STANDARDIZATION OF INDIRECT HAEMAGGLUTION TEST FOR MONITORING *PASTEURELLA MULTOCIDA* ANTIBODIES

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ABSTRACT

Indirect haemagglutination (IHA) test was standardised for monitoring *Pasteurella Multocida* antibodies. It was observed that human blood group O erthrocytes, 1: 16 dilution of bacterial supernant to coat erthrocytes and 30 minutes of supernatant-erthrocytes interaction had improved the sensitivity of the IHA. While either of the serum diluent such as normal saline or phosphate buffered saline (pH 7.2) and final concentration of the sensitised erthrocytes had no effect on the IHA antibody titre. However, concentration of sensitised erythrocytes was inversely proportional to the reading time of IHA results. With these standards, the results of IHA are reliable with 100% reproducibility.

INTRODUCTION

Indirect haemagglutination (IHA) test is a sinsitive, reliable and economical serodiagnostic method (Philip, 1965). It is extensively used for detection of antibodies against Pasteurella spp. (Manning, 1982; Anonymous, 1992). Indirect haemagglutination test is also used for typing of Pasteurella spp. (Biberstien *et al.*, 1960; Manning, 1982). The polysaccharide of many bacterial species including *P. multocida* and *P. haemolytica* adsorb erythrocytes without coupling agents (Herman, 1980). Human blood group O erythrocytes are recommended for the antigen adsorption (Bain *et al.*, 1982). The poor sensitivity and reproducibility of IHA is not an uncommon problem. It might due to serum diluent, source of erthrocytes, amount of bacterial lipopolysaccharide (LPS) adsorbed erythrocytes etc. This project is designed to explore the factors responsible for the aforesaid limitations of the test.

MATERIALS AND METHODS

Bacterial antigen and hyperimmune serum preparation

Pasteurella Multocida (Roberts type 1) was procured from Veterinary Research Institute, Lahore. Eight hours old culture of *P. multocida* was subcutaneously inoculated in mice. The heart blood of mice (200 microlitre) was thickly inoculated on blood agar plate and incubated at 37°C for 20 hours. The morphlogy of all colonies of bacteria were uniform. Moreover, the bacteria of the growth were gram negative, coccobacilli and bipolar. The uniform colonies of the bacterial growth and morphological features indicated the purity of the culture. The pure growth was washed in 4 mL of saline solution (0.85% sodium chloride aqueous solution). The *P. multocida* suspension was heated at 56°C for 30 minutes in water bath to dissolve the bacterial capsule (Surface lipopolysaccharied antigin). This suspension was spun at 600 g for 60 minutes. The supernatant was decanted and used for sensitization of erthrocytes. The hyperimmune serum against *P. multocida* was prapared in rabbits (Fraser *et al.*, 1983)

Sensitization of erythrocytes

Erythrocytes were sensitised with the supernatant as described by Fraser *et al.* (1983). In summary, 0.2 mL of washed packed erythrocytes suspension in 4 mL of the bacterial supernatant was incubated at 37°C for 2 hours. The erythrocyte suspension was spun twice at 300 g for 3 minutes. The packed erythrocytes were resuspended in the saline solution.

Indirect haemagglutination (IHA) test

The IHA test was performed to monitor antibodies against *P. multocida* as described by Bain *et al.* (1982). In summary, 40 microliter of diluent (saline solution) was poured in all wells of immunoplate (Flow Laboratories). The serum (40 μL) was added in first well of the row and was serially diluted up to well No. 11. The sensitised erythrocyte suspension (40 μL) was poured into each well of the immunplate.

In this study, effects of various factors including serum diluent (normal saline and phosphate buffered saline-PBS, pH 7.2), source of erythrocytes (human blood group O, chicken and sheep), concenteration of lipopolysaccharide (LPS) to sensitise the erythrocytes (undiluted), 1:4, 1:8, 1:16), LPS-erythrocytes interaction time (30 and 120 minutes) and dilution of

Table 1: Effect of source of erythrocytes on sensitivity of indirect haemagglutination test

Source of erthrocytes (n=8)	Distribution of samples on the basis of indirect haemagglutinating antibody titre								GMT
	2^{-4}	2^{-5}	2^{-6}	2^{-7}	2^{-8}	2^{-9}	2^{-10}	2^{-11}	
Sheep	0	0	0	0	8	0	0	0	256
Chicken	0	0	0	0	8	0	0	0	256
Human	0	0	0	0	0	8	0	0	512

In this experiment, undiluted LPS-erythrocyte interaction time was 120 minutes. The results with sensitised sheep erythrocytes were perceived in 70 minutes, with human erythrocytes in 45 minutes and with chicken in 20 minutes

Table 2: Effect of sensitised erythrocytes with diluted lipopolysacchride on sensitivity of IHA test

Source of LPS (n=8)	Distribution of samples on the basis of indirect haemagglutinating antibody titre								GMT
	2^{-4}	2^{-5}	2^{-6}	2^{-7}	2^{-8}	2^{-9}	2^{-10}	2^{-11}	
Pure	0	0	0	0	0	8	0	0	512
1:4	0	0	0	0	0	0	8	0	1024
1:8	0	0	0	0	0	0	0	8	2048
1:16	0	0	0	0	0	0	0	8	2048

In this experiment, interaction time of LPS with human blood group-O erythrocytes was 120 minutes.

serum on the sensitivity of indirect haemagglutination test, were evaluated. The results of various experiments were statistically analysed (Villegas and Purchase, 1989).

RESULTS AND DISCUSSION

Indirect haemagglutination (IHA) test is extensively used to monitor the sera of vaccinated or pasteurella carrier animals. Workers are using the test with variable results. It was observed that either of the serum diluent, normal saline (0.85% sodium chloride) or 0.5M Phosphate buffered saline (pH 7.2), had undetectable difference in the IHA antibody titre against *P. multocida*. The normal saline being economical was used in all the remaining experiments.

The erythrocytes of different animal species are variable in respect of their density, size, shape and surface molecules (Melvin, 1982). In routine, human blood group O erythrocytes are sensitised with bacterial polysaccharide (Fraser *et al.*, 1983). In this study, sheep erythrocytes gave one log lower titre than that of human erythrocytes (Table 1), while chicken erythrocytes showed results similar to that of sheep erythrocytes but result was interpretable within 20 minutes, (25 minutes earlier to that of human and sheep erythrocytes). This could be due to larger in size and higher density of chicken erythrocytes than that of

sheep and human erythrocytes. The human blood group O erythrocytes do not contain A, B, or AB antigen on their surface (William, 1981), which might had supported the adsorption of required amount of the polysaccharide. This could be plausible reason of routine use of human blood group O erythrocytes. Present study has revealed that due to the varying chemistry of surface molecules, the chicken or sheep erythocytes could have adsorbed the polysaccaride comparatively more efficiently, contributing to low sensitivity of the test.

The lipopolysaccharide (LPS) adsorbs on erythrocytes without coupling agents (Philip, 1965). Erythrocyte sensitization with undiluted supernatant of *P. multocida* washing showed four fold low titre of antibodies in the test as compared to that of sensitised with 1:16 diluted supernatant (Table 2). It might be due to over adsorption of LPS on the surface of erythrocytes which might be responsible for the poor sensitivity of the test.

The LPS production by *P. multocida* is influenced by bacterial genes, type of medium used for growth, number of passages on the laboratory medium and physical factors for bacterial growth (Anonymous, 1992). In the present study, the bactera was activated by mice inoculation followed by cultivation on blood agar. Then 20 hours growth was harvested in 4 mL saline. Adaptation of this method may help production

Table 3: Effect of lipoplysaccride-erythrocytes interaction time on sensitivity of IHA test.

LPS-erythrocytes interaction time (in min.) (n=8)	Distribution of samples on the basis of indirect haemagglutinating antibody titre								GMT
	2^{-4}	2^{-5}	2^{-6}	2^{-7}	2^{-8}	2^{-9}	2^{-10}	2^{-11}	
30	0	0	0	0	0	8	0	0	512
120	0	0	0	0	8	0	0	0	256

In this experiment, Human blood group-O erythrocytes were sensitised with undiluted bacterial supernatant or LPS.

Table 4: Effect of antiserum dilution on the sensitivity of IHA test.

Dilution of antiserum (n=8)	Distribution of samples on the basis of indirect haemagglutinating antibody titre								GMT
	2^{-4}	2^{-5}	2^{-6}	2^{-7}	2^{-8}	2^{-9}	2^{-10}	2^{-11}	
Undiluted	0	0	0	0	0	8	0	0	512
1:4	0	0	0	8	0	0	0	0	128
1:16	0	8	0	0	0	0	0	0	32

In this experiment, human blood Group-O erythrocytes were sensitised with undiluted LPS for 120 minutes and results were recorded in 45 minutes.

of consistent amount of LPS. In the subsequent experiments, LPS was diluted at the rate of 1:16 for sensitization of erythrocytes. However, further investigation is needed to determine the quantitative level of LPS required to sensitise the erythrocytes for optimum sensitivity.

In the conventional technique, erythrocytes suspension in undiluted supernatant of bacterial washing (containing capsular lipopolysaccharide) is incubated at 37°C for 2 hours. It was observed that this incubation time for 30 minutes had improved the sensitivity of the test (Table 3). It might be due to the fact that required amount of LPS might have coated within this period.

In routine, 1% sensitised erythrocytes are recommended for the test (Bain *et al.*, 1982; Anonymous, 1992). It was observed that with this erythrocytes suspension, the IHA antibody titre was perceived within 45 minutes, but at the same time, it resulted reading problem i.e., in the highest serum dilution showing HA activity, some of the erythrocyets settled in the centre while others showed HA activity. However, tilting the immunoplate for 2 minutes resulted flow of settled erythrocytes in wells showing non-haemagglutination. In this way the highest dilution of serum showing either clear haemagglutination or maintainence of erythrocyte's position on tilting the immunoplate, was recorded as titre of IHA antibodies. Subagglutinating level of serum IHA antibodies reacting with the antigen on the sensitised erythrocytes might be

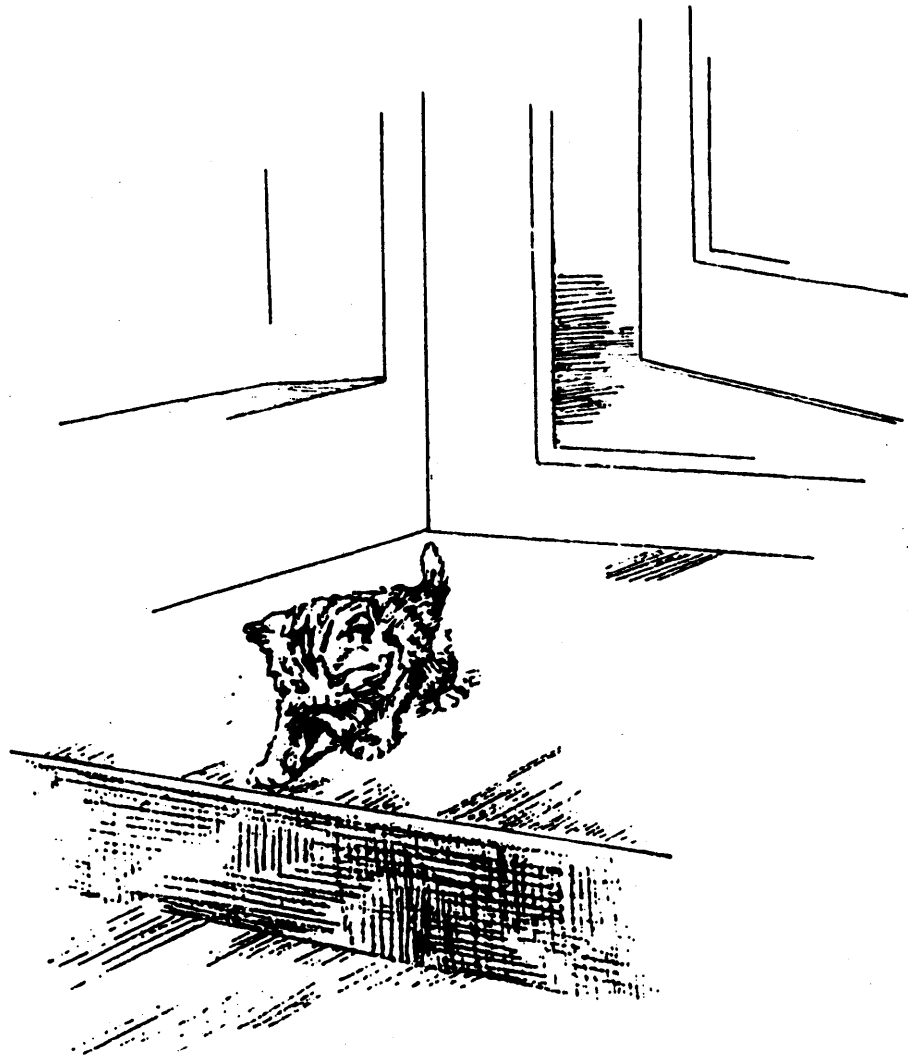a plausible reason of resistance of the erythrocytes flow.

In the standardised IHA, dilution of the sera proportionately reduced IHA antibody level. The results of IHA antibodies in the sera showed 100% reporducibility (Table 4).

It is concluded that source of erythrocytes, amount of LPS adsorbed on the erythrocytes and time of LPS-erythrocytes interaction had influenced the sensitivity of the IHA test. The results of the study are highly valuable in comparing different laboratory results of IHA antibodies against *P. multocida*.

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