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Assay of BLf using Sandwich ELISA and its Comparative Study of Holstein-Friesian Crossbred and Sahiwal with Poda Thurpu Cow Milk

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ABSTRACT

Bovine lactoferrin (BLf) is a multifunctional bioactive protein found in high concentration in milk. The present study was done to evaluate, isolate and compare the quantity of BLf present in the milk of exotic cross bred and indigenous breeds of cow. Milk samples were collected from exotic HF cross bred and indigenous breeds like Sahiwal and Poda thurpu. The quantification of BLf was done by using sandwich enzyme-linked Immunosorbent assay (ELISA). The processed milk samples of each of three breed were then subjected to cation exchange column chromatography using Sephadex C-50 resin. The eluted fractions obtained from the chromatography were used to run sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) along with a protein marker to confirm the identity of BLf. The estimated BLf values (µg/mL) from ELISA were found as 306.47±8.91, 369.06±14.04 and 403.9±20.10 in HF cross bred, Sahiwal and Poda thurpu cow breed. Further electrophoresis results showed a clear band was formed at 80KDa approximately when compared with the standard protein marker with a more prominent band in indigenous breeds than HF cross bred where a thin band was seen.

HIGHLIGHTS

- Lactoferrin (Lf) is a iron binding protein which shows various bioactive functions.
- BLf content was highest in indigenous breed cow milk than in exotic cross bred cow milk.
- Among indigenous breeds, BLf concentration was found to be higher in Poda thurpu cow milk followed by Sahiwal cow milk.

Keywords: Bovine lactoferrin, Poda thurpu, ELISA, Sephadex C-50, SDS-PAGE

Lactoferrin (LF) is a biologically active iron-binding glycoprotein with a molecular weight of 75 to 80 KDa. It is present in many biological fluids and is widely distributed in colostrum and milk (Tsakali *et al.,* 2014). It is a multifunctional bioactive protein with various antimicrobial properties.. Several studies have demonstrated the potential antiviral, antifungal, and antiparasitic activity of LF toward a broad spectrum of species (Wakabayashi and Takase, 2006), deriving mostly from its ability to bind iron (Jenssen and Hancock, 2009). In addition, it interacts with molecular and cellular components of both hosts and pathogens (García-Montoya *et al.,* 2012). Lactoferrin is also considered to be an important host defence molecule during infant development (Jenssen and Hancock, 2009). The lactoferrin content in milk varies between different

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mammalian species and, within a given species, between lactation periods (Levay and Viljoen, 1995) Lactoferrin concentration in bovine milk ranges from 20 to 200 mg/ mL^{-1}, depending on the lactation period (Law and Reiter, 1977). Lf in milk is in dissolved state, in aqueous form, it is stabilized by hydrogen bonding, hydrophobic and hydrophilic interactions, disulphide bonds and ligand bindings (Brisson *et al.,* 2007).

Recent studies have shown various methods to evaluate concentration of BLf. A competitive enzyme-linked immunosorbent assay (ELISA) was established by Chen and Mao (2004) to determine the Lf concentration in milk by using bovine LF antiserum and BLf-Biotin conjugate. An automated SPR-biosensor assay was described for the quantitation of lactoferrin in protein isolates, milk, colostrum and lactoferrin-supplemented infant formula (Indyk and Filonzi, 2005). Latest methods such as micro-batch ion-exchange resin extraction method coupled with reverse-phase HPLC and one step RP-HPLC method for determining concentration of BLf in bovine milk samples have also been used (Pochet *et al.,* 2018 and Tsakali *et al.,* 2019). Various factors like breed, lactation stage as well as daily milk production influence the Lf concentration (Tsuji *et al*., 1990 and Cheng *et al.,* 2008).

BLf have been purified from bovine milk and colostrums by numerous methods such as CM-TP column chromatography, two-step ultrafiltration process followed by fast flow strong cation exchange chromatography system and fast protein liquid chromatography using CM Sephadex C-50 column and identity was confirmed using SDS- PAGE with band formation at 80KDa (Yoshida *et al*., 2000; Lu *et al.,* 2007; Moradian *et al.,* 2015, Vijayan *et al.,* 2017 and Shaz murtuza *et al*., 2021). However limited studies are done with regard to BLf quantification and comparision between exotic cross bred and indigenous cattle breeds. The milk of native cow breeds is superior in terms of proteins profile (Ashok kumar *et al.,* 2018). The present study was aimed to evaluate lactoferrin content by using sandwich ELISA and purify the lactoferrin from the milk of exotic crossbred and indigenous breed of cattle *i.e*. Sahiwal and Poda thurpu breed using cation exchange chromatography technique so that the lactoferrin content can be determined between the three breeds.

MATERIALS AND METHODS

Milk samples of HF cross bred and Sahiwal were collected from Livestock Farm Complex, College of Veterinary Science, Rajendranagar, Hyderabad, Telangana State and milk samples of Poda thurpu were procured from Achampet mandal of Nagarkurnool district, Telangana state.

Determination of BLf concentration

BLf concentration in skimmed milk samples was evaluated by sandwich ELISA method using a commercial bovine lactoferrin ELISA quantification kit (Kinesis Dx, Krishgen Biosystems). The procedures were performed according to the instructions of the manufacturer. 50 µL of standards which was prepared using the provided standard concentrations and diluents and around 40 µL of samples were pipetted into respective wells. Then 10 µL of biotinylated bovine lactoferrin antibody was added into each sample well but not in blank and standard wells. After addition of above antibody 50 µL of HRP conjugate was added into sample and standard well but not in the blank well). The plate was covered and incubated for 1 hour at 37ºC in incubator. The plate was washed 4 times with 1 X wash buffer and residual buffer was removed by tapping plate on absorbent paper. After washing 50 µL of TMB substrate A and 50 µL of TMB substrate B was added respectively. The plate was incubated for 10 min at 37ºC in dark. After incubation 50 µL of stop solution was added after which the wells turned from blue to yellow in colour. The absorbance was read at 450 nm within 15 min after adding the stop solution. The final absorbance of the samples was measured using an ELISA plate-reader (Bio rad). A standard curve was constructed by plotting the O.D. values for each standard on the vertical (Y) axis against the concentration on the horizontal (X) axis and the concentration of samples corresponding to the mean absorbance were obtained from the standard curve. The obtained values from native and cross bred cow milk has been tabulated properly and analyzed for finding significance ($P < 0.05$) among the breeds as per the procedures of Snedecor and Cochran (1994) by using statistical package for social sciences (SPSS – 25 software).

Isolation of bovine lactoferrin

The collected milk samples were processed for the isolation of BLf (Yoshida *et al.,* 2000 and Moradian *et al*., 2014). Acid whey was prepared by acidifying the diluted skimmed milk. This acid whey was filtered and neutralized The neutralized whey was subjected to ammonium sulphate precipitation initially by half saturation (0-45%) by adding ammonium sulphate and was brought to 45-80 percent saturation later. The sample fractionated was then dialysed using Himedia dialysis membrane 50. The insoluble materials in the dialysed protein solution were removed by centrifugation and the clear solution (supernatant) was then used for column chromatography. The ion exchange column was prepared using CM-Sephadex C-50 (Sigma Aldrich). Before addition of sample, the column was equilibrated with equilibration buffer (10mM sodium phosphate buffer with 250 mM NaCl). Flow rate of equilibration was maintained at 0.80 mL /min (10 drops / min). The sample obtained after dialysis was then loaded on to the equilibrated column and a flow rate of 0.33mL / min (4 drops /min) was maintained.

The bound protein was subsequently released from the resin using an isocratic separation technique involving varying molarities of NaCl. Elution buffers I, II, and III were 10mM sodium phosphate buffers containing 0.4 M NaCl, 0.6 M NaCl, and 0.8 M NaCl, respectively. The OD_{280} value of each fraction collected was measured in a spectrophotometer (Labomed INC, UVD 3200). The fractions with a minimum OD of 0.065 were recovered from the eluted samples. The absorbed protein was then eluted with elution buffer II, following the same process as before. After collecting the fraction with minimum OD of 0.065, the same protocol was followed with elution buffer III until OD became constant value. The samples with peak OD values were pooled and used for further confirmation of the protein by SDS-PAGE (Laemmli, 1970).

RESULTS AND DISCUSSION

The mean values of BLf (µg/mL) concentration in HF cross bred and indigenous breeds like Sahiwal and Poda thurpu was estimated as 306.47+8.91, 369.06+14.04 and 403.9+20.10 respectively. The significantly ($P < 0.05$) higher BLf concentration was observed in milk samples of Poda thurpu cow breed followed by Sahiwal cow milk. BLf concentration was recorded significantly ($P < 0.05$) lowest in HF cross breed among the experimental cow breeds. However, there was no significant ($P < 0.05$) difference observed between indigenous breeds like Sahiwal and Poda thurpu cow breeds. The results were closely related to the value obtained by Chen and Mao (2004) where BLf concentration was 466 µg/mL in cow milk. However, the concentration of BLf in HF cross bred and Sahiwal were contradictory where the BLf concentration were less than results obtained by Chen and Mao (2004) which may be due to genetic variations of the cattle breeds.

Table 1: Mean values of Lactoferrin (µg/mL) of milk among experimental cows of cross bred and Indigenous breeds

Sl. No.	Cow breeds		Lactoferrin (µg/mL)
1	Cross bred	Holstein Friesian	306.47 ± 8.91^{b}
2	Indigenous	Sahiwal	369.06 ± 14.04^{a}
3	Indigenous (Native breed of Telangana)	Poda thurpu	403.9 ± 20.10^{a}

Values are Mean ± SE (n=10); One way ANOVA (SPSS); Means with different alphabets as superscripts differ significantly ($P < 0.05$).

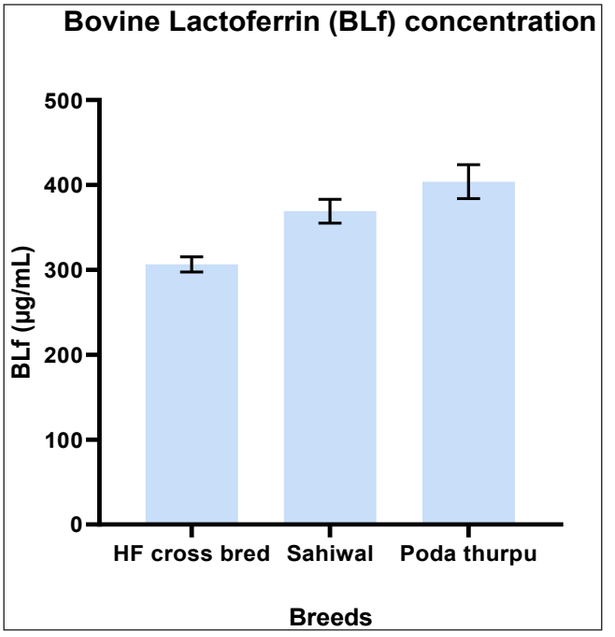

Fig. 1: Graph showing mean values of BLf (µg/mL) concentration of milk among experimental cows of cross bred and Indigenous breeds

The presence of BLf in eluted fraction was confirmed using SDS-PAGE. After destaining, a single band was observed in the electrophoretic profiles of the eluted fractions of all the three experimental cow breeds at the same position as the position of molecular weight of the BLf i.e. approximately 80 KDa as shown in the figure. This confirmed the identity and purity of BLf of all the three experimental cow breeds. However, a clear protein band was observed in Poda thurpu cow milk sample and less distinct faint protein band was noticed in Sahiwal and HF cross bred cow milk. The results were in strong resemblance with various studies where a single band was formed at approximately 80 KDa (Calvo and Batistaviera, 1994; Yoshida *et al*., 2000; Lu *et al.,* 2007 and Conesa *et al.,* 2008).

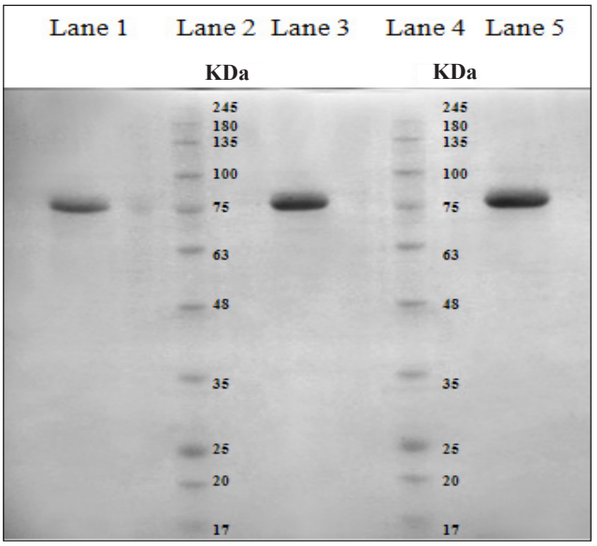

Fig. 2: Electrophoretic mobility of eluted Bovine Lactoferrin fraction

Lane 1: Eluted fraction isolated from HF cross bred cow milk containing BLf; **Lane 3**: Eluted fraction isolated from Sahiwal cow milk containing BLf; **Lane 5**: Eluted fraction isolated from Poda thurpu cow milk containing BLf; **Lane 2 and 4**: Standard protein marker.

CONCLUSION

The BLf concentration in milk samples of HF cross bred and indigenous cow milk differ significantly ($P < 0.05$) and higher concentration was found in indigenous breeds followed by HF cross bred. However, there was no significant difference in Lf concentration of native breeds. BLf isolated from all the three experimental cow breeds by using FPLC. Further it was evident by SDS-PAGE analysis that a more distinct, clear protein band was observed in Poda thurpu cow milk sample and less distinct faint protein band was noticed in Sahiwal and HF cross bred cow milk. The present study revealed that the BLf content was highest in Poda thurpu cow milk and least in HF cross bred cow milk, establishing the superiority of native breeds.

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