INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
1560–8530/2005/07–3–441–444
http://www.ijab.org

Standardization of Indirect Haemagglutination Test for Titration of Antibodies Against *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Escherichia coli* Isolated From Bubaline Mastitis

SAJJAD-UR-REHMAN, M. ATHAR, A. SHAKOOR, G. MUHAMMAD AND A. A. BUTT
Faculty of Veterinary Science, University of Agriculture, Faisalabad–38040, Pakistan

ABSTRACT

An Indirect Haemagglutination (IHA) test was successfully developed and standardized to determine the antibody response against *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Escherichia coli* isolated from milk of buffaloes affected with mastitis. Sonicated antigens showed variable antibody titres in all the groups with the ranges of 1:16 to 1:256 in rabbits and 1:16 to 1:128 in buffalo-calves. Human group 'O' and sheep RBCs both showed equally good and parallel antibody titres which indicated that any of these erythrocytes may be successfully used to determine the antibody titre. However, sheep RBCs were found better due to the fact that Human group 'O' sensitised RBCs may stay better for 3–4 hours as compared to 7 days stability of sheep RBCs fixed with glutaraldehyde. Moreover, it is mention worthy that 2% sensitised RBC suspension showed results within 30 minutes compared to 1% sensitised RBCs which showed results in 2 h. In view of more economical terms, the optimum test conditions favourable for titrating antibody against *S. aureus*, *Str. agalactiae*, and *E. coli* sheep RBC glutaraldehyde fixed sensitised with sonicated antigen may be recommended in 1% suspension. Further studies may be conducted to determine and prove the efficacy of IHA test.

Key Words: Standardization; Haemagglutination; Titration; Antibodies; *Staphylococcus aureus*

INTRODUCTION

Mastitis is regarded as the major production-limiting disease of dairy animals worldwide (Lightner *et al.*, 1988; Giraudo *et al.*, 1997; Kossaibati *et al.*, 2000). It not only affects the quality but also quantity of the milk (DeGraves & Fetrow, 1993; Sordillo *et al.*, 1997). Worldwide, annual losses caused by this disease are nearly $35 billion (Ratafia, 1987). *Staphylococcus aureus* is the most important aetiological agent followed by *Streptococcus agalactiae* and *Escherichia coli* as per some of the studies conducted heretofore in Pakistan (Hashmi, 1978; Sahi, 1983; Razzaq, 1998). Several preventive strategies have been applied to minimize mastitis in cows, including hygienic cleaning procedures, disinfection, antibiotic therapies, culling, and vaccination. In order to find out the best vaccine to control mastitis, estimation of serum antibody titres for a certain duration is mandatory to establish its effectiveness for that period. Worldwide, antibody titration is being conducted through antigen-specific enzyme-linked immunosorbant assay (ELISA) (Opdebeeck & Norcross, 1984; Nickerson *et al.*, 1993; Nordhaug *et al.*, 1994; Giraudo *et al.*, 1997; Han & Park, 2000). In a country like Pakistan, where facilities to conduct such tests are lacking, other methods need to be evaluated and standardized. Direct bacterial agglutination, haemagglutination, haemagglutination inhibition and indirect haemagglutination tests are being used for antibody titration against various bacterial antigens to fulfil the requirement. However, none of the test was standardized for common mastitis pathogens (*S. aureus*, *Str. agalactiae*, & *E. coli*). The present study was, therefore, conducted to develop and standardize indirect haemagglutination test for titration of antibodies against the aforementioned bacterial pathogens causing mastitis. It is hoped that the results of the present study will open new vistas in the future mastitis research endeavours.

MATERIALS AND METHODS

Indirect haemagglutination (IHA) test was standardized with certain modifications to assess antibody titres against *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Escherichia coli* isolated from milk of mastitic buffaloes (Bing *et al.*, 1967; Sawada *et al.*, 1981; Tamura *et al.*, 1985). The test was conducted separately with 10 rabbits and 5 buffalo-calves in each group.

Isolation and biocharacterization of mastitis pathogens. Isolation and biocharacterization of culture isolates (Table I) from buffaloes affected clinically with mastitis was conducted following the procedures described by National Mastitis Council, Inc. USA (1990). Gram-positive, catalase-positive, coagulase-positive and α/β-haemolysis producing cocci were regarded as the virulent isolate of *S. aureus* with API Staph biochemical profile of 6736153 (CMb-2). Gram-

Table I. Description of bacterial isolates recovered in different samples collected from clinical mastitic cases of buffaloes around Faisalabad district.

Sample No.	Source	Morphological Characters
CMb-1	Milker's hands	G+ve/Cocci in clusters
CMb-2	Mastitic milk	G+ve/Cocci in clusters
CMb-3	Udder washing	G+ve/Cocci and G-ve/Coccobacilli
CMb-4	Teat canal	G+ve/Diplococci
CMb-5	Mastitic milk	G+ve/Cocci in short chains
CMb-6	Teat canal	G+ve/Micrococci
CMb-7	Milker's hands	G+ve/Cocci in clusters
CMb-8	Udder washing	G+ve/Cocci and G-ve/Coccobacilli
CMb-9	Teat canal	G+ve/Cocci singly
CMb-10	Udder washing	G+ve/Cocci and G-ve/Coccobacilli
CMb-11	Milker's hands	G+/Cocci in clusters
CMb-12	Mastitic milk	G-ve/Coccibacilli

CMb = Clinical Microbiology
G+ve = Gram-positive
G-ve = Gram-negative

positive, catalase-negative, Esculin-negative, CAMP-positive, and β-haemolytic cocci were selected as virulent isolate of *Str. agalactiae* with API 20 Strep biochemical profile of 3462414 (CMb-5). Whereas Gram-negative, citrate-negative, lactose-positive, and in triple sugar iron slants butt-forming bacilli were selected as virulent isolates of *E. coli* with API 20E biochemical profile of 5154552 (CMb-12). The pathogenicity of *E. coli* was further confirmed by ileal loop ligatin assay (Altwegg & Bockemühl, 1998). The purified virulent isolates were then preserved in trypticase soy broth containing 20% glycerol at –70°C (Muhammad, 1992).

Preparation of hyperimmune sera. Whole culture formalin-inactivated antigens of *S. aureus*, *Str. agalactiae* and *E. coli* containing 1.2×10^9 cells mL^{-1} each were prepared (Watson & Davies, 1993; Giraudo *et al.*, 1997). Sterility of each antigen preparation was checked by streaking a loopful of the culture onto blood agar and MacConkey's agar plates, and Thioglycolate broth and incubating for 24–48 h. Ten rabbits were selected to raise the hyperimmune sera (Malik, 2001). Progressively increasing doses of antigens were given intravenously on day 1, 3, 5, 7, and 9. Fourteen days after the last injection, the rabbits were bled to collect the sera. Five buffalo-calves (4–6 months of age) were inoculated with 2 mL of formalin-inactivated antigen separately. Second injection was repeated after 7 days and blood wad collected after 14 days of last injection. Serum was separated, heat inactivated to 56°C for 30 minutes in water bath and stored under –20°C.

Preparation of sonicated antigens. Twenty-four hour broth cultures of *S. aureus, Str. agalactiae,* and *E. coli* were prepared. The cultures were centrifuged at 2,000 rpm at 4°C for 30 minutes for bacterial sedimentation and the sediment was washed 3 times with 0.15M PBS (pH 7.2). The bacterial counts were then adjusted spectrophotometrically at 1.2×10^9 cells mL^{-1}. These bacterial suspensions were then subjected to sonication (Rapidis 600; Ultrasonics Ltd., USA) at 20 kHz, 105 W for 5 minutes. The sonicated suspensions were then centrifuged at 2,000 rpm at 4°C for 30 minutes. The resulting supernantents were then used as antigens for sensitising Human group 'O' (–ive) (HGO) and sheep RBCs which were subsequently used for IHA test. The protein concentrations of these suspensions were adjusted to 200 µg mL^{-1} using refrectometer (Baush & Lomb, New York, USA) prior to sensitising the RBCs.

Preparation of glutaraldehyde-fixed sheep RBCs. Fresh sheep RBCs (S-RBCs) were washed by centrifugation (1,500 rpm for 5 minutes) three times with three volumes of normal saline (0.85% NaCl). After the last washing, the packed cells were resuspended in 0.15M PBS (pH 7.2) to yield a 20% suspension (v/v) and were stored at 4–10°C. A 25% solution of glutaraldehyde (GA) (Sigma Chemica Co., St. Louis, USA) was used to prepare 1% solution in PBS and then chilled to 4°C. A 20% suspension of washed S-RBCs was mixed with an equal volume of 1% GA and the mixture was incubated at 4°C for 30 minutes with gentle stirring. The mixture was then centrifuged at 1,500 rpm for 5 minutes. The pelleted fixed S-RBCs were resuspended in PBS, washed 3 times with PBS and resuspended in PBS to yield a 20% suspension of GA-fixed S-RBCs (GA-S-RBCs).

Sensitization of Human Group 'O' (-ive) and Glutaraldehyde-fixed Sheep RBCs. Four aliquots each for HGO-RBCs and GA-S-RBCs were made. Of these, three aliquots from each were sensitised with *S. aureus*, *Str. agalactiae*, and *E. coli* sonicated antigens whereas fourth aliquots were kept as unsensitized RBCs. Briefly, a 20% suspension of RBCs was mixed with an equal volume of sonicated antigen and double volume of 0.15M PBS. The mixture was incubated at 37°C for 30 minutes with occasional shaking. The sensitised cells were washed 3 times with PBS and resuspended to yield a 1% suspension for IHA test.

Procedure for IHA. All 96 wells of U-bottom Titreteck microplates (Flow Labs., UK) were dispensed with 50 µL of normal saline using the microdispenser, while the wells of column 12 stood as control. Using the Titreteck manual microdispenser, 50 µL of each serum sample (inactivated by placing in water bath at 56°C for 30 minutes to eschew the chances of non-specific reactions) was transferred from first row of wells to the second row, mixed well and transferred the same amount from second row of wells to third row, and so on, thereby diluting each sample as 1:2, 1:4, 1:8, 1:16, 1:1024.

After having the 2-fold dilutions, 0.05 mL 1% sensitised HGO-RBCs were dispensed in each well with an automatic multichannel microdispenser except the wells of last columns that stood control to which 0.05 mL of 1% unsensitized HGO-RBCs were added. The plates were gently tapped for a number of times to ensure an even suspension of RBCs. The plates were incubated at room temperature for 2 h. The end point having the maximum

dilution of each serum sample caused a distinct haemagglutination giving a characteristic reticulum-settling of erythrocytes throughout the bottom whereas no haemagglutination was manifested by peculiar central button-shaped settling of erythrocytes. The IHA antibody titre of each serum sample was narrated as the reciprocal of its end point dilution. Thus the IHA antibody titres of all the serum samples were recorded.

RESULTS AND DISCUSSION

Antibody titres against *S. aureus* in rabbits ranged from 1:16 to 1:128 as determined through human group 'O' (HGO-RBCs) and glutaraldehyde-fixed sensitised sheep RBCs (GA-SRBCs). Similarly, in calves, the titre range was 1:16 to 1:128 as shown in Table II. Antibody titres against *Str. agalactiae* ranged from 1:32 to 1:128 in rabbits and calves irrespective of the source of the sensitised RBCs used in the performance of IHA test (Table III). Antibody titre against *E. coli* showed variable results (Table IV), where HGO-RBCs showed minimum starting titre of 1:64 in rabbits and 1:32 in calves; whereas, GA-SRBCs showed minimum starting titre in rabbits as 1:32 and 1:16 in calves. The maximum limit of antibody titre remained the same at 1:128 in both cases of rabbits and calves (Table IV).

Table II. Indirect haemagglutination titres against *Staphylococcus aureus* inoculated in rabbits and buffalo-calves

Source of RBCs	Sensitization Status	Ranges of Antibody Titres		Geomean Titre (GMT)	
		Rabbits	Calves	Rabbits	Calves
Human Group 'O' Sheep (GA-fixed)	Sensitized	1:16– 1:128	1:16– 1: 256	68.6	157.6
	Unsensitized	0 - 1:2	0 – 1:2	–	–
	Sensitized	1:16 –1:128	1:16 – 1:256	73.3	181.0
	Unsensitized	0 – 1:4	0 – 1:2	–	–

Table III. Indirect haemagglutination titres against *Streptococcus agalactiae* inoculated in rabbits and buffalo-calves

Source of RBCs	Sensitization Status	Ranges of Antibody Titres		Geomean Titre (GMT)	
		Rabbits	Calves	Rabbits	Calves
Human Group 'O' Sheep (GA-fixed)	Sensitized	1:32 – 1:128	1:32 – 1:256	97.0	207.9
	Unsensitized	0 – 1:2	0 – 1:2	–	–
	Sensitized	1:32 –1: 128	1:32 – 1:128	104.0	111.4
	Unsensitized	0 – 1:4	0 – 1:2	–	–

Table IV. Indirect haemagglutination titres against *Escherichia coli* inoculated in rabbits and buffalo-calves

Source of RBCs	Sensitization Status	Ranges of Antibody Titres		Geomean Titre (GMT)	
		Rabbits	Calves	Rabbits	Calves
Human Group 'O' Sheep (GA-fixed)	Sensitized	1:64 – 1:128	1:32 – 1:256	84.4	194.0
	Unsensitized	0 – 1:2	0 – 1:2	–	–
	Sensitized	1:32 – 1:128	1:16 – 1:256	119.4	238.9
	Unsensitized	0 – 1:4	0 – 1:2	–	–

As far as the geomean antibody titres (GMT) are concerned, there was no much variation in the determination of antibody titres against Staphylococci and Streptococci, however, in *E. coli* the GMT antibody titre varies with apparently higher titres through HGO-RBCs compared to GA-SRBCs. The practical implication indicated that HGO-RBCs did not work after 3-hours of sensitisation compared to the GA-SRBCs which showed the reproducibility of results even after 12 hours of sensitisation.

Concludingly, the optimum test conditions for conducting IHA test includes the GA-fixed sheep RBCs with 1% final suspension may be recommended to evaluate antibody titre against *S. aureus*, *Str. agalactiae*, and *E. coli* in rabbits and calves. Moreover, the test conditions standardized for conducting IHA test may provide less time consuming and reproducible results for future studies.

RERERENCES

Altwegg, M. and J. Bockemühl, 1998. *Escherichia* and *Shigella*. In: *Topley and Wilson's Microbiology and Microbial Infections.* 9th Ed. Vol. II. pp. 946–7. Arnold, London

Bing, D.H., J.G.M. Weyand and A.B. Stavitsky, 1967. Haemagglutination with aldehyde-fixed erythrocytes for assay of antigens and antibodies. *Proc. Soc. Exp. Biol. Med.,* 124: 1166–70

DeGraves, F.J. and J. Fetrow, 1993. Economics of mastitis and mastitis control. *Vet. Clinics of North America–Food Animal Practice,* 9: 421–34

Giraudo, J.A., A. Calzolari, H. Rampone, A. Rampone, A.T. Giraudo, C. Bogni, A. Larriestra and R. Nagel, 1997. Field trials of a vaccine against bovine mastitis. 1. Evaluation in heifers. *J. Dairy Sci.,* 80: 845–53

Han, H.R. and H.M. Park, 2000. Effects of adjuvants on the immune response of staphylococcal alpha toxin and capsular polysaccharide (CPS) in rabbit. *J. Vet. Med. Sci.,* 62: 237–41

Hashmi, H.A., 1978. *Subclinical Mastitis in Cattle and Buffaloes. M.Sc. (Hons.) Thesis,* College of *Veterinary Science*, University of Agriculture, Faisalabad–Pakistan

Kossaibati, M.A., M. Hovi and R.J. Esslemont, 1998. Incidence of clinical mastitis in dairy herds in England. *Vet. Rec.,* 143: 649–53

Lightner, J.K., G.Y. Miller., W.D. Hueston and C.R. Dorn, 1988. *Food animal economics*: Estimation of the costs of mastitis using National Animal health Monitoring system and milk somatic cells count data. *J. Am. Vet. Med. Assoc.,* 192: 1410–3

Malik, B.S., 2001. *A Laboratory Manual of Veterinary Microbiology. Part-II. Immunology and Serology.* p. 13. CBS Publishers & Distributors, New Delhi, India

Muhammad, G., 1992. Staphylococci of bovine mammary gland: Conventional and molecular dynamics of infections, plasmid stability, reproducibility, and interspecific conjugal transfer of antibiotic resistance. Ph.D. Dissertation, *Department of Vet. Preventive Medicine*, The Ohio State Univ., Ohio, USA

National Mastitis Council, Inc., USA, 1990. *Microbiological Procedures for the Diagnosis of Bovine Udder Infection.* National Mastitis Council, Inc., Arlington, Virginia, USA

Nickerson S.C., W.E. Owens and R.L. Boddie, 1993. Effect of a *Staphylococcus aureus* bacterin on serum antibody, new infectins and mammary histology in non-lactating cows. *J. Dairy Sci.,* 76: 1290–7

Nordhaug, M.L., L.L. Nesse, N.L. Norcross and R. Gudding, 1994a. A field trial with an experimental vaccine against *Staphylococcus aureus* mastitis in cattle. 1. Clinical parameters. *J. Dairy Sci.,* 77: 1267–75

Opdebeeck, J.P. and N.L. Norcross, 1984. Comparative effect of selected adjuvants on the response in the bovine mammary gland to staphylococcal and streptococcal antigens. *Vet. Immunol. Immunopathol.*, 6: 341–51

Razzaq, A., 1998. *Comparative efficacy of Vetimast®, Tetra-Delta®, and Akamycin-D® in mastitis of buffalo in and around Lahore. M.Sc. Thesis, College of Vet. Science*, Lahore, University of Agriculture, Faisalabad.

Sahi, K.B., 1983. *Bacteriology of Milk taken from Apparently Normal Buffalo Udder. M.Sc. Thesis, Department of Microbiol., Faculty of Vet. Sci*., Univ. of Agri., Faisalabad.

Sawada, T., R.B. Rimler and K.R. Rhoades, 1981. Indirect haemagglutination test that uses glutaraldehyde-fixed sheep erythrocytes sensitised with extract antigens for detection of *Pasteurella* antibody. *J. Clin. Microbiol.*, 15: 572–6

Sordillo, L.M., K. Shafer-Weaver and D. DeRosa, 1997. Immunobiology of the mammary gland. *J. Dairy Sci.*, 80: 1851–65

Tamura, Y., H. Makie and S. Tanaka, 1985. An indirect haemagglutination test for the detection of antibodies to *Clostridium chauvoei*. *Vet. Microbiol.*, 10: 315–24

Watson, D.L. and H.I. Davies, 1993. Influence of adjuvants on the immune response of sheep to a novel *Staphylococcus aureus* vaccine. *Vet. Microbiol.*, 34: 139–53

Zia, M.A., M. Athar., M.N. Sarwar and K. Muhammad, 1995. Indirect haemagglutination (IHA) test for the detection of antibodies against *Clostridium chouvoei* (black quarter disease). *Pakistan J. Livestock Poult.*, 1: 121–3

(Received 01 October 2004; Accepted 10 March 2005)