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Direct sandwich ELISA to detect the adulteration of human breast milk by cow milk

Mahmoud K. Eldahshoury and Ian P. Hurley*
School of Health, Leeds Beckett University, Leeds LS13HE, United Kingdom

ABSTRACT

The demand for commercially available human breast milk has significantly increased in recent years. For various reasons, a significant amount of commercially available human breast milk is being adulterated with other types of milk. This fraudulent practice poses a threat to consumers' health due to potential adulterants such as cow milk, which may put the infant at risk due to intolerance or allergy. A direct sandwich anti-bovine IgG ELISA has been developed for the sensitive and specific detection of cow milk in adulterated human breast milk. This assay uses polyclonal anti-bovine IgG antibody as a capture antibody and monoclonal anti-bovine IgG-alkaline phosphatase antibody as a detection antibody. Once optimized, the assay was found to be highly sensitive, and specific to bovine IgG. The assay had no significant cross-reaction with human breast milk, indicating that it was highly specific. The anti-bovine IgG ELISA was able to detect the presence of cow milk in adulterated human breast milk with a detection limit of 0.001% cow milk. The developed assay was highly reproducible (coefficient of variation <10%). The developed direct sandwich anti-bovine IgG ELISA is simple, reliable, and reproducible, making it an ideal test for this purpose.

Key words: human breast milk adulteration, sandwich ELISA, IgG, food adulteration

INTRODUCTION

Human breast milk (**BM**) is an essential nutritional source for newborns, providing an ideal balance of protein, fat, carbohydrates, and nutrients for growth and development. Additionally, BM provides the infant with antibodies in the first few days after birth (passive immunity). This immunity provides protection against childhood allergies and a variety of chronic illnesses such as arthritis, diabetes, and childhood leukemia (Nuzzi et al., 2021; Yi and Kim, 2021; Meek and Noble, 2022). Given the benefits of breastfeeding, many mothers who are unable or unwilling to breast-feed their children still want to use human BM to feed their infant. As a result, lactating women began selling their BM to new parents via the internet or milk banks (Schafer et al., 2018). There are other groups of consumers who may purchase BM, including bodybuilders who use it as a super food to enhance muscle recovery and mass (Dowling and Grant, 2021).

In the United Kingdom, the practice of selling BM online is not illegal, but it raises many questions about the safety of the milk. It is unlikely to be offered as a pasteurized product, and even if it were, the milk would lose most of its passive immunity benefits (antibodies could be denatured). Only the nutritional value would be retained, which would only contribute to the benefits of breastfeeding in part. An analysis of 102 products marketed as human BM revealed that 10% of these products were adulterated with cow milk (based on qualitative detection of bovine DNA; Keim et al.,2015). Cow milk can be harmful to neonates who are allergic or intolerant to cow milk proteins such α_S caseins, β-LG, or α-LA.

Various nonimmunological assays have been developed to identify the species of origin in milk products. These assays rely on the detection of either cow α_{S1}-CN using isoelectric focusing in polyacrylamide gel analysis, or α-LA, and β-LG (the 2 most abundant whey proteins) using MALDI or HPLC (Manzo et al., 2017; Zajác et al., 2021; Rysova et al., 2022). Isoelectric focusing, MALDI, and HPLC assays have a detection limit of adulteration with respect to cow milk of 0.5, 5, and 1%, respectively (Borková and Snášelová, 2005; Calvano et al., 2012; Špoljarić et al., 2013).

The PCR has also been employed to detect milk and dairy product adulteration; however, basic PCR analysis is typically not quantitative (Haddad and Abu-Romman, 2020). This downside could be overcome using real-time PCR; but it is time-consuming and laborious (Giglioti et al., 2022).

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*Corresponding author: i.p.hurley@leedsbeckett.ac.uk

Immunological methods currently used to detect milk adulteration use polyclonal antibodies against β-CN, α_{S1}-CN, β-LG, or κ-CN (Borková and Snášelová, 2005). These assays show some limitations when differentiating between closely related species such as cow and buffalo (Manzo et al., 2017). Targeting bovine IgG as an analyte has been found to be more specific and sensitive when detecting adulteration by cow milk. Two immunoassays targeting cow IgG (indirect competitive and indirect sandwich ELISA) were developed to detect adulteration of dairy products and showed detection limits of 0.1 and 0.01 μg/mL of bovine IgG, respectively (Hurley et al., 2004, 2006).

The typical application of cow milk speciation assays is for monitoring adulteration in dairy products (e.g., cheese; Ren et al., 2014). Similarly, the proposed assay could be used to detect adulteration with cow milk in other milk products such as camel, goat, soy, oat, and almond milk; however, the main purpose of this paper is to present a sensitive and selective test for the detection of cow milk in commercially produced adulterated BM. The developed approach involved using a direct sandwich anti-bovine IgG ELISA to specifically identify and quantify the amounts of cow milk used to adulterate BM.

MATERIALS AND METHODS

Preparation and Source of Materials

No animals were used in this study, as commercial milk products were used. The study received ethical approval from Leeds Beckett University Research Ethics Committee.

Unless otherwise stated, all reagents were acquired from Sigma Chemical Company Ltd. Fresh whole cow milk, skimmed cow milk, UHT whole cow milk, and goat milk were obtained from a local shop (Morrisons brand, the United Kingdom) and used immediately. Human BM was obtained from a consenting volunteer and used immediately after collection.

Stock antigen solution was prepared by reconstituting lyophilized bovine IgG (Sigma I-5506) with PBS (NaCl, 140 m*M*; KCl, 3 m*M*; Na_2HPO_4, 2 m*M*; $KHPO_4$, 10 m*M*; pH 7). Polyclonal anti-bovine IgG (Sigma B1645) was obtained as a solution containing 15 m*M* sodium azide. Monoclonal anti-bovine IgG-alkaline phosphatase (**ALP**) antibody (Sigma A7554) was provided as a solution in 0.05 *M* Tris buffer, pH 8.0, containing 1% BSA, 1 m*M* $MgCl_2$, 50% glycerol, and 15 m*M* sodium azide. Bovine IgG (Sigma I-5506), goat IgG (Sigma I5256), and human IgG from serum (Sigma I2511) were supplied as a lyophilized powder then reconstituted using PBS. P-nitrophenyl phosphate substrate (Sigma P7998) was supplied as a ready to use solution. All reagents were stored as recommended by the providing company.

Optimization of Direct Sandwich Anti-Bovine IgG ELISA Antisera

Optimum dilutions were assessed based on checkerboard titrations to choose the optimum dilution of polyclonal Ab anti-bovine IgG (Sigma B1645) as a capture antibody and monoclonal anti-bovine IgG-ALP (Sigma A7554) as a detector Ab, to maximize the sensitivity of the assay, and to ensure the maximal working range. The optimal dilution of both the capture and detector antibodies was determined based on a strong signal versus low noise in addition to the maximum signal/noise ratio (**S/N**). The S/N was determined using the following equation:

$$\text{S/N ratio} = \frac{\text{OD of the signal side}}{\text{OD of the noise side}}.$$

Direct Sandwich Anti-Bovine IgG ELISA Method

Nunc MaxiSorp flat-bottom (Thermo Scientific) were coated overnight at 4°C with 100 μL/well of polyclonal anti-bovine IgG in PBS. The plates were then blocked for 1 h at 37°C with 250 μL/well of 3% (wt/vol) dried nonfat milk (Morrisons brand, the United Kingdom) in PBS then washed 3 times with ELISA wash buffer (PBS + 0.01% Tween 20). In quadruplets, 100 μL/well of bovine IgG (10 μg/mL) was added as top standard and diluted down the plate in PBS to obtain a 10-fold serial dilution followed by an incubation step for 30 min at 37°C. The samples added varied depending on the samples being tested in each of the performed assays. These samples were either whole-fat cow milk, semi-skimmed cow milk, skimmed cow milk, goat milk, or human BM. These samples were subjected to a 10-fold serial dilution from neat in PBS. After being washed 3 times using ELISA wash buffer, 100 μL/well of diluted monoclonal anti-bovine IgG-ALP in PBS was added to the plates and incubated for 30 min at 37°C. After performing another washing step as previously described, the plates were incubated with 100 μL/well of P-nitrophenyl phosphate substrate for 15 min at 37°C. The reaction was stopped by the addition of 50 μL/well of 3 *M* NaOH, and the absorbance was measured at 405 nm using a microplate reader. Each experiment was repeated 3 times to ensure reproducibility.

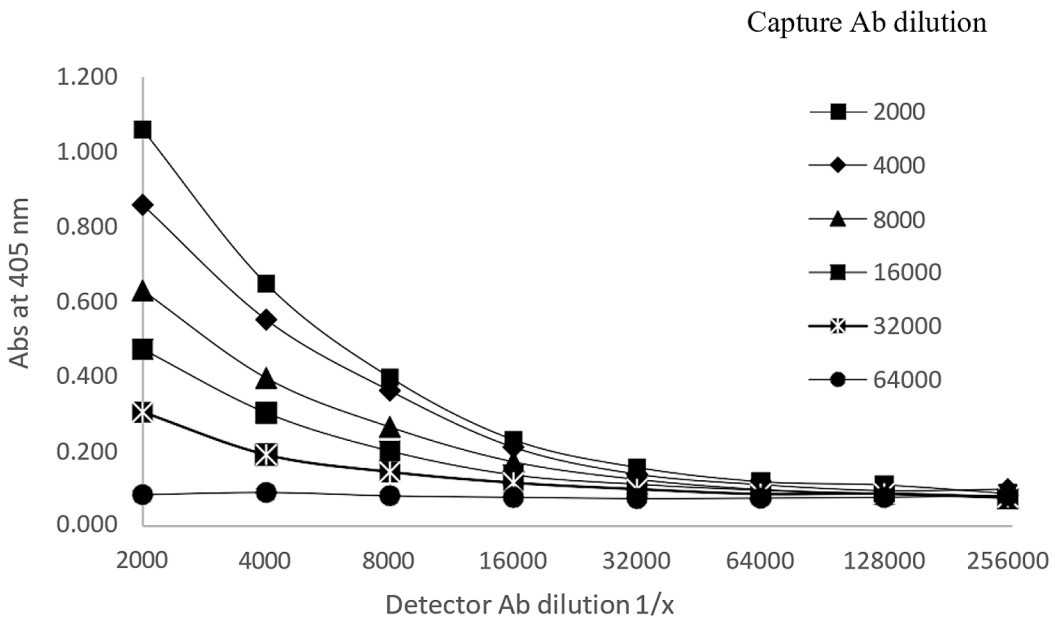

Figure 1. Optimization of capture and detector antibody (Ab) dilution of direct sandwich anti-bovine IgG ELISA. Wells were coated with different dilutions of both capture and detector antibodies. Sample was 1.0 μg/mL bovine IgG. Absorbance (Abs) was measured at 405 nm to assess the optimum dilutions of the antibodies.

Percentage Adulteration by Cow Milk

These assays were carried out as described above; however, they differed in the samples and diluents used. The sample used was cow milk acting as the adulterant, while the diluents used were either goat or human BM. Cow milk is a common adulterant in dairy products and the ELISA should be specific for cow milk. The human BM acted as the potential milk that could be a target for adulteration. The use of the goat milk as a diluent served as another potential target for adulteration with cow milk to further assess the specificity of the assay. The percentage of cow milk adulteration ranged from 0.00001 to 100%. These spiked samples were prepared by 10-fold dilutions of cow milk using either goat milk or human BM as diluent to create a series of standards.

These assays were performed to assess the assay's validity, specificity, and sensitivity (in terms of limit of detection) to detect the adulteration of goat and human BM with cow milk in real applications. Each experiment was repeated 3 times to ensure reproducibility.

RESULTS

Optimization of the Assay

To determine the optimal dilutions of both the capture antibody (polyclonal anti-bovine IgG) and the detector antibody (monoclonal anti-bovine IgG), an optimization assay was performed. The optimal dilution of both the capture and detector antibodies was 1:2,000 based on the results obtained, as they provided a strong signal versus low noise in addition to the maximum S/N (Figure 1).

Sensitivity of the Assay

Once optimized, the assay was highly sensitive to bovine IgG and showed a limit of detection as low as 0.01 μg/mL ($P = 0.004$) with a working range of 0.01 to 10 μg/mL (Figure 2). The assay was also highly sensitive in detecting bovine IgG when tested with cow milk and showed a limit of detection of 1/10,000 dilution with a working range of 1/10,000 to 1/10 dilution (Figure 3).

Specificity of the Assay

We assessed the specificity of the assay by testing its performance with bovine, caprine, and human IgG samples. The assay detected bovine IgG with no cross-reaction with the tested species (Figure 4). We further evaluated the specificity of the assay by testing its performance with goat milk, human BM, and different forms of cow milk (whole, semi-skimmed, and skimmed) at various concentrations. Only cow milk was detected, indicating the potential to detect cow milk in a mixture, as there is no cross-reaction with goat or human IgG (Figure 5).

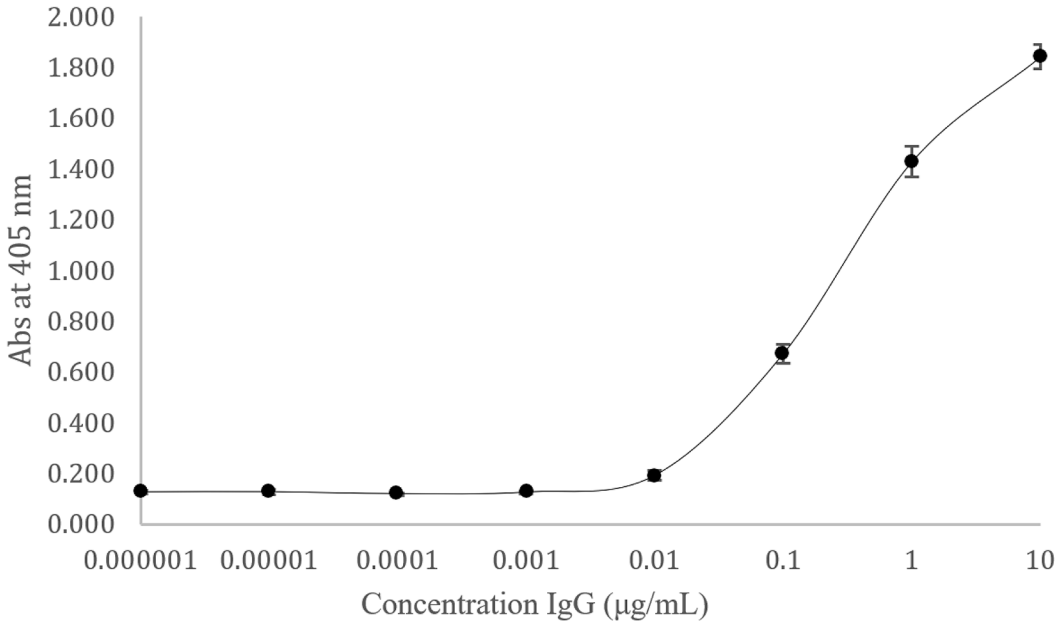

Figure 2. Optical density at 405 nm values of bovine IgG standard curve using direct sandwich anti-bovine IgG ELISA. The dilution of the capture and detector antibodies was 1/2,000. Data points are the mean ± SD. This test was carried out in triplicate (n = 3). Abs = absorbance.

Limit of Detection in Adulterated Samples

The direct sandwich anti-bovine IgG ELISA was then used to assess its ability to detect bovine IgG in adulterated milk samples by mixing goat milk or human BM with cow milk at different percentages of adulteration. This assay detected up to 0.001% adulteration ($P < 0.01$) by cow milk in goat milk (Figure

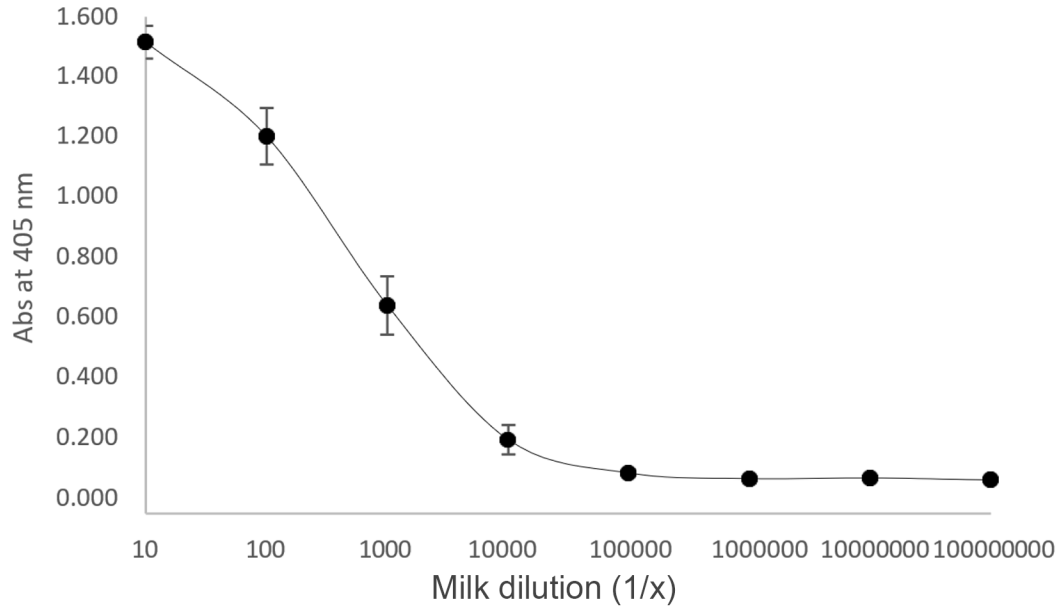

Figure 3. Optical density at 405 nm values of whole cow milk standard curve using direct sandwich anti-bovine IgG ELISA. The dilution of the capture and detector antibodies was 1/2,000. Data points are the mean ± SD. This test was carried out in triplicate (n = 3). Abs = absorbance.

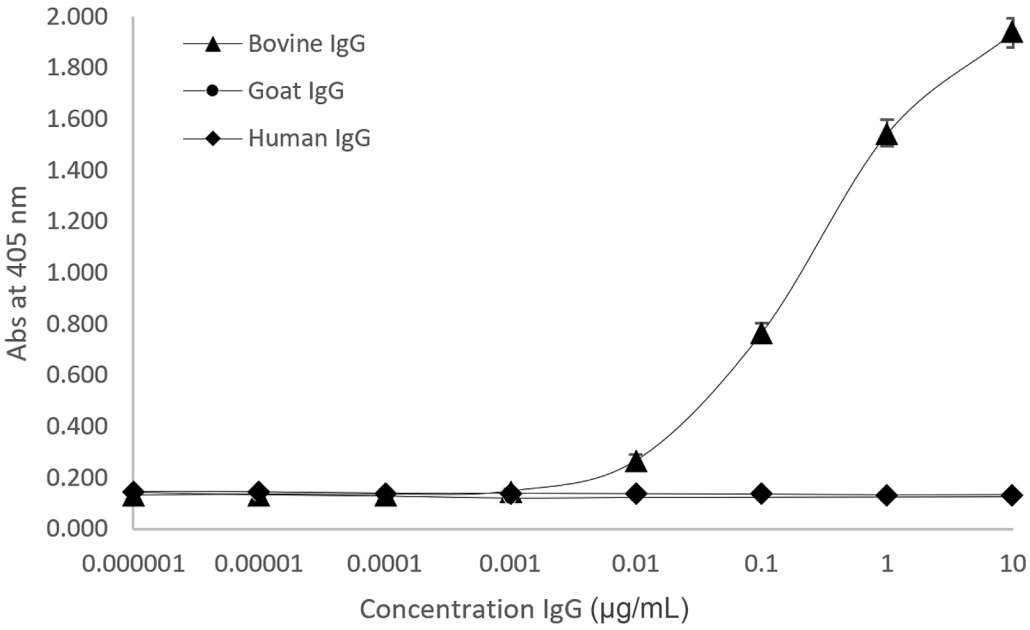

Figure 4. Optical density at 405 nm of various dilutions of bovine, goat, and human IgG using anti-bovine IgG ELISA. The dilution of the capture and detector antibodies was 1/2,000. Data points are means ± SD. This test was carried out in triplicate (n = 3). Abs = absorbance.

6). The assay also detected human BM adulteration with whole, semi-skimmed, and skimmed cow milk at a level of 0.001% ($P = 0.05$), indicating a sensitive assay (Figure 7).

DISCUSSION

The main objective of this study was to develop an assay to test commercial human BM to monitor wheth-

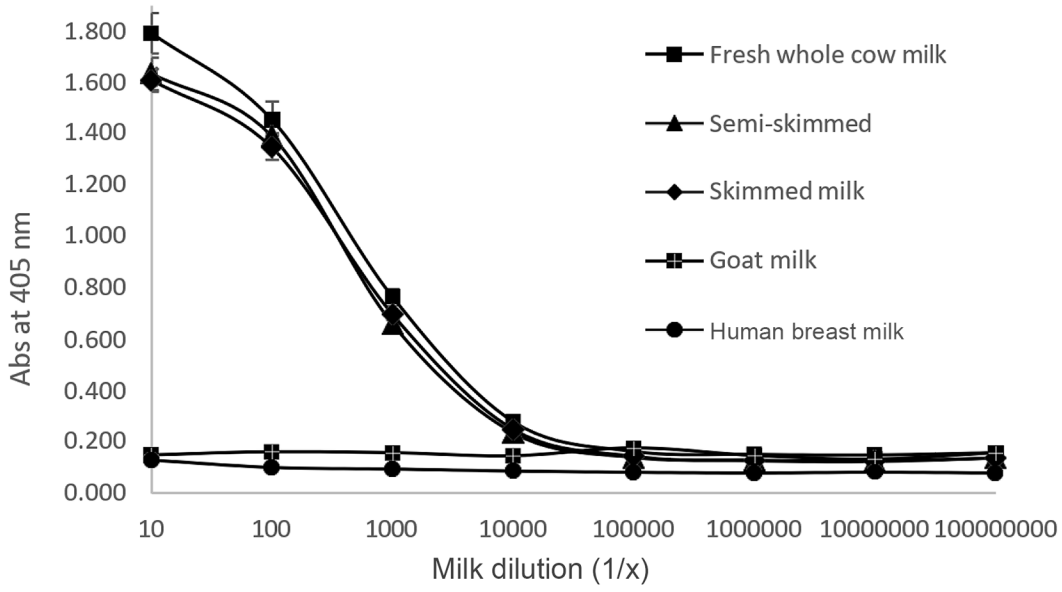

Figure 5. Optical density at 405 nm of various dilutions of fresh whole, semi-skimmed, and skimmed cow milk, goat milk, and human breast milk using direct sandwich anti-bovine IgG ELISA. The dilution of the capture and detector antibodies was 1/2,000. Data points are means ± SD. This test was carried out in triplicate (n = 3). Abs = absorbance.

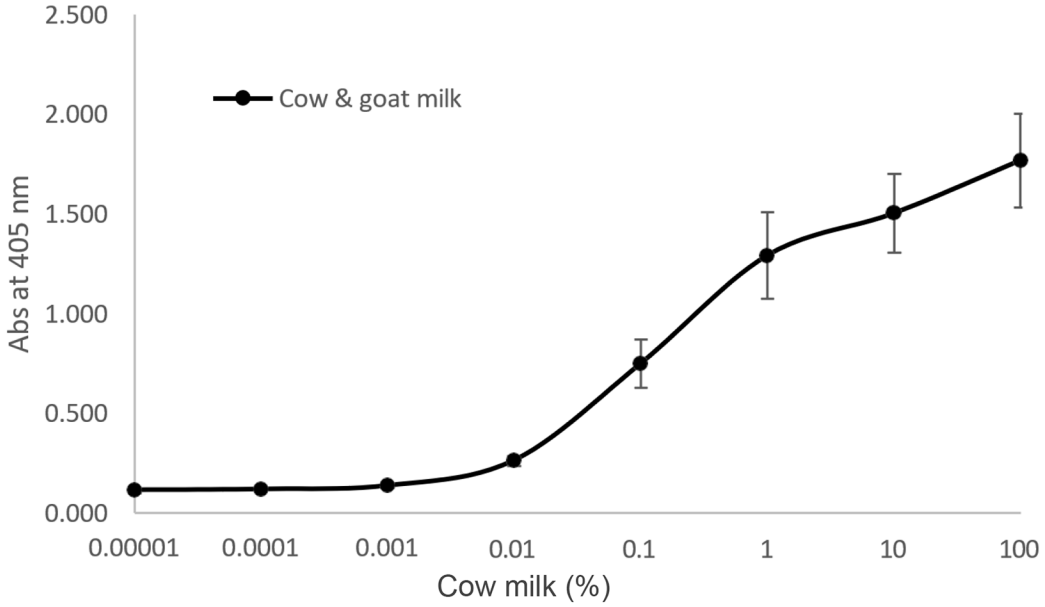

Figure 6. Detection of cow milk mixed with goat milk at various adulteration dilutions using direct sandwich anti-bovine IgG ELISA. The dilution of the capture and detector antibodies used was 1/2,000. The data points are the mean ± SD. This test was carried out in triplicate (n = 3). Abs = absorbance.

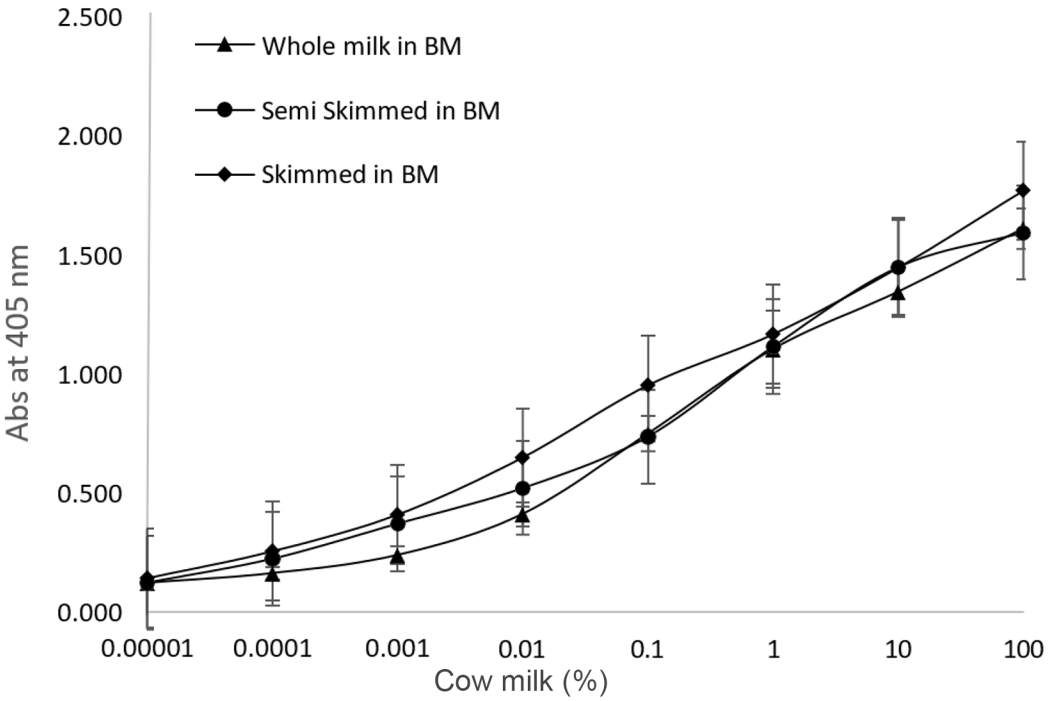

Figure 7. Detection of whole, semi-skimmed, and skimmed cow milk after mixing them individually with human breast milk (BM) at various adulteration dilutions using direct sandwich anti-bovine IgG ELISA. The dilution of the capture and detector antibodies was 1/2,000. The data points are the mean ± SD. This test was carried out in triplicate (n = 3). Abs = absorbance.

er it has been adulterated with cow milk. Previous attempts to develop speciation assays for the identification and measurement of cow milk have been made to detect the adulteration with cow milk in dairy products (Ren et al., 2014)

During specificity testing, the developed assay demonstrated its ability to identify the adulteration of goat milk with cow milk, suggesting that it may also be used to detect cow milk adulteration of other milk products. However, testing the adulteration of human BM with cow milk was the main objective of this assay.

Many immunoassays have been developed to detect cow milk, but they were targeting milk proteins, similar to caseins (Ren et al., 2014; Cosenza et al., 2019); which are poorly immunogenic and susceptible to proteolysis (Hurley et al., 2004). In the developed direct sandwich ELISA assay, we selected IgG as the target protein due to its species-specific epitopes, high immunogenicity, and the abundance of IgG in blood, meat, milk, and other tissues and secretions (Vidarsson et al., 2014). Previous research had also suggested that IgG could be used as a species-specific target antigen in milk products authentication assays (Hurley et al., 2004).

The performance of any ELISA should be evaluated in terms of its specificity and sensitivity, so assay reagent optimization was carried out to increase both of these parameters. Another goal of optimization is to prevent cross-reaction between the capture and detector antibodies, which could lead to erroneous results (Mousavi et al., 2016). After optimization, the developed assay showed a detection limit of 0.01 μg/mL for bovine IgG detection with no cross-reaction with caprine or human IgG stock samples, showing that it is particularly sensitive and specific to bovine IgG, at least with the 2 species tested.

It has also detected bovine IgG in fresh whole cow milk with a detection limit of 1/10,000 dilution with no cross-reaction with either goat or human BM.

When used to detect adulteration by cow milk, this assay showed a detection limit of 0.001% adulteration of goat milk with whole cow milk and 0.001, 0.001, and 0.001% adulteration of human BM with whole, semi-skimmed, and skimmed cow milk, respectively.

These findings show a higher sensitivity in the detection of cow milk than both the established indirect competitive ELISA cow milk detection limit of 0.1% using monoclonal anti-bovine IgG antibody and the indirect competitive ELISA with a detection limit of 1% using anti-bovine β-CN monoclonal antibody (Hurley et al., 2004; Ren et al., 2014).

The direct sandwich anti-bovine IgG ELISA was unable to detect bovine IgG when tested with dried skimmed cow milk and long-life cow milk. This limitation could be attributed to the denaturation of IgG in milk following ultra-high temperature pasteurization (Krishna et al., 2021). This issue could be addressed by developing heat-denatured epitope-specific antibodies or by employing an alternative technique, such as PCR; however, the expense of producing dried skimmed cow milk and long-life cow milk is high, making them unsuitable products for adulteration (Azad and Ahmed, 2016).

Although the developed assay has not been tested directly with dried cow skim milk, we believe it cannot be identified because it was used as a blocking buffer and showed no reactivity. Similarly, the developed assay has not been tested with long-life (UHT) cow milk. However, even if the assay was unable to detect its presence, we consider it unlikely that BM is at risk of adulteration from such a product.

In conclusion, the direct sandwich anti-bovine IgG ELISA was able to detect the presence of whole, semi-skimmed, and skimmed cow milk in adulterated human BM with a detection limit of 0.001%. The developed assay has also shown a detection limit of 0.001% for adulteration of goat milk with cow milk. These findings suggest that this assay is a highly sensitive and specific assay for the detection of the adulteration of human BM with cow milk. Our study also highlights the potential of the assay to be applied to the detection of cow milk adulteration in other commercial milk products. The ELISA has the potential to be developed into a kit for routine human BM monitoring, particularly in milk banks. We also believe that with further development, the assay could be used to detect cow milk adulteration in dairy products derived from goat, sheep, buffalo, and camel, as well as soy, almond, and oat milks.

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ORCIDS

Mahmoud K. Eldahshoury https://orcid.org/0000-0003-3067-2556
Ian P. Hurley https://orcid.org/0000-0002-1735-2761