

DEVELOPMENT AND STANDARDIZATION OF A BLOCKING ELISA BASED ON MONOCLONAL ANTIBODY TO *PASTEURELLA MULTOCIDA*

PANKAJ KUMAR* and ARVIND KUMAR

Department of Veterinary Microbiology, College of Veterinary Sciences
Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar-125 004

Received: 03.12.2013; Accepted: 26.12.2013

ABSTRACT

A blocking enzyme-linked immunosorbent assay (ELISA) based on monoclonal antibody was developed and standardized for the detection of antibodies to *Pasteurella multocida* in animals vaccinated against haemorrhagic septicaemia. Serum antibodies in vaccinated animals were titrated using a monoclonal antibody raised against whole bacterial sonicated antigen. The test was developed in such a manner that it allowed binding of specific bio-reagents i.e. serum antibodies and antispecies-horse radish peroxidase enzyme conjugate only and the binding of monoclonal antibody was blocked specifically by blocking of sites on the antigen by serum antibodies. The procedure for development of the specific blocking ELISA and its use on estimation of serum antibodies are described.

Key words: Blocking ELISA, *Pasteurella multocida*, monoclonal antibody, vaccination monitoring.

Pasteurella multocida is a Gram negative bacterium and is the causative agent of fowl cholera, haemorrhagic septicaemia (H.S.) and swine atrophic rhinitis. This organism can be classified into 5 capsular serogroups A, B, D, E and F (Rhodes and Rimler, 1989) and 16 somatic serotypes 1-16 (Heddleston *et al.*, 1972). H.S. is common to all wet tropical countries in Asia and still remains as one of the most serious animal diseases causing huge economic losses to livestock farmers (De Alwis, 1999). In India, H.S. is prevalent in almost all states of the country with a high bovine mortality and other species of animals (Singh *et al.*, 1996). Singh *et al.* (2008) calculated the annual economic losses in India due to *P. multocida* infection in all species alone to the tune of Rs. 225 million. However, due to poor reporting and surveillance systems, losses due to H.S. probably are actually greater than that have been reported.

Vaccination against H.S. is the method of choice for control of the disease. Therefore, ELISA based serological test is of importance for monitoring of vaccination. A single dilution ELISA for estimation of H.S. immunity status based on serum antibodies titres has been reported (Kumar *et al.*, 2003). However, blocking ELISA using *Pasteurella* specific antibodies may improve specificity of the test. Blocking ELISA has been successfully used in monitoring FMD (Chenard *et al.*, 2003). In the present study, development and

standardization of a blocking ELISA based on anti – *Pasteurella* monoclonal antibody is described.

MATERIALS AND METHODS

Antigen: *P. multocida* B:2 P52 culture was streaked on two blood agar plates and the plates were incubated over night at 37°C. The bacterial colonies were taken from blood agar with the help of a loop in a 15ml centrifuge tube containing 10ml of phosphate buffer. The tube was shaken gently to make a homogenous bacterial suspension. Formalin was added to this bacterial suspension to reach the final concentration of 0.5% v/v. The suspension was stirred on a magnetic stirrer for 30 min and was then left at room temperature for overnight. To assure the sterility of the antigenic preparation, a loopful of suspension was streaked on to blood agar plate and incubated. No growth was observed after 24 hrs of incubation at 37°C. The formalin killed *P. multocida* suspension was sonicated at 35 amplitude/30 seconds for five times. The sonicated antigen was then centrifuged at 1000 g for 5 min. The supernatant was collected and stored at -20°C till used.

Monoclonal Antibodies: A monoclonal antibody against *P. multocida* was used. The monoclonal antibody secreting hybridoma cell line was developed in the laboratory against whole cell lysate of sonicated antigen. On immunoblotting it reacted with a 28kDa protein band, that is for TonBm, which supplies energy for iron transport. The antibody present in mice ascetic fluid was used in the study.

*Corresponding author: drpankaj42@gmail.com

Vaccinated Animals: For standardization of the test, pooled serum sample (2 of cattle and 3 of buffaloes) was used. The serum samples of cattle and buffaloes that gave antibody titre $>2.7 \log_{10}$ in a monoclonal antibody based ELISA were selected for preparation of the pooled serum samples. The titres of pooled sera were 1.131 and 1.039 for cattle and 1.496, 1.314 and 1.269 for buffaloes. The serum samples of buffaloes and cattle vaccinated with H.S. vaccine (H.S. alum precipitated vaccine) provided by State ELISA Laboratory, Sonipat were used.

Standardization of the ELISA: The test was standardized in a manner that it allowed binding between the antigen and the anti-*P. multocida* monoclonal antibody only. The antispecies- HRPO conjugate may cross react with antibodies of another species. In the test, anti-*P. multocida* antibodies in the cattle serum were allowed to bind the antigen and block antigenic sites on the antigen. In case of cross reactivity between anti-mouse HRPO conjugate and cattle serum antibodies, the conjugate may erroneously bind to the cattle serum antibodies and may lead to false negative results. The monoclonals should not bind to the antigen when cattle serum has anti-*Pasteurella* antibodies and blocking the antigenic sites and therefore, the colour should not develop. However, in the event of unblocked cross reactivity between the cattle serum antibodies and the anti-mouse -HRPO conjugate, the colour would develop despite blocking of binding of the anti-*P. multocida* monoclonal antibody.

To develop a specific test new born calf serum (NBCS) known to be free from anti-*P. multocida* antibodies was used as a blocking agent in ELISA diluent. Various percentages of the new born calf serum were tested and the lowest percentage that completely blocked cross reactivity between cattle/buffalo serum and the anti-mouse HRPO conjugate was used in the blocking ELISA. The plates were coated with the optimum concentration of the sonicated antigen of *P. multocida* and the vaccinated animal serum in 1/10 dilution was allowed to bind to the coated antigen. The serum @50 $\mu\text{l/well}$ was added and the plates were incubated at 37°C for 1 hour. The wells were washed and dried and the anti-mouse antibody-HRPO conjugate in optimum dilution was added @50 $\mu\text{l/well}$. The plate was again incubated and then washed and dried as done below. The test was developed using 3,3,5,5 tetramethyl benzidine as a

substrate, stopped by acidification and read at 450 nm in an ELISA reader.

Blocking ELISA: The wells of a 96 well polystyrene flat bottom ELISA plate (Maxisorp, Nunc.) were coated with @50 $\mu\text{l/well}$ of optimum dilution (1:1000) in PBS-T with 2% NBCS (PAA laboratories) the sonicated antigen of *P. multocida* (1:1000) and kept overnight at 4°C. The wells were washed thrice with ELISA washing buffer and blotted dry. Various two fold dilutions of the vaccinated animal serum sample were added in duplicate @50 $\mu\text{l/well}$. The antibodies in vaccinated animal serum were allowed to bind and block sites on the bacterial antigen by incubating at 37°C for 1 hour. To assess the blocking of the sites of the antigen, the monoclonal antibody in optimum dilutions (1:500) was added @50 $\mu\text{l/well}$ and the plate was incubated at 37°C for 1 hour. The monoclonal antibody failed to bind when all antigenic sites on the antigen were blocked by the antibodies of the vaccinated animal serum. Upon dilution of the vaccinated animal serum more and more antigenic sites became available to the binding of the monoclonal antibody. The highest dilution of the vaccinated animal serum that blocked 50% sites was described as antibody titre (The highest dilution of the vaccinated animal serum that gave optical density value half in comparison to the positive control well). The positive control well contained a serum known to be negative for anti-*P. multocida* antibodies and therefore no site was blocked and colour developed due to binding of the anti-*P. multocida* monoclonal antibody with the antigen. The wells were washed thrice with ELISA washing buffer and blotted dry. For detecting the binding of the monoclonal antibody to the free sites, anti mouse antibody-HRPO conjugate in optimum dilution was added @50 $\mu\text{l/well}$ and incubated at 37°C for 1 hour. The plate was again washed and dried and the test was developed using 3,3,5,5 tetramethyl benzidine as a substrate (1:500) in substrate buffer. The color development was stopped by acidification and read in ELISA reader at 450 nm.

The values of optical density were recorded. The antibody titres were described as the reciprocal of log dilution of the vaccinated animal serum that gave 50% blocking of color development as compared to positive control. In the positive control, the cattle serum without any *Pasteurella* antibodies was used in the step when vaccinated animal serum was added to the test wells.

Table 1
Cross reactivity between cattle serum antibodies and anti-mouse antibody-HRPO conjugate

Serial two fold dilution of pooled vaccinated cattle & buffalo serum	Cross reactivity between cattle serum antibodies and anti-mouse antibody-HRPO conjugate (OD value)	Blocking of cross reactivity between cattle serum antibodies and anti-mouse antibody-HRPO conjugate		
			Wells coated with NBCS (OD value)	Wells coated with monoclonal antibody (OD value)
1/10	0.411	2% NBCS	0.042	1.460
1/20	0.396		0.040	1.562
1/40	0.266	3% NBCS	0.042	1.526
1/80	0.228		0.042	1.501
1/160	0.110	5% NBCS	0.045	1.699
1/320	0.036		0.041	1.644
1/640	0.008	10% NBCS	0.042	1.663

NBCS=New born calf serum

Percent inhibition=100- (Test well OD/Positive control well) x100.

RESULTS AND DISCUSSION

Cross reactivity between cattle serum antibodies and anti-mouse antibody-HRPO conjugate was observed. When the cattle serum antibodies bound to coated antigen were detected using anti-mouse antibody-HRPO conjugate high OD values were observed. The values showed a decreasing trend when the cattle serum antibodies were serially two fold diluted (Table 1). To block this cross reactivity between cattle serum antibodies and anti-mouse antibody-HRPO conjugate, various percentage of *Pasteurella* antibody free NBCS was added in the ELISA diluents. Addition of 2% calf serum blocked the cross reactivity (Table 1).

The 50% inhibition was between 1/80 and 1/160 dilutions. The exact dilution giving 50% inhibition was determined by plotting the percent inhibition versus dilution (Table 2). This way the blocking ELISA titre with the

monoclonal antibody was recorded as 2.04 log₁₀. In the single dilution ELISA, antibody titre 2.0log₁₀ is taken as protective antibody titre (Kumar *et al.*, 2003). The monoclonal antibody estimated the protective antibody levels in the vaccinated animal serum. The blocking ELISA antibody titres are more correlated to protection in foot-and-mouth disease (Chenard *et al.*, 2003). Therefore, the monoclonal antibody based blocking ELISA may be more specific ELISA for estimation of HS immunity status.

REFERENCES

- Chenard, G., Miedema, K., Moonen, P., Schrijver, R.S. and Dekker, A. (2003). A solid-phase blocking ELISA for detection of type O foot-and-mouth disease virus antibodies suitable for mass serology. *J. Virol. Methods* **107**: 89-98.
- De Alwis, M.C.L. (1999). Haemorrhagic Septicaemia. ACIAR Monograph No. 57. Australian Center for International Agricultural Research, Canberra, Australia.
- Heddleston, K.L., Gallagher, J.E. and Rebers, P.A. (1972). Fowl cholera: gel diffusion precipitin test for serotyping *Pasteurella multocida* from avian species. *Avian Dis.* **16**: 25-936.
- Kumar, A., Dahiya, G. and Kakkar, N.K. (2003). Single dilution ELISA for estimation of serum antibody levels against haemorrhagic septicaemia. *Haryana Vet.* **42**: 51-55.
- Rhoades, K.R. and Rimler, R.B. (1989). Fowl cholera. In: *Pasteurella* and Pasteurellosis. Adlam, C., Rutter, J.M. (eds.). Academic Press Inc., California.
- Singh, V.P., Kumar, A.A., Srivastava, S.K. and Rathore, B.S. (1996). Significance of H.S. in Asia: India. International Workshop of diagnosis and control of H.S. Bali, Indonesia, May 28-30, 1996.
- Singh, V.P., Sinha, D.K., Gupta, S.K. and Chauhan, R.S. (2008). Prevalence of HS in India. Vth Annual Scientist Meet on Haemorrhagic Septicaemia. 5-6th September. Guwahati, Assam, India.

Table 2
Blocking ELISA results of monoclonal antibody

Dilutions of pooled vaccinated cattle & buffalo serum	OD value	Percent inhibition
1/10	0.069	83.7
1/20	0.032	92.5
1/40	0.043	89.8
1/80	0.039	90.8
1/160	0.370	12.4
1/1320	0.388	8.1
Positive control	0.422	-