

*Full Length Research Paper*

# **Studies on the interaction between IgA, lactoferrin and lysozyme in the breastmilk of lactating women with sick and healthy babies**

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**The IgA, lactoferrin and lysozyme levels in breastmilk samples of mothers with healthy babies and mothers whose babies developed septicaemia were analyzed by the enzyme – linked immunosorbent assay. The IgA level in the milk of mothers with healthy babies decreased from  $183.48 \pm 114.91$  mg/l in colostrum to  $147.75 \pm 114.6$  mg/l in mature milk while the level in mothers whose babies developed septicaemia rose from  $221.22 \pm 115.46$  mg/l in colostrum to  $267.81 \pm 77.89$  mg/l in mature milk. This rise in IgA level was marched with a rapid increase in lysozyme levels and with a gradual fall in the lactoferrin level. This correlated with an increase in the lysozyme level and a decrease in the lactoferrin levels. This correlation was statistically significant ( $p < 0.05$ ). There was a gradual decrease in IgA level in the breastmilk of mothers with healthy babies and this corresponds to a gradual increase in the lysozyme and lactoferrin levels. This decrease correlated significantly with an increase in the lysozyme level. The lactoferrin levels also recorded a gradual rise as the milk transits from colostrum to mature milk. This rise however, did not correlate significantly with the fall in IgA level. There was however no correlation between lysozyme and lactoferrin in both milk the samples assayed.**

**Key words:** Lactoferrin, lysozyme, septicaemia, IgA, lactoferrin, breastmilk, correlation.

## **INTRODUCTION**

Human milk is the most appropriate and nutritionally balanced protective food for an infant. Breast milk and colostrum contain significant amounts of cell and humoral factors that protect the neonate from a variety of infections (Lawton, 1977) including diarrheal diseases, otitis media (Dewey et al., 1995) and respiratory illnesses (Howie et al., 1990). Breast milk not only provides optimal nutrition to infants but also supplies a range of bioactive factors that are involved in the protection against many invading pathogens (Lawton, 1977). This latter function is reflected by the immunologic composition of breast milk and is vital for newborns as their mucosal and systemic immune systems are not fully developed at birth. The importance of these compounds in breast milk has led the American academy of pediatrics (AAP) to recommend

that all infants be exclusively breastfed for the first 6 months and that breastfeeding continue with supplements of solid foods during the next 6 months of life (A.A.P., 1997).

Three proteins that are found in relatively high concentrations in breast milk and confer immunologic benefits to infants are secretory immunoglobulin A (sIgA), lactoferrin, and lysozyme. IgA in breast milk is in the molecular form of sIgA and is therefore more resistant to proteolytic activity of the gastrointestinal tract (Rodriguez-Palmero et al., 1999). sIgA prevents the adherence of bacteria to mucosal surfaces and neutralizes toxins from microorganisms (Rodriguez-Palmero et al., 1999). Lactoferrin also works at the mucosal sites and has demonstrated anti-inflammatory and antimicrobial activities, such as competing with bacteria for ferric iron and preventing the growth of microorganisms. Lysozyme is a protein that lyses bacteria and may work synergistically with lactoferrin and sIgA in antibacterial

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functions (Garofalo and Goldman, 1999).

The importance of this work stems from the fact that despite the acceptance and adoption of exclusive breastfeeding worldwide, infections and neonatal illnesses leading to their hospitalization is still common. The objective of this study is therefore to determine the levels and changes in levels of IgA, lactoferrin and lysozyme in the breastmilk from mothers with sick babies compared with mothers with healthy babies.

## MATERIALS AND METHODS

### Study area

The study was conducted in four selected tertiary hospitals and two secondary health centres located in five local Government areas of Kaduna State in North Central Nigeria. The hospitals include Ahmadu Bello University Teaching Hospital, (A.B.U.TH) from Shika, Giwa LGA, Gambo Sawaba General Hospital Kofar Gayan Zaria LGA, Barau Dikko Specialist Hospital Kaduna North LGA, Yusuf-Dansoho General Hospital in Kaduna South LGA, Samaru Government Health Centre and Ahmadu Bello University Health services (UHS – Sick Bay) in Sabon Gari LGA.

### Experimental design

This study use cross-sectional design with a descriptive approach, aimed at all neonates admitted into the selected hospitals within a period of one year from August 2006 to July 2007. A total of 384 samples of breastmilk were collected from 384 lactating mothers for analysis of some factors such as IgA, lactoferrin and lysozyme. A total of 185 mothers whose babies were admitted into the special care baby units with suspected cases of septicaemia were sampled (Figure 1).

Septicaemia was confirmed by blood culture as reported in an earlier work by the same authors (Ella et al., 2009b). Control breastmilk samples were obtained from 193 women with apparently healthy neonates attending selected post – natal clinics in Kaduna State (Figure 1).

### Inclusion criteria

The study covered neonates (age 0 – 28 days) admitted into the intensive care baby unit of the hospital with suspected symptoms of septicaemia and confirmed by a positive blood culture. These were classified as sick babies. On the other hand, babies with no record and episode of septicaemia confirmed by verbal interviews with their mothers were classed as healthy babies and their mother considered controlled as mothers.

The breastmilk samples were collected from mothers of the babies sampled with septicaemia. Control milk samples were obtained from other mothers whose babies had no incidence of neonatal septicaemia.

### Sample collection

A total of five to ten milliliters of breast milk was obtained from 378 lactating mothers attending the hospitals selected for the study as indicated in experimental design. Milk samples collected from mothers 0 – 5 days post – partum was considered colostrums while those collected within 6 – 15 days were categorized as transitional milk and the milk collected from 16 days upward post-partum

classified as mature milk. The assistance of nurses on duty was employed to aseptically collect the samples. The breast nipples were cleaned and blotted dry with sterile distilled water to clean the teats. The milk samples (colostrum, transitional and mature milk) were collected into sterile sample bottles and transported on ice to the laboratory for analysis.

### Ethical considerations

Ethical permit was obtained from the Ahmadu Bello University Teaching Hospital, Zaria's ethical board as well as the Kaduna State Ministry of Health for the study. Verbal consent was obtained from mothers for the collection of breastmilk. Permission was also granted by the Director of Medical Services for the inclusion of the Ahmadu Bello University Health Services (Sick Bay) in the study.

### Lactoferrin and lysozyme ELISA assay procedure

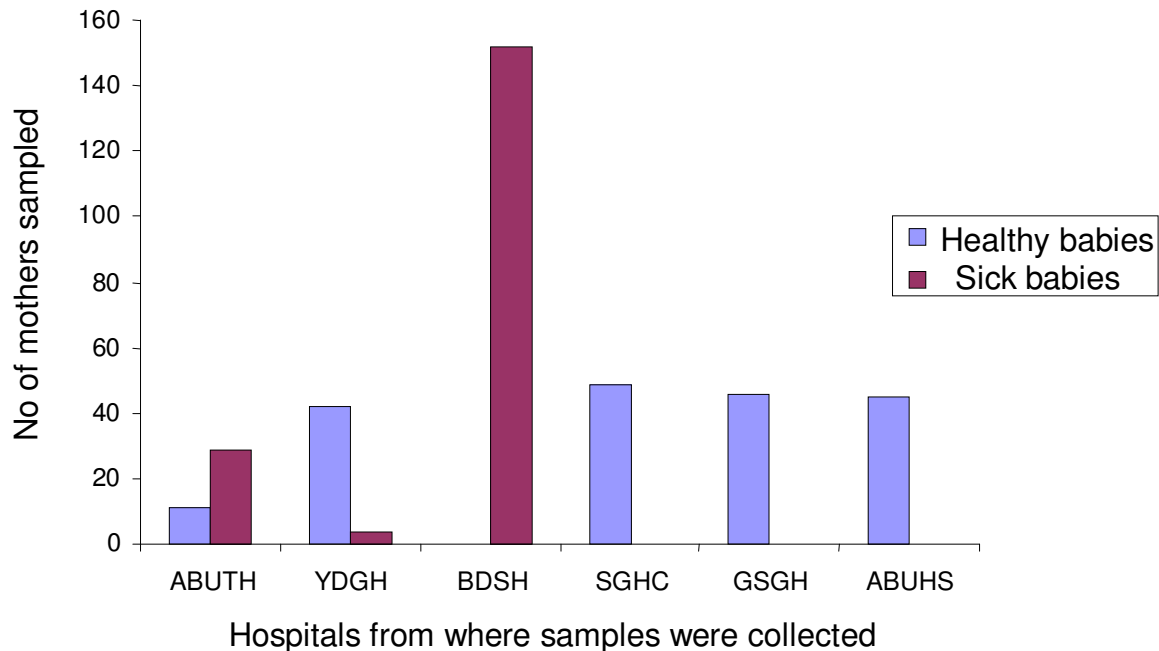
The AssayMax Human ELISA kit was obtained from ASSAYPRO (USA) for both tests. All the reagents were allowed to warm up to room temperature (25°C) before use in accordance with the manufacturer's instruction. The lactoferrin kit contents were plates pre-coated with polyclonal antibody against human lactoferrin, human lactoferrin standard, biotinylated lactoferrin antibody, while the lysozyme kit comprises of pre-coated with polyclonal antibody against human Lysozyme, human Lysozyme standard. Other components of the kits include peroxidase conjugate streptavidin, sample diluent, wash buffer, chromogen, substrate and the stop solution. The process followed the manufacturer's instruction and the plates were read at a wavelength of 450 nm, using the Microplate reader (SIGMA Diagnostic ELISA Reader).

### IgA ELISA assay procedure

The Human IgA ELISA core kit (KOMA BIOTECH) was obtained for the assay. The was kit comprised of coating antibody (Affinity purified anti-human IgA), detection antibody (HRP conjugated goat anti-human IgA), human reference serum protein (IgA), color development reagent A (TMB {tetramethylbenzidine} solution) and color development B (substrate solution). In the assay, microplate wells (Linbro/titertek – Flow Labs) consisting of 96 wells were coated by transferring 100 ul of the coating antibody at a ratio of 1:100 using 50 mM bicarbonate (pH 9.5) to each well. The plates were first incubated at room temperature (25°C) for 1 h and further incubated at 5 – 8°C for 24 h to allow for effective binding. At the end of binding, the plates were aspirated and washed four times with the washing solution (10 mM phosphate, 0.14 M NaCl and 0.05% Tween 20 pH 7.4).

Excess liquid was removed by damping over a series of absorbent tissue paper and 100 ul of the blocking solution (10 mM phosphate, 0.14 M NaCl and 1% BSA; pH 7.4) was added to each well. The plates were further incubated at room temperature (25°C) for one hour. At the end of incubation, the wells were aspirated and washed four times with the washing solution. The wells were blotted again and 100 ul of the standards (500, 250, 125, 62.5, 31.3, 15.6 and 7.8 ng/ml) were added to wells 2 to 8, respectively, with well one left as blank. The test samples were diluted 1:150 and added to the other wells in 100 µ volume. The plates were then incubated again for one hour at room temperature. This was followed by washing and blotting of the excess liquid. A total of 100 µ of the detection antibody (secondary antibody) ratio 1: 50000 was added to each well with the exception of the first blank well and the plates were incubated at room temperature for one hour.

The plates were then washed again to remove the unbound detection antibody and excess liquid removed as before. This was



**Figure 1.** Profile of breastmilk in relation to health of babies from various hospitals: ABUTH – Ahmadu Bello University Teaching Hospital, YDGH Yusuf – Dansoho General Hospital; BDSH – Barrau Dikko Specialist Hospital; SGHC – Samaru Government Health Centre; GSGH – Gambo Sawaba General Hospital and ABUHS – Ahmadu Bello University Health Services.

**Table 1.** Determination of correlation between IgA, lactoferrin and lysozyme levels from mothers with sick babies.

	IgA	Lactoferrin	Lysozyme
IgA	r: 1.000	r: 0.18 p: 0.02*	r: 0.33 p <0.0001*
Lactoferrin	r: 0.18 p: 0.02*	r - 1.000	r: 0.02 p: 0.79**
Lysozyme	r: 0.33 p <0.0001*	r: 0.02 p: 0.79**	r - 1.000

r – correlation coefficient; \*significant on  $p < 0.05$  level; IgA – immunoglobulin A.

followed by the addition of  $100 \mu$  of the color development solution (TMB solution – 60 ml + substrate solution 125 ml that is, ratio 1: 2) to all the wells including the blank well and further incubation at room temperature for 40 min. The reaction was stopped after 40 min with the stop solution ( $2M H_2SO_4$ ) and the absorbance read at 450 nm using the microplate reader (SIGMA Diagnostic ELISA Reader).

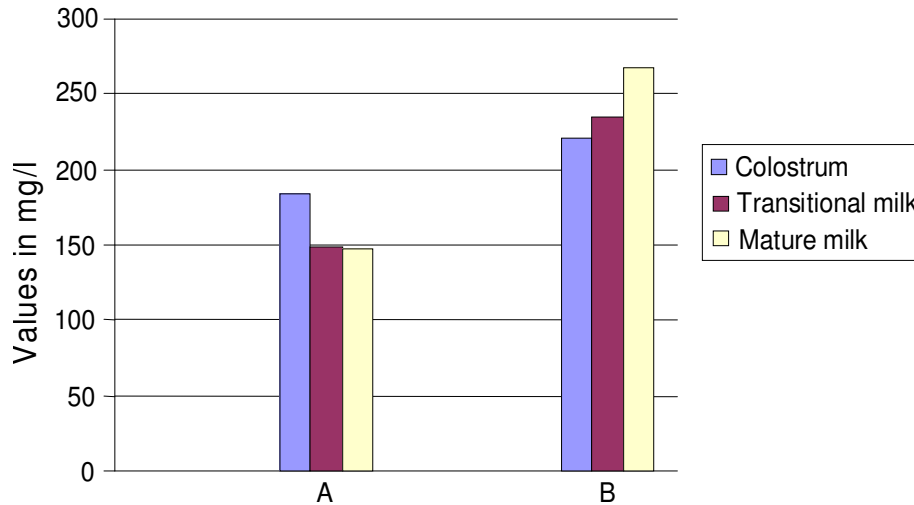
#### Data and statistical analysis

The data generated were analyzed statistically using version 9.1.3 of SAS (statistical analysis system) package - SAS 9.1.3. Means were compared by the student's *t*-test analysis of means to determine the level of significance. Correlation was also calculated between IgA, lactoferrin and lysozyme.

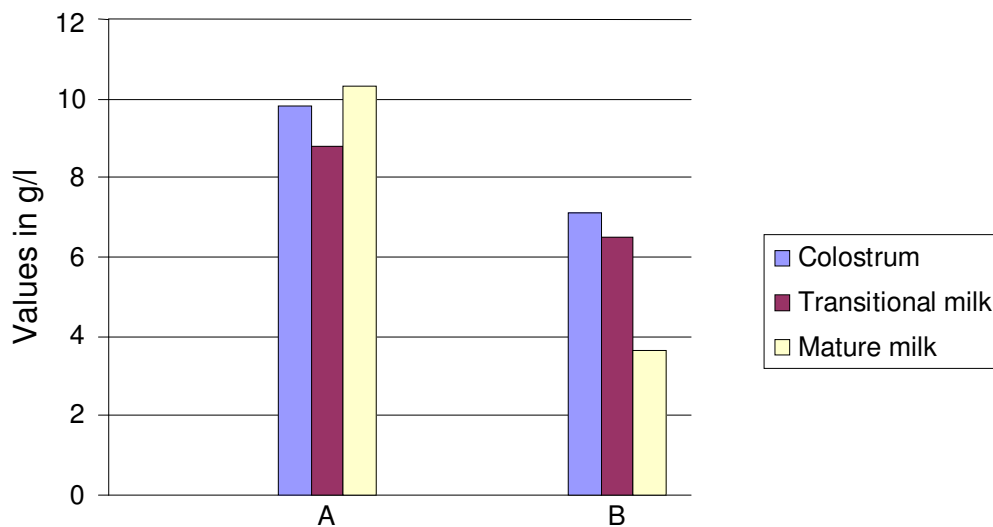
## RESULTS

It was observed in the milk of mothers whose babies developed septicaemia that IgA levels rose rapidly from  $221.22 \pm 115.46$  mg/l in colostrum to  $267.81 \pm 77.89$  mg/l in mature milk. This rise in IgA level was marched with a rapid increase in lysozyme levels with a gradual fall in the lactoferrin level. This decrease in IgA level was correlated with an increase in the lysozyme level and a decrease in the lactoferrin levels. This correlation was statistically significant ( $P < 0.05$ ) (Table 1).

The result shows that the gradual decrease in IgA level in the breastmilk of mothers with healthy babies corresponds to a gradual increase in the lysozyme and



**Figure 2.** IgA levels in mothers with healthy and sick babies. A - mothers with healthy babies; B - mothers with sick babies.



**Figure 3.** Lactoferrin levels in mothers with healthy and sick babies. A - mothers with healthy babies; B - mothers with sick babies.

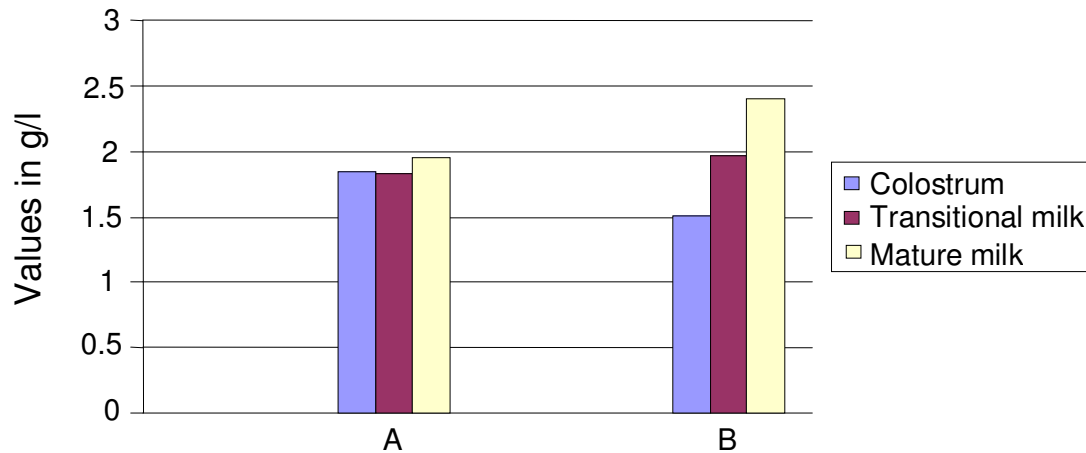
lactoferrin levels (Figures 2, 3 and 4). This decrease correlated significantly ( $P < 0.05$ ) with an increase in the lysozyme level. The lactoferrin levels also recorded a gradual rise as the milk transits from colostrum to mature milk. This rise however did not correlate significantly ( $P > 0.05$ ) with the fall in IgA level (Table 2). There was however no correlation between lysozyme and lactoferrin ( $P > 0.05$ ) in both the milk samples assayed.

## DISCUSSION

IgA is abundant in milk and provides the immunoglobulin

on the mucosal surfaces and in many body fluids including breastmilk. In the mothers with healthy infants, the IgA level was highest in the colostrum and decreased as the milk transits to mature milk. On the other hand, lactoferrin and lysozyme were observed to increase as the milk matured from colostrum to mature milk. The result presents lactoferrin and lysozyme as the main bioactive property of transitional and mature milk. This may not be surprising as IgA in milk is of maternal origin and secreted in response to maternal stimulations. This passive immunity becomes gradually replaced as the infant builds up its own protection.

IgA is the major immunoglobulin known to be present



**Figure 4.** Lysozyme levels in mothers with healthy and sick babies. A - mothers with healthy babies; B - mothers with sick babies.

**Table 2.** Determination of correlation between IgA, lactoferrin and lysozyme levels from mothers with healthy babies.

	<b>IgA</b>	<b>Lactoferrin</b>	<b>Lysozyme</b>
IgA	r: 1.000	r: 0.07 p: 0.31**	r: 0.34 p<0.0001*
Lactoferrin	r: 0.07 p: 0.31**	r:1.000	r: 0.01 p: 0.88**
Lysozyme	r: 0.34 p<0.0001*	r: 0.01 p: 0.88**	r: 1.000

Key: r - correlation coefficient; \*significant on p< 0.05 level; IgA – immunoglobulin A.

abundantly in breastmilk, thus allowing the mother to pass some form of passive protection to her infant. In the milk samples of mothers with healthy babies, IgA levels decreased as the milk transition from colostrum to mature milk. This noted decrease has been reported earlier (Ella et al., 2009b). A decrease in IgA level correlated with an increase in the lysozyme level in the milk of the mothers with healthy babies. The gradual rise in lysozyme levels in the milk samples was also found in another work by Ella et al. (2009a). This gradual rise is an indication that in healthy mothers with healthy babies, the maternal IgA protection becomes gradually replaced by lysozyme, with a broad spectrum antibacterial enzymatic activity (Noble, 2000) unlike IgA, which is usually more specific in its action of protection. It was also noticed in this work that lactoferrin level gradually increased with the rise in lysozyme level indicating that the action of lysozyme is complemented with that of lactoferrin. The existence of synergy between lysozyme and lactoferrin agreed with the work of Leitch and Willcox (1999) and Kanyshkova et al. (2003).

However, in the milk samples obtained from mothers

whose babies developed sepsis, IgA levels increased with milk transition to mature milk. This rise in IgA could probably have resulted from immune stimulations in the mothers in response to infections and invariably a risk factor for the child. This was also reported in the findings of Finn et al. (2002) and by Ella et al. (2009b). The latter also correlated with a significant rise in lysozyme, indicating a relation between IgA and lysozyme (Martinez and Carroll, 1980; Losnedahl et al., 1998). The marked increase in IgA and lysozyme reflects a punctuation of the immune response in the mother which is thus, reflected in the breastmilk levels of these bioactive molecules. It would be expected that the build up of these bioactive molecules will culminate in the protection of the infant and result in mild infection in the infant.

## Conclusion

From the above findings, it can be concluded that in the milk samples of mothers with sick babies, there exist a correlation between high IgA and low lactoferrin as well

as IgA and lysozyme levels. However, there was no correlation between lactoferrin and lysozyme levels. Similarly, there exists a correlation between mean IgA and lysozyme levels in the milk from mothers with healthy babies. However, there is no correlation between IgA and lactoferrin as well as between lactoferrin and lysozyme levels.

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