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SHORT COMMUNICATION

Optimization of In-House Indirect ELISA for *Pasteurella multocida* B: 2 Based on Recombinant Outer Membrane Protein H to Detect Post Vaccination Immune Response in Bovine in Pakistan

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A B S T R A C T

To estimate the immune response against *Pasteurella multocida* B:2 in cattle and buffalo, different serological assays have been used as agglutination and indirect haemagglutination assay (IHA), but none is highly sensitive. Enzyme Linked Immunosorbent Assay (ELISA) has been effectively used to determine the immune status in vaccinated cattle and buffalo. It was developed by using purified recombinant outer membrane H protein (rOmpH) as coating antigen of *P. multocida* B: 2. The optimum condition for this assay was 1/800 coated rOmpH antigen (1.22 ug/mL), 1/400 dilution of primary sera and 1/5000 dilution of the conjugate. The cut-off value was calculated as 0.101. The sensitivity and specificity of developed ELISA was 92 and 86% respectively. Positive and negative predictive value was 87.42 and 91.6% respectively. This finding suggests that an in-house indirect ELISA is highly sensitive than IHA and can be used to monitor the bovine antibodies against hemorrhagic septicemia in Pakistan.

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INTRODUCTION

Pasteurella multocida, causative agent of hemorrhagic septicemia, is a gram negative and opportunistic pathogen of upper respiratory tracts of wild and domestic animals (Fowler and Mikota, 2006). It has economic importance because of endemic and epizootic diseases in domestic and wild animals, including snuffles in rabbit, fowl cholera in poultry, atrophic rhinitis in swine and hemorrhagic septicemia (HS) in cattle and buffalo (Chandrasekharan, 2002).

In India, a study was conducted for estimation of economic losses due to HS, which concluded that morbidity losses account for 23% of total losses and remaining 77% due to the mortality of animals. At the Indian national level, the losses were estimated ₹5255 crores (Sing *et al*., 2014). There is no diagnostic test available in Pakistan to monitor the immune status of vaccinated cattle and buffalo. The world organization for animal health reference for HS recommends that IHA titer from 1:160 to 1:1280 indicates the exposure of HS in affected herds. In previous studies, different forms of antigens as whole cell, capsular or heat extracted antigen of *P. multocida* were used for coating on ELISA plate (Hatfaludi *et al.*, 2010). In the current study, we used rOmpH, which is highly immunogenic protein of *P. multocida* B: 2 as a coating antigen in ELISA. ELISA is best technique used to detect the immune status of diseased and healthy animal against hemorrhagic septicemia (Kharb, 2015). For the validation of new diagnostic tests, different statistical tools are used to classifying cases as positive and negative as Mean + 3SD and standard curve. Indirect haemagglutination (IHA) was used as a gold standard test for the evaluation of developed diagnostic test in the present study. The present study was conducted to develop the in house indirect ELISA to check the immune status of healthy and diseased population of bovine. This is the first ever study to evaluate the immune status of bovine in Pakistan.

MATERIALS AND METHODS

Preparation of recombinant outer membrane H protein as coating antigen: The *Pasteurella multocida* B:2 was isolated from natural outbreak of HS and the *ompH* gene was amplified having 1002 bp. It was further

cloned into expression vector pET 40-b (+) and transformed into BL21 (DE3) cells for the expression of recombinant rOmpH protein. The cells were induced with 1 mM of IPTG and got maximum expression in soluble cytoplasmic fraction of the cell followed by the sonication of cells. The rOmpH was purified by HisPur™ Ni-NTA Spin Columns and characterized by SDS-PAGE and Immunoblotting analysis by anti-His antibodies. The recombinant rOmpH protein had molecular weight of 36 KDa and used as coating antigen in in-house ELISA.

Calf sera and animal ethics: The positive sera were raised by immunization of 100ug rOmpH in two calves, kept at Ravi campus of University of Veterinary and Animal Sciences, Lahore, Pakistan by providing standard conditions, and all experimental approaches were according to ethics. However, two claves were immunized with PBS to raise the negative sera. The titers of antibodies were checked by agar gel precipitation test (AGPT) method as described by Abernathy and Heiner, (1961). To evaluate the ELISA kit, 350 bovine sera samples were collected from different farms of Lahore and tested the sera samples by indirect haemagglutination assay to declare sera as positive and negative as per standard protocol (Anonymous, 2004).

Indirect ELISA: Immuno plates (Nuncimmuno ™ plate, Denmark) were used for the coating of rOmpH as coating antigen. The rOmpH protein was diluted in coating buffer (0.1M carbonate and bicarbonate pH: 9.6). The plates were incubated at 4°C overnight and washed with washing buffer (phosphate buffer with tween 20 of 0.05% -PBST). To reduce the non-specificity of results, plates were blocked with blocking buffer (1% Bovine serum albumin dissolved in PBS-T) for two hour. The primary antibodies were diluted in blocking buffer and incubated the plates for one hour at room temperature. Plates were washed with washing buffer three times. The conjugate (HRP sheep Anti-Bovine IgG, Thermoscientific, USA) were diluted in blocking buffer and incubated the plates for one hour at room temperature and washed the plates with washing buffer three times. Tetramethylbenzidine (TMB, Thermoscientific, USA) was used as a substrate and optical density (OD) read at 450nm after stop the reaction using ELISA plate reader (Multiskan FC, Thermoscientific, USA).

Optimization of the indirect ELISA: Checker board titration method was used to optimize the reagents as described by Crowther (2001). The rOmpH was coated on plate with different dilutions from 1:50 to 1: 25600 with 2-fold dilutions. The primary antibodies were 2-fold diluted with blocking buffer from 1:50 to 1:3200 as shown in Table 1. The optimized dilution 1:5000 of secondary antibodies as conjugate (Thermo-scientific, USA, Catalog # PA1-84664) was used in reaction.

Calculation of cut-off value: The cut-off value was calculated by the sample over positive ratio described by Crowther (2001). The formula for cut-off value is described as: Cut-off value = Mean OD_{450} of negative sera + three standard deviations of Mean OD_{450} of negative sera.

Sensitivity and specificity of ELISA: The samples (n=302) were collected for the estimation of sensitivity and specificity of in-house ELISA kit from different farms of Lahore. As per history, all animals were previously vaccinated against HS. The antibodies titer were determined by the gold standard test IHA and developed in-house ELISA kit based on cut-off value as described above. The sensitivity and specificity were determined by given formula: Sensitivity =100×TP/TP+ FN; Specificity= =100×TN/TN+FP. Positive and negative predictive value was calculated by described formula: PPV=100×TP/ (TP+FP); NPV=100×TN/ (FN+TN) as shown in Table 2.

RESULTS AND DISCUSSION

The serum sample was considered to be positive if IHA titer is higher than 1:16 while the titer of negative sera is to be equal or less than 1:4 as shown in Table 4. The optimum condition for ELISA was 100uL of 1/800 rOmpH antigen (1.22ug/mL), 100uL of 1:400 dilution of primary antibodies in blocking buffer (1 % BSA in PBST) and the optimum dilution for conjugate (Sheep anti-bovine IgG HRP) was 100uL of 1:5000 diluted in blocking buffer. The standard curve was standardized when OD_{450} value of last dilution of positive sera sample was equal to the OD_{450} value of negative sera. The standard equation {X = (OD of samples – 0.4847)/0.005} was used to calculate the standard values.

Cut-off value calculated by mean of negative sera samples at OD_{450} was 0.052, and the value of three standard deviation of mean of negative was 0.049. However, the set calculated cut-off value was 0.101. On the base of cut-off value, seropositive and seronegative samples were decided. If the OD_{450} value of sample sera is more than 0.101 that is to be considered as seropositive and the sample with less than 0.101 OD_{450} value is

Table 1: Checker board titration method (CBT): the antigen was diluted 2-fold horizontally from 1 to 11 rows, while antibodies diluted 2-fold vertically from A to G. The area in blue from column D1 to D5 was indicated the ideal OD_{450} values (2.023, 2.106, 1.804, and 1.878) of antigen and antibodies titrating maximally. The however 6th column and row E indicating, decrease in OD_{450} values (1.464, 1.388, 1.395, 1.264, 1.212, 1.212) due to decrease in antigen and antibodies concentration

	50	100	200	400	800	1600	3200	6400	12800	25600	51200	
	1	2	3	4	5	6	7	8	9	10	11	12
A	3.43	3.49	3.01	2.44	2.02	1.62	1.32	1.22	1.12	0.64	0.39	0.02
B	3.48	3.36	2.98	2.59	2.11	1.51	1.11	0.91	0.83	0.52	0.52	0.03
C	2.44	2.33	2.53	2.18	1.80	1.50	1.40	0.99	0.72	0.50	0.41	0.01
D	2.47	2.32	2.47	2.10	1.88	1.60	1.60	0.99	0.72	0.30	0.21	0.04
E	1.46	1.39	1.40	1.26	1.21	1.21	1.22	1.20	1.21	0.42	0.36	0.02
F	1.45	1.33	1.28	1.16	0.94	0.95	0.96	0.85	0.88	0.34	0.20	0.03
G	1.26	1.18	1.05	1.04	0.51	0.41	0.22	0.11	0.11	0.01	0.09	0.02
H	0.07	0.07	0.05	0.05	0.04	0.03	0.02	0.01	0.03	0.02	0.01	0.02

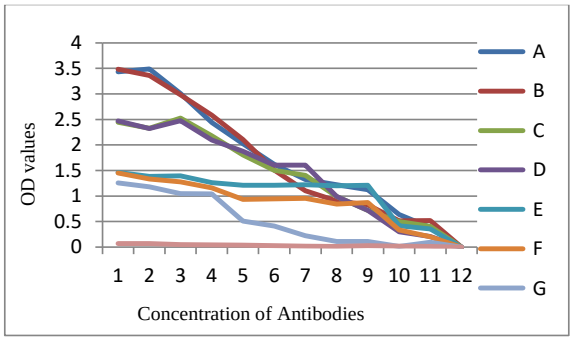

Fig. 1: Graphical representation of Checkerboard titration method; Graph relating OD values for different antigen concentrations against dilution series of antibody, as diluted the antibodies (1 to 11) antigen (A to G) the OD 450 values also decrease gradually.

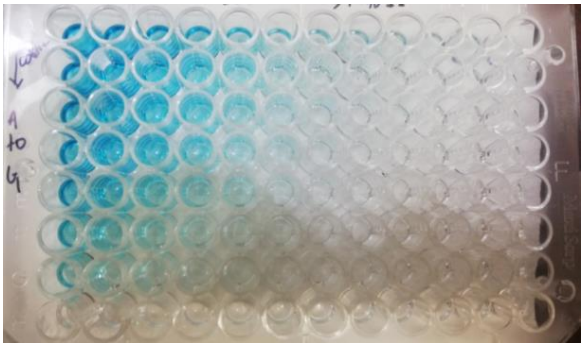

Fig. 2: In checkerboard titration, antigen and antibodies diluted horizontally and vertically respectively. The intensity of color decrease gradually as antigen and antibody diluted.

Table 2: Sensitivity and Specificity was calculated by two by two table as given below

	IHA +ve			IHA -ve		
ELISA +ve	139	True positive	TP	20	False positive	FP
ELISA -ve	12	False negative	FN	131	True negative	TN

Sensitivity= 100×TP/TP+ FN= 92%; Specificity= 100×TN/TN+FP= 86%
PPV=100×TP/ (TP+FP) = 87.42%; NPV=100×TN/ (FN+TN) = 91.60%

considered as seronegative as previously described (Pruvot *et al.*, 2013; Dogra *et al.*, 2015). Field sera samples were tested by IHA and ELISA and calculated the sensitivity and specificity according to the statistical formula given by Samad *et al.*, 1994). According to the two by two table, true positive (TP) and true negative samples were 139 and 131 respectively while false positive and false negative samples were 20 and 12, respectively. According to the formula, the calculated sensitivity was 92% and specificity was 86% as described (White et *al.*, 2016). Positive and negative predictive value was 87.42 and 91.6% respectively.

From study it was concluded that ELISA is highly effective and sensitive test than IHA to detect the immune status of vaccinated cattle and buffalo in Pakistan. It was concluded that rOmpH of *P. multocida* B: 2 is immunogenic and can be used as coating antigen in optimization of ELISA (Takada-Iwao *et al.*, 2007). According to the OIE hemorrhagic septicemia manual, the immune response of cattle was determined by IHA. In this study, field isolates were first tested by IHA and then analyzed by in house indirect ELISA. The IHA positive sera were lower than the ELISA positive sera and concluded that the sensitivity of ELISA is higher than IHA. This study suggested that the ELISA would be more powerful than IHA for classifying positive or negative samples as described by Tankaew *et al.* (2017). There is no ELISA kit available in Pakistan to detect the immune response of vaccinated cattle and buffalo. Before that IHA test was used to monitor the immune status of bovine against HS but due to low sensitivity of IHA, we developed in-house ELISA kit which would be able to monitor the immune status of bovine and decrease the economic losses. The developed ELISA is economical, easy to perform and can be used to screen many samples at a time

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Authors contribution: Ali Ahmad Sheikh and Aamir Ghafoor conceptualize the idea. Abida Mushtaque worked on research and methodology. Wasim Shehzad assisted in purification of protein. Muhammad Rizwan Khan collected the bovine sera samples from different farms of Lahore, Pakistan.

Table 3: OD $_{450}$ values calculated by ELISA. The OD values highlighted in blue color were indicating as test sample is negative by ELISA and positive by IHA test while OD values highlighted in green color were indicating as test sera sample is positive by ELISA and negative by IHA test

Value	1	2	3	4	5	6	7	8	9	10	11	12
A	1.7257	1.2631	1.8543	0.0489	0.0861	0.0340	0.0560	1.6640	0.0765	0.0750	0.0461	0.0351
B	0.0653	0.0670	0.7283	2.0777	0.0560	1.4679	0.0670	0.0207	0.0947	1.5075	1.9367	2.1165
C	0.0656	0.0355	1.0874	0.0540	1.4432	0.0297	1.1181	0.0653	1.5483	0.0248		
D	0.0430	1.0216	0.0760	2.0199	0.5903	0.0683	1.5460	1.6826	0.0329	0.0697		
E	2.0631	1.7622	0.0928	1.5371	1.1169	2.0082	1.1179	0.0348	1.2518	1.3501		
F	0.0345	1.3425	0.0456	1.0255	1.6539	1.0778	1.6732	0.0670	0.0760	0.0564		
G	0.0422	0.0581	1.6732	0.5830	1.9496	1.0890	0.0500	0.0243	0.0650	0.0991		
H	1.8470	2.0550	0.0501	0.0848	1.0145	1.4930	0.0932	0.0188	0.0333	0.0414		

Table 4: IHA units calculated by IHA test: The serum sample was considered to be positive if IHA titer is higher than 1:16 while the titer of negative sera is to be equal or less than 1:4

IHA Units	1	2	3	4	5	6	7	8	9	10
A	5	4	0	5	0	0	0	0	0	0
B	4	0	4	0	0	5	0	1	0	4
C	0	0	5	0	4	5	1	1	5	1
D	0	4	0	5	4	0	5	5	0	1
E	5	5	1	5	5	5	0	0	4	4
F	0	5	4	4	5	5	5	0	1	1
G	0	1	0	4	0	5	5	1	0	0
H	1	1	1	1	1	0	1	5	1	0

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