# Characterization, quantification, and assessment of immune protection potential of secretory immunoglobulin A in colostrum samples from Saudi women

*Adel Almogren,* MSc, PhD.

## ABSTRACT

الأهداف : توصيف وتقييم الكمي والوظيفي لمختلف أشكال sIgA في لبأ المرأة السعودية .

الطريقة : تمت الدراسة في 23 امرأة سعودية بمتوسط عمر 4±26 عام . وقد تم جمع 20 مليلتر من اللبأ من كل امرأة في ظرف 48 ساعة بعد الولادة . تم تحديد مستويات sIgA و sIgM بواسطة اختبار إليزا وتم تنقية الأجسام المضادة الإفرازية بعد تجميع العينات بطريقة تدريجية باستخدام الإستشراب الثيوفيلي الهلامي و استشراب جاكلين – أغاروز وترشيح سيفارسيل 300 الهلامي . تم الحصول على كريات الدم العدلات والوحيدات من الدم المحيطي بطريقة العزل المتدرج لتقييم الانفجارات التنفسية التي تسببها الأجسام المضادة و ذلك عن طريق اللمعان الكيميائي .

النتائج : بلغ متوسط تركيز sIgA1، sIgA2، sIgM كان كالآتي : 0.053 ملغ/مل، 0.047 ملغ/مل و 0.067 ملغ/مل على التوالي . لم يكن هناك فرق في تركيز sIgA1 و sIgA2 لكن تركيزهما كان أعلى بكثير عن تركيز sIgM. بينما كان 60% من IgA1 موجودة في شكل ثنائي و 30% كانت في شكل ثلاثي فإن الجزء الأعظم ( 85% ) من sIgA2 كان في شكل ثنائي . كانت الأنماط السوية للجسم المضاد A الإفرازي والجسم المضاد A المصلي متطابقة تقريباً في تفعيل انفجارات تنفسية في العدلات والوحيدات .

خاتمة : أن الشكل الثنائي هو الشكل السائد في عينات اللبأ و الجسم المضاد A الإفرازي ويتماثل وظيفياً مع الجسم المضاد A المصلي .

**Objectives:** To characterize, quantify, and assess the function of colostral secretory immunoglobulin A (sIgA) in Saudi women.

**Methods:** This prospective study was performed between March 2009 and February 2010 at King Khalid University Hospital, Riyadh, Saudi Arabia. Twenty milliliters of colostrum sample was collected from each of 23 healthy Saudi women (mean age 26±4 years) included in the study within 48 hours postpartum.

Levels of sIgA and sIgM were determined by enzyme-linked immunosorbent assay (ELISA) and purification of secretory antibodies from pooled clarified sample was performed by thiophilic-gel chromatography, Jacalin-agarose chromatography, and Sephacryl S-300 gel filtration. Antibody induced respiratory burst in peripheral blood neutrophils and monocytes was assessed by chemiluminescence.

**Results:** The median concentration of sIgA1 was 0.053 mg/ml, sIgA2 0.047 mg/ml and sIgM 0.067 mg/ml with interquartile ranges of 0.308, 0.158 and 0.150. The levels of antibodies were no different. Whereas, 60% of IgA1 was present in dimeric and 30% in trimeric form; the major bulk of sIgA2 (85%) were comprised of the dimeric form. Both sIgA and serum IgA were able to induce effective and almost identical respiratory bursts in neutrophils and monocytes.

**Conclusion:** Dimeric forms of sIgA were the predominant antibodies in colostrum samples and sIgA antibodies exhibited functional similarity with serum IgA.

*Address correspondence and reprint request to: Dr. Adel Almogren, Department of Pathology, Immunology Unit, College of Medicine & University Hospitals, King Saud University, PO Box 2925, Riyadh 11461, Kingdom of Saudi Arabia. Tel. +966 (11) 4671843. Fax. +966 (11) 4671925. E-mail: almogren@ksu.edu.sa*

Immediately after birth, the neonate is exposed to a variety of microorganisms and requires effective protection during this very critical early period of life. The immune system particularly the mucosal immunity in the neonate is poorly developed at birth as indicated by relatively small amounts of secretory immunoglobulin A (sIgA) and sIgM antibodies present in secretions from exocrine glands at this stage.[1] An adequate supply of these antibodies through mother's milk is therefore essential for acquiring mucosal immunity during the initial months of life.[2] Immunoglobulin A antibody plays a critical role in mucosal immunity to infectious diseases.[3] Although abundant in breast milk considerably larger amounts of sIgA are present in colostrums.[4] Secretory IgA present in human milk performs as the first line of defense in protection against pathogens invading mucosal surfaces[5] because of its wide diversity in binding to a large number of antigens.[6,7] Two IgA subclasses IgA1 and IgA2 have been described, IgA1 is predominantly found in serum whereas IgA2 is more abundant in colostrums.[8] Between the 2 naturally existing forms, IgA2 is less susceptible to degradation by bacterial proteases and provides relatively better mucosal immunity.[9] Because of being resistant to digestion by gastric enzymes,[10] sIgA can not only effectively prevent infections due to several enteric pathogens, but it can also counter the allergenic potential of the ingested proteins.[11] The concentrations of sIgA in colostrum reported in the literature ranges between 1.6-85.9 mg/ml.[12] This wide variability may be due to differences in the timing of sample collection. Concentration of IgA in human colostrum as high as 13.4±5.9 g/L on day one has been reported, which was shown to have been dropped to 2.3±2.0g/L at the end of the first week, and remained at low levels until 3 weeks of the study period.[13] These findings highlight the importance of early breast feeding after birth. The same study found low levels of IgG and IgM antibodies in human milk and their concentrations did not vary during the 3 weeks period suggesting that IgG and IgM antibodies are minor components of human milk. Immunoglobulin A content of maternal breast milk may have a variable influence on neonate's immunity as concentration of IgA in colostrum has been shown to vary in different geographic locations influenced by the exposure to environmental endotoxins.[14,15] This study was performed to assess structural and functional variations in sIgA and sIgM present in colostrums from Saudi women.

**Methods.** This was a prospective study performed at King Khalid University Hospital, Riyadh, Saudi

Arabia between March 2009 and February 2010. After obtaining the ethical approval, the colostrum samples were collected within 48 hours postpartum from 23 healthy multigravidae consenting Saudi women (mean age 26±4; range 23-29 years). Women suffering from Mastitis or Rhagades were excluded from the study to avoid blood contamination of the sample in order to prevent secretory IgA mixing with the serum IgA. Previously described method[16] for collection of colostrum samples was used except for the use of alcohol swabs as it tends to induce dryness and bleeding due to cracking of nipple skin. Sterile pads were soaked in warm sterile water and applied directly to nipples. Twenty milliliters of colostrum sample was collected from each woman and stored at -20ºC.

*Measurement of sIgA and sIgM antibodies.* An aliquot from each colostrum sample was subjected to enzyme-linked immunosorbent assay (ELISA) for determination of the concentration of secretory immunoglobulins both sIgA and sIgM antibodies. Microtitre plates (Nunc Immuno-Plate, Denmark) were coated with capturing antibody (polyclonal anti-human α chain; Caltag, USA for sIgA or polyclonal anti-human μ chain for sIgM, Sigma, UK) at known optimum concentration (5-10µg/ml) in phosphate buffered saline (PBS) pH 7.2 and incubated overnight at 4ºC. After incubation, the plate was washed 3 times with PBS with 0.1% v/v Tween[20] (PBST) at pH 7.2 and banged dry on paper towels between each wash. The wells were blocked with 5% Bovine Serum Albumin (BSA) as blocking buffer. After blocking, 100µl of sample was added to each well in triplicate and incubated for one hour at room temperature. After 3 washes with PBST, the complex was then detected using an appropriate alkaline phosphatase conjugate (monoclonal anti-human α chain conjugated with alkaline phosphatase for sIgA; Southern Biotechnology, USA or monoclonal anti-human μ chain conjugated with alkaline phosphatase for sIgM; Sigma, UK) and incubated for one hour at room temperature. The plate was washed 3 times before the addition of p-nitrophenyl phosphate (p-npp). After development of color the ELISA plate was read at 405 nm using an ELISA plate reader (Model Mrx Ii, Dynex Technologies, USA).

*Characterization and purification of sIgA antibodies in colostrum samples.* Colostrum samples were thawed at 37ºC and mixed with 10ml of isotonic saline (0.9% NaCl). The diluted colostrum was centrifuged and separated into 3 distinct phases: the fatty layer, intermediate aqueous phase (containing soluble factors) and the cell pellet. The defatted clarified colostrum was collected from the intermediate phase. Samples

were pooled and the secretory immunoglobulins (polymeric and dimeric SIgA1, SIgA2, secretory IgM) were separated from a clarified colostrum preparation using thiophilic-gel chromatography, jacalin-agarose chromatography, and Sephacryl S-300 gel filtration as described previously.[17]

*Isolation of peripheral blood monocytes and neutrophils.* Peripheral blood monocytes and neutrophils were isolated for assessment and comparison of the effect of sIgA binding Fc alpha receptors expressed on both the cell types. Mononuclear cells (PBMC) were isolated from 10ml of heparized peripheral blood by centrifugation over the standard density medium, Lymphoprep (Nycomed As, Norway). In a universal container, a 7 ml of Lymphoprep was overlaid by the blood sample and centrifuged at 800g for 20 minutes at room temperature. Using a pasture pipette mononuclear, cells at the interface were carefully removed and washed 3 times in PBS (with 1% BSA, 0.1% sodium azide). Finally, the pellet was resuspended in Roswell Park Memorial Institute (RPMI) medium .

For isolation of monocytes, ethylenediaminetetraacetic acid (EDTA) was used as an anticoagulant. Ten parts of EDTA blood were mixed with one part of 6% Dextran T70 in 0.9% sodium chloride solution and allowed to stand at room temperature for 35 minutes. The upper, leukocyte-rich, plasma layer was then drawn off and layered over Nycodenz Monocyte medium (Nycomed, Norway). After centrifugation at 600 g at room temperature for 15 minutes, the monocyte layer at the interface between the sample and the Nycodenz Monocytes medium, was drawn off. The cells were washed and checked for purity and viability using trypan blue.

Neutrophils were isolated using previously described method[18] by inclusion of an additional 15ml layer of a higher density medium under 10ml of Lymphoprep in the standard protocol used for isolation of peripheral blood mononuclear cells. This denser medium comprised of a solution of 7% Ficoll 400 (Pharmacia) and 17.67% sodium diatrozoate (Sigma) and had a density of 1.119 g/ml. After centrifugation, peripheral blood mononuclear cells were recovered from sample/ Lymphoprep interface, and neutrophils were recovered from the Lymphoprep/high density medium interface. After the isolation, cells were checked for purity and viability using trypan blue.

*Chemiluminescence assay.* Specific microtitre-plate chemiluminescence wells (Microlite 1 Removawell Strips, Dynatec Laboratories Inc. El Paso, USA) were coated overnight at 4ºC with IgA, at a concentration of 10µg/ml in PBS pH 7.2. The well strips were then washed 3 times with PBS. The 100µl Luminol solution (prepared by dissolving 1 mg of luminol [Sigma, UK] in 100µl of 0.5M sodium hydroxide and making up to 50ml in Hank's Buffered Salt Solution [HBSS], 0.1% bovine serum albumin [BSA], 20mM HEPES) was added immediately to each well followed by 50µl of purified cells at $10^6$ cells/ml. The chemiluminescence emitted was read in a microtitre plate luminometer (LB96P, MicroLumat, EG & G Berthold, Milton Keynes, UK) continuously for one hour at 37ºC and the acquired data were analyzed using excel software. All samples were run in triplicate and the average was used to construct the chemiluminescence graph.

*Statistical analysis.* Data were statistically analyzed using SPSS computer software version 19. Since the data were not normally distributed, Mann Whitney U test was applied for comparative analysis and the concentrations of immunoglobulins were expressed as median values with interquartile ranges (IQR). A p value of either equal to or less than 0.05 was considered significant.

**Results.** Figure 1 shows the concentrations of secretory immunoglobulins in the colostrum samples collected from 23 women within the first 48 hours postpartum. The median concentrations of sIgA1 was 0.053 mg/ml, sIgA2 0.047 mg/ml, and sIgM 0.067 mg/ml with interquartile ranges of 0.308, 0.158, and 0.150, respectively. Comparative analysis did not reveal any statistically significant difference between the levels of antibodies in colostrum samples (IgA1 versus IgA2
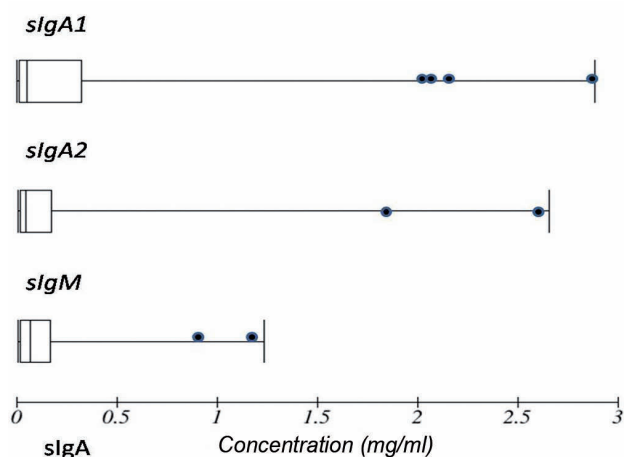


**Figure 1 -** Measurement of secretory immunoglobulin A1 (sIgA1), sIgA2, and sIgM in colostrum samples collected from 23 women within 48 hours postpartum by enzyme-linked immunosorbent assay (ELISA) in triplicate. Each dot depicts an outlier. Comparative analysis using Mann Whitney U test did not reveal any significant difference between the secretory immunoglobulins tested.

[*p*=0.968], IgA1 versus IgM [*p*=0.912], and IgA2 versus IgM [*p*=0.896]). Following the protocol, sIgA and sIgM were obtained with a high degree of purity.

**Figure 2** describes the SDS-polyacrylamide gel electrophoresis (SDS-PAGE) run under non-reducing conditions showing separation of different molecular forms of serum and secretory IgA1and sIgM. **Table 1** shows the yield of total IgA and various isoforms of the pooled colostrum samples after being subjected to different quantitative and qualitative procedures. After purification with T gel, 3.5mg/ml of total sIgA
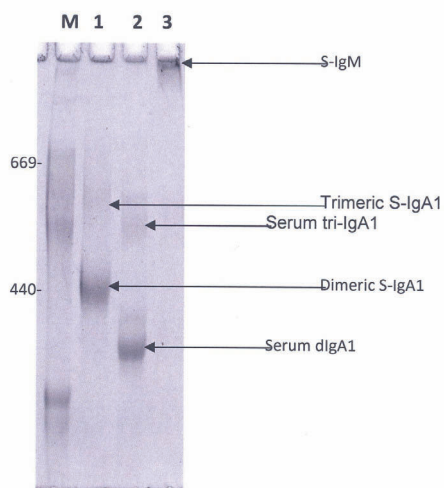
was obtained. Further characterization of sIgA after being subjected to Jacalin-agarose eluted fraction and fall though resulted in detection of 1.62 mg/ml of sIgA1 and 0.86 mg/ml of sIgA2, respectively. These isoforms comprised of 56.2% sIgA1 and 42.1% sIgA2 of the total recovery which was 98.3%. Further characterization with S-300 gel filtration of sIgA1 revealed that dimeric form of sIgA1 formed the major bulk (60%) of the sIgA1 antibodies which was present at a concentration of 1.48 mg/ml compared to the trimeric form which was present at a concentration of 1.11 mg/ml constituting 30% of the isoforms. Dimeric form of sIgA2 constituted 85% and the polymeric form 8% of the sIgA2 antibodies with the concentrations of 1.45 mg/ml and 0.3 mg/ml, respectively.

**Figure 3** shows comparative chromatogram showing the difference between dimers of sIgA1 and serum IgA1 by gel filtration on Superdex 200. The dimer of sIgA1 eluted as a single symmetrical peak from the Superdex 200 demonstrates its homogeneity. The sIgA1 dimer was eluted ahead of serum dimeric IgA1 representing it greater size. In addition, the 2 forms of IgA1 showed different electrophoretic mobility when subjected to SDS-PAGE. Shown on the top right of **Figure 3** is a Western blot probed with anti IgA1 monoclonal antibody demonstrating the difference in electrophoretic mobility between these 2 forms.



**Figure 2 -** Comparative 4% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) run under non-reducing conditions for separation of different molecular forms of serum and secretory immunoglobulin A1 (sIgA1). M - Markers, lane 1 - sIgA1 mixture, lane 2 - serum dimeric IgA1 and trimeric IgA1, and lane 3 - sIgM.

**Table 1 -** The final yield and recovery of secretory immunoglobulin A1 (sIgA1) and sIgA2 purified from colostrum after different purification stages.

| Steps | IgA concentration mg/ml (total yield) | IgA recovery (%) |
|---|---|---|
| Clarified colostrum | 2.34 (54.3) | |
| Modified T gel | 3.5 (52.7) | 97% |
| *Jacalin-agarose* | | |
|   Eluted fractions sIgA1 | 1.62 (29.6) | sIgA1 56.2% |
|   Fall-through sIgA2 | 0.86 (22.2) | sIgA2 42.1% |
| | | Total (98.3%) |
| S-300 gel filtration of sIgA1 | 1.48 (17.8) | sIgA1$_{dimeric}$ 60% |
| | 1.11 (8.9) | sIgA1$_{trimeric}$ 30% |
| | 0.56 (2.96) | Mixture 10% |
| S-300 gel filtration of sIgA2 | 1.45 (18.9) | sIgA2$_{dimeric}$ 85% |
| | 0.3 (1.8) | sIgA2$_{polymeric}$ 8% |
| | 0.22 (1.6) | Mixture 7% |
| sIgA - secretory immunoglobulin A | | |



**Figure 3 -** Comparative chromatogram showing the difference between dimeric secretory IgA1 (sIgA1$_{dimeric}$) and serum dimeric IgA1 (dIgA1) by gel filtration on Superdex 200. At the top right of the figure is a Western blot probed with anti-IgA1 monoclonal antibody demonstrating the difference in electrophoretic mobility between these 2 forms.

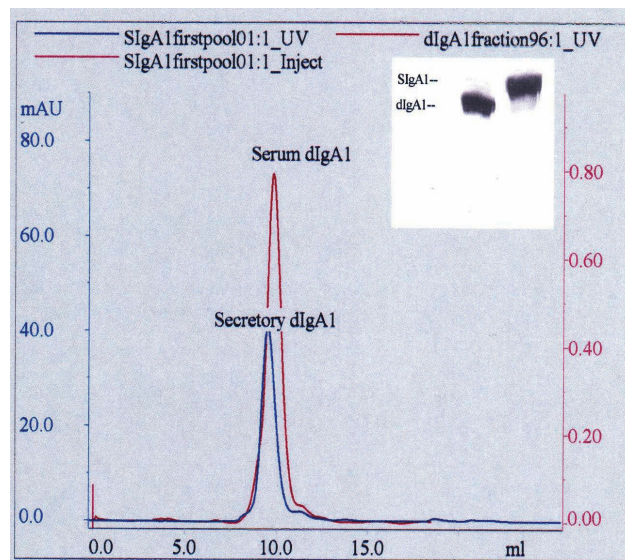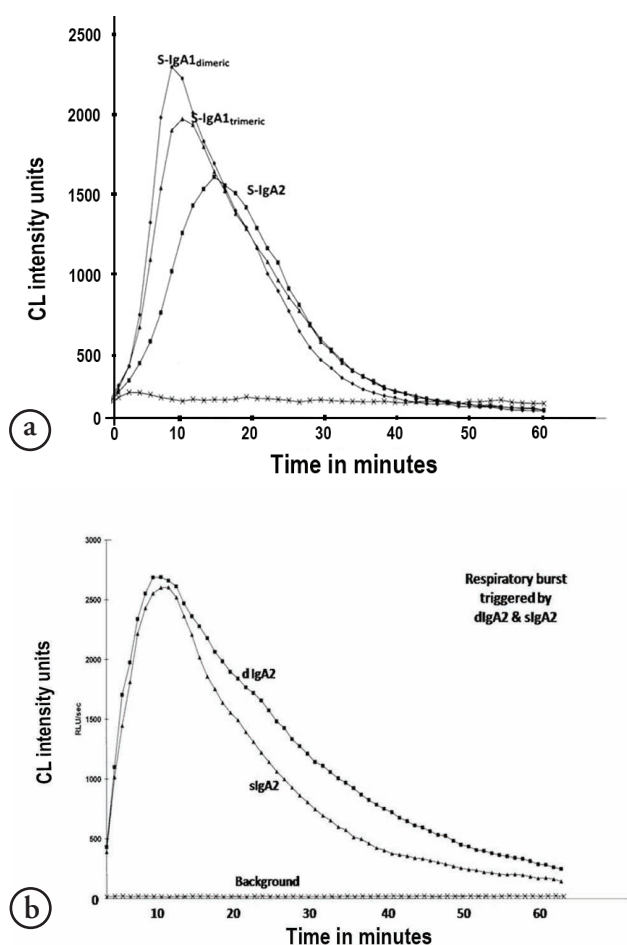**Figure 4** - Luminol enhanced chemiluminescence. **a)** Comparison of respiratory burst of neutrophils triggered by dimeric secretory (sIgA1), trimeric secretory IgA1 (sIgA1) and sIgA2. The traces represent the mean values of triplicate samples. Luminol enhanced chemiluminescence. **b)** Comparison of the respiratory burst of monocytes triggered by serum diameric IgA2 (dIgA2) and secretory IgA2 (sIgA2). The traces represent the mean values of triplicate samples

With regards to the functional assessment, **Figure 4a** describes the measurement of chemiluminescence depicting sIgA induced triggering of respiratory burst in neutrophils. Both sIgA isotypes were very effective in triggering a chemiluminescence response from neutrophils. Dimeric or trimeric forms of sIgA1 triggered similar respiratory bursts by neutrophils that peaked after 10 minutes. On the other hand, sIgA2 generated a burst with different kinetics to that elicited by sIgA1, reaching a maximum after 15 minutes.

**Figure 4b** shows the comparison of monocyte respiratory bursts induced by sIgA2 and its counterpart in serum. Both the antibodies were capable of inducing an effective and almost identical respiratory burst in

monocytes. Similarly no differences were observed between neutrophil respiratory bursts induced by sIgA1 and serum IgA1 (data not shown).

**Discussion.** Quantitative assessment of antibodies in the colostrum samples collected within 48 hours postpartum from Saudi women revealed presence of a considerable amounts of sIgA in colostrum samples. These findings are in agreement with previously published data describing sIgA as a major component of colostrum, and its concentration rapidly decreased by the end of first week.[13] High levels of sIgA in colostrum compared to their levels in mature milk is an established phenomenon that have been well documented[14,19] highlighting the importance of colostrum as a rich source of sIgA antibodies. On the contrary, there is evidences suggesting that there is no difference between the levels of total sIgA and specific antibodies against certain microbes in colostrum and milk indicating that human milk is as effective in providing protection as colostrums.[20] Colostrum content of sIgA has been shown to differ between Swedish and Estonian mothers.[14] The level of sIgA in colostrum samples detected in the present study was however lower than both the ethnic groups indicating a regional variation. It is therefore possible that variations in sIgA levels in colostrum may contribute to the regional variations in the immune protection provided by sIgA with regards to acquisition mucosal immunity.

Thiophilic chromatography has been traditionally applied for purification of IgG antibodies.[21,22] The optimization and modification of the conditions of thiophilic gel (T gel) synthesis for IgA performed in the present study served as an important initial step in efficient purification of sIgA. The procedure not only eliminated most of the non-immunoglobulin serum or whey proteins but also contributed in concentrating the immunoglobulins. In addition, a major disadvantage associated with Jacalin-agarose is that it binds proteins such as C1-esterase inhibitor[23] human serum albumin[24] and human α 2-HS glycoprotein,[25] which was overcome by the prior application of T gel that does not bind these proteins. This not only resulted in a higher yield of the purified sIgA, but also allowed homogeneous separation of different molecular forms. In milk and saliva, the proportions of dimeric and tetrameric sIgA has been reported as approximately 3:2.[26] The higher yield of the 2 isotypes of IgA from colostrum in the present study easily allowed estimation of relative abundance of different molecular forms where the dimeric sIgA1 represented approximately 60-65% and trimeric sIgA1 was 35-40% which was similar to the

ratio reported for milk and saliva. On the contrary, dimeric sIgA2 comprised of 90% of the molecular forms. Moreover, there were no differences from those reported perviously[27] in the percentages of sIgA1 and sIgA2 in colostrum samples detected in the present study. Despite the prevailing ambiguity on the exact role of sIgA, the antibody is considered to be the key factor in maintaining mucosal defense.[28,29] It may be evident from the fact that a significant correlation has been reported to exist between the concentration of sIgA and resistance to infection.[30] The property of sIgA antibodies to act as a potent opsonin as indicated by their ability to induce respiratory burst in granulocytes appears to be a crucial in handling microorganisms on the mucosal surfaces.[31] In the present study, the potential of sIgA to induce respiratory burst on neutrophils and monocytes was assessed and compared with serum IgA. Despite differences in structures of sIgA and serum IgA functional similarity was observed between the 2 forms. Similarly, no difference was observed in induction of respiratory burst between dimeric and trimeric molecular forms of sIgA and their counter parts in the serum. Similar observation was also made for sIgA2 antibodies in the colostrum samples. A study comparing the potential of serum IgA and sIgA to induce neutrophil respiratory burst in the past has also failed to show any difference between the 2 types of IgA antibodies.[32] Compared with sIgA1 the delayed induction of respiratory burst in neutrophils by sIgA2 observed in the present study has also been reported previously,[32] which has been attributed to the rigidity of sIgA2 due to lack of hinge region resulting in interference of sIgA2-FCαR interaction. The ability of sIgA to act as an effective opsonin may have important consequences in the backdrop of limited availability of complement components on the mucosal surfaces.

Colostrum serves as the first line of defense in a new born and is a key factor in maintenance of gastrointestinal homeostasis. Similarly, reproducibility of IgA induction of respiratory burst in phagocytic cells should be established as it was performed only once in the present study. Large scale studies are recommended to further evaluate and define normal ranges of not only immunoglobulins, but also other immune parameters in colostrum samples of the local population.

In conclusion, dimeric forms of sIgA1 and sIgA2 were found to be major antibodies in colostrum samples of Saudi women. Following the purifications protocol described previously, sIgA was obtained with a high degree of purity. The functional assessment of sIgA revealed that sIgA antibodies did not differ from their counterparts in the serum as assessed by their ability to induce respiratory burst. Both sIgA1 and sIgA2 antibodies were found to be effective opsonins indicating that these antibodies may have a significant role in providing mucosal protection against microorganisms.

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