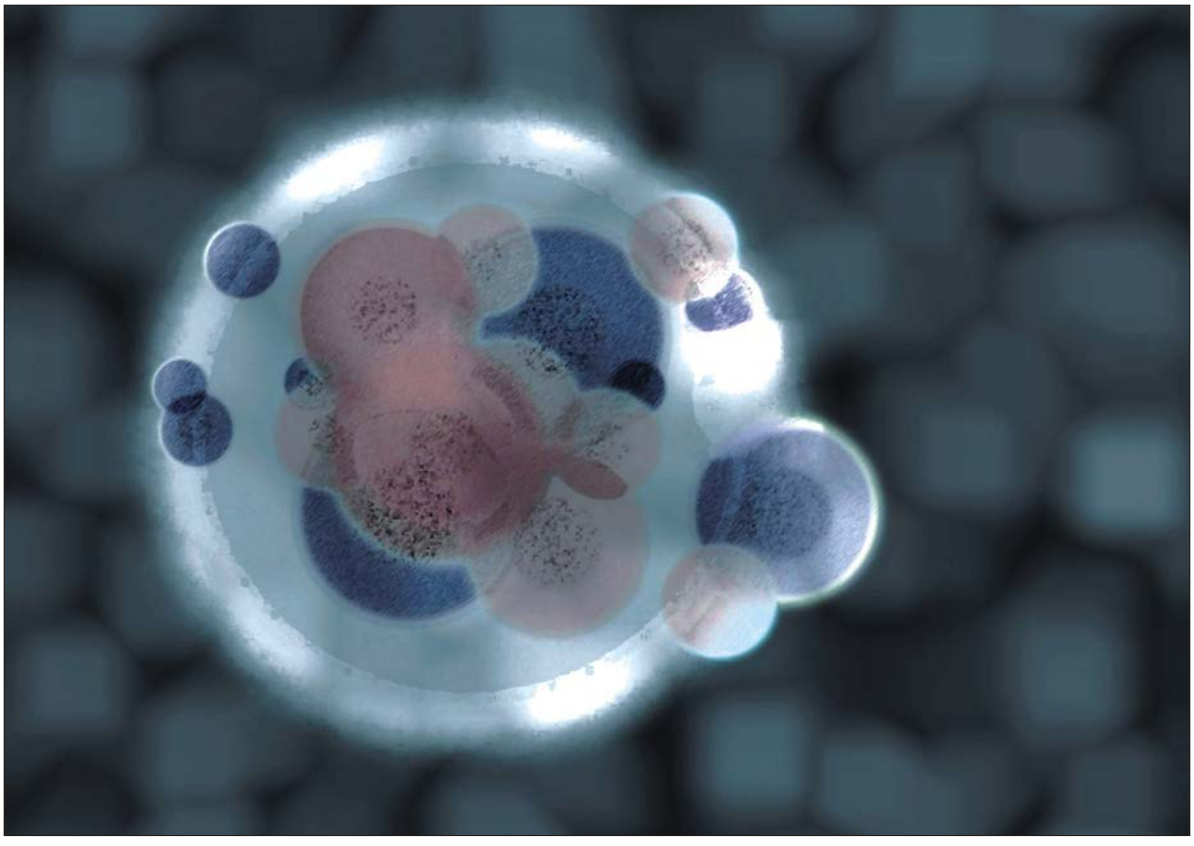

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Single Radial Hemolysis Test for Detection of Anti-tick Antibodies in Buffaloes

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Abstract: Single radial hemolysis test for the detection of antibodies against *Boophilus microplus* was developed. Optimum test conditions were determined on the basis of clear hemolysis and maximum haemolytic zone diameter by using human, sheep and rabbit erythrocytes; complement dilutions and temperature. There was a non significant difference ($p > 0.05$) in average haemolytic zone diameters among the erythrocytes of three species used but qualitative analysis indicated that human group "O" erythrocytes at the rate of 2 percent in agar gel medium gave better results. A 1:2 dilution of complement in vernal buffer at the rate of 8 percent in agar gel medium was found to be optimally appropriate. The combination of over night refrigeration and subsequent incubation at 37°C in a humid chamber for 8-12 hours gave good results regarding the quality of the zones of hemolysis which were well delineated.

Key words: *Boophilus microplus,* single radial hemolysis, optimum conditions.

Introduction

Single radial hemolysis (SRH) test is frequently used to detect the antibody titres against various bacterial and viral infections (Charan *et al*., 1981; Rehman *et al*., 1990). It is a reliable and specific test (Serafim *et al*., 1981; Gaidamovich *et al*., 1981); not affected by non-specific inhibitors and is simple to perform (Zahur *et al*., 1994). But so far the test has not been employed to detect antibodies against any of the parasite. In the present studies, optimum test conditions for SRH test were determined for assaying antibodies against *Boophilus* (B.) *microplus*.

Materials and Methods

Preparation of Antigen: Adult semi engorged female ticks, *B. microplus* were obtained from the tick colony maintained by the Department of Veterinary Parasitology, University of Agriculture, Faisalabad-Pakistan (Akhtar and Hayat, 1993). They were given several washings with phosphate buffered saline (PBS, pH 7.2). Mid guts of the ticks were dissected and mid gut antigen was prepared following Opdebeeck *et al*. (1988). Briefly, the guts were disrupted in an ultrahomogenizer (Ultra-Turrax, Janke and kunkel) in 0.15 M PBS, containing 1 mM disodium EDTA, sonicated (Rapid 600, Ultra-Sonic, Ltd.) for 10 minutes in 30-60 seconds bursts and centrifuged (1500 rpm/60 minutes). The supernatant thus collected was used as an antigen.

Sensitization of erythrocytes: Erythrocytes from human group "O" were collected, washed and suspended in PBS and sensitized with *B. microplus* mid gut antigen following the method adopted by Akhtar and Hayat (2000). Briefly, washed human group "O" erythrocytes were sensitized with the antigen (2 mL per 0.01 mL of washed packed erythrocytes, diluted in 2 mL PBS). The suspension was kept at 37°C for 60 minutes. The excessive antigen was removed by centrifugation (1500 rpm/5 minutes). The sensitized erythrocytes were washed twice with PBS.

Washed sheep and rabbit erythrocytes were sensitized with chromium chloride following the method described by Bansal *et al*. (1986). Briefly, One volume of Chromium chloride (1:4000) in vernal buffer was mixed with two volumes of 5 percent washed erythrocytes suspension and two volumes of mid gut antigen. The suspension incubated for 10 minutes at 37°C was centrifuged (1500 rpm/5 minutes) and the supernatant was discarded. The packed cell volume of sensitized erythrocytes were washed twice with PBS.

Serum samples: Twelve serum samples of knowm IHA antibody titre from buffaloes vaccinated with mid gut, *B. microplus*, cell culture vaccine (Akhtar *et al*., 1999) were used in the present study .

Preparation of Agar-gel medium: One gram of Noble agar (Difco Laboratories, Detroit, Michigan, USA) was properly dissolved in 99 mL of vernal buffer. A 0.1% sodium azide (W/V) was added to the Noble agar suspension and autoclaved at 0.7 kg/CM^2 for 15 minutes and was maintained at 45-46°C in water bath.

Use of different species Erythrocytes: One percent Noble agar suspension was taken in three equal aliquotes (20 ml each) in three test tubes maintained at 45-46°C in water bath. In one tube, 0.4 ml of sensitized human group "O" erythrocytes was mixed to attain a final concentration of 2 percent in Noble agar, similarly in second and third tube, 0.4 ml of sensitized sheep and rabbit erythrocytes was mixed, respectively. A 1.6 ml of complement (1:2 diluted in vernal buffer) was added in each tube to have a final concentration of 8 percent complement. After gentle mixing the contents of each tube were layered on the petri plates separately in thin layer (2-3 mm thin). The medium was allowed to solidity for five to ten minutes.

Twelve sera were subjected to SRH test on each type of erythrocytes using filter paper discs (paper vicks for electrophoresis; Beckman Instruments, INC. California, USA) 7 mm in diameter. The serum samples were absorbed on the filter paper discs and were applied in triplicate on the different erythrocytes media. Two control discs were also applied, one soaked in hyper immune sera and other in PBS. After eight to twelve hours incubation in a humid chamber the results were recorded.

Complement: Fresh guinea pig serum was used as the source of complement. One percent Noble agar suspension was divided into three aliquotes (30 mL each). Each aliquote was mixed with different dilution of the complement at the rate of 8 percent viz fresh, diluted 1:2 and 1:5 in vernal buffer with human group "O" sensitized erythrocytes at the rate of 2 percent. After gentle mixing gel, was layered onto clean petri plates and allowed to solidify for five to ten minutes.

Table 1: Average haemolytic zone diameter (mm) using different species erythrocytes

Sheep	Rabbit	Human group `O`
7.5	8.5	8.5
8.5	9.0	9.0
8.5	9.0	9.0
7.5	7.5	8.0
10.5	10.5	10.5
5.5	6.5	6.5
6.5	6.5	6.5
9.5	9.5	10.5
10.5	10.5	10.5
10.5	11.0	11.0
-ve	-ve	-ve
-ve	-ve	-ve

-ve = Negative

Table 2: Average haemolytic zone diameter (mm) using Complement dilutions

Fresh	1:2	1:5
8.5	10.0	8.5
9.5	9.5	9.5
9.0	9.5	9.0
7.5	7.5	7.5
10.0	12.5	10.5
11.0	12.5	11.0
11.5	13.0	11.5
7.5	7.5	7.5
8.5	8.4	8.4
9.0	9.0	9.0
-ve	-ve	-ve
-ve	-ve	-ve

-ve = Negative

Table 3: Averae haemolytic zone diameter (mm) under different temperture conditions

37°C	4°C for 12 hours and at 37°C for 8-12 hours
10.0	10.0
9.0	9.5
9.5	9.5
8.2	8.2
10.5	10.5
8.0	7.5
7.5	7.5
12.0	12.5
12.5	12.5
13.0	13.0
-ve	-ve
-ve	-ve

-ve = Negative

Serum samples were applied to the agar gel medium in triplicate and kept at 37°C in the humid chamber. The results were recorded after eight to twelve hours.

Temperature conditions: One percent Nobel agar (40 mL) was mixed with human group "O" sensitized erythrocytes at the rate of 2 percent along with complement (1:2 diluted in vernal buffer) at the rate of 8 percent. The suspension was layered onto the clean petri plates after gentle mixing.

Serum samples were applied on to agar gel medium in duplicate and were subjected to different temperature conditions. i.e., one set was kept at 4°C over night prior to incubation at 37°C and the second set was kept directly at 37°C in humid chamber for eight to twelve hours. The plates were observed for appearance of haemolytic zones at two hours intervals and average haemolytic zone diameter were recorded.

Results and Discussion

Optimum test conditions for SRH test were determined on the basis of clear hemolysis and maximum haemolytic zone diameter by using erythrocytes of different species viz; human, sheep and rabbit, complement dilutions and temperature conditions.

Out of 12 samples, 10 were found positive to SRH test. Chromium chloride coated sensitized sheep and rabbit erythrocytes showed average haemolytic zone diameter ranging from 5.5 to 10.5 mm and 6.5 to 11.0 mm; respectively. Human group `O` erythrocytes showed average haemolytic zone diameter ranging from 6.5 to 11 mm (Table 1). There was a non significant difference ($p>0.05$) in average haemolytic zone diameters among the erythrocytes of three species used, yet qualitative variations helped in deciding the better erythrocytes for use in SRH test. It was observed that in case of sheep and rabbit erythrocytes, the colour changed from red to brown after sensitization which may be due to the use of chromium chloride as a coupling agent. These observations agreed with those of Guo *et al*. (1982) who reported that sheep and chicken erythrocytes treated with sodium-periodate were better than those treated with chromium chloride or even without any treatment. Zahur *et al*. (1994) also observed change in color of sheep erythrocytes after sensitization while applying SRH test to detect antibodies against *Mycoplasma mycoides subspecies capri*. Moreover, in case of sheep erythrocytes the haemolytic zones gave hazy appearance and were poorly demarcated. The sensitized rabbits erythrocytes gave a non homogenous suspension in the agar gel medium. The rate of erythrocyte lysis was higher throughout the agar gel medium when rabbit erythrocytes were used, which hindered the measurement of haemolytic zone formed by the antigen antibody interaction initiated by complement. Such variations were not observed when human group "O" erythrocytes were used as they did not require treatment with coupling agent. Rehman *et al*. (1990) and Zahur *et al*. (1994) also suggested the use of human group "O" erythrocytes without coupling agent for SRH against *Pasteurella multocida* and *Mycoplasma mycoides subspecies capri*, respectively.

Twelve sera were applied to the agar gel medium having 2 percent sensitized human group "O" erythrocytes containing different dilutions of complement i.e., fresh, complement diluted 1:2 and complement diluted 1:5 in vernal buffer. Out of 12 samples, 2 were found negative. The average haemolytic zones were comparable in the agar gel medium containing fresh as well as complement diluted to 1:5 ranging from 7.5 to 11.5 mm in diameter whereas the complement diluted to 1:5 showed higher average haemolytic zone diameter ranging from 7.5 to 13.00 mm (Table 2). So 1:2 dilution of complement in vernal buffer used at the rate of 8 percent in agar gel medium was found to be optimally appropriate for SRH test. Charan *et al*. (1981) and Rehman *et al*. (1990) suggested the use of 1:5 diluted complement which may partially be due to the difference in titre of the complement. No significant difference ($p>0.05$) was observed in average haemolytic zone diameters between fresh and complement diluted 1:5 in agar gel medium but the difference was significant when compared with complement diluted 1:2.

Twelve sera were applied in duplicate on the agar gel medium containing 1:2 diluted complement and human group "O" and were subjected to different temperature conditions i.e., direct incubation at 37°C in a humid chamber for 8-12 hours and in

other case petriplates were refrigerated at 4°C in humid chamber for 12 hours and then incubated at 37°C for eight to twelve hours. The average haemolytic zone diameter were comparable, ranging from 7.5 to 13.00 mm in both the cases but the haemolytic zones were of better quality and clearly delineated in the later case i.e., combination of 4°C for 12 hours prior to incubation at 37°C for eight to twelve hours (Table 3). Various temperature conditions have been recommended by different workers for SRH test. In this study the results indicated no significant difference ($p>0.05$) regarding the average haemolytic zone diameters at low temperature conditions. The results were obtained earlier when the plates were kept directly at 37°C in a humid chamber for 8-12 hours but the combination of over night refrigeration and subsequent incubation at 37°C in a humid chamber gave good quality of the zones of hemolysis which were well delineated. It probably allows the diffusion of serum around the impregnated discs.

From the results it could be concluded that SRH can be used for the detection of anti tick and other parasite antibodies. The advantage of the test lie in the e and does not require elaborate equipments. The test can, therefore, be used in the field where minimal facilities are available.

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