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Research Article

Development and Evaluation of latex agglutination test for Haemorrhagic Septicaemia and Anthrax

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Abstract

In this present investigation, two rabbits were taken and one was vaccinated with *Bacillus anthracis* then another one was vaccinated with *Pasteurella multocida*. Wild Ag of organism inoculated in to the vaccinated rabbits measured the efficiency of both vaccines. 5ml of blood sample was bled from each vaccinated rabbits by cardiac puncture method. Serum was separated from both blood and immunoglobulin from both serum samples were precipitated by using $(\text{NH}_4)_2\text{SO}_4$. IgG molecules were separated and purified from precipitate by dialysis method. Folin's estimated the IgG concentration phenol method and the concentration of IgG (50mg/ml) adjusted with saline to 1mg/ml. Commercially available latex beads (10%) are purchased in Sigma Company and diluted to 1% using carbonate-bicarbonate buffer. Then the purified IgG molecules were coated on the latex and stored at 4°C. Coated IgG was challenge with both positive and negative samples. It given agglutination in positive samples (blood sample containing antigen) and agglutination could not occur in negative sample (control).

Keywords: *Bacillus anthracis*; *Pasteurella multocida*; blood sample; dialysis; Folin's estimation. vegetation.

Introduction

Latex agglutination is a diagnostic method for detecting method for detecting various diagnosis causing microbial agents such as: *Salmonella*, *Shigella*, *Proteus*, *E. coli*, *Gonococci* and *Meningococci* etc. Soluble proteins react with their antibodies to produce precipitate at optimum antigen-antibody concentrations. If the antigen is particulate, the antigen and antibody complex agglutinates. In this method, the antigen is coated onto an inert matrix i.e. Latex. The antigen bound to the latex acts similar fashion as particulate antigen.

This antigen coated latex when mixed with antibody agglutinates, which can be visualized by the necked eye. Singer, *et. al.*, (1956) was the first to describe the use of latex particles for the diagnostic test, and they used the particles follow changes in the titre of rheumatoid factor in patient's samples. This method is still in use today, and this technology is now the basis of the many commercially available kits for a wide range of analytes. The basic principles of latex reagents for agglutination tests, among the latexes used, partially hydrolyzed styrene-acrylamide copolymer latex were the best with respect to test

efficiency and storage stability. (Kawaguchi, *et. el*, 1989). The principle of a novel latex agglutination test based on combined results of electrophoretic mobility and particle aggregation measurements were described by Radomska-Galant, *et. al.*, (2003). Masson, *et. al.*, have done considerable work on developing stable, reactive, covalently attached antibody particle reagents (Masson, *et. al.*, 1981). They have stressed the importance of using antibody fragments as opposed to whole antibodies to overcome problems caused by nonspecific aggregation, and they have described procedures for making particles with the antibody fragments. To cover the sites not occupied by the antibody or antigen increases particle reagent specificity and stability. Particle reagents are usually stored in glycine-buffered saline. Latex particles coated with HAS (Dezelic, *et. al.*, 1971).

Latex agglutination can identify organisms within a few minutes, and its sensitivity and specificity are comparable to those of staphylococcal coagulation and better than those of immuno-electrophoresis (Thirumoorthi, *et. al.*, 1979). Laflar and Looney have used the alternative procedure of antigen-coated particles to measure the concentration of the corresponding antibody. They used the streptolysin o-coated 0.14-micrometer polystyrene latex from Behring's Rapi-TEX ASO kit and used them in a method for measuring the antibody concentration on the Du Pont aca discrete clinical analyzer. With an endpoint measurement, the detection limit was 100 IU/ml with 10% coefficient of variation at the 200 IU/ml medical decision level. This assay principle can be used for many serological testes.

Polyethylene glycol certainly increases the sensitivity in some assays, but it can lead to self-aggregation, especially of antibody-coated particle reagents (Masson, *et. al.*, 1981). pH is a very important factor in LAT. If the P_H is below 4 or above 11, no specific immunoreactivity of the particles can be detected. (Dezelic, *et. al.*, 1971). Particle enhanced immunoassays stabilized by hydration forces: a comparative study between IgG and F (Ab) 2 immunoreactivity. This research throws light upon the use of hydration forces as a new approach to stabilizing immunoassay reagents

that are colloiddally unstable in physiological reaction buffers. (Molina-Bolivar, *et. al.*, 1998).

A polystyrene latex (0.81 µm) suspension was used as a carrier particle for hydatid antibodies in the test. The latex particles were sensitized with hyper immune hydatid antiserum raised in rabbits. The hydatid antibody-sensitized latex particles were used for the detection of hydatid antigens in serum. The results of the study showed that the LAT could detect the circulating hydatid antigen in (72%) patients with surgically confirmed CE (Devi, *et. al.*, 2003). A method for preparing polystyrene latexes for slide agglutination test is described. The latexes were used for preparing latex slide tests for evaluating C-reactive protein and myoglobin. Effects of such factors as pH, temperature, antibody concentration, and latex particles concentration, on sensitization and agglutination have been studied by Gasparian, *et. al.*, 2001). The polystyrene latex particles are made by emulsion polymerization. (Craig, *et.al.*, 1998). The polymerization of the styrene monomer is carried out in an aqueous emulsion of the styrene monomer using surfactants such as sodium-dodecyl-sulfate or Aerosol. Potassium per sulfate is the usual free radical initiator for the polymerization. The polymer is formed in the droplets of monomer-surfactant in the water. Changing the concentration of the surfactant most easily controls particle size.

Haemorrhagic septicaemia (HS)

HS is a disease caused by *P. multocida* and the same pathogen causing fowl cholera in birds. The observations of Bollinger and Kitt in 1978 on epidemic disease affecting wild hogs, deer and later cattle in the neighborhood of Munich constitute the first work of importance on a grope of disease attacking several species of animals, and known collectively as HS. Kitt was successfully in isolating the causative organism and transmitting the disease to mice and pigeons. Pasteur obtained a similar organism in 1880 from fowl cholera, by Loeffler in 1882 from swine plague (1886) from septic pleuropneumonia of calves, and by numerous workers during the next few years from diseases in other animals.

Reviewing the literature, Regamey, (1939) traced ten cases that were probably, and 6 cases, which he thinks were certainly, due to this organism. Of the 6 genuine cases, pleurisy, 2 by meningitis, and one by local lesion following the scratch of the cat characterized 3. Of more recent years genuine cases have been recognized, some of them recorded (Foerster, 1938), and some of them not. Allot and his colleagues (1944) reported 6 cases, 3 following cat bites and 3 following dog bites. 3 of the cases were complicated by osteomyelitis. Localized disease is commonly followed by recovery, but generalized cases have usually proved fatal. Infection may occur without accompanying illness; in our own experience, for example, an animal house attendant who carried *Pasteurella septica* in his nose for several months remained quit well.

Pathogenicity

No true exotoxin produced. Virulence subject to alteration. Causes fowl cholera in birds. Other members of this group produce HS in pigs, cattle, sheep, rabbits, mice, rats, reindeer, buffaloes and other animals. Experimental inoculations reproduce the disease in these animals. Subcutaneous of a 24 hours' broth culture in to a mouse proves fatal in 18-72 hours. Local edema and congestion; often no other signs; microscopically bacilli present in enormous numbers in blood and viscera. If a small dose is given and animal does not die for 4-7 days, there is often a fibrinopurulent pericarditis, a layer of fibrin over the pleura, and partial consolidation of the lungs. Bacilli are numerous in blood and organs.

Diagnostic techniques

Haemorrhagic septicaemia (HS) is an acute, highly fatal, septicaemic disease of cattle and buffaloes caused by certain serotypes of *Pasteurella multocida* (Bain, *et. al.*, 1982). Presently, two serotypes are recognised – the Asian serotype and the African serotype. Three serotyping methods are available- the 'capsular' typing method using an indirect haemagglutination (IHA) test (Carter, 1955), 'somatic' typing using agglutination, and the agar gel immunodiffusion (AGID) test (Heddleston

, *et. al.*, 1972). The Asian strains belong to capsular type B only while the African strains are types B and E (Mustafa, *et. al.*, 1978). Using the somatic typing methods, all strains belong to type 6 by the agglutination tests and type 2 by the AGID test.

Cultural and biochemical methods

A suitable medium for the growth of *Pasteurella* is casein/sucrose/yeast (CSY) agar containing 5% blood. The composition of this medium is casein hydrolysate (3 gm), sucrose (3 gm), yeast extract (5 gm), sodium chloride (5 gm), anhydrous dipotassium hydrogen orthophosphate (3 gm), and distilled water to 1 litre. The pH is adjusted to 7.3-7.4, after which 1.5% agar is added. The medium is autoclaved at 1 bar for 15 minutes. After cooling to 45-50°C, 5% calf blood (antibody-free *P. multocida*) is added (Wijewardana, *et. al.*, 1986).

Materials and Methods

Collection of test animal

The 5 rabbits and the 6 mice were taken from the *Institute of animal health and veterinary biologicals, Palode, Thiruvananthapuram*, as experimental animal chosen for this present study and acclimatized our good environmental condition.

Collection of organism

The mother inoculams of the organisms such as, *Bacillus anthracis* and *Pasteurella multocida* were taken from the *Institute of animal health and veterinary biologicals, Palode, Thiruvananthapuram*, as experimental study.

Culture of organism

Pasteurella multocida

Method

The *P.maltocida* was streaked in to the petri plate containg the yeast agar media and incubated for 24 hours at 37°C. And then from that the organism was

inoculated in to the Rouex flask containing yeast agar media for 24-48 hours at 37°C. From that, the *P.maltocida* was hearvisted by using formyl saline. That is called as crued sample

Bacillus anthracis

Method

The *Bacillus anthracis* was streaked in to the petri plate containing the blood agar media and incubated for 24 hours at 37°C. And then from that the organism was inoculated in to the Rouex flask containing yeast agar media for 24-48 hours at 37°C.

Preparation of antigen

Preparation of *Bacillus anthracis* antigen (Serial dilution method):

The biochemical tested *Bacillus anthracis* was taken as one loopful of culture and inoculated in a 10ml of saline containing screw capped test tube and mixed well, and marked as 10⁻¹ using marker pen. And then from that tube, 1ml of sample was taken and transferred to the 9ml of saline containing tube using sterlized syringe, and marked as 10⁻². Similarly 1ml was transferred to the other tubes by serial dilution method and named as 10⁻³, 10⁻⁴, 10⁻⁵, 10⁻⁶, 10⁻⁷, 10⁻⁶, 10⁻⁷, 10⁻⁸, 10⁻⁹ and 10⁻¹⁰.

The tubes from 10⁻⁵ to 10⁻¹⁰ was taken for the consideration, and 1ml of saline was taken from each tubes and added to the respected sterilized petri plates marked 10⁻⁵ to 10⁻¹⁰ and then the nutrient agar solution was poured in to the petri plates and then the petri plates were rotated clockwise and anti-clockwise 2-3 times for spreading the sample. And incubated the plates in to the incubator at 37°C for 24-48hours. And the serially diluted tubes were stored into the freezer at 4°C. After the incubation period over, the petri plates are taken and the no. Of colonies were counted by using colony counter. And the colonies were tabulated.

Sl. NO.	DILUTION	NO. COLONIES
1.	10 ⁻⁵	TNTC
2.	10 ⁻⁶	TNTC
3.	10 ⁻⁷	110
4.	10 ⁻⁸	27
5.	10 ⁻⁹	TLTC
6.	10 ⁻¹⁰	TLTC

So 10⁻⁷ dilution was taken for injection to the mice.

Preparation of the *Pasteurella multocida* antigen

10ml of crude sample was taken and centrifuged at 2000-3000rpm for 10 min. Then the supernatant was discarded. Pellet was collected and to that 8.5% NaCl was added as 10ml and mixed well. And than the sample was heated in a boiling water bath at 100°C. Then the sample was cooled and then centrifuged at 2000-3000rpm for 30min. Finally the supernatant was collected. And the supernatant was stored at 4°C and this supernatant was used as antigen.

Production of hyperimmune serum in rabbit:

Vaccination

Raising the antibodies against *Anthrax*

For vaccination of anthrax, 2 young and non immunized rabbits were taken and the *Bacillus anthracis* vaccine were taken as 2.0ml and injected to the each rabbit as 1.0ml by subcutaneous injection for rising the antibodies against *Bacillus anthracis* in the immune system between one week interval at 3-4 times. For testing whether the immune system is responded or not, the live *Bacillus anthracis* is injected as 1.0ml to the vaccinated rabbit by subcutaneous injection. If the

immune system produces the antibodies against the *Bacillus anthracis* antigen the rabbits will alive if not the rabbits will die. But after injection of the 110 spores to the already immunized rabbits, the rabbits are alive so I confirmed that the antibodies produced against the particular antigen.

Raising the antibodies against HS

For vaccination of HS, 2 young and nonimmunized rabbits were taken and *Pasteurella maltocida* the vaccine were taken as 2.0ml and injected to the each rabbit as 1.0ml by intramuscular injection for rising the antibodies against *Pasteurella maltocida* in the immune system between one week interval at 3-4 times. For testing whether the immune system is responded or not, the live *Pasteurella maltocida* is injected as 1.0ml to the vaccinated rabbit by intramuscular injection. If the immune system produces the antibodies against the *Pasteurella maltocida* antigen the rabbits will alive if not the rabbits will die. But after injection of the crude sample to the already immunized rabbits, the rabbits are alive so I confirmed that the antibodies produced against the particular antigen.

Bleeding of rabbits and separation of serum:

The blood was bled carefully in a rabbits by intracardiacally as 10ml and bled blood was immediately transferred into the screw-capped test tubes and the test tubes were kept in a slanting position for separation of serum for 24 hours at room temperature. And then the serum was collected using micropipette and transferred in to the eppendroff tubes. And the remaining blood was centrifuged for 3000rpm at 30 minutes. And then the serum was collected by using micropipette and stored at 4°C.

Separation of igg by $(nh_4)_2so_4$ precipitation method:

1. The stored serum was taken and centrifuged at 1000g minutes for 20-30min. at 4°C.
2. Pellet was discarded.
3. And then supernatant was collected and cooled at 4°C and stirred slowly for few minutes.

4. Saturated $(NH_4)_2SO_4$ was added to the serum drop wise manner to produce 35-45% final saturation.
5. Then stirred at 4°C for 1-4 hours or over night using the magnetic stirrer.
6. Then the stirred solution was taken and centrifuged at 2000-4000rpm for 15-20minutes at 4°C.
7. The supernatant was discarded.
8. The pellet was collected and dissolved the pellet in 10-20% original volume in a PBS with mixing using Pasteur pipette.
9. And dialyzed the solution against the required 0.01M phosphate buffer at 4°C.

Purificatin of igg by using dialysis method

1. The stored solution was taken and filled in a dialysis bag.
2. The bag was kept on higher side to allow the fluid to come out of the bag.
3. And the fluid is collected in a container containing 0.01M-carbonate bicarbonate buffer (pH-8).
4. The purified IgG present in the dialysis bag was collected.
5. The collected IgG was taken and the concentration of IgG was estimated by Folin's phenol method.

Estimation of igg antibodies by folin's phenol method

1. Taken 7 clean and dry test tubes and marked the tubes $S_1, S_2, S_3, S_4, S_5, T_1, T_2,$ and B using marker pen.
2. To the tubes marked $S_1, S_2, S_3, S_4,$ and $S_5,$ 0.2ml, 0.4ml, 0.6ml, 0.8ml and 1.0ml of standard solution was taken with corresponding concentration ranging from 20-100 microgram respectively.
3. The IgG solution was taken as 0.5-1.0ml in to the named T_1 and $T_2.$

4. All the test tubes were made up to 1.0ml with saline.
5. Blank also prepared simultaneously taking 1.0ml of saline.
6. To all the tubes freshly prepared reagent (Alkaline copper tartarate) was added as 4.5 ml.
7. And kept in a boiling water bath at 37°C for 10 minutes.
8. Then 0.5 ml of Folin's reagent was added to all the test tubes and mixed vigorously.
9. The blue color developed was read at 640 nm using spectrophotometer.
10. And the results were tabulated.
11. Using the results, a graph was drawn by taking optical density of the IgG on Y-axis and the concentration of the IgG on X-axis.
12. Using the graph, the concentration of the IgG was calculated.
13. And the concentration of the IgG was adjusted as 1mg/1ml using saline.

Coating of igg on latex beads:

Prepared 1% suspension of carbonate-bicarbonate buffer (P_H-9.6). Then added 1% latex beads to the 0.1ml of IgG (1mg/ml) and mixed thoroughly and kept over night at 4°C. Washed the beads 3 times with Carbonate-Bicarbonate buffer at 20-30 minutes time interval between each washing to remove the excess amount of latex beads and kept over night at 4°C. That is called as IgG-Latex complex. . Finally prepared 0.35% (v/v) suspension on coating buffer. And then the IgG-Latex suspension is tested using infected animals blood.

Challenging of produced kit against samples: collection of sample

Disease free mice were taken, mice injected with *pasteurella multocida* and *Bacillus anthracis* .The animal left out for incubation period of 24 hours .then 2ml of blood samples collected from each group of mice and it is tested against produced kit

Testing of kit by slide agglutination

For Heaemorrhagic Septicaemia and Anthrax

Two Clean dry glass slides were taken and marked as A and B. Blood is collected from infected animal was taken in slide A and non infected animals blood was taken in slide B. To each slides anti bodies coated latex beads were added and mixed well with tooth pick. After 3-5 min the results were observed.

Results and Discussion

Agglutination occurred in slide A and slide B. So it is denoted as presence of Ag in the samples. In slide C agglutination not occurred, it is denoted as absence of Ag in the blood sample. Morphological and Molecular diagnosis for *Bacillus anthracis* and *Pasteurella multocida*:

Two types of antigens are used for this experiment respectively *Bacillus anthracis* and *Pasteurella multocida*. Previously both this Ag diagnosed by microscopic observation, cultural character, serological diagnosis such as agar gel immuno diffusion, immuno fluorescence but it took longer duration for the experiment, and also it is diagnosed by some molecular biology method especially PCR analysis but it is very costlier methods. So this Ags require a new diagnostic procedure for reducing the duration and cost. So this technique can be a entirely different and low cost and low time consuming procedure.

Biochemical identification of *Bacillus anthracis* and *Pasteurella multocida*:

The both Ags, first biochemically identified. So for identification the following test are done and following results were observed. *Pasteurella multocida* is G -ve appeared as pink color, rod shaped bacteria under microscopic field and it is given +ve results to Catalase test, H₂S test, Indole test and Carbohydrate reduction test & as same it given -ve results to MR and VP test.

Table.1 Biochemical tests for *Pasteurella multocida* and *Bacillus anthracis*

S.No	Test name	<i>P. multocida</i>	<i>B. anthracis</i>
1.	Gram staining	G -ve	G +ve
2.	Catalase test	+ve	-ve
3.	H ₂ S test	+ve	-ve
4.	Indole test	+ve	-ve
5.	MR test	-ve	-ve
6.	VP test	-ve	-ve
7.	Carbohydrate reduction test	+ve	-ve

Bacillus anthracis is G +ve appeared as violet color, rod shaped bacteria under microscopic field and it is given +ve results to MR test and -ve results to Catalase test, H₂S test, Indole test, VP test and Carbohydrate reduction test.

The Ag prepared from both organisms, prepared Ag was injected into 2 different disease free rabbits and IgG Ab against the Ag isolated from blood sample of vaccinated rabbits and it is purified by dialysis method.

The purified IgG Ab coated with polystyrene beads and tested against both Ag given agglutination (+ve reaction). Another blood sample does not contained Ag was tested with produced kit, it could not given any agglutination (control). The efficiency of kit tested against *Brucella* and *Salmonella*.

This LAT was first described by Singer et al., (1956). Viral diagnosis through this technique was first introduced by Fichman.,(1956). He used this technique for identification of hydatid disease. Later this technique developed by Masson et al., (1981), then this technique developed for years by various researchers for finding various diseases.

Main advantage of LAT was, it will take only low time for it results Thirumoorthi, *et. al.*, (1979). *Bacillus anthracis* is a causative agent for anthrax it leads to death finally. *Pasteurella multocida* is a causative agent for hemorrhagic septicemia is an

acute highly septicemia disease of cattle and buffaloes. Human abscesses are characterized by extensive edema and fibrosis. Encapsulated organisms resist phagocytosis. Endotoxin contributes to tissue damage.

For identification of both organisms various techniques used such as, microscopic observation, cultural character, serological diagnosis such as agar gel immuno diffusion, immuno fluorescence. But these techniques are needed more requirement, time consuming and skilled person. Molecular biology technique like PCR analysis also used for diagnosis but it is very costlier technique. So we need a new technique for this disease analysis that can be more effective, low time consumable and low costlier. So for that LAT can be used for diagnosis of particular organism. For both this antigens, the prepared kit given agglutination reaction (positive results). And also it taken only low time, all manufacturing cost of this is very low comparing to other techniques. So it can be successful technique for identification of Anthrax and HS in following years.

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