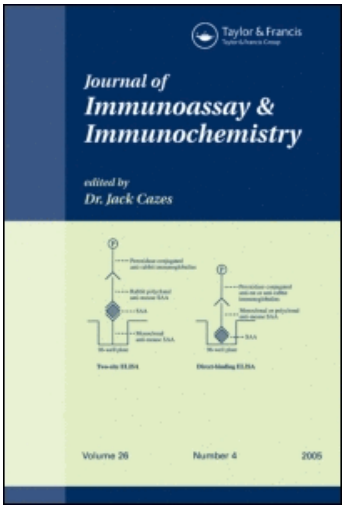

Journal of Immunoassay and Immunochemistry

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

A Sensitive Sandwich ELISA for Buffalo Prolactin

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To cite this Article Panchal, Manoj , Muralidhar, K. and Gupta, S. K.(2007) 'A Sensitive Sandwich ELISA for Buffalo Prolactin', Journal of Immunoassay and Immunochemistry, 28: 2, 147 — 154
To link to this Article: DOI: 10.1080/15321810701212062
URL: http://dx.doi.org/10.1080/15321810701212062

Journal of Immunoassay & Immunochemistry, 28: 147–154, 2007

ISSN 1532-1819 print/1532-4230 online
DOI: 10.1080/15321810701212062

A Sensitive Sandwich ELISA for Buffalo Prolactin

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Abstract: Two murine monoclonal antibodies have been developed against the buffalo prolactin (buPRL). These were designated as 1501 and 1504. Using two MAbs and anti-buPRL rabbit serum, an analysis was performed for the development of a sandwich ELISA (sELISA). The 1504/buPRL/anti-buPRL rabbit serum system was found feasible for sELISA. The sELISA had a sensitivity of 156 pg/mL. The buffalo serum sample showed a parallelism with the standard curve. The intra- and inter-assay coefficients of variance were 8.4% and 9.06%, respectively. These data proved the validity of the assay.

Keywords: buPRL, Sandwich ELISA, Monoclonal antibodies

INTRODUCTION

Buffalo (Bubalus bubalis) is the key animal in the dairy economy of India. Several aspects of buffalo reproductive physiology are unclear. Unavailability of specific homologous immunoassays has hampered our understanding of its reproductive physiology. Prolactin (PRL) is one of the hormones which plays an important role in reproduction. Prolactin knockout female mice have been shown to be sterile because of failure of implantation.[1] Recently, studies on

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artificial insemination in buffaloes have indicated that factor(s) responsible for implantation of embryos plays a crucial role in its success.[2] Prolactin exhibits a diversity of activity in vertebrates.[3] Its diversity has been attributed to its structural heterogeneity.[4] Different size and charge isoforms of prolactin have been reported in different species of mammals. Because of the presence of different isoforms in the serum/plasma samples, it is impossible to say which particular isoform of prolactin is related to the particular physiological state of the species. Assays of different isoforms or particular groups of isoforms of PRL has always been emphasized, especially in cases like prolactin and growth hormone as being crucial to understand its actions. Such types of assays are possible only by a probe which can recognize a particular isoform or group of isoforms. Monoclonal antibody based assays are useful in such situations. An epitope created by a different isoform can be specifically recognized by a monoclonal antibody. In this report, a sandwich ELISA for prolactin has been developed and applied to the serum samples of buffaloes.

EXPERIMENTAL

Production of Monoclonal Antibodies For Buffalo PRL

A 1 mg/mL solution of buPRL in 0.9% NaCl was mixed with an equal volume of Freund's complete adjuvant (FCA) and emulsified. Five female mice were immunized by injecting, subcutaneously, the emulsion equivalent to 50 μg buPRL per mouse. The same amount of buPRL was given, i.p., with incomplete Freund's adjuvant for boosters. Two boosters were given, i.p., at intervals of one month each. Fifteen days after the 2nd booster, higher doses (150 μg-300 μg PRL in saline) were given, i.p., for three consecutive days. Twenty four hours after the last injection, the spleen was surgically removed and the spleen cells were released using forceps. The spleen cells were mixed with Sp2/o cells in a 1:4 ratio. The cell suspension was washed with serum free DMEM (Sigma) medium. Fusion was initiated by adding 0.5 mL of 50% PEG 1500 (Sigma) solution (prewarmed to 37°C) into the tube. After a minute, the suspension was diluted and plated in a 24-well plate. Presence of antibody in the culture supernatant was checked by a direct binding ELISA. 200 μL of culture supernatant was aspired from each well of the plates and used as a primary antibody in the ELISA. The cells from positive wells were collected and diluted separately in 20% DMEM so that concentration of cells was 1 cell/200 μL. The rest of the suspension was transferred to a flask. 100 μL of the diluted suspension was added to each well of a 96-well plate and left in a CO_2 incubator for 10–14 days, until macroscopic colonies were visible. 200 μL of the medium was aspirated from the positive wells and replaced with fresh medium. This 200 μL of culture supernatant was used in an ELISA to note the presence of antibody.

SDS-PAGE

Samples were analyzed by a 15% polyacrylamide gel, using Atto made apparatus with glass sheet (11 × 13 cm). All the buffer systems followed were according to Laemmli et al.[5]

Western Blot

At the end of the run, the gel was transferred to a nitrocellulose paper, electrophoretically, at a constant current of 10 mA, overnight, according to the procedure of Towbin et al.[6] 1504 culture supernatant or 1:500 diluted rabbit a/s to buPRL, developed earlier, was used.[7]

Direct Binding Enzyme Linked Immunosorbent Assay (ELISA)

Antigen in a 100 μL volume was coated onto 96-well ELISA plates (Greiner Bio-One GmbH, Germany) and the plates were left overnight for incubation at 4°C. The rest of the steps were carried out at room temperature. The next day, each well was washed thrice, each time with 250 μL of 10 mM phosphate, pH 7.2/0.5% Tween 20 (PBT). Blocking was done with 1% casein/100 mM phosphate, pH 7.2, for 1 hr. At the end of this time, 100 μL of a 1:10,000 diluted rabbit anti-buPRL serum in 0.1% casein/100 mM phosphate, pH 7.2, was added to all the wells and the plates were incubated for 3 hr. After removing the primary antiserum, 100 μL of anti-rabbit IgG-HRP conjugate, diluted 1:1,000 in 0.1% casein/100 mM phosphate, pH 7.2, was added and incubation continued for 1 hr. Each incubation step was followed by washing in PBT, as above. The volume of 100 μL substrate, 1 mg OPD/50 mM citrate buffer, pH 5.5/0.06% H_2O_2 was added to each well and left for 15 min incubation at room temperature. The reaction was stopped by addition of 25 μL of 1 M oxalic acid. The color was read at 490 nm using an ELISA plate reader (ECIL, India).

Ascites Development for Hybrid

A syngenic mouse was injected, i.p., with 0.5 mL of pristane (Sigma) and left for 7–10 days. At the end of this time, $1-5 \times 10^6$ cells, washed and suspended in plain DMEM (no FCS/protein was present), were injected, i.p., and the mice were left for 10–14 days. During this period, the peritoneal cavity started swelling. The peritoneal fluid was tapped out by an 18G needle into a 15 mL centrifuge tube. The mouse was left for another 2–3 days for the next tapping. Each batch of acsites was centrifuged to separate the plasma from the cells. After adding 0.02% azide and making suitable aliquots, the

supernatants were kept at −20°C till use. The cells were stored in 10% DMSO and 90% FCS to be injected further into another syngenic mouse.

Sandwich ELISA

Ascites of 1504 was diluted 1:5,000 in 0.1M NH_4HCO_3. Then, 100 μL of the solution was coated onto wells of the ELISA plates. Wells were washed once with 10 mM phosphate buffer/0.05% Tween 20. Different dilutions of buPRL or serum samples were placed into wells. The plate was kept aside for 2 hr. At the end of this period, the plate was washed once with PBT. In each well of the plate, 100 μl of 1:10,000 diluted anti-buPRL serum was added and the plate was kept for 2 hr. At the end, plate was washed thrice with PBT. HRP-conjugated anti-rabbit IgG (Genei, Banglore, India), was placed in each well and kept for 1 hr. Then, 1 hr later, the plate was washed thrice with PBT. 100 μL of substrate containing H_2O_2/OPD/50 mM citrate, pH 5.5, was added for color development. The plate was read at 490 nm by an ELISA plate reader (ECIL, India).

Intra- and Inter-Assay Coefficients

To determine intra-assay coefficient of variation, a serum sample was run in 6 replicates. Inter-assay coefficient of variation was determined by analyzing aliquots from a single sample in 7 differrent assays.

RESULTS

Monoclonal anti-PRL Antibodies Characterization

Two clones were obtained, i.e., 1501 and 1504, from the fusion experiment for buPRL. 1501 and 1504 were analysed by Western blot (Figs. 1 & 2). Two and three bands were detected, respectively, by 1501 and 1504, while anti-buPRL serum detected 7 size isoforms. These bands were identified as 45K and 23K for 1504 and 23K, 45K, and 26K for 1501. The 45K and 23K were dimer and monomer, respectively. The 7 bands recognized by the anti-PRL serum were 66K, 45K, 29K, 26K, 23K, 18K, and 14K.

Direct Binding ELISA

The ascites developed for 1504 was analysed by a direct binding ELISA. The dilution of ascites by 1:2,000 could detect 10 ng/mL of buPRL while the same concentration was detected by 1:10,000 diluted anti-buPRL serum (Figs. 3 & 4).

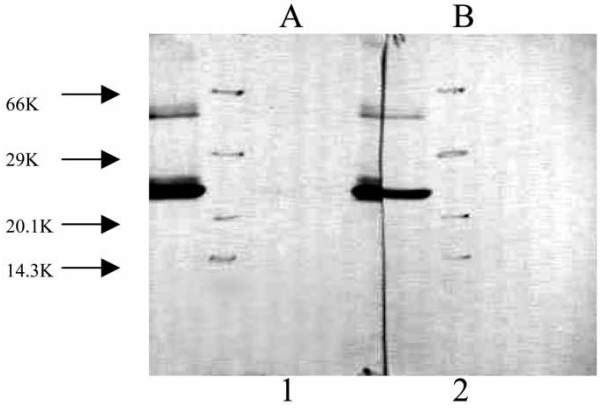

Figure 1. Western blots of buPRL. The monoclonal 1501 (1) and 1504 (2) were used as primary antibody for the development of the blot. Marker protein has been marked on the blots.

Standard Curve and Parallelism of Assay

The sandwich ELISA was developed by the same ascites and anti-PRL serum (Fig. 5). Using different combination of dilutions, 1:5,000 and 1:40,000 were used, respectively, for 1504 and anti-PRL serum. The standard concentration of the assay used were 78.125, 156.25, and 312.5 pg/mL, 625 pg/mL, 1250 pg/mL, 2,500 pg/mL, 5,000 pg/mL, 10,000 pg/mL, 20,000 pg/mL, and 40,000 pg/mL. There was parallelism between the standard curve and the serum dilutions (Fig. 5).

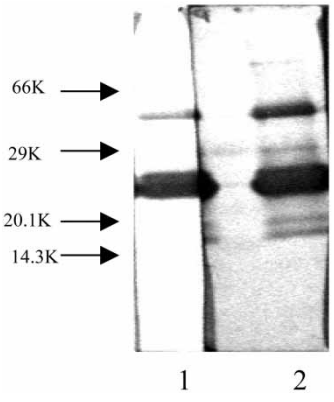

Figure 2. Western blot of SDS PAGE of buPRL. Blot 1 developed by 1501; blot 2 developed by anti-buPRL serum.

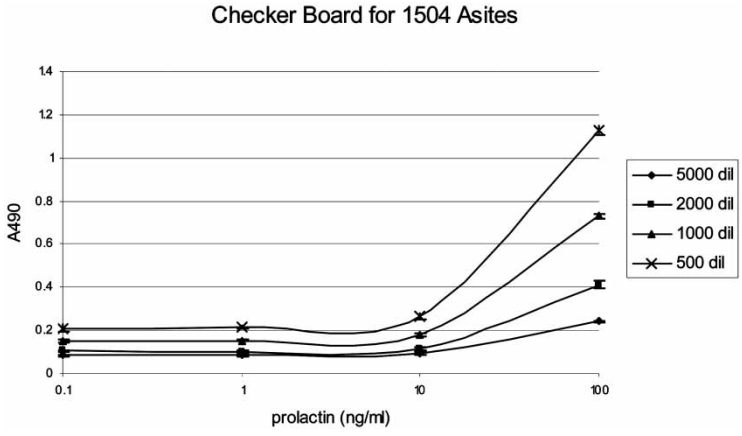

Figure 3. Direct binding ELISA to check the optimum dilution of 1504 ascites.

Sensitivity of Assay

Sensitivity of the assay is defined as blank $\pm$ SD. The sensitivity was measured as 156 pg/mL. The buffalo serum samples were used at different dilutions.

Intra- and Inter-Assay Coefficient of Measurement

The maximum intra-assay CV measured was 8.4%. Inter-assay CV, obtained by a sample in 7 different assays, was 9.06%.

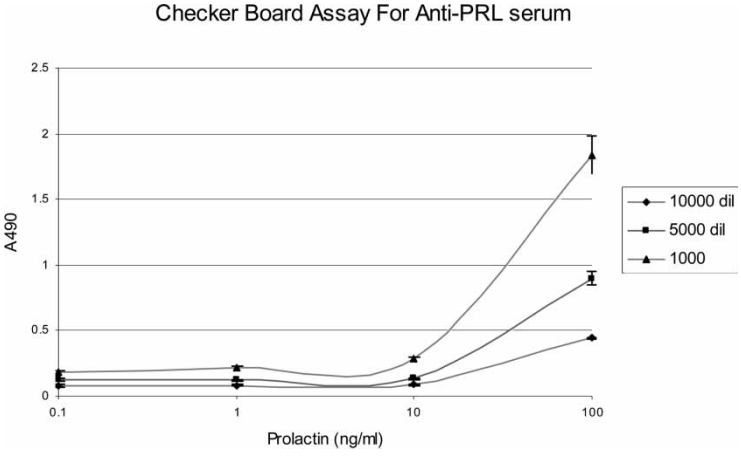

Figure 4. Direct binding ELISA to check the optimum dilution of anti-buPRL serum.

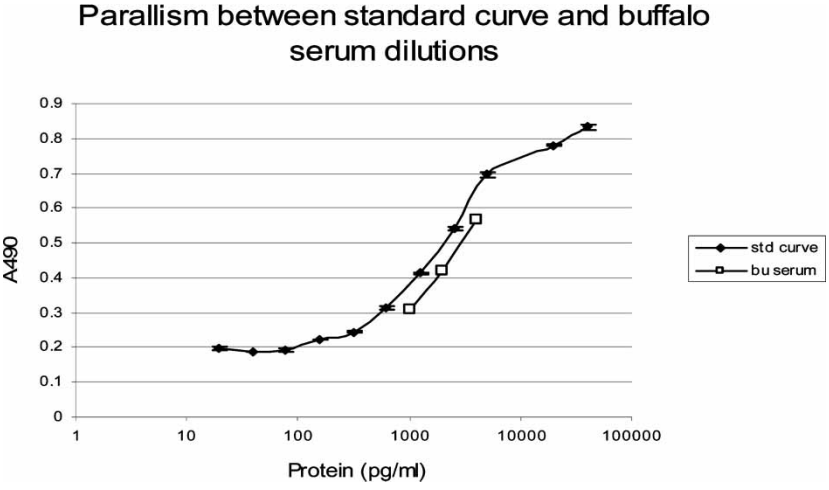

Figure 5. The graph shows the parallism between standard curve of sandwich ELISA and different dilutions of serum sample. Values of concentrations for serum sample were taken arbitrarily to show parallelism.

DISCUSSION

Western blot, developed by anti-buPRL serum and 1504, showed that 1504 recognised only 2 bands, i.e., 23K and 45K, out of 7 bands of buPRL recognized by anti-buPRL serum. Therefore, 1504 recognised only a particular subset of size isoforms of buPRL.

On a direct binding ELISA, 10 ng/mL could be detected by 1:1,000 dilution and 1:10,000 dilution of 1504 ascites and anti-PRL serum, respectively, while sandwich ELISA could increase the sensitivity to 0.156 ng/mL using dilutions of 1:5,000 and 1:40,000 for 1504 ascites and anti-buPRL serum, respectively. This shows that buPRL has a lower affinity for the ELISA plate surface than IgG present in 1:5,000 dilution of ascites. Therefore, the higher amount of IgG bound onto the surface of the ELISA plate increased the binding and sensitivity of buPRL.

Different dilutions of serum samples showed parallelism with the standard curve of buPRL and intra- and inter-assay CVs were less than 10%. These data prove its validity for serum samples.

Buffalo pituitary prolactin has been quantitated by assays developed earlier, using anti-buPRL serum or anti-oPRL serum.[7–9] There is no assay available which was developed based on buPRL monoclonal antibody. There is a scope for development of MAb based assays of hormone like prolactin which has a different size and charge, as well as phosphorylated and sulphated forms.[4,7,10] Such difficulties of measurement of different isoforms have been discussed earlier.[11] But, still, there is no assay for

determination of a particular isoform, except for one which could not recognize glycosylated porcine PRL.[12] Each assay which can recognize a particular group of isoforms is important to monitor the changes of that particular group during different physiological states.

This is the first ELISA based on MAb of buPRL. Because of its peculiar behaviour, several aspects of the buffalo reproductive physiology are not known. Such assays are important to elucidate the importance of prolactin in reproductive physiology.

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Received September 30, 2006
Accepted October 28, 2006
Manuscript 3215