Food Chemistry 351 (2021) 129311

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

# Adulteration of cow's milk with buffalo's milk detected by an on-site carbon nanoparticles-based lateral flow immunoassay

Rajan Sharma [a,*], Archana Verma [b], Nitin Shinde [a], Bimlesh Mann [a], Kamal Gandhi [a], Jan H. Wichers [c], Aart van Amerongen [c]

[a] Dairy Chemistry Division, ICAR-National Dairy Research Institute, Karnal 132 001, India
[b] Animal Genetics & Breeding Division, ICAR-National Dairy Research Institute, Karnal 132 001, India
[c] BioSensing & Diagnostics, Wageningen Food & Biobased Research, Wageningen University & Research, Bornse Weilanden 9, 6708 WG Wageningen, The Netherlands

ARTICLE INFO

*Keywords:*
Lateral flow immunoassay
Carbon nanoparticles
Adulteration
Buffalo's milk
Cow's milk

ABSTRACT

A competitive lateral flow immunoassay using amorphous carbon nanoparticles (CNPs) and non-immunoglobulin antigen has been developed for the rapid detection of adulteration of cow's milk with buffalo's milk. Purified polyclonal antibodies against a specific buffalo's milk protein fraction were conjugated to CNPs and sprayed on a conjugate pad. The test line consisted of buffalo's skimmed milk proteins (1.6 μg/cm), while the control line contained anti-rabbit antibodies raised in goat (0.5 μg/cm). In the test procedure milk sample is mixed with 100 mM borate buffer (pH 8.8 containing 1% BSA and 0.05% Tween 20) and pipetted onto the sample-cum-conjugate pad. A black/grey test line can be observed if the sample is free from buffalo's milk. The sensitivity of the test i.e. no visible test line is 5% adulteration of cow's milk with buffalo's milk. The test has applicability at the milk receiving stations and can be applied to heated milk samples.

## 1. Introduction

Lateral flow immunoassay (LFIA) has been established as an effective analytical tool in the health sector for the rapid diagnostic of various physiological conditions (van Amerongen, Veen, Arends, & Koets, 2018). Being a rapid technique, LFIA is also finding applications in monitoring the quality of raw food material. The technique is of immense use to assess the quality of perishable foods such as milk and meat prior to their processing. Lateral flow immunoassays have been developed for rapid detection of, e.g. antibiotics residues (Lata, Sharma, Naik, Rajput, & Mann, 2016; Naik, Sharma, Mann, Lata, Rajput, & Surendra Nath, 2017), aflatoxin M1 (Anfossi et al., 2013; Wang et al., 2018), melamine (Zhong et al., 2016) microbial toxin (Upadhyay & Nara, 2018) and allergens (Wang et al., 2015) in milk. LFIA has also been applied for rapid detection of adulteration of soymilk in bovine milk (Gautam, Sharma, Lata, Rajput, & Mann, 2017). In recent times, this technique has also found applications for species authentication of milk because milk from some species is being marketed at premium prices due to perceived health effects, for their typical sensory properties or due to their intended use (Genis, Sezer, Bilge, Durna, & Boyaci, 2020; Ullah, Khan, Ali, & Bilal, 2020).

The species authentication of milk is also a requirement of regulatory bodies of many countries. The European Union has regulation (European Union Regulations, 2018) for the detection of cow's milk components in cheese made from milk of minor species. The Indian regulatory authority has defined standards of milk of different species such as cow, buffalo, goat, sheep and camel (FSSAI Regulations, 2011) to regulate their sale to consumers. This has led to price variations in milk of various species and thus encourages admixing of one species of milk with another. This is a type of economically motivated adulteration and although such mixing may not affect food safety, but it leads to mislabelling and thus compromises consumers' preference. India is the largest producer of buffalo's milk contributing to 49% and the second-largest producer of cow's milk contributing to 47% of the total milk production of 187.7 million tons (DAHD Report, 2019). Almost all the constituents in buffalo's milk have higher levels than in cow's milk. It is nearly twice as rich in fat and about 30% higher in total solids than cow's milk. The differences in chemical constituents and physico-chemical properties of both cow's and buffalo's milk make them suitable for certain specialized products (Aneja, Mathur, Chandan, & Banerjee, 2002). Most of the well-known cheese varieties in the world are normally produced from cow's milk (Arora & Khetra, 2017).

* Corresponding author.
*E-mail addresses:* Rajan.Sharma@ICAR.gov.in (R. Sharma), jan.wichers@wur.nl (J.H. Wichers), aart.vanamerongen@wur.nl (A. van Amerongen).

https://doi.org/10.1016/j.foodchem.2021.129311
Received 23 July 2020; Received in revised form 26 December 2020; Accepted 4 February 2021
Available online 20 February 2021

Further, cow's milk yields a soft coagulum, making it suitable for preparation of *Chhana* which is used as the main ingredient for some of the Indian dairy sweets like sandesh, rasogolla, rasmalai, etc. There has also been a shift in the preference of consumers for cow's milk over buffalo's milk due to various reasons including therapeutic aspects and, therefore, some leading dairy companies in India are exclusively marketing liquid cow's milk (Das, 2015; ICICI Securities, 2018). To cater the demand of cow's milk, addition of water to buffalo's milk and passing it as a cow's milk, i.e. 100% adulteration, is being practiced (Sharma, Rajput, & Barui, 2013). Dairy industries as well as regulatory bodies are looking for simple and rapid methods for the detection of buffalo's milk in cow's milk supplies. In the past, tests have been developed for detection of buffalo's milk in cow's milk which include a PCR-based test (De et al., 2011) and an agglutination assay (Verma, 2019). LFIAs have been developed for the detection of the presence of cow's milk in goat's, sheep's or buffalo's milk (Galan-Malo, Mendiara, Razquin, & Mata, 2018; Liu et al., 2019). These LFIA have been developed using gold nanoparticles (AuNPs) by using antibodies that had been raised either against bovine immunoglobulin (Galan-Malo et al., 2018) or casein (Liu et al., 2019). The use of carbon nanoparticles (CNPs) in LFIA has not been broadly exploited. Earlier, it has been reported that use of CNPs as label in LFIA increased the signal-to-noise ratio which has been mainly attributed to contrast of dark black colour of carbon against white nitrocellulose membrane (Aktas, Wichers, Skouridou, van Amerongen, & Masip, 2019; Posthuma-Trumpie, Wichers, Koets, Berendsen, & van Amerongen, 2012).

In this research article, a buffalo's milk specific protein fraction has been isolated for raising polyclonal antibodies and a LFIA has been developed using amorphous CNPs. The main aim of the present research work was the development of a rapid and sensitive LFIA by employing antibodies raised against the buffalo's milk protein fraction using CNPs as label agent. These antibodies can detect the presence of buffalo's milk in cow's milk at less than a 5% adulteration level.

## 2. Materials and methods

### 2.1. Materials

Glass fiber based sample-cum-conjugate pad (Grade 8951) and cotton linter fiber based absorbent pad (Grade 270) were obtained from Ahlstrom-Munksjö, Helsinki, Finland. Three different types of nitrocellulose membrane used in the study were purchased from GE Healthcare Life Sciences, Pittsburgh, PA, USA (FF80HP and FF120HP) and Sartorius Stedim Biotech, Göttingen, Germany (UniSart CN 140). Amorphous CNPs (carbon black) were from Orion Engineered Carbons, Houston, TX, USA. Goat anti-rabbit IgG (Fc fragment specific) was obtained from Jackson Immuno Research, Inc. West Grove, PA, USA. Goat anti-rabbit antibodies conjugated with horseradish peroxidase (anti-rabbit IgG–HRP), 3,3′,5,5′-tetramethylbenzidine (TMB), boric acid, sodium tertraborate, trehalose, bovine serum albumin (BSA) (heat shock fraction, protease free), albumin from chicken egg white (OVA) were purchased from Sigma-Aldrich, St. Louis, Missouri, USA. Throughout the study, Type I water ($\leq$18.2 MΩ cm) was used (PURELAB® Water Purification System, ELGA Lab Water, High Wycombe, UK). Pure buffalo's milk and cow's milk samples were collected from the Livestock Research Center, ICAR-National Dairy Research Institute, Karnal, India. Pooled milk samples were collected and brought into the laboratory in glass bottles and stored at 4 °C and were used within 24 h.

### 2.2. Preparation of anti-buffalo polyclonal antibodies against a buffalo's milk specific protein fraction

Polyclonal antibodies were raised against buffalo's milk's specific protein fraction as per the protocol suggested by Jairam and Nair (1980) with modification. In brief the modified method is as follows:

#### 2.2.1. Isolation of buffalo's milk specific protein (antigen) and its characterization

Whole buffalo's milk was centrifuged (Sigma 3 K15, Osterode am Harz, Germany) at 3000×*g* (4 °C for 30 min) and cream plug formed at the top was carefully removed and discarded. Thirty millilitres of skimmed milk thus obtained was centrifuged (Sigma 3K15, Osterode am Harz, Germany) at 10,700×*g* (4 °C for 30 min). The supernatant was discarded and the protein pellet obtained was re-suspended in the same volume of water. The preparation was stored at 4 °C and was used for immunization of rabbits. For comparison, a similar kind of preparation was also prepared using cow's milk. The protein content in the preparation was estimated using the Kjeldahl method (ISO 8968-4, 2016).

#### 2.2.2. Preparation of anti-buffalo polyclonal antibodies

Adult male albino rabbits (New Zealand White) with body weights ranging from 1.5 to 2 kg were used for the production of polyclonal antibodies using the buffalo's milk specific protein as the immunogen. Immunization was done for 6 weeks on three consecutive days in a week using both intravenous (i/v) and intraperitoneal (i/p) routes. In the first week, the immunogen was injected via the intravenous route 0.5 mL on day 1, 2 and 1.0 mL on day 3. In the second week, 1.0 mL on day 1 via the intraperitoneal route, subsequently 1.0 mL and 1.5 mL i/v on day 2 and 3. In the third week, 1.5 i/p, 1.5 i/v and 2.0 i/v on day 1,2,3, respectively. In the fourth week: 2.0 (i/p), 2.0 (i/v) and 2.5 (i/v) on day 1, 2 and 3. In the fifth week: 2.5 (i/p), 2.5 (i/v) and 3.0 (i/v) on day 1, 2 and 3. Sixth week: 3.0 (i/p), 3.0 (i/v) and 3.5 (i/v).

Blood samples from each rabbit were collected prior to immunization as well as after the 4th week of immunization in 15 mL sterile falcon tubes and kept undisturbed at 4 °C for 12 h. The tubes were then centrifuged at 1200×*g* (Kubota, Tokyo, Japan) to get the red blood cells settle down and the obtained antiserum was tested for titre by mixing it with an equal amount of diluted (1:10, aqueous) buffalo's and cow's skimmed milk on a glass slide. The formation of an agglutination reaction within 15–20 s confirmed the formation of antibodies and the immunization was continued for the rest of the rabbits for subsequent weeks.

After the completion of immunization schedule, blood samples were collected and serum was prepared as described above. The IgG fraction of the anti-buffalo antiserum was purified using a protein A column (Miller & Stone, 1978).

#### 2.2.3. Quality of anti-buffalo antibody preparation

The quality of the prepared anti-buffalo antibodies was assessed using an enzyme-linked immunosorbent assay (ELISA) (Lata et al., 2016). Dilutions of antiserum, purified antibody and pre-immune serum were made (1:100, 1:200, 1:500, 1:1000, 1:2000, 1:4000 and 1:8000, 1:16,000) and these dilutions were added to wells of a microtiter plate coated with 100 μL buffalo's milk specific protein fraction (0.6 μg/mL), followed by washing and addition of anti-rabbit IgG–HRP. After removal of unbound anti-rabbit IgG-HRP, TMB substrate was added to develop the colour and the absorbance reading was done at 450 nm in microplate reader (Infinite F200Pro, Männedorf, Switzerland).

### 2.3. Conjugation of anti-buffalo antibodies to amorphous CNPs

A 1% aqueous suspension of amorphous CNPs was sonicated in ultrasonic bath (VWR Singapore Pte Ltd. Singapore) for 1 h at 40 KHz at room temperature. Subsequently, a 0.2% carbon suspension was prepared in borate buffer (5 mM, pH 8.8) and the preparation was sonicated for 5 min (Bioruptor Plus, Diagenode SA, Seraing, Belgium). For conjugation the purified anti-buffalo antibody preparation was desalted using 0.5 mL spin columns (7 K MWCO, Zeba Spin Desalting Columns, Thermo Scientific, Rockford, USA). Protein content in the desalted preparation was 1.85 mg/mL estimated using the spectrophotometer (DeNovix DS-11 FX, Wilmington, USA). One millilitre of 0.2% carbon suspension was mixed with the purified antibody preparation (protein:

1.85 mg/mL) to a final protein concentration of 350, 175, or 87.5 μg/mL. The content was stirred at 4 °C for 12 h on a magnetic stirrer and subsequently centrifuged at 13636 × g (4 °C, 15 min, Sigma 2 K15, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany). The pellet was washed three times with washing buffer (borate buffer, 5 mM, pH 8.8 containing 1% BSA) and suspended in 100 mM borate buffer (pH 8.8 containing 1% BSA). The conjugate was stored at 4 °C until further use. Conjugation of CNPs with anti-buffalo antibodies was verified by running the lateral flow assays strips containing a control line having goat anti-rabbit IgG (0.25 mg/mL) in 100 mM borate buffer (pH 8.8 containing 1% BSA and 0.05% Tween 20). .

### 2.4. Optimization of parameters for the preparation of lateral flow assay strips

For optimization of the lateral flow assay for detection of buffalo's milk in cow's milk, several parameters were studied by using half-lateral flow (HLF) assay strips. HLF strips were prepared on backing cards having a pressure sensitive adhesive and consisting of a 2.5 cm nitrocellulose membrane (FF80HP) and 3.5 cm absorbent pad. First of all, nitrocellulose membrane was pasted on a backing card followed by pasting an adsorbent pad that overlapped 2 mm on the nitrocellulose membrane. Firm and reproducible attachment was accomplished by using a laminator (LM5000™ Clamshell Laminator, Biodot Inc., Irvine, CA, USA). The architecture of the HLF strip is given in the Supplementary Data section. The test line (diluted buffalo's skimmed milk containing various concentrations of milk protein) was dispensed on the nitrocellulose membrane by a Linomat IV TLC dispenser (Camag, Muttenz, Switzerland) at a distance of 14 mm from the origin of the nitrocellulose membrane. Finally, 5 mm wide strips were cut using a programmable cutter (CM5000™ Guillotine Cutter, Biodot Inc., Irvine, CA, USA) and packed in aluminum pouches (Nefab, Barneveld, The Netherlands) along with silica desiccants (Multisorb Technologies, Inc., NY, USA). The strips were stored at room temperature until use.

### 2.5. Detection of buffalo's milk in cow's milk using HLF assay strip

Cow's milk was spiked with buffalo's milk in the range of 1 to 100%. Ten microliters of such prepared samples were added to 80 μL 100 mM borate buffer (pH 8.8 containing 1% BSA and 0.05% Tween 20) in a well of a microplate assay plate. Prior to the assay, 10 μL ten times diluted in 100 mM borate buffer (pH 8.8 containing 1% BSA and 0.05% Tween 20) carbon colloidal conjugate containing different levels of anti-buffalo antibodies was added to this mixture. Prepared HLF strips were placed in the well of a microwell assay plate. After 10 min the test line was visually inspected and the strips were scanned using flatbed scanner (Epson Perfection V600 Photo, Seiko Epson Corporation, Nagano, Japan) at an 8 bit grayscale and a resolution of 1220 dpi. The intensity of the test line was evaluated using the TotalLab Quant (TotalLab Ltd., Newcastle-Upon-Tyne, UK) in terms of total pixel grey value. Subsequently, pixel grey value per unit area was calculated by dividing the total pixel grey value by the test line pixel area. Each test was repeated three times and the absence of a visually perceptible test line was taken as the detection limit.

### 2.6. Preparation of complete lateral flow (CLF) assay strips

Different segments of the lateral flow assembly were pasted on a backing card having a pressure sensitive adhesive (Gautam et al., 2017) using the laminator. Anti-buffalo antibody-carbon nanoparticle conjugate diluted with dilution buffer (100 mM, borate buffer, pH 8.8, containing 1% BSA and 3% Trehalose) was sprayed (20 μL/cm) on a conjugate pad by using a Biodot Dispense Platform (Biodot Inc., Irvine, CA, USA). Test line (diluted buffalo's skimmed milk containing 400 μg/mL protein) and control line (anti-rabbit antibodies raised in goat having a protein concentration of 250 μg/mL) were dispensed on the nitrocellulose membrane by a Linomat IV TLC dispenser (Camag, Muttenz, Switzerland). Finally, 5 mm wide strips were cut using a programmable cutter and packed in aluminum pouches (Nefab, Barneveld, The Netherlands) along with silica desiccants (Multisorb Technologies, Inc., NY, USA). The strips were stored at room temperature until use.

### 2.7. Detection of buffalo's milk in cow's milk using CLF assay strip

Cow's milk was spiked with buffalo's milk in the range of 1 to 100%. The fractions were ten times diluted in 100 mM borate buffer (pH 8.8 containing 1% BSA and 0.05% Tween 20). One hundred microliter of diluted sample was dispensed on the sample-cum-conjugate pad of the lateral flow strip that had been placed in a plastic cassette. After 10 min strips were removed from the cassettes and bands at the test and control lines were visually inspected and scanned and the detection limit was assessed as described earlier (Section 2.5).

## 3. Results and discussion

### 3.1. Preparation of polyclonal antibodies against the buffalo's milk protein fraction

The development of LFIA for the detection of buffalo milk in cow milk is summarized in Fig. 1. One of the most important steps in the development of an immunoassay is the preparation of robust and specific antibodies against the analyte of interest. In the past various antigenic fractions of buffalo's milk like skimmed milk, micellar casein, individual caseins (Jairam & Nair, 1980) and sedimented buffalo's milk specific protein fraction (Verma, 2019) were used by different researchers for the production of antisera to develop a test for the detection of buffalo's milk as an adulterant in cow's milk. Agglutination assay was developed for the detection of buffalo milk in cow's milk using these antisera (Verma, 2019). Based on these earlier findings, in the present study, a specific buffalo's milk protein fraction has been used for raising antibodies. This protein fraction was obtained from 30 mL skimmed buffalo's milk by centrifugation at 10,700×*g* for 30 min at 4 °C. The pellet was collected and resuspended in 30 mL water. The protein content in this diluted fraction was found to be 3.0 ± 0.3 mg/mL. This protein fraction of buffalo's milk was used for raising polyclonal antibodies in rabbits. The identification of the specific proteins in this buffalo's milk fraction (pellet) is part of another study.

Antiserum collected after 4 weeks was subjected to agglutination reaction with diluted buffalo's and cow's milk and results are presented in Fig. 2A. The result clearly indicates the agglutination reaction of antiserum with buffalo's milk and not with cow's milk, which confirms the specificity of the antiserum towards buffalo's milk. The agglutination reaction of the antiserum with buffalo's milk occurred within 5 *sec* while cross-reactivity of the antiserum was not observed with cow's milk even up to 15 min. Similar results were also observed in immunodiffusion experiment (Fig. 2B) wherein the formation of precipitating lines of the antiserum with either pure buffalo's milk or its mixture with cow's milk at various levels was observed. This indicates the specific reaction of the antiserum with buffalo's milk. Whereas, no precipitating line was observed with pure cow's milk. Further, it may be noted that the intensity of the precipitating lines decreases with the decreasing quantity of buffalo's milk in the mixture. Following the full immunization schedule of the rabbits, the total pool of antiserum was subjected to protein A affinity chromatography for purification of the IgG fraction and the protein content of the purified antibodies was found to be 1.82 ± 0.05 mg/mL. The quality of these anti-buffalo antibodies was checked by indirect ELISA. For comparison, pre-immune serum and antiserum were also taken and the results are presented in Fig. S1. It is clear that, pre-immune serum had little activity towards the target analyte. On the other hand, in the case of antiserum and purified antibodies, absorbance values were around 0.87 and 0.72, respectively at zero dilution and decreased with dilutions. This indicated that antibodies in the antiserum

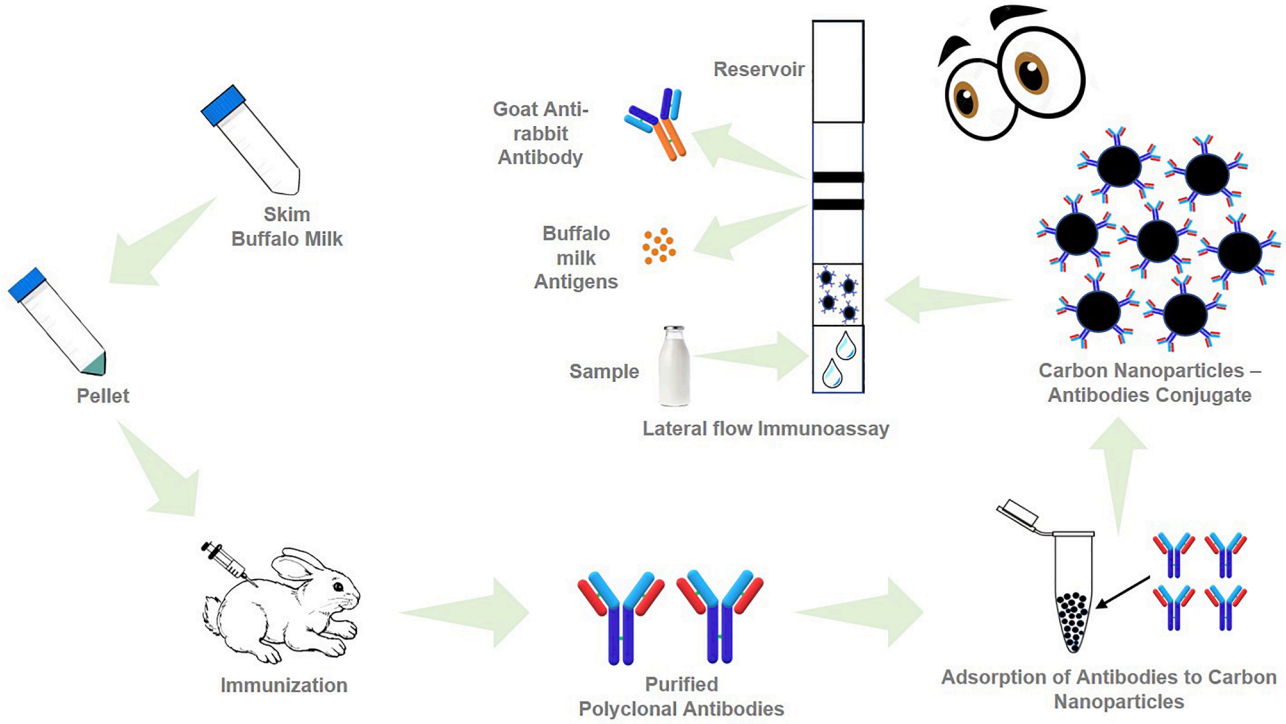


**Fig. 1.** Summary of development of lateral flow immunoassay for detection of buffalo's milk in cow's milk.

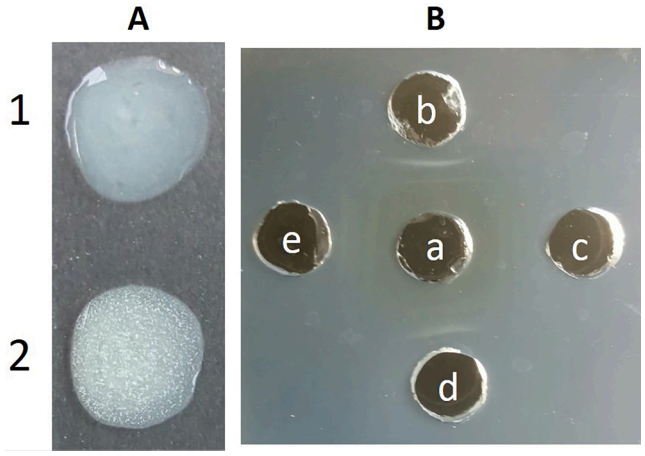


**Fig. 2.** Checking the quality of antiserum. A. Incubation of equal volumes of the antiserum raised in rabbit against the protein fraction isolated from buffalo's milk with diluted (1:10) skimmed milk from cow (1) and buffalo (2) milk. Agglutination can be seen for buffalo's milk, B. Analysis of the interaction between the antiserum raised in rabbit against the protein fraction isolated from buffalo (a) and buffalo's skimmed milk (b), cow's skimmed milk (c), buffalo's skimmed milk mixed with cow's skimmed milk (50:50) (d), and buffalo's skimmed milk mixed with cow's skimmed milk (25:75) (e).

as well as in the affinity purified preparations have reacted with the target analyte i.e. the buffalo's milk protein fraction which had been coated onto the wells of the ELISA plate.

### 3.2. Development of the LFIA for the detection of buffalo's milk in cow's milk

In this study, we developed a competitive format of the LFIA because preliminary results did not provide satisfactory results with the sandwich format. The design of the competitive LFIA was similar to design described earlier (Gautam et al., 2017). One of the main steps in the development of this LFIA was the successful conjugation of antibodies to a coloured label and we have used colloidal carbon in this study. For initial experiments wherein antibodies and antigen loading on CNPs and test line, respectively, were optimized, only lateral flow strips with nitrocellulose membranes and absorbent pads were used, the so called HLF strips (Fig. 3A). To reduce development time, sample pad as well as conjugate pad were not used in these prepared HLF strips. Optimization studies were done in the wells of a microtitre plate containing diluted sample as well as carbon nanoparticles-antibody conjugate (CNP-C). The percent buffalo's milk in cow milk, where the test line was absent, was taken as the sensitivity of the assay. Test line intensities that were significantly less than the intensity of the 100% cow's milk control could be interpreted as adulterated with a percentage between the test significance level and the absolute sensitivity of the test.

#### 3.2.1. Optimization of antibody loading on CNPs using HLF

For the development of the LFIA for the detection of buffalo's milk in cow's milk, antibodies against a specific buffalo's milk protein fraction were conjugated with CNPs. Such CNPs have been used in our laboratory (Wageningen laboratory) from 1993 onwards (van Amerongen et al., 1993) and were reported to have a size range of 160–180 nm (Aktas et al., 2019). The size is larger than the conventionally used AuNPs (±40 nm). CNPs have been reported as a very sensitive label as compared to AuNPs and latex beads amongst other characteristics based on their dark colour against a white background (Gordon & Michel, 2008; Linares, Kubota, Michaelis, & Thalhammer, 2012). Conjugation was done by mixing de-salted antibodies with CNPs in 5 mM borate buffer at pH 8.8. It has been reported that the surface of CNPs is quite hydrophobic in nature (Ciaurriz, Bravo, & Hamad-Schifferli, 2014) and the immobilization of antibodies on the surface of particles can be accomplished by simple physical adsorption that will be followed by rearrangement of the protein molecules enabling stable hydrophobic interactions with the surface (Posthuma-Trumpie et al., 2012). In such non-covalent conjugation, the role of a low ionic strength buffer such as the 5 mM borate buffer at pH 8.8 is important, because it renders the protein molecules unstable, thereby promoting the binding of the

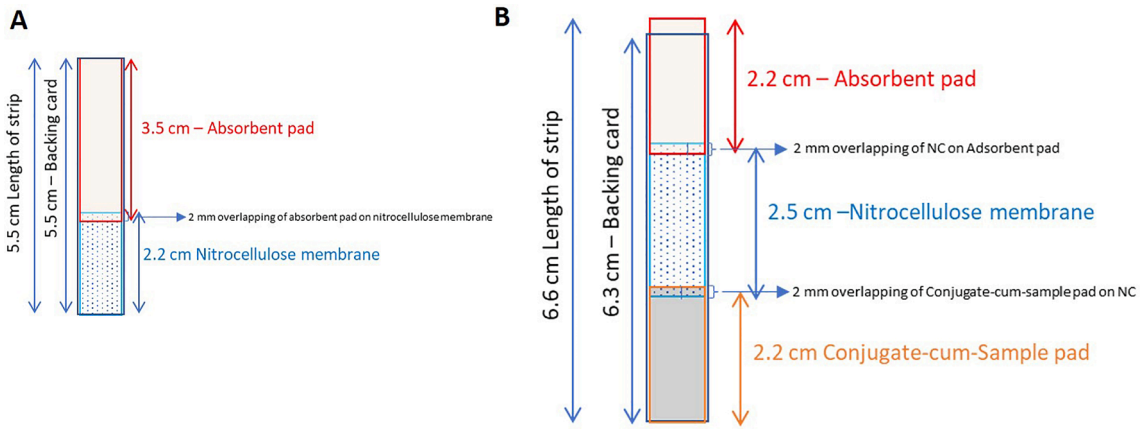


**Fig. 3.** Architecture of the half- (A) and complete (B) lateral flow immunoassay strip.

protein molecules onto the CNPs that have a slight negative surface charge. Using the buffers with high ionic strength will neutralize the negative charge due to the presence of positively charged cations, causing early co-agglutination of the particles. Once a protein molecule is immobilized on the surface, the charge of that protein provides colloidal stability to the CNPs suspension. Earlier publications have also indicated the use of borate buffer at a similar molarity (Aktas et al., 2019; Noguera et al., 2011; Suárez-Pantaleón, Wichers, Abad-Somovilla, van Amerongen, & Abad-Fuentes, 2013). CNP-C once prepared were found to be stable for at least a year at 4 °C in 100 mM borate buffer (pH 8.8) containing 1% BSA. Since in the present competitive design of the LFIA the CNP-C will interact with antigen in the sample as well as on the test line, the quantity of antibodies loaded on CNPs would impact the sensitivity. Thus, the load of antibodies conjugated to the CNPs was optimized by comparing their sensitivity. Three levels of antibody loading on CNPs (350, 175 and 87.5 μg/mL in a 0.2% (w/v) suspension of CNPs) were evaluated and the sensitivity of the LFIA was assessed (Fig. 4A). Although at a particular adulterant percentage the intensity of the test line slightly reduces upon decreasing the amount of adsorbed antibody, the visual detection limit was similar in all cases (≥5%). The CNP-C preparation with 175 μg/mL was selected for further experiments. This was done as a balance between a good intensity of the test line on a strip with 0% adulteration and the attempt to use as less antibody as possible. In all these experiments, a FF80HP nitrocellulose membrane was used and antigen loading on the test line was 1.6 μg/cm. BSA (1%) was used to block unoccupied sites on the CNP-Cs and as the blocking-on-the-run protein while running tests. To be sure that BSA did not interfere with the bovine reagents in the test, we also used OVA (1%) as a blocking protein (Fig. S2). The results show that OVA seem to decrease the competition as is evident from decreased intensity of test line in control as well as in subsequent mixtures vis-à-vis when BSA was used as blocking protein. Further, it may be noted that the decrease in test line intensity was less than 50% in presence of 1% buffalo's milk in cow's milk as compared to pure cow's milk indicating OVA is interfering in the competition. But more than 80% decrease in the intensity was reported with BSA in the similar experiment (Fig. 4A). Therefore, BSA was further used as the blocking protein in the assay.

### 3.2.2. *Optimization of antigen loading on test line using HLF strip*

The antigen loading on the test line was also optimized. In our preliminary experiment, we have used the buffalo's milk protein fraction that was used as the immunogen (section 2.2.1) for creating the test line. However, subsequently, it was observed that even buffalo's skimmed milk can be used as antigen for test line preparation. Since skimmed buffalo's milk is easy to prepare, subsequent experiments were focused on the optimization of the spraying of the skimmed buffalo's milk sample. Skimmed buffalo's milk contains around 4% protein (Hooda, Mann, Sharma, & Bajaj, 2020) and the antigen load on the test line was

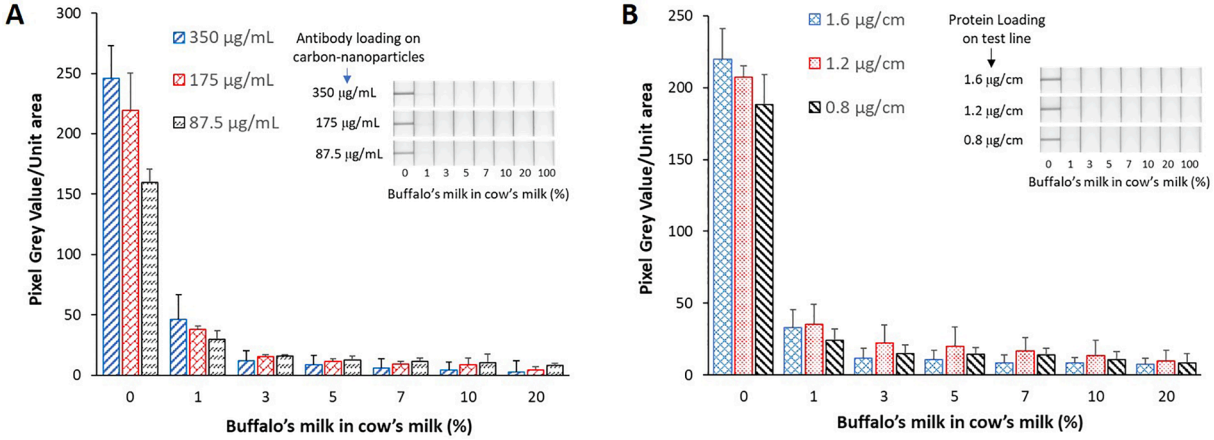


**Fig. 4.** Optimization of antibody loading on carbon nanoparticles (CNPs) and capture antigen loading on test line using half-lateral flow assay strip. A. Anti-buffalo antibodies were conjugated to CNPs at three different levels [350, 175 and 87.5 μg/mL CNPs (0.2%, w/v)] and the antigen loading on the test line was 1.6 μg/cm. The conjugates were evaluated for detection of buffalo's milk in cow's milk using lateral flow immunoassay strips. B. Three levels of the buffalo milk protein antigen was sprayed on the test line (1.6, 1.2 and 0.8 μg/cm) and lateral flow immunoassay strips were evaluated for the detection of buffalo's milk in cow's milk. FF80HP nitrocellulose membranes were used. Line intensities (pixel grey value/unit area) recorded after 1 h (n = 3) are shown and have been subtracted from buffalo's milk test line intensity. Extra sites on CNPs were blocked using bovine serum albumin. The test strips are presented as an insert (top right).

calculated on the basis of the protein content in skimmed milk. While standardizing antibody loading on CNP-C, the test line was made of 1.6 μg/cm antigen (Fig. 4A). For further standardization of antigen load on the test line, three different antigen concentrations were taken, i.e. 1.6, 1.2 and 0.8 μg/cm. The results are presented in Fig. 4B. It may be noted that test line intensity has decreased significantly (>83%) even in the presence of 1% buffalo's milk in all three cases. The intensity decreased to more than 89% in case of milk samples containing 3% buffalo's milk and these lines could hardly be seen by the naked eye. Visually, the test line was not perceptible at 5% adulteration of cow's milk with buffalo's milk, irrespective of the test line antigen loading used. It was decided to choose a capture protein concentration on the test line of 1.6 μg/cm, since the intensity of the test line in control milk sample was sufficiently intense to be visible under field conditions.

### 3.2.3. Construction of the CLF assay strip

The architecture of the CLF assay strip is presented in Fig. 3B. In this design, we have used sample-cum-conjugate pad instead of individual sample and conjugate pads. Sample-cum-conjugate pad was used for convenience purpose as instead of assembling two different pads only one pad is required. As sample-cum-conjugate pad is made of glass fiber with water binder, the material has sufficient water absorption (63 mg/cm$^2$) and wicking rate (1.5 s/cm). During our initial experiment, sample-cum-conjugate pad of different grade (water absorption: 46 mg/cm$^2$ and wicking rate 6 s/cm) was also studied, however, it did not provide the satisfactory results mainly due to a delayed response. The sample-cum-conjugate was pre-treated with coating buffer containing 100 mM borate buffer containing 1% (w/v) BSA and was sprayed with diluted CNP-C (0.02%, w/v). The diluent used was borate buffer (100 mM, pH 8.8) containing 1% BSA & 3% trehalose. The purpose of trehalose is to preserve the antibodies during the shelf-life of the strips, mainly by lowering the water activity in the environment by acting as a humectant. Further, the presence of trehalose in the environment serves to smoothen the re-suspension of the dry CNP-C. In literature, both trehalose as well as sucrose has been widely used in the conjugate pad preparation (van Amerongen et al., 2018). Test line and control line were drawn towards the distal end of the strip; 3 mm apart. An absorbent pad with good water absorption was able to accommodate the 100 μL of diluted milk sample. All the components of the lateral flow strip were assembled on the backing card and strips were cut at 0.5 cm width.

Commercially, various types of nitrocellulose membranes are available having different properties in terms of flow speed and surfactant content, factors that have their impact on test parameters such as duration and sensitivity. In this study, three types of nitrocellulose membranes were used with different characteristics, FF80HP and FF120HP and UniSart CN140. Selection of a nitrocellulose membrane was done after optimization of two other critical parameters i.e. antibody loading on the CNPs and antigen loading on the test line. In choosing the right nitrocellulose membrane to be used, speed and sensitivity parameters were taken into consideration. The time of appearance as well as the intensity of the test line was recorded at fixed intervals for 20 min. The appearance of the control line reached a maximum intensity within 10 min in all cases. The results are presented in Fig. 5 and indicated that with all the combinations, the definite presence of buffalo's milk in cow's milk, i.e. no visible line, could be detected at ≥to 5%. The intensity of the test line decreased to more than 68% in all cases on admixing of 1% buffalo's milk in cow's milk. In the case of 3% buffalo's milk in cow's milk, the intensity of the test line decreased further to around 14% of the control value (pure cow's milk). On visual inspection, it was observed that the test line completely disappeared when buffalo's milk equal to or at more than a 5% level was admixed in cow's milk in all cases. This also demonstrates the robustness of the design and fact that the application of antigen on test line and loading of antibodies on conjugate was optimum. Finally, we have selected the FF120HP membrane (Fig. 5) as the test line intensity was brightest in case of control milk samples as compared to other membranes. The bright test line in control milk would be advantageous under field conditions (Fig. 6).

We found that 100 μL of 10 times diluted sample with 100 mM borate buffer (pH 8.8 containing 1% BSA and 0.05% Tween 20) provided satisfactory results. It was expected that the presence of fat globules in milk could affect the results as indicated from previous publications (Gautam et al., 2017; Lata et al., 2016; Naik et al., 2017). In these publications the use of direct skimmed milk has been recommended for application on lateral flow strips. This is mainly due to the hindrance created by fat globules in the flow through the nitrocellulose membrane. Since in the present work, ten times diluted milk samples have been used, no such obstruction of flow was observed and this will certainly be an advantage while using the strip under field conditions. To check the robustness of the method, heated milk samples (85 °C for 1 min) were also analyzed for detection of the presence of buffalo's milk in cow's milk. It appeared that the sensitivity of the developed LFIA is not

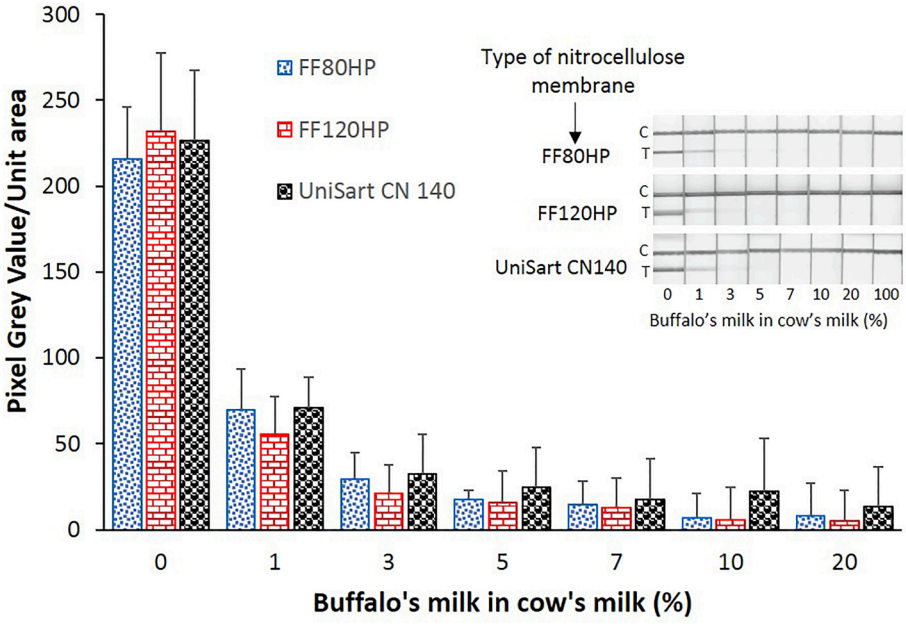


**Fig. 5.** Optimization of type of nitrocellulose membrane on lateral flow immunoassay strips. Three different types of commercially available nitrocellulose membranes (FF80HP, FF120HP and UniSart CN 140) were evaluated for the detection of buffalo's milk in cow's milk. Decrease in test line intensities may be noted with the increasing concentration of buffalo's milk in milk samples. Loading of anti-buffalo antibodies loading on carbon nanoparticle conjugates was 175 μg/mL (0.2%, w/v). Antigen loading on test line was 1.6 μg/cm. Following removal of the strips from the cassettes (after 1 h), line intensities were recorded (n = 5) and are shown as pixel grey value/unit area corrected for 100% buffalo's milk control. The test strips are presented as an insert (top right).

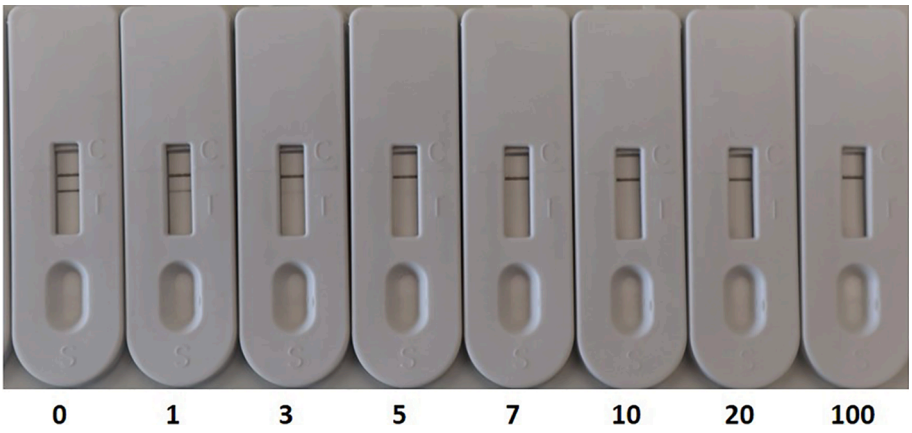


**Fig. 6.** Results with optimized lateral flow immunoassay strip in cassette format for the detection of buffalo's milk in cow's milk. Decrease in test line intensities may be noted with the increasing concentration of buffalo's milk in milk samples. Test line intensity is completely abolished in presence of 5% buffalo's milk in cow milk. Strips were prepared using FF120HP nitrocellulose membrane, antigen loading on test line was 1.6 μg/cm and anti-buffalo antibody was conjugated to carbon nanoparticles at 175 and μg/mL (0.2%, w/v) level. Extra sites on CNPs were blocked using bovine serum albumin. Numeric values indicate buffalo's milk mixed with cow's milk (%).

affected (Fig. S3) on its application in heated milk samples indicating the robustness. This indicated that the antigenic proteins in the sample are still recognized by the antibody molecules in the specific IgG fraction. Thus, the developed test can also be applied for examining such adulteration in pasteurized milk. Although, we have not done the experiment by subjecting mixture of buffalo's milk and cow's milk to UHT conditions, but the applicability of test on UHT cow milk has been verified in commercial samples (data not presented). Further, the developed strips have been validated with milk obtained from at least two breeds of buffalo's milk (Murrah and Nili Ravi), three bovine breeds (Sahiwal, Tharparkar, Karan Fries), blind samples (250 samples) and market cow milk samples. Results were satisfactory and in case of blind samples, 100% accuracy was observed (data not presented). Beside this, the strips have also been tested with goat milk and it was found that presence of goat milk did not interfere with the results. The properly sealed strips in aluminium pouches were stable for at least 20 weeks at ambient temperature.

## 4. Conclusion

In the present study, a lateral flow immunoassay with colloidal carbon nanoparticles as the signal label was developed for the detection of buffalo's milk in cow's milk. The method is rapid as well as sensitive enough for its application under field conditions to segregate adulterated milk in milk supplies (sensitivity from 1% and higher). Although a specific protein fraction of buffalo's milk was used for the development of polyclonal antibodies, it was demonstrated that skimmed buffalo's milk can be used as the capture ligand at the test line. The application of non-immunoglobulin antigen allows the application of assay even in heated milks samples.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Acknowledgements

This work was supported by National Agricultural Higher Education Project, Indian Council of Agricultural Research, New Delhi, India.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2021.129311.

## References

Aktas, G. B., Wichers, J. H., Skouridou, V., van Amerongen, A., & Masip, L. (2019). Nucleic acid lateral flow assays using a conjugate of a DNA binding protein and carbon nanoparticles. *Microchimica Acta, 186*(7), 426. https://doi.org/10.1007/s00604-019-3544-0.

Aneja, R. P., Mathur, B. N., Chandan, R. C., & Banerjee, A. K. (2002). Technology of Indian milk products: Handbook on process technology modernization for professionals, entrepreneurs and scientists. *Technology of Indian milk products: Handbook on process technology modernization for professionals, entrepreneurs and scientists*. Delhi: Dairy India Yearbook. https://www.cabdirect.org/cabdirect/abstract/20033113926.

Anfossi, L., Baggiani, C., Giovannoli, C., Biagioli, F., D'Arco, G., & Giraudi, G. (2013). Optimization of a lateral flow immunoassay for the ultrasensitive detection of aflatoxin M1 in milk. *Analytica Chimica Acta, 772*, 75–80. https://doi.org/10.1016/j.aca.2013.02.020.

Arora, S., & Khetra, Y. (2017). Chapter 42 – Buffalo milk cheese. In P. L. H. McSweeney, P. F. Fox, P. D. Cotter, D. W. Everett, P. L. H. McSweeney, P. F. Fox, … D. W. Everett (Eds.), *Cheese* (4th ed., pp. 1093–1101). San Diego: Academic Press. https://doi.org/10.1016/B978-0-12-417012-4.00042-9.

Ciaurriz, P., Bravo, E., & Hamad-Schifferli, K. (2014). Effect of architecture on the activity of glucose oxidase/horseradish peroxidase/carbon nanoparticle conjugates. *Journal of Colloid and Interface Science, 414*, 73–81. https://doi.org/10.1016/j.jcis.2013.09.039.

Report, D. A. H. D. (2019). *Annual Report 2018–19*. New Delhi, India: Department of Animal Husbandry, Dairying and Fisheries Ministry of Agriculture & Farmers Welfare Government of India.

Das, S. (2015). Milking the value chain. Business Standard. https://www.business-standard.com/article/management/milking-the-value-chain- 115072801372_1.html/Accessed 28 May 2020.

De, S., Brahma, B., Polley, S., Mukherjee, A., Banerjee, D., Gohaina, M., … Goswami, S. L. (2011). Simplex and duplex PCR assays for species specific identification of cattle and buffalo milk and cheese. *Food Control, 22*(5), 690–696. https://doi.org/10.1016/j.foodcont.2010.09.026.

European Union Regulations (2018). Commission implementing regulation (EU) 2018/150 – of 30 January 2018 – amending Implementing Regulation (EU) 2016/1240 as regards methods for the analysis and quality evaluation of milk and milk products eligible for public intervention and aid for private storage. 34.

Regulations, F. S. S. A. I. (2011). *Food safety and standards (food products standards and food additives) regulations, 2011*. Government of India, New Delhi, India: Ministry of Health and Family Welfare. https://www.fssai.gov.in/upload/uploadfiles/files/Food_Additives_Regulations.pdf.

Galan-Malo, P., Mendiara, I., Razquin, P., & Mata, L. (2018). Validation of a rapid lateral flow method for the detection of cows' milk in water buffalo, sheep or goat milk. *Food Additives & Contaminants: Part A, 35*(4), 609–614. https://doi.org/10.1080/19440049.2018.1426886.

Gautam, P. B., Sharma, R., Lata, K., Rajput, Y. S., & Mann, B. (2017). Construction of a lateral flow strip for detection of soymilk in milk. *Journal of Food Science and Technology, 54*(13), 4213–4219. https://doi.org/10.1007/s13197-017-2890-3.

Genis, D. O., Sezer, B., Bilge, G., Durna, S., & Boyaci, I. H. (2020). Development of synchronous fluorescence method for identification of cow, goat, ewe and buffalo milk species. *Food Control, 108*, 106808. https://doi.org/10.1016/j.foodcont.2019.106808.

Gordon, J., & Michel, G. (2008). Analytical sensitivity limits for lateral flow immunoassays. *Clinical Chemistry, 54*(7), 1250–1251.

Hooda, A., Mann, B., Sharma, R., & Bajaj, R. (2020). Physicochemical characterisation of native micellar casein concentrates from buffalo and cow skim milk harvested using microfiltration. *International Journal of Dairy Technology, 73*(4), 781–789. https://doi.org/10.1111/idt.v73.410.1111/1471-0307.12711.

ICICI Securities (2018). ISec Report: Rise of premiumisation in milk consumption. Company data, ICICI Securities.http://content.icicidirect.com/mailimages/DairySector-2018.htm. Accessed 12 May 2020.

ISO 8968-4 (2016). Milk and milk products — Determination of nitrogen content — Part 4: Determination of protein and non-protein nitrogen content and true protein content calculation (Reference method). https://www.iso.org/obp/ui/#iso:std:iso:8968:-4:ed-2:v1:en/Accessed July 18, 2020.

Jairam, B. T., & Nair, P. G. (1980). Modified procedure for production of Hansa test serum. I. Trials with various components of buffalo milk with or without adjuvants and methods of immunization. *Indian Journal of Dairy Science, 33*(1), 51–56.

Lata, K., Sharma, R., Naik, L., Rajput, Y. S., & Mann, B. (2016). Lateral flow assay-based rapid detection of cephalexin in milk: Cephalexin detection in milk by lateral flow. *Journal of Food Quality, 39*(1), 64–73. https://doi.org/10.1111/jfq.12175.

Linares, E. M., Kubota, L. T., Michaelis, J., & Thalhammer, S. (2012). Enhancement of the detection limit for lateral flow immunoassays: Evaluation and comparison of bioconjugates. *Journal of Immunological Methods, 375*(1–2), 264–270. https://doi.org/10.1016/j.jim.2011.11.003.

Liu, B., Si, J., Zhao, F., Wang, Q., Wang, Y., Li, J., Li, C., & Li, T. (2019). Rapid detection of cow milk adulteration/contamination in goat milk by a lateral flow colloidal gold immunoassay strip. *Journal of Dairy Research, 86*(1), 94–97. https://doi.org/10.1017/S0022029919000116.

Miller, T. J., & Stone, H. O. (1978). The rapid isolation of ribonuclease-free immunoglobulin G by protein A-sepharose affinity chromatography. *Journal of Immunological Methods, 24*(1), 111–125. https://doi.org/10.1016/0022-1759(78)90092-3.

Naik, L., Sharma, R., Mann, B., Lata, K., Rajput, Y. S., & Surendra Nath, B. (2017). Rapid screening test for detection of oxytetracycline residues in milk using lateral flow assay. *Food Chemistry, 219*, 85–92. https://doi.org/10.1016/j.foodchem.2016.09.090.

Noguera, P., Posthuma-Trumpie, G. A., van Tuil, M., van der Wal, F. J., de Boer, A., Moers, A. P. H. A., & van Amerongen, A. (2011). Carbon nanoparticles in lateral flow methods to detect genes encoding virulence factors of Shiga toxin-producing Escherichia coli. *Analytical and Bioanalytical Chemistry, 399*(2), 831–838. https://doi.org/10.1007/s00216-010-4334-z.

Posthuma-Trumpie, G. A., Wichers, J. H., Koets, M., Berendsen, L. B. J. M., & van Amerongen, A. (2012). Amorphous carbon nanoparticles: A versatile label for rapid diagnostic (immuno)assays. *Analytical and Bioanalytical Chemistry, 402*(2), 593–600. https://doi.org/10.1007/s00216-011-5340-5.

Sharma, R., Rajput, Y. S., & Barui, A. K. (2013). Rapid methods for detection of adulteration in milk - present status. *Indian Dairyman, 65*(4), 99–102.

Suárez-Pantaleón, C., Wichers, J., Abad-Somovilla, A., van Amerongen, A., & Abad-Fuentes, A. (2013). Development of an immunochromatographic assay based on carbon nanoparticles for the determination of the phytoregulator forchlorfenuron. *Biosensors and Bioelectronics, 42*, 170–176. https://doi.org/10.1016/j.bios.2012.11.001.

Ullah, R., Khan, S., Ali, H., & Bilal, M. (2020). Potentiality of using front face fluorescence spectroscopy for quantitative analysis of cow milk adulteration in buffalo milk. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy, 225*, 117518. https://doi.org/10.1016/j.saa.2019.117518.

Upadhyay, N., & Nara, S. (2018). Lateral flow assay for rapid detection of Staphylococcus aureus enterotoxin A in milk. *Microchemical Journal, 137*, 435–442. https://doi.org/10.1016/j.microc.2017.12.011.

van Amerongen, A., Veen, J., Arends, H. A., & Koets, M. (2018). Lateral Flow Immunoassays. In S. K. Vashist, J. H. T. Luong, S. K. Vashist, & J. H. T. Luong (Eds.), *Handbook of Immunoassay Technologies* (pp. 157–182). Massachusetts: Academic Press. https://doi.org/10.1016/B978-0-12-811762-0.00007-4.

van Amerongen, A., Wichers, J. H., Berendsen, L. B. J. M., Timmermans, A. J. M., Keizer, G. D., van Doorn, A. W. J., … van Gelder, W. M. J. (1993). Colloidal carbon particles as a new label for rapid immunochemical test methods: Quantitative computer image analysis of results. *Journal of Biotechnology, 30*(2), 185–195. https://doi.org/10.1016/0168-1656(93)90112-Z.

Verma, A. (2019). Detection of buffalo milk in cow milk using Hansa test serum. In R. Sharma, A. K. Singh, R. Singh, R. Sharma, A. K. Singh, & R. Singh (Eds.), *Technologies available at NDRI for Commercialization* (p. (p. 47).). Karnal, India: Institute Technology Management Unit, ICAR-National Dairy Research Institute.

Wang, C., Peng, J., Liu, D.-F., Xing, K.-Y., Zhang, G.-G., Huang, Z., … Lai, W.-H. (2018). Lateral flow immunoassay integrated with competitive and sandwich models for the detection of aflatoxin M1 and Escherichia coli O157:H7 in milk. *Journal of Dairy Science, 101*(10), 8767–8777. https://doi.org/10.3168/jds.2018-14655.

Wang, Y., Deng, R., Zhang, G., Li, Q., Yang, J., Sun, Y., … Hu, X. (2015). Rapid and sensitive detection of the food allergen glycinin in powdered milk using a lateral flow colloidal gold immunoassay strip test. *Journal of Agricultural and Food chemistry, 63*(8), 2172–2178. https://doi.org/10.1021/jf5052128.

Zhong, Y., Chen, Y., Yao, L., Zhao, D., Zheng, L., Liu, G., … Chen, W. (2016). Gold nanoparticles based lateral flow immunoassay with largely amplified sensitivity for rapid melamine screening. *Microchimica Acta, 183*(6), 1989–1994. https://doi.org/10.1007/s00604-016-1812-9.