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Are camel milk proteins convenient to the nutrition of cow milk allergic children?

Elsayed I. El-Agamy[a,*], Mohsen Nawar[a], Sherif M. Shamsia[b], Sameh Awad[a], George F.W. Haenlein[c]

[a] Department of Dairy Science, Faculty of Agriculture, Alexandria University, El-Shatby, Alexandria, Egypt
[b] Department of Food & Dairy Science and Technology, Faculty of Agriculture (Damanhour), Alexandria University, Egypt
[c] Department of Animal & Food Science, University of Delaware, Newark, DE 19717-1303, USA

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ABSTRACT

Camel, cow and human milk proteins were prepared and analyzed by two different gel electrophoretic techniques. The immunological cross-reactivity between camel and cow milk proteins was tested using immunoblotting and enzyme-linked immunosorbent assay (ELISA) techniques. Camel milk proteins have unique electrophoretic patterns that are completely different from cow and human milk proteins. When specific antisera to camel milk proteins were applied in immunoblotting (Western blot) analysis, results showed the absence of immunological cross-reactivity between camel and cow milk proteins. Similar results were obtained when sera from some children allergic to cow milk were tested for the specificity of their immunoglobulin E (IgE) to camel milk proteins. The study concluded that the absence of immunological similarity between camel and cow milk proteins can be considered an important criterion from the nutritional and clinical points of view, since camel milk may be suggested as a new protein source for nutrition for children allergic to cow milk and can be used as such or in a modified form.

1. Introduction

The best nutritional option for newborn infants is mother's milk; however, some infants may not be exclusively breast fed during the first months of life. In that case, another substitute or alternative must be provided as cow milk. This substitution results in an allergic disease known as cow milk protein allergy (CMPA) in 2–6% of children (El-Agamy, 2007). Nowadays, most common alternatives are soy and extensively hydrolyzed milk proteins formulae (El-Agamy, 2007). However, there is evidence that 10–20% of children allergic to cow milk do not tolerate soy derivatives (Businco et al., 1992; Maldonado et al., 1998; Zeiger et al., 1999) and some cases of high immunological reaction to extensively hydrolyzed formulae have been reported (Businco et al., 1989; Sampson et al., 1992; de Boissieu and Dupont, 2002). Meanwhile, several international studies (Spuergin et al., 1997; Vereda et al., 2006; Duarte et al., 2008; Shamsia et al., 2008) have shown that some infants and children who are allergic to cow milk will also suffer an allergic reaction to buffalo, goat, sheep, donkey and mare milk proteins due to the presence of positive immunological cross-reaction with their counterparts in cow milk. On these bases, the identification of a suitable protein source for children allergic to cow milk represents an important goal for both nutritionists and pediatricians. According to FAO statistics, there are about 19 million camels in the world. Nowadays, camel milk production is in progress in many countries in both Asia and Africa due to increased demand. Pasteurized milk and other dairy products made from camel milk are available in the markets in Gulf area and Mauritania (El-Agamy, 2006). Therefore, the present

* Corresponding author. Tel.: +20 3 590 8338; fax: +20 3 590 8338.
E-mail address: elsayed_elagamy@yahoo.com (E.I. El-Agamy).

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study aims at evaluating the suitability of camel milk for the nutrition of children allergic to cow milk by studying the antigenic characteristics of camel milk proteins and their immunological cross-reactivity with cow milk proteins.

2. Materials and methods

2.1. Milk samples

Seventy individual camel (*Camelus dromedaries*) milk samples, representing lactation period of first to sixth month after parturition, were obtained from 10 farms at El-Alamin, El-Saloom and Bourg El-Arab areas around Alexandria, similar number of Holstein cow milk samples were collected from the herd of Faculty of Agriculture, Alexandria University, Egypt. Thirty human milk samples were collected from healthy volunteer women at Alexandria, Egypt.

2.2. Human sera

Sera were prepared from blood samples (2–5 ml) of 40 children aged 6 months to 8 years. The allergic subjects were chosen according to their family history, positive skin prick test (SPT) and assay of serum total and specific IgE . The subjects had signs and symptoms of allergy after ingestion of cow milk or its products, such as rash, urticaria, wheezing, vomiting, angioedema, gastroesophageal reflux, colic, diarrhea and abdominal pain. Symptoms disappeared after 2–3 days of elimination of cow milk or its products from diets. Children were hospitalized at El-Shatby children hospital, Alexandria University, Egypt.

2.3. Animals

Six New Zealand White rabbits were obtained from the farm of Faculty of Agriculture, Alexandria University, Egypt.

2.4. Chemicals

Protein marker (low molecular weight, 14.4–97 kDa), ultrapure agarose, nitrocellulose membrane (0.45 μm), Tricine buffer and all chemicals used in gel electrophoresis were from Bio-Rad (Richmond, CA 94804, USA). Polyvalent antiserum of goat anti-rabbit IgG or rabbit anti-human IgE labeled with horse radish peroxidase, 3,3′-diaminobenzidine, *O*-phenylendiamine, H_2O_2 (30%), Tween 20, Freund's adjuvants were from Sigma (St. Louis, MO 63178, USA). Normal calf rennet was obtained from Dairy Pilot Plant, Alexandria University, Egypt.

2.5. Preparation of caseins

Pooled samples of whole camel milk were skimmed by centrifugation at 5000 × *g* for 20 min at 4 °C. Casein was prepared from skim milk by precipitation with 1 M HCl (Swaisgood, 1992). Prepared casein was kept at −30 °C until analysis.

2.6. Preparation of whey proteins

Pooled samples of skimmed milk were warmed to 40 ° and renneted (1 ml/l). Clear whey was obtained by centrifu gation at 10,000 × *g* for 15 min at 4 °C, then concentrate by polyethyleneglycol (20,000 K) and stored at −30 °C unt used (Marshall, 1982).

2.7. Alkaline native-polyacrylamide gel electrophoresis

Milk proteins were diluted 1: 3 (v/v) with buffer 0.05 M Tris–HCl, pH 6.8, then mixed in the ratio 1:1 (v/v) with sam ple buffer 0.5 M Tris–HCl, pH 6.8, containing glycerol (7.5% bromophenol blue (0.5%) and subjected to electrophoresi (Hames and Rickwood, 1990). The running buffer consiste of 0.192 M glycine and 0.025 M Tris. Runs were carried out a 150 V until the end of electrophoresis. Electrophoresis wa performed using Mini-Protean II cell (Bio-Rad) and protei bands were stained in the gels using Coomassie blue R-25 (0.1%).

2.8. Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Milk proteins were diluted in the same manner as thos of native-polyacrylamide gel electrophoresis (PAGE) an diluted samples were mixed in the ratio 1:1 (v/v) with sam ple buffer 0.5 M Tris–HCl, pH 6.8, containing glycerol (7.5% sodium dodecyl sulphate (SDS) (2%), β-mercaptoethanc (5%) and bromophenol blue (0.5%) and subjected to hea in a boiling water bath at 100 °C for 10 min. Sample were cooled at room temperature, centrifuged at 10,000 × for 10 min to remove any insoluble material, and the loaded onto the gel using the discontinuous buffer syster (Laemmli, 1970). The running buffer consisted of 0.192 M glycine, 0.025 M Tris and SDS (0.1%). Runs were carrie out at 125 V in stacking gel then increased to 175 V unt the end of electrophoresis. Electrophoresis was performe using Mini-Protean II cell (Bio-Rad) and protein bands wer stained in the gels using Coomassie blue R-250 (0.1%).

2.9. Protein molecular mass determination

Molecular masses (kDa) of separated proteins on SDS PAGE were determined according to the method describe by Weber and Osborn (1969) using the standard protei marker.

2.10. Antisera production (immunization)

Polyvalent antisera to camel milk proteins were pre pared according to the procedure described by Clause (1988). Rabbits were first immunized, with 0.5 ml of anti gen (5 mg/ml sterile NaCl, 0.9%) in suspension with 0.5 m complete Freund's adjuvant by intramuscular injection i several sites at week 1. At weeks 3 and 5, each anima was injected intradermaly with a booster dose 0.5 ml o antigen (1 mg/ml) in suspension with 0.5 ml incomplet Freund's adjuvant. The sera were tested for antibody pro duction before the third immunization. The animals wer bled about 14 days after the last immunization. Blood wa

taken from rabbits and the antiserum titre was measured using immunodiffusion technique of Jurd (1981). Antisera were stored at −30 °C until used.

2.11. Immunoblotting (Western blot)

After SDS-PAGE, transfer of separated proteins from the gel onto the nitrocellulose membrane (0.45 μm, Bio-Rad) was achieved by electrophoretic elution using a 0.025 M Tris, 0.192 M glycine and methanol (200 ml/l) at 100 V for 1 h with Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). To verify the protein transfer, the gels were stained by Coomassie blue R-250. The blotted membranes were blocked with gelatin (1%) and washed three times with Tris-buffered saline (0.05 M Tris, 0.15 M NaCl). Membranes were then incubated overnight at room temperature with polyvalent antiserum to camel casein or whey proteins (WPs) diluted 1:10 with Tris-buffered saline. To detect the antigen–IgG complex, a second incubation was then carried out with polyclonal antiserum of goat anti-rabbit–IgG peroxidase conjugate diluted 1:1000 with Tris-buffered saline. Color developed in the presence of H_2O_2 with 3,3′-diaminobenzidine as a substrate (Holen et al., 2001). All the washing steps used Tris-buffered saline containing Tween 20 (2 ml/l).

2.12. Enzyme-linked immunosorbent assay

IgE-ELISA was performed as described by Holen et al. (2001) using 96-well, round-bottom, microtitre plates (Falcon Laboratory ware, CA 93030, USA). Plates were coated with 50 μl per well of 20 μg/ml of cow or camel (casein or WP). Fifty microlitres of serum sample from each patient were added. Polyclonal antiserum of rabbit anti-human IgE (Fc specific) horseradish peroxidase conjugate (Sigma) was added to each well. The reaction was developed with *O*-phenylendiamine–H_2O_2 (Sigma). Absorbance was measured at 490 nm in a Titertek Multiskan spectrophotometer. Healthy non-allergic individuals were included as controls. ELISA inhibition was expressed as (absorbency of control − absorbency of Ag/Ab complex/absorbency control) × 100.

3. Results

3.1. Gel electrophoresis of milk proteins

Native-PAGE electrophoretic patterns of camel, cow and human milk caseins (Fig. 1A) showed that each type of casein has a unique electrophoretic pattern. Camel and cow milk caseins showed the appearance of three fractions differ in their migration positions. Human casein was separated also into three fractions, which differed markedly from those of camel or cow casein with respect to their migration positions. One of these fractions was the major (β-CN) fraction and other two were minor fractions, as indicated by small arrows. Different casein fractions were identified on the gel as described in our previous study (El-Agamy et al., 1997). Cow milk β-CN and αs-CN were the fastest, whereas human caseins were the slowest in migration on the gel. This feature reveals the differences in types

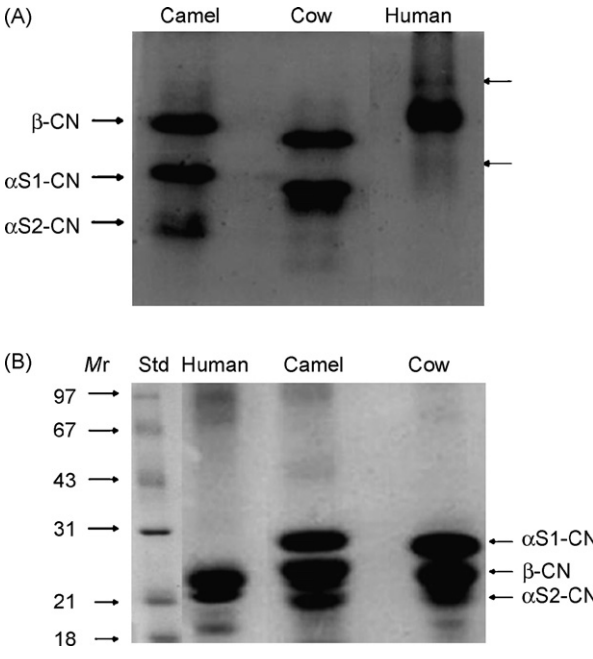

Fig. 1. (A) Alkaline native-PAGE of acid camel, cow and human milk caseins. Anode is toward bottom of photograph. (B) SDS-PAGE of acid camel, cow and human milk caseins. Std: standard protein marker. Anode is toward bottom of photograph.

and density of the charges among the three types of milk caseins. After applying SDS-PAGE, the molecular masses of β-CN, α_{s1}-CN and α_{s2}-CN were estimated at 26, 28, and 23.6 and 24, 25.5, and 22.9 kDa for camel and cow milk caseins, respectively. For human β-CN and α_{s1}-CN, the molecular masses were 23 and 21.6 kDa, respectively (Fig. 1B). The study by Kappeler (1998) showed that molecular masses of camel α_{s1}-CN, α_{s2}-CN and β-CN are 24.8, 22 and 24.9, respectively.

Alkaline native-PAGE of WPs prepared from the three types of milk is shown in Fig. 2A. The electrophoretic patterns showed also the distinguished differences among different WPs.

α-Lactalbumin (α-la) in both cow and human milk had about the same migration position, but had faster migration in camel milk. Neither camel nor human WPs pattern had β-lactoglobulin (β-lg), whereas its band was dominant in cow milk WPs. It was noticed that cow milk β-lg separated into two different genetic variants, as indicated by small arrows. A study on molecular bases by Kappeler (1998) revealed that camel milk is free of β-lg. SDS-PAGE electrophoretic pattern (Fig. 2B) showed that human WPs were characterized by the presence of high-intensity α-la and lactoferrin bands, whereas α-la and blood serum albumin (BSA) bands were dominant in camel milk. The molecular masses of WPs were estimated after separation on SDS-PAGE (Fig. 2B). Camel α-la was estimated at 15 versus 14.4 kDa for cow and human α-la. The molecular mass of camel, cow and human BSA was estimated at 66.2 kDa. It was reported that camel BSA has a molecular mass of 66 kDa (Farah, 1993) and bovine BSA has a molecular mass of 66.2 kDa (El-Agamy et al., 1996).

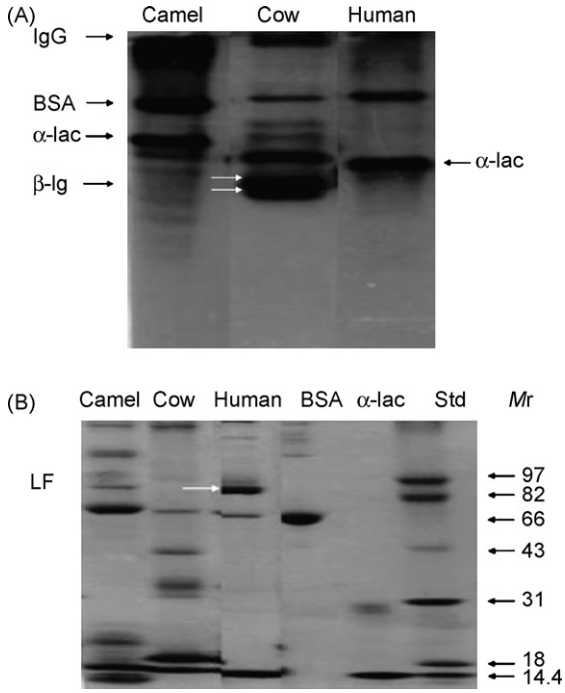

Fig. 2. (A) Alkaline native-PAGE of camel, cow and human milk whey proteins. Anode is toward bottom of photograph. (B) SDS-PAGE of camel, cow and human milk whey proteins. LF; lactoferrin; α-la: α-lactalbumin; BSA: bovine serum albumin. Std: standard protein marker. Anode is toward bottom of photograph.

In order to evaluate the antigenic relationship between camel and cow milk proteins, different immunological techniques were applied. When specific antiserum to camel milk caseins was used in Western blot (Fig. 3A), it was noticed that all fractions of camel milk casein were detected on the nitrocellulose membrane, whereas no detection of any band belonging to cow milk caseins was observed. This result revealed the antigenic dissimilarities between both types of casein due to their complete structural differences. Similarly, very limited immunological cross-reactivity between camel and cow milk WPs was found (Fig. 3B). The compositional and structural differences

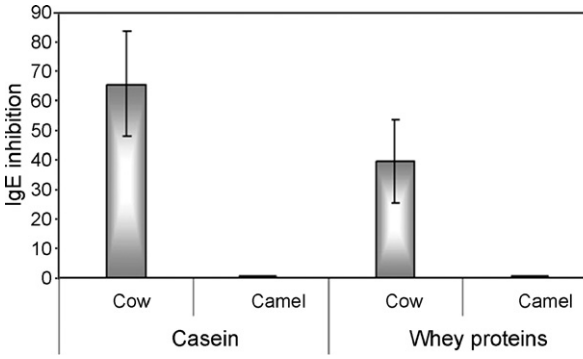

Fig. 4. IgE-ELISA inhibition of cow and camel milk proteins.

between casein fractions (Farah, 1993; Kappeler, 1998) an WPs (Farah, 1993; El-Agamy et al., 1996; Kappeler, 1998) c camel and cow milk proteins were established.

To investigate whether IgE from allergic children rec ognized epitopes on camel milk proteins, we set up ELIS inhibition experiments, using cow or camel milk casein an WPs as the antigens. Results obtained by ELISA inhibitio are shown in Fig. 4. Cow milk proteins were able to cause high level of IgE inhibition with all the sera tested, rangin from 41 to 83% (mean 65.8%) and 23–59% (mean 39.8%) fo casein and WPs, respectively. On the contrary, when came milk proteins were tested as inhibitors, the IgE reactivit against casein and WPs was zero.

4. Discussion

For all newborn infants, mothers' milk will alway be the ideal nutrition because it best ensures health short- and long-term development as well as enhance the immune functions and is hypoallergenic (Wold an Adlerberth, 1998). However, some infants may not be exclu sively breastfed during the first months of life, potentiall leading to a reduction in overall health status and the earl onset of allergic diseases in some infants (Exl, 2001). Th present study is a trial to gather more information about th suitability of camel milk for cows' milk allergic children. I order to pursue such goals, the molecular and immunolog

Fig. 3. (A) SDS-PAGE followed by immunoblotting (Western blot) of camel and cow milk caseins. Lanes 1–3: camel milk casein; lane 4: cow milk casei Polyvalent antiserum to camel casein was applied in the blot. (B) SDS-PAGE followed by immunoblotting (Western blot) of camel and cow-milk whe proteins. Lanes 1–3: camel milk whey proteins; lane 4: cow milk whey proteins. Polyvalent antiserum to camel whey proteins was applied in the blot.

cal similarities between camel and cow milk proteins were studied.

The electrophoretic analyses of camel, cow and human milk revealed that milk proteins in each type of milk have their own characteristics due to their distinguished behavior in migration positions and molecular masses. On the bases of this behavior, it is expected that the amino acid and structural composition will be different. These results are in agreement with those reported by Farah (1993) and Kappeler (1998). On the other hand, although camel and cow milk caseins showed the appearance of equal fractions on the native-PAGE gel, variations in migration behavior of all fractions were observed. This mainly reflects the charge differences between both types of caseins. Farah and Farah-Riesen (1985) reported that camel casein was separated into three main fractions on native-PAGE gel and these fractions are similar in number to those of cow milk casein but different in migration positions. It is interesting that the electrophoretic patterns of camel and cow caseins showed equality of β-CN and αs-CN fractions in their intensities. On the contrary, human casein pattern revealed the dominance of β-CN. The study by Kroening et al. (1998) showed that human casein is mainly β-CN and αs-CN is present in very low ratio. The high ratio of β-CN in human milk casein reflects its higher digestibility rate in the infant's gut, since human milk β-CN is more sensitive to peptic hydrolysis than αs-CN (Abou-Soliman, 2005). Meanwhile, it is taken into account that the higher the ratio of αs-CN in cow milk, the higher the incidence of allergy (hypersensitivity reaction) in children (Taylor, 1986). Therefore, the hypoallergenicity of human milk is due, at least in part, to the high ratio of β-CN and low ratio of αs-CN. The electrophoretic patterns revealed that β-lg is present in cow milk but absent in both camel and human milk. Kappeler (1998) reported that camel milk is free of β-lg, which is considered one of the major antigens of cow milk proteins responsible for the incidence of hypersensitivity reactions (allergy) in infants (Lara-Villoslada et al., 2005; El-Agamy, 2007). Therefore, it is expected that camel milk proteins may cause little hypersensitivity reactions.

According to the molecular characterizations, camel milk proteins have unique properties, which reflect their own composition and structure than cow and human milk proteins. In order to confirm such findings, another immunological criterion was applied. Western blot analysis revealed the absence of shared antigenic properties between camel and cow caseins and each has its own antigenic determinants, which form its unique structure. These results are in agreement with those reported by Restani et al. (1999). Camel and cow milk WPs showed limited immunological similarities represented in BSA and other peptides. This means that BSA shares a limited sequence in its primary structure similar to that of camel milk protein. The immunological similarity between serum albumins in both camel and cow milk may be due to the fact that BSA is being derived from blood and not synthesized in the mammary gland, i.e., not organ-specific protein (Fox and McSweeney, 1998). However, the impact of such immunological similarity on allergy can be neglected. Compared with β-lg and α-la, BSA has no significant role in the hypersensitivity reaction (El-Agamy, 2007). Therefore, it is expected that the allergenicity mode of milk proteins is different between cow and camel milk.

ELISA test is frequently used for the determination of IgE-binding epitopes, using respectively allergen-specific polyclonal animal sera or human patient sera. This method is very sensitive, allowing the detection of minute amounts of antigen. ELISA inhibition test measures monovalent and polyvalent IgE epitopes that may be allergenic (Fritsche, 2003). We examined the antigenic epitopes of camel and cow milk proteins (casein and WPs) using positive sera to cow milk proteins allergenicity.

All tested sera showed the specific IgE recognition of cow milk proteins. This is expected because the serum samples were obtained from subjects allergic to cow milk proteins. However, there was no IgE recognition at all to the epitopes of either casein or WPs of camel milk when these sera were incubated with each one. These results reveal the antigenic dissimilarities between camel and cow milk proteins. In another study (Restani et al., 1999) using immunoblotting technique, IgEs from children allergic to cow milk are capable of recognizing most parts of milk proteins from mammals bred in European countries, such as sheep, goat and buffalo, while no serum IgEs were reacting with camel milk proteins. In this study, no information was recorded about camel species or breed as well as milk composition. It is well known that camel milk varies not only in gross composition but also in components structure among different breeds and species (*Camelus dromedaries and Camelus bactrianus*). In the same study, pasteurized camel milk was used and the cross-reactivity between bovine and camel milk proteins was tested using specific antiserum against bovine milk proteins not to camel milk proteins.

On the bases of all findings in the present study, it can be concluded that this *in vitro* trial provides further evidences that cow and camel milk protein epitopes appear to be quite different. It was observed that diluted skimmed camel milk is being used by nomads in the deserts of Egypt, Sudan, Mauritania, Kenya, China and Kazakhstan for feeding their babies. Therefore, camel milk might be a promising new protein source for children allergic to cow milk protein and camel milk infant formulae can be taken into account. Camel milk has an adequate amount of essential amino acids similar to that of cow milk (El-Agamy et al., 1997), in addition to the absence of β-lg that is one of the most dominant cow milk allergen. The implication of this result on nutrition properties of camel milk should be emphasized.

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