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Humoral immune response in cattle and buffaloes vaccinated against *Pasteurella multocida* B: 2 with reference to levels of B Lymphocytes and IL-2R$^+$

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ABSTRACT

HS vaccines have been developed from time to time using whole bacteria or their components The variation in duration of immunity, kinetics and isotype of antibody and humoral and cellular immune responses with regards to vaccine have been poorly understood. There are repeated incidences of vaccination failure inspite of vaccinations done at regular intervals. This work was envisaged to study the role of humoral immunity by emphasizing on levels of B lymphocytes *in vitro* and their markers in unvaccinated and vaccinated cattle and buffaloes against *P. multocida* B: 2 (strain P52) at different time intervals. On immunoflourescence staining using monoclonal antibodies for B lymphocytes and activated lymphocytes (IL-2R$^+$), the distinct fluorescence pattern was seen on the periphery of both B lymphocytes and activated B lymphocytes. Flowcytometry was done to evaluate the levels of B lymphocytes and activated lymphocytes (IL-2R) in blood from 10 cattle and 10 buffaloes which were vaccinated with alum precipitated HS vaccine. There has been a decrease in the level of B Lymphocytes over a period of time from zero days to 90 days post vaccination. But there was no significant variation in B lymphocytes levels and activated lymphocyte (IL-2R$^+$) levels between prevaccinated and post vaccinated cattle and buffaloes at different time intervals (30 day, 60 day, 90 days and 120 days) as revealed by ANOVA. However a significant increase was revealed by paired t-test in activated lymphocytes (IL-2R$^+$) levels at 30 days and 120 days post vaccination.

1. INTRODUCTION

HS vaccines have been developed from time to time using whole bacteria (conventional vaccine from P52 strain of *Pasteurella multocida*) or their components The variation in duration of immunity, kinetics and isotype of antibody and humoral and cellular immune responses with regards to vaccine have been poorly understood. There are repeated incidences of vaccination failure inspite of vaccinations done at regular intervals. IL-2 plays a major role in the activation of humoral immune response by binding to IL-2R and stimulating various immune cells. The interleukin-2 (IL-2) / IL-2 receptor (IL-2R) system is the main regulatory determinants of T cell reactivity. The primary role of interleukin-2 (IL-2) is to expand the population of activated T lymphocytes [1]. It is the main cytokine controlling the proliferation of anti-CD3-stimulated CD4 T lymphocytes [2]. Binding of IL-2 to its receptor was initially demonstrated to be critical for inducing the proliferation of T cells *in-vitro* by Hatakeyama in 1989 [3]. IL-2 also stimulates the cytotoxic activity of CD8 T lymphocytes and NK cells, proliferation and Ig production by activated B lymphocytes, and some monocyte functions [4]. IL-2R signaling leads to the activation of many genes associated with cell proliferation such as c-myc and fos [5]. So signaling via the IL-2R complex would be critical for mounting effective immune responses *in vivo*. This work was envisaged to study the role of humoral immunity by emphasizing on levels of B lymphocytes *in vitro* and their markers in unvaccinated and vaccinated cattle and buffaloes against *P. multocida* B: 2 (strain P52) at different time intervals.

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2. MATERIALS AND METHODS

2.1.Estimation of levels of B lymphocytes in the peripheral blood in pre- and post-vaccinated cattle and buffaloes

2.1.1Collection of blood sample

Blood samples of 20 pre-and post-vaccinated cattle and buffaloes which were vaccinated with commercially available alum precipitated HS vaccine were collected from an organized dairy Farm in Ludhiana at monthly intervals up to 4 months post-vaccination. The collection of blood was done aseptically from the jugular vein of animal (using sterile 18 G needle) in a sterile tube containing 2% EDTA.

2.1.2 Isolation of blood leukocytes

Isolation of leukocytes was done by density gradient centrifugation using leukocyte separation method by Ficoll Hypaque (Sigma-Aldrich, USA) as per the standard method [6]. Briefly, the blood sample was centrifuged at 2500 rpm for 15 mins and buffy coat was separated and overlaid onto ficoll hypaque and centrifuged @ 2000 rpm for 30 min at room temp. After centrifugation the white layer of mononuclear cells at the junction of plasma and Ficoll Hypaque was collected and washed thrice with equal volume of RPMI-1640 by centrifugation at 3000rpm for 15 mins. Finally, the cell pellet was resuspended in RPMI-1640 to determine the cell viability and concentration.

2.1.3 Determination of cell viability and concentration

The number of viable cells in a cell suspension was determined by trypan blue dye exclusive method. 50µl of trypan blue solution (0.1% w/v in DW) was added to 5µl of cell suspension and 45µl PBS. This mixture was then loaded onto haemocytometer and viable cells (unstained) and dead cells were counted. The percentage of viable cells was determined and the viable cell concentration was adjusted to 2×10^6 cells/ml.

2.1.4 Indirect staining using monoclonal antibody

Standard immunofluorescent staining protocol of Hudson and Roitt [7] was followed with minor modifications. Briefly, primary α-bovine B cells (VMRD, Inc. USA) monoclonal antibody was diluted (1:200) in PBS. The primary antibody (50µl) was added to the 50µl of the cell suspension and mixed gently. The mixture was incubated for 1 hr at 37°c and then washed in RPMI-1640 medium and then re-suspended the cells in 50 µl of secondary Goat anti-mouse FITC antibody (Genei, Bangalore). The mixture was incubated for 1 hr at 4°C in dark and washed with PBS. The fluorescence was observed under fluorescent microscope (Olympus BX61, Nikon).

2.1.5 Counting of cell using flowcytometer

After binding with primary antibody, cells were fixed with 1% paraformaldehyde solution and held at 4°C protected from light. Prior to analysis on flowcytometer, two washings with PBS were given and then resuspended the cells in 50 µl of secondary antibody (Goat anti-mouse FITC antibody). The mixture was incubated for 1 hr at 4°C in dark and washed with PBS. Finally the cells were suspended in 250µl PBS and readings were taken on flowcytometer (BD FACS Calibur, Germany having FL-I filter). CellQuest Pro software was used to acquire, store and analyze data.

2.2 Levels of activated B lymphocytes (IL-2R+) in the peripheral blood in pre- and post-vaccinated cattle and buffaloes

2.2.1 Indirect staining using monoclonal antibody

Collection of blood sample and peripheral leukocyte isolation were the same as described earlier. Primary α-bovine CD25 (IL-2R) (VMRD, Inc. USA) monoclonal antibody was diluted (1:200) in PBS. The primary antibody (50µl) was added to 50µl of the cell suspension and mixed gently. The mixture was incubated for 1 hr at 37°C and then washed in RPMI-1640 medium and then resuspended the cells in 50 µl of secondary antibody *i.e.* Goat anti-mouse FITC antibody (Genei, Bangalore). The mixture was incubated for 1 hr at 4°C in dark and washed with PBS The fluorescence was observed under fluorescent microscope (Olympus BX61, Nikon).

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3. RESULTS AND DISCUSSION

3.1 Levels of B lymphocytes in the peripheral blood in pre- and post-vaccinated cattle and buffaloes

On immunoflourescence staining using monoclonal antibody (α-bovine B cells), the fluorescence was seen on the periphery of B lymphocytes (Fig 1).

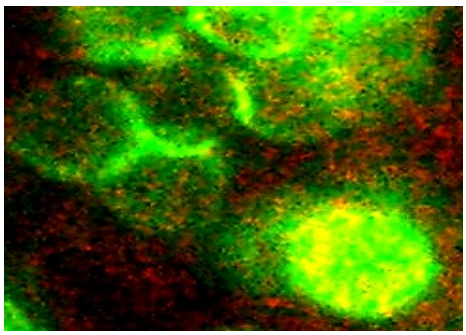
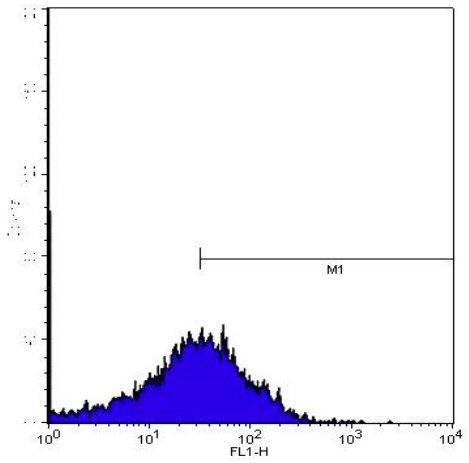


Fig. 1: Immunofluorescence on B lymphocytes.

Levels of B lymphocytes in cattle and buffalo calves vaccinated with alum precipitated HS vaccine were analyzed by flow cytometery (Fig 2).

The mean B lymphocytes levels at 0 day, 30 days, 60 days, 90 days and 120 days post vaccination were 39.40±13.59, 28.38±9.89, 29.77±12.45, 27.04±14.27 and 30.31±15.19 respectively (Table 1). There was gradual fall in levels of B lymphocytes (Fig 3). However, there was no significant variation found in B lymphocyte levels between prevaccinated and post vaccinated cattle and buffaloes at different time intervals (0 day, 30 day, 60 day, 90 days and 120 days) as revealed by ANOVA.



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M1	32, 9910	4409	44.09	44.09	81.33	66.66	93.72	58.82	51

Fig.2: B lymphocytes cell population observed using Flowcytometer.

Table 1: B lymphocyte levels in the peripheral blood in pre- and post-vaccinated cattle and buffaloes by flow cytometry.

Animal no.	Percentage of B lymphocytes levels				
	0 day	30 days	60 days	90 days	120 days
1.	16.21	27.14	38.8	11.55	30.16
2.	22.11	31.51	12.02	27.36	15.22
3.	36	37.46	11.25	19.59	35.47
4.	39.59	15.15	43.06	21.97	14.65
5.	60.04	33.56	29.57	19.98	35.24
6.	53.24	13. 90	26.94	43.58	13.7
7.	44.09	26.64	35.33	18.6	41.47
8.	50.77	41.69	41.24	53.71	56.62
9.	32.67	-	-	-	18.52
10.	39.35	-	-	-	-
Mean± SD	**39.40± 13.59**	**28.38± 9.89**	**29.77± 12.45**	**27.04± 14.27**	**30.31± 15.19**

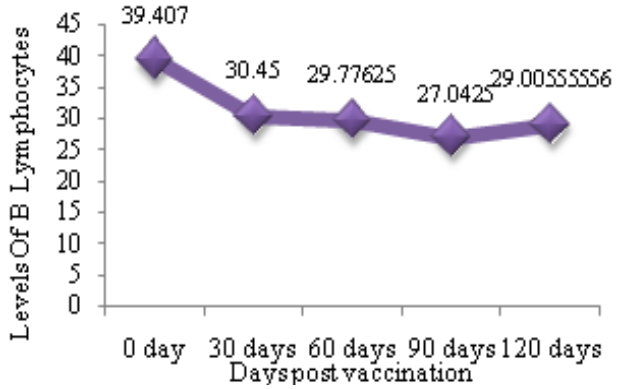


Fig. 3: Levels of B lymphocytes in the peripheral blood at different intervals by flowcytometer.

3.2 Levels of activated lymphocytes (IL-2R$^+$) in the peripheral blood in pre- and post-vaccinated cattle and buffaloes

On immunoflourescence staining using monoclonal antibody (α-bovine CD25), a distinct pattern of fluorescence was seen on the periphery of activated lymphocytes (IL-2R$^+$) (Fig 4). Levels of activated lymphocytes (IL-2R$^+$) in cattle and buffalo calves vaccinated with alum precipitated HS vaccine were analyzed by flow cytometery (Fig 5).

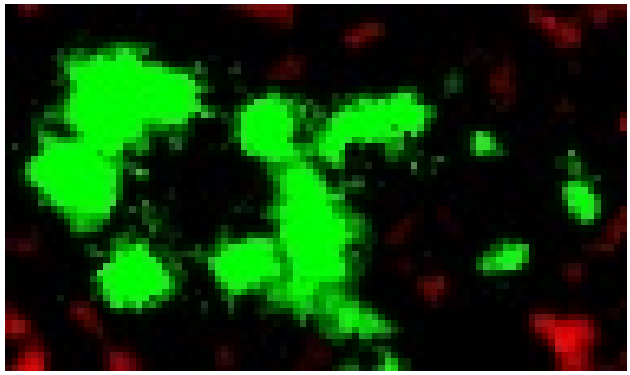
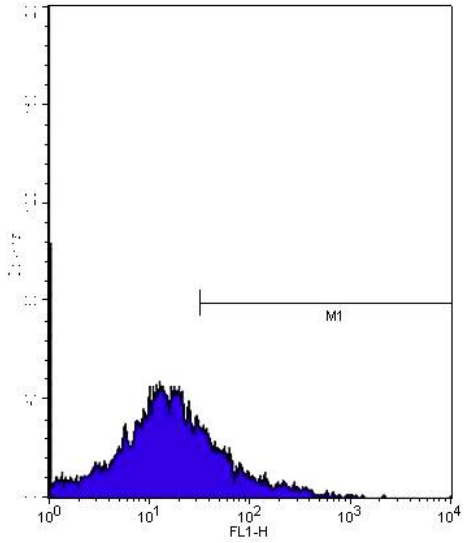


Fig. 4: Immunoflorescence on activated B lymphocytes (IL-2R+).



Histogram Statistics

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Patient ID:
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Total Events: 10000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
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M1	32, 9910	2178	21.78	21.78	96.08	67.99	236.41	55.23	32

Fig. 5: Activated B lymphocytes (IL-2R+) cell population observed using Flowcytometer.

The mean levels of activated lymphocytes at 0 day, 30 days, 60 days, 90 days and 120 days post vaccination were 17.25±8.65, 25.13±5.2, 22.18±8.30, 21.47±5.31 and 27.08±11.35, respectively (Table 2).

A significant increase was revealed by paired t-test in activated lymphocytes (IL-2R$^+$) levels at 30 days and 120 days post vaccination (Fig 6). But there was no significant variation in activated lymphocyte (IL-2R$^+$) levels between prevaccinated and post vaccinated cattle and buffaloes at different time intervals (30 day, 60 day, 90 days and 120 days) as revealed by ANOVA.

Table 2: Activated lymphocyte (IL-2R+) levels in the peripheral blood in pre- and post-vaccinated cattle and buffaloes by flow cytometry.

Animal No.	Percentage of Activated lymphocytes (IL-2R+) levels				
	0 day	30 days	60 days	90 days	120 days
1	11.02	21.47	22.12	23.48	20.21
2	15.31	31.07	11.15	21.38	23.6
3	11.57	20.81	32.72	17.18	13.24
4	13.46	25.83	23.52	31.34	30
5	12.9	17.13	14.36	26.22	28.81
6	18.61	29.58	29.21	12.84	46.63
7	37.59	23.97	-	22.07	-
8	17.6	31.2	-	19.71	-
9	-	-	-	19.02	-
Mean±SD	**17.25±8.65**	**25.13±5.2**	**22.18±8.30**	**21.47±5.31**	**27.08±11.35**

No information is available in the literature related to the levels of activated lymphocytes (IL-2R) in vaccinated animals. The IL-2 binds with IL-2R and stimulates various immune cells. The interleukin-2 (IL-2)/IL-2 receptor (IL-2R) system is the main regulatory determinant of T cell reactivity. The primary role of IL-2 is to expand the population of activated T lymphocytes [1]. IL-2 is the main cytokine controlling the proliferation of lymphocytes [2]. Binding of IL-2 to its receptor is critical for inducing the proliferation of T cells *in vitro* [3]. IL-2 also stimulates the cytotoxic activity of CD8 T lymphocytes and NK cells, proliferation and Ig production by activated B lymphocytes and some monocyte functions [4].

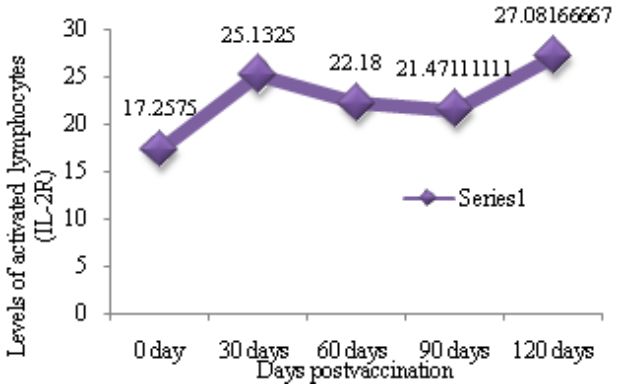

Fig. 6: Levels of IL-2R lymphocytes in the peripheral blood at different intervals.

4. CONCLUSIONS

This study concludes that there is no significant variation in the levels of B lymphocytes in prevaccinated and post vaccinated cattle and buffaloes over a time period (0 day, 30 day, 60 day, 90 days and 120 days). An increase in the levels of activated lymphocytes (IL-2R$^+$) was observed at 30 days and 120 days post vaccination. But there was no significant variation in levels of activated lymphocytes (IL-2R$^+$) between prevaccinated and post vaccinated cattle and buffaloes over a period of time.

5. ACKNOWLEDGEMENTS

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