaria *et al.*, 2019). According to the results of the present study and the positive results of strip kit validation in comparison with data obtained from ELISA and HPLC methods, as well as the significant advantages of this method, this method can be used in the diagnosis of AFM1 in milk.

The superiority of the present study with similar studies is that the rapid method has been confirmed with the reference method (ELISA-HPLC) and the validity of the study has been increased. Also, by preparing 12 spike samples with the standard method, in dilutions higher and lower than Iran's permitted limit, measurements were made using STRIP test, and it was used to reconfirm the diagnosis that 100% of rapid test kits with samples Spike read together. Among the limitations of the study, we can point out that parts of the measures leading to the results were expensive and time-consuming due to working with the HPLC device.

Conclusion

The results obtained from this research showed that the STRIP test has high sensitivity and shows a good agreement with the results of the ELISA test, and the initial results of the test were well confirmed by ELISA. The STRIP test is highly effective in identifying AFM1 in milk samples. Its cut-off level matches the Iranian

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standard, making it a reliable option. Additionally, this method offers numerous advantages over more expensive, time-consuming, and complex techniques like ELISA and HPLC. Therefore, using the STRIP test is strongly recommended for preliminary sample analysis.

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Authors' contributions

all authors contributed to the design and implementation of the research, to the analysis of the data and to the writing of the manuscript.

Conflicts of interest

There is not any conflict of interest.

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