ANTIGENS OF *Pasteurella multocida* ISOLATES FROM RABBIT AND THEIR IMMUNOGENICITY

By

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THESIS

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Dedicated to my loving
parents and beloved brothers

DECLARATION

I hereby declare that this thesis entitled **"Antigens of Pasteurella multocida Isolates from Rabbit and Their Immunogenicity"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title of any other University or Society.

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Mannuthy,

25.11.1994.

CERTIFICATE

Certified that the thesis entitled **"Antigens of Pasteurella multocida Isolates from Rabbit and Their Immunogenicity"** is a record of research work done independently by **Shri. S. Manoharan** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to him.

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We, the undersigned members of the Advisory Committee of **Shri. S. Manoharan**, a candidate for the degree of Master of Veterinary Science in Microbiology, agree that the thesis entitled **"Antigens of Pasteurella multocida Isolates from Rabbit and Their Immunogenicity"** may be submitted by Shri. S. Manoharan, in partial fulfilment of the requirement for the degree.

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INTRODUCTION

It is needless to point out the paramount importance of protein, a principal component constituting the human food items. The main sources of protein particularly for non-vegetarian are meat, egg and fish besides, milk and legumes consumed mainly by vegetarian to meet the protein requirement. As the human population swells up year by year, there will be need for a proportionate increase in the requirement of protein as well and consequently alternate ways and means other than the conventional sources are to be found out to augment the availability of protein.

In the recent past rabbits which were used as laboratory animals for experimental purpose and reared as pet animals are being used for establishing rabbitries, an enterprise considered as highly profitable and remunerative venture for elite farmers since,

a. Rabbits can be fed with high forage diet and low grain diets that are largely non-competitive with human food requirements.

b. Rabbits have a high feed conversion efficiency.

c. They have a high growth rate, attaining market weight of two kg or above at about eight weeks of age.

d. Rabbits have the potential of being in a constant state of reproduction.

e. Rabbit meat is of high quality nutritious product and,

f. Rabbits are suited to both small scale (backyard) and to large scale commercial production.

With all these aforesaid desirable aspects broiler rabbits with high production potential can be more productive only if good health coverage is provided.

Recently there have been many reports of health problems in rabbits and reasons for this could be attributed to the intensive farming methods adopted, management of over-crowded flocks in limited area without proper ventilation, frequent handling and shifting of animals etc.

Rabbits are prone to diseases caused by bacteria, viruses, fungi and parasites. Common bacterial diseases of rabbits are pasteurellosis, tularemia, yersiniosis, necrobacillosis, salmonellosis, listeriosis, tuberculosis, treponematosis and staphylococcosis. Among these, pasteurellosis caused by Pasteurella multocida takes a heavy toll in many rabbitries due to the acute nature of the disease and its high morbidity and mortality rates. The clinical manifestations of this disease are snuffles, pneumonia, otitis media, conjunctivitis, pyometra, orchitis, abscesses and generalised septicemia. On the other hand, this

bacteria is also demonstrated as a dormant resident in the upper respiratory tract of apparently healthy rabbits.

The devastating effect of this disease on rabbits is so high and hence warrants detailed studies on serotypes involved, its pathogenic propensities, treatment, prevention and control methods.

Majority of informations available on the serology and immunology of P. multocida are from studies conducted with isolates from livestock and birds. Recently serotypes of P. multocida infecting rabbits have received attention and much research on the serology and immunology are in progress.

Various methods such as maintenance of pasteurella free flock by practising strict and scrupulous hygienic measures and proper managemental practices, timely segregation of infected animals and their treatment with appropriate antibiotics, development of genetically resistant strain of rabbits and regular immunization of animals with proper vaccine, have been advocated for prevention and control of this disease in rabbitries. But none of the above practices except immunization is fool-proof when applied individually. The application of methods developed and used to study the immunobiology of P. multocida from divergent species would facilitate a better understanding of this disease in rabbits and possibly form a basis for future immunization practices.

Bacterins, live attenuated organisms, mutants and cell free immunogens were all been on trials for immunization of rabbits and each of them was reported with varying results. The lacuna in the success of immunization trials was attributed to the fact that the serotypes are immunologically distinct and they are able to cross-infect divergent species.

The P. multocida infections have been reported from many rabbitries in this state. Earlier in the Department of Microbiology few strains of P. multocida were isolated from various rabbitries. These isolates were characterized and were shown to be virulent and pathogenic to mice. The present work is undertaken to study the antigenic relationship and immunogenicity of P. multocida isolates from rabbits and to compare with that of P. multocida vaccine strain P-52. The programmes of present work envisages -

a. Assessment of antigen mosaicity of the isolates.

b. Comparison of the antigen profiles of the various isolates.

c. Production of antisera and its use in the study of serological behaviour.

d. The immunogenicity assay of different antigens in mice.

Review of Literature

REVIEW OF LITERATURE

Pasteurella multocida: Nomenclature

Revolta (1877) investigated an outbreak of a disease, now known as fowl cholera and he was the first to report upon the causal agent of the disease as bacteria although he did not isolate the agent or assign it any name. Bollinger (1878) was the earliest to describe the bacteria causing haemorrhagic septicemia in cattle. Perroncito (1878) isolated and described the causative agent of fowl cholera as Bacillus, for the first time.

Following its first description, organisms similar to the causal agent of fowl cholera, was isolated by various investigators from different animal host species viz., chicken (Pasteur, 1880), pigs (Salmon, 1886), rabbits (Smith, 1887) and bison (Gochenour, 1924). It was further observed that fowl cholera, haemorrhagic septicemia of cattle, epidemic disease affecting wild pigs and snuffles of rabbits, were caused by similar organisms and Kitt (1885) named this organism as *Bacterium bipolar multocidum* to emphasize the multihost character of this organism. Considering the close similarity of the organisms isolated from divergent species and common pattern of disease produced, Hueppe (1886) named

the organism as Bacterium septicemae haemorrhagicae, while another name Bacillus bipolaris septicus was assigned to this organism by some other investigators (Kruse, 1896 and Hauduroy et al., 1937).

Trevisan (1887) proposed the generic name Pasteurella in recognition of Pasteur's work on the causal agent of fowl cholera and his choice of the generic name for this organism was followed by several others (Lignieres, 1900; Bergey et al., 1923; Topley and Wilson, 1929 and Rosenbusch and Merchant, 1939).

Lignieres (1900) proposed specific name for each organism according to the animal it attacked, thus the name of the organism from fowls was Pasteurella aviseptica, from pigs Pasteurella suiseptica, from cattle Pasteurella boviseptica, from sheep Pasteurella oviseptica and from rabbits Pasteurella lepiseptica. As these organisms behaved as if they belonged to a single species, Topley and Wilson (1929) suggested that they should all be referred to by the name of Pasteurella septica. Based on a study conducted using 114 different strains of Pasteurella, Rosenbusch and Merchant (1939) suggested the species name for this organism as Pasteurella multocida and they proposed the grouping of all indole positive, non-hemolytic hemorrhagic septicemia organisms in this species.

The name P. multocida was used in the sixth (Breed et al., 1948), seventh (Breed et al., 1957) and eighth (Buchanan and Gibbons, 1974) editions of Bergey's Manual of Determinative Bacteriology. In the above editions of Bergey's Manual, the genus Pasteurella was placed in the family Enterobacteriaceae as one of the genera of uncertain affiliations. Hussaini (1975) published a review article on the taxonomy of P. multocida. In the ninth edition of Bergey's Manual of Systematic Bacteriology genus pasteurella has been placed in the family Pasteurellaceae among the different families under the section five (Facultatively anaerobic gram negative rods) (Mannheim, 1984). The family pasteurellaceae included the genera like Pasteurella, Haemophilus and Actinobacillus. The species included in the genus Pasteurella are P. multocida, P. pneumotropica, P. haemolytica, P. ureae, P. aerogenes, P. gallinarum and P. anatipestifer.

Pasteurellosis

The term pasteurellosis although still often used, is somewhat outmoded and misleading as it fails to distinguish between diseases caused by different Pasteurella species, or between the various forms of infection that occur. The disease pasteurellosis caused by P. multocida is differently known in divergent species as fowl cholera in birds,

haemorrhagic septicemia in cattle, buffalo, sheep and fallow deer, atrophic rhinitis in pigs and snuffles in rabbits.

P. multocida is found in a wide variety of animals and has its main habitat in the respiratory tract (Smith, 1955). Strains of P. multocida from different sources vary in their virulence for experimental animals as well as natural hosts (Carter, 1967). The P. multocida is pathogenic for a wide variety of animals, producing anything from a harmless inapparent infection to a rapidly fatal haemorrhagic septicemia (Smith and Philips, 1990).

The P. multocida infections have been reported from different species of animals and birds viz., cattle (Dhanda and Lall, 1958; Prince and Smith, 1966; Carter, 1967), chicken (Dorsey and Harshfield, 1959; Heddleston 1962), deer (Jones, 1982), dog (Vaissaire et al., 1989), ducks (Hilbert and Tax, 1938), pigs (Kim et al., 1986), quail (Polero, 1988), sheep (Hancock, 1991), turkey (Heddleston and Rebers, 1972; and Lu et al., 1988). According to Smith and Philips (1990), dogs and cat strains do not play any important pathogenic role in their normal hosts.

P. multocida infections in animals are often precipitated by stress factors and diseases produced are generally septicemic or pneumonic although other forms occur.

In subacute respiratory disease concurrent infections with other infectious agents may exist (Flatt, 1974).

P. multocida infections in rabbits

The ailment caused by P. multocida infection in rabbits, is generally known as pasteurellosis and a number of clinical forms are manifested for this disease as snuffles, enzootic pneumonia, otitis media, pyometra, orchitis, abscesses, conjunctivitis as well as generalized septicemia (Webster, 1924b and Flatt, 1974).

Snuffles

This has been reported from rabbits since the species was used as experimental animals (Alexander et al., 1952) and this condition caused by P. multocida has been under extensive investigation by several workers. The earliest record of the isolation of this organism was from cases of snuffles in rabbit (Ferry, 1913). Ferry and Hoskins (1920) reported that snuffles was caused by P. multocida and Staphylococcus aureus combined infections.

DeKruif (1923) working with rabbits suffering from snuffles observed that this organism is a normal inhabitant in the upper respiratory tract of animals. McCartney and Olitsky

(1923) reported chronic inflammation of nasal passages and the para nasal sinuses in snuffles

Webster (1924a) reported seasonal influence on the incidence of snuffles with peaks in the fall and spring and lowest in summer. He further observed that rabbits were asymptomatic carriers of P. multocida in the nasal cavity and stress on the part of the host allowed the bacteria to multiply, thus initiating episodes of overt clinical disease. The relationship between snuffles and the other clinical forms of pasteurellosis were studied and reported by Webster (1925) and Smith (1927). Smith (1927) further reported that snuffles was the most commonly observed disease in domestic rabbits and morbidity ranged from 20 to 70 per cent.

According to Hagen (1958), the spreading of P. multocida from dam to the offspring occurred via the respiratory route, shortly after birth. Mortality rate even upto 50 per cent by snuffles in rabbits has been reported by Ward (1973).

Enzootic pneumonia

Acute deaths from this form of disease have been observed for many years. The condition is reported to take the form of an acute fibrinopurulent pneumonia and pleuritis often with terminal septicemia (Smith, 1927 and Hagen, 1959).

Webster (1925) demonstrated that pneumonia in rabbits was invariably associated with P. multocida infection. Pneumonia with pleuritis and terminal septicemia was reported to be the cause of death in rabbits suffering from Pasteurellosis (Hagen, 1958). Alexander et al. (1952) reported that the mortality rates due to pneumonia varied from 5 to 50 per cent.

Otitis media

Smith and Webster (1925) observed that the otitis media experimentally produced by intranasal inoculation of P. multocida to susceptible rabbits was indistinguishable from naturally occurring cases. They further observed that staphylococcus species, Bordetella species and other bacteria could be associated with P. multocida in natural cases of otitis media.

Fox et al. (1971) isolated P. multocida from 88 out of 91 rabbits with otitis media. They have further reported that a low percentage (2%) of rabbits with otitis media, developed torticollis. P. multocida was isolated by Kunstyr and Neumann (1985) from pus and nasal mucous membrane from 18 NewZealand White rabbits having torticollis, otitis and empyema of one or both ears.

Genital infection

Genital infections in rabbits include metritis, or pyometra in the doe and orchitis and epididymitis in the buck (Flatt, 1969). These conditions occur frequently in adults/young adults and is observed more often in does and less frequently in bucks.

Venereal transmission occurs when infected bucks breed uninfected does or vice versa. Rabbits with acute and subacute infection are seldom observed to have a serous, mucus or mucopurulent vaginal discharge. Bucks may also show enlargement of one or both testicles (Flatt, 1974).

Abscesses

Davis (1917) described subcutaneous abscesses in rabbits from colonies experiencing epidemics of enzootic pneumonia and snuffles.

The reported sites of abscess formation were lung, brain, heart, testicles or any organ or tissue of the body. Consequent to the abscess formation, rabbits invariably developed septicemia and death (Flatt, 1974). The etiology and pathology of pulmonary abscesses in rabbits slaughtered in Northern Italy abattoir was reported by Guarda et al. (1991).

They observed P. multocida as one of the etiological agent responsible for this condition.

Conjunctivitis

According to Blount (1957), conjunctivitis caused by P. multocida has received little attention as the incidence of this clinical form of the disease has not been reported regularly. The condition is said to be quite common in rabbits following P. multocida infection. The clinical signs included, swollen eyelids and reddened conjunctiva with mucopurulent exudation (Flatt. 1974). In chronic cases, the condition finally subsides but the exudation continues resulting in epiphora.

Septicemia

Septicemia with P. multocida is reported to be a common sequellae to several forms of Pasteurellosis and is considered as the cause of mortality in affected animals (Smith, 1887 and Davis, 1917).

Nakagawa et al. (1986) observed the prevalence of P. multocida in rabbits belonging to breeding colonies and reported that the rate of isolation of bacteria increased with age of rabbits. Similar observations were made by Lukas et al. (1987) and according to them the prevalence and

isolation rate of P. multocida vary considerably depending on the age, sex and health status of the rabbit, and the technique used for detection.

In Kerala, Nair et al. (1987) reported that pasteurellosis and coccidiosis were the two important diseases causing high mortality in rabbits. Devi et al. (1990) also reported pasteurellosis as the most important single disease which caused highest mortality in rabbits over six weeks of age.

Dillehay et al. (1991) reported the isolation of P. multocida serotype A:3 from Flemish Giant rabbits suffered from an outbreak of pasteurellosis and this serotype was shown to be less virulent to NewZealand White rabbits.

Sheela (1992) isolated and characterized few pathogenic strains of P. multocida from rabbits with respiratory infections. P. multocida were isolated from 16 out of 39 dead and one out of four sick rabbits which were clinically suspected for Pasteurellosis by Katoch et al. (1993).

Serotyping of P. multocida

P. multocida has been the subject of numerous antigenic and serologic studies. Serologic properties of the

bacteria are based mainly on the capsular and cell wall antigenic characteristics.

Lal (1927) compared P. multocida strains from divergent species using complement fixation test and noted cross-reactions between bovine and ovine strains and to a lesser degree between swine and avian strains, although no cross reactions was found between these two main groups. Strains from rabbits were unrelated to either group.

Cornelius (1929) attempted a serological classification using agglutination and agglutinin absorption techniques and placed P. multocida strains into four groups as type I, II, III and IV. Ochi (1934) divided 72 strains of P. multocida from various species, into four types viz., A, B, C and D by employing tube agglutination test and noted that type A belongs to cases of fowl cholera, type B were from cases of haemorrhagic septicemia and those of types C and D were isolated from pigs and sheep respectively. Khalifa (1934) was the first to correlate serological and biochemical reactions in the classification of this organism, employing passive immunization tests in rabbits. From his studies he could establish a relationship between the biotypes A, B and C which could be distinguished by the fermentation of mannitol, arabinose and xylose. Biotype A of avian origin were quite distinct from biotype B. Biotype C was serologically

heterogenous and subdivided into two as Ci of pig strains and Cii of rabbits.

Yusef (1935) employed precipitin-absorption techniques for classifying P. multocida strains and grouped them into three serotypes I, II and III. These serotypes were comparable with the serotypes described earlier by Cornelius (1929) except for type IV which was possibly included with serotype II.

Pirosky (1938) extracted polysaccharide antigen from smooth cultures of P. multocida and demonstrated their serologic specificity by means of precipitation test. He suggested that the specificity of the antigens might help in the serological grouping of the species.

Based on conventional agglutination procedure and sugar fermentation of xylose, arabinose, and dulcitol, Rosenbusch and Merchant (1939) divided strains of P. multocida into three principal groups as I, II and III.

Little and Lyon (1943) classified 30 strains of P. multocida into three types as type 1, 2 and 3 by employing slide agglutination and passive immunization tests in mice.

Roberts (1947) demonstrated four serological types of P. septica by mouse cross protection tests and designated them

as type I, II, III and IV. The antiserum used in the test was raised in rabbits by injecting intravenously with broth culture grown at 38°C and killed by heating at 56°C for 1 h, twice weekly and a total of eight injections were given with doses ranging from 0.5 to 5 ml. He further observed that the strains of P. multocida causing haemorrhagic septicemia in cattle and water buffaloes belonged to type I. Apart from the above four types he also noted the prevalence of antigenic variants.

Capsular or 'K' antigen

Carter (1952) identified three groups or types of P. multocida by means of a precipitation test employing saline extracted capsular substances as antigen. The precipitation test which he described required considerable amounts of serum and antigen, and moreover cross reaction were often recorded.

Carter and Annau (1953) analysed the type specific capsular polysaccharide of P. multocida and demonstrated the presence of hyaluronic acid by depolymerisation studies. Bain (1953) also recovered a viscous polysaccharide comprising equal amounts of hexosamine and uronic acid from type II and type IV of Roberts.

Carter (1955) demonstrated indirect haemagglutination test (IHA) and described it as reliable and superior over

precipitation test for serological characterisation of P. multocida. In this method, he adsorbed capsular soluble antigen onto human erythrocytes and the sensitised erythrocytes were used for haemagglutination test with specific antiserum. By this method, he could determine four capsular types of P. multocida viz., A, B, C and D. He also made a comparison of these serotypes with that of Roberts type I, II, III and IV which were reported to be identical with Carter's types B, A, C and D respectively.

Bain (1955) observed that P. multocida producing haemorrhagic septicemia in Asian cattle contained a capsular glycoprotein.

Carter (1957), Carter and Subronto (1973) employed an acriflavine test for quick identification of capsular type D. The capsule of mucoid type A strains was easily disintegrated by staphylococcal hyaluronidase (Carter, 1958). They have suggested that the presence of capsular hyaluronic acid could be the reason for the inagglutinability of many freshly isolated strains of P. multocida.

Briefman and Yaw (1958) separated monosaccharides from the capsular substances of type 1 and 3 of Little and Lyon. They also identified pentose as ribose and hexose as galactose, tentatively. Bain and Knox (1961) recovered a

fraction containing protein with some polysaccharide and lipopolysaccharide from Roberts capsular type I strains.

The infrequent occurrence of type C was observed and hence dropped subsequently (Carter and Bain, 1960 and Carter, 1963). Carter (1961) had further identified nine strains of P. multocida recovered from cases of bovine haemorrhagic septicemia in Central Africa, as members of the new capsular type and designated it as type E.

Namioka and Murata (1961a) compared the efficiency of slide agglutination test with haemagglutination test to detect the capsular types of P. multocida using formalinized antigen and reported that the results of slide agglutination test paralleled with that of haemagglutination test.

Avian, swine and human strains of P. multocida were either A or D when typed for capsular antigen by IHA test (Carter, 1967).

The lipopolysaccharide of P. multocida type A, B and D were extracted from moderate harvests of bacteria by phenol method and were used in IHA test for the detection and measurement of antibody against the specific LPS, by Carter and Rappay (1963). They further reported that the least amounts of LPS to yield maximal haemagglutination were in the range of 2 to 10 ug per millilitre of 1 per cent erythrocytes.

Norrung (1963) had reported the use of slide agglutination test to type capsular antigens and he could group 45 strains into type A (25 strains), type D (nine strains) and the rest were untypable. Perreau and Petit (1963) isolated glycolipid or lipopolysaccharide antigen from type E strains.

Three different antigens viz. beta, alpha complex and gamma specific to capsular substances of P. multocida were identified by Prince and Smith (1966). Chemically the beta antigen was a type specific polysaccharide from iridescent and mucoid variants of P. multocida which was shown to be responsible for indirect haemagglutination reactions while the alpha complex was identified as cell wall associated, heat labile, polysaccharide-protein complex which is immunogenic. The lipopolysaccharide gamma antigen derived from all variants of P. multocida was also found to be associated with the cell wall.

According to Carter (1967) capsular type A strains are associated on the North American continent with fowl cholera, pneumonia in cattle and pigs as well as with primary and secondary infections in a wide variety of animals. Type B strains with haemorrhagic septicemia of cattle and buffaloes in Asia and Australia. Type D strains with primary and secondary infections in a wide range of animal and type E

strains in cattle of Central Africa, with haemorrhagic septicemia. Strains from human origin were mainly attributed to type A and to a less extent to type D. He further reported that in rabbits, the results of capsular typing by indirect haemagglutination grouped, 11 of 27 isolates into type A, three into type D and the remaining were untypable.

Penn and Nagy (1974) identified two major antigenic components of P. multocida types B and E as capsular antigen and endotoxin by subjecting the organisms to saline and phenol water extraction respectively. For characterisation of these antigens, they have employed immunodiffusion, immunoelectrophoresis and tube agglutination tests.

Carter and Rundull (1975) opined that major capsular substance of mucoid variants of type A P. multocida was hyaluronic acid as the capsule production was inhibited in a medium containing hyaluronidase.

Penn and Nagy (1976) have further demonstrated that when aqueous solution of the capsular material of P. multocida precipitated with cetyl pyridinium chloride by addition of polar organic solvents, the precipitate contained polyanions, probably high molecular weight acidic polysaccharide.

Capsular antigen from P. multocida strain P-1059 of Turkey was isolated by Kodama et al. (1981). They purified

the polysaccharide antigen from crude capsular extract obtained by heating at 56°C in 2.5 per cent saline by precipitation with cetyl pyridinium chloride.

Hippe and Schliesser (1981) investigated 71 strains of P. multocida from rabbits with upper respiratory disease, serologically using indirect haemagglutination test and reported that 70 strains belonged to capsular type A and one strain to type D.

Manning (1984) subjected whole cells and lipopolysaccharides of 10 isolates of P. multocida from laboratory rabbits to chemical and serological analysis. He showed that LPS of most of these isolates possessed pyrogenic potency and the mean protein content was about 1.6 per cent. He further reported the LPS (probably capsular polysaccharide) are responsible for the type specificity which formed the basis for the A, B, D and E classification of this organism. They have also detected a good antibody response in rabbits by ELISA and IHA test when the animals were injected with boiled P. multocida culture or lyophilized lipopolysaccharide.

Physico chemical characteristics of capsular material obtained from P. multocida were studied by Lin et al. (1988) and was shown to contain protein 55.5 $\pm$ 12 per cent, carbohydrate 4.7 $\pm$ 1.5 per cent, phospholipids 10.9 $\pm$ 2.5 per

cent some nucleic acids and O-acetyl group. The molecular weight estimated by SDS-PAGE analysis was reported to be 2.5 x 10^5 Da.

Somatic or 'O' antigen

Davies (1956) was the first to extract lipopolysaccharide from Pasteurella pestis, having endotoxic properties and it was the first report about somatic antigen from Pasteurella species.

Namioka and Murata (1961b) studied the somatic antigens of P. multocida employing formalinized, heated, alcohol treated and hydrochloric acid treated cells as antigen. Agglutination and absorption studies conducted with antiserum raised against the 'O' antigens showed that 'O' antigens prepared by hydrochloric acid treatment could alone represent the true 'O' antigen. They could further differentiate this 'O' antigen into common and specific and thus divided cultures belonging to types A and D of carter into two types according to this 'O' antigen.

By the use of agglutination and absorption tests Namioka and Murata (1961c) established six somatic groups and they identified P. multocida serotypes by listing first with number standing for the specific somatic antigen followed by the capital letter standing for the specific capsular antigen.

Ten major somatic groups and number of subgroups within each major 'O' group were later identified by Namioka and Bruner (1963). They further reported that most strains belonging to serotype 5:A were from fowl cholera and the serotypes 1:A, 3:A, 1:D, 2:D and 4:D were isolated from pneumonia of swine or sheep. An additional somatic group (11) was further reported by Namioka and Murata (1964).

Heddleston et al. (1966) were the first to show that the gel diffusion test could be used to identify specific somatic antigens of P. multocida isolated from fowl cholera. They have employed antisera raised in chicken and rabbits against the bacteria for performing the precipitation test.

Heddleston et al. (1972) could identify 16 different 'O' antigens for P. multocida isolates using supernates of cell suspension treated at 100°C for 1 h as antigen and thus 'O' antigens presumably included the 11 'O' groups which were described earlier by Namioka and Murata.

Carter (1972) had reported that treatment of avian strain of P. multocida with hyaluronidase made the organism readily agglutinable and could be used in agglutination test to identify their somatic antigens.

Heddleston et al. (1972) had used heat stable antigens extracted with formol saline in gel diffusion test to study

the somatic antigen content of P. multocida from fowl cholera. They could group the isolates into five somatic types viz. type 1, 3, 4, 5 and 6. Further they have compared the behaviour of heat stable antigens of their groups by gel diffusion and immunoelectrophoresis with partially purified LPS-protein complex (endotoxin).

Buxton and Fraser (1977) summarised the serotypes encountered in animals which were modified from Namioka and Bruner (1963) and Carter (1967). The prevalent serotypes in diverse host species are said to be 7:A, 6:B and 6:E (cattle), 1:D and 4:D (sheep); 1:A, 3:A, 5:A, 1:D, 2:D. 4:D and 10:D (pigs); 3:D (cat); 1:A (mice); 5:A (chicken, turkey and duck); 8:A (chicken) and 9:A (turkeys).

Heat stable somatic antigen of P. multocida of rabbit isolates was tested by gel diffusion test employing antisera raised in chicken and was shown that majority of the isolates were serotype 12 and the rest were serotype 3(Brogden, 1980).

Mushin (1980) reported that he was unable to serotype the rabbit isolates of P. multocida by gel diffusion test because all the strains he tested showed common 'O' antigen. Whereas the avian strains of P. multocida were divided into 23 distinct types based on bacteriocin typing.

Bhasin and Shaw (1980) reported the antigenic complexity of somatic antigens by crossed immunoelectrophoresis and they found in an envelope-cytoplasm preparation of serotype 1 atleast 19 cell envelope and 55 cytoplasmic antigens.

Carter and Chengappa (1981) proposed a capsular: somatic scheme of designation of serotypes based on the Carter:Heddleston system. The H.S. strains under this scheme, were designated as B:2.

Chengappa et al. (1982) performed capsular and somatic serotyping on 79 cultures of P. multocida from rabbits and reported that 74 were capsular type A as determined by staphylococcal hyaluronidase decapsulation test and five were type D by acriflavine flocculation test. In somatic serotyping type 12 was predominant and the types 1, 3, 4 and 11 were less frequent as determined by the gel diffusion test.

Lu et al. (1983) serotyped 111 isolates recovered from healthy and diseased rabbits by Carter and Heddleston typing systems. The results of 48 isolates from healthy rabbits showed that serotype 12:A was 33%, nontypable:A (10%) and nontypable:D (10%) and in diseased rabbits the serotypes were,

12:A (32%), untypable:A (30%) and 3:A (16%) and they concluded that serotype 12:A was predominant.

Brogden and Rimler (1983) studied the serotyping of P. multocida isolates from birds and mammals over two decades using gel diffusion test and reported the serotypes 1 and 3 as most prevalent.

Rimler et al. (1987) studied the serology and immunology of P. multocida isolates from septicaemic fallow deer, and reported that they belonged to capsular type B and somatic types 3 and 4.

The FAO/APHCA workshop on haemorrhagic septicemia (H.S.) held in Sri Lanka in 1979 recommended the use of Namioka:Carter system for designating P. multocida serotypes. Thus the Asian H.S. serotype was designated as 6:B and the African H.S. serotype as 6:E and a non-H.S. producing B strain originally isolated from Australia as 11:B (De Alwis, 1987).

The use of agar gel diffusion test to determine the somatic antigens of P. multocida from divergent species of animals was reported by Wu et al. (1987) and they observed that somatic antigen contents of the bacteria were shared between isolates from different species.

Smith and Phillips (1990) designated the serotype of a strain of P. multocida by stating first the somatic and then the capsular group..

Rimler and Wilson (1994) examined the strains of P. multocida from outbreaks of haemorrhagic septicemia in bison and calves by capsule and somatic serotyping methods and DNA finger printing. They reported that the serotypes of bison, dairy calves and beef calves were B:3,4 and serotype B:2 was from beef calves only.

Characterisation of other antigens of P. multocida

Syuto and Matsumoto (1982) purified a protective antigen by chromatographic methods from the saline extract of crude soluble material of P. multocida of Turkey. This protective antigen contained four protein peaks and a substantial amount of carbohydrate. Upon SDS-PAGE analysis the purified antigen presented three protein bands with molecular weights of 44000, 31000 and 25000 kilo Dalton (kDa) respectively and one carbohydrate band. The carbohydrate band did not correspond to any of the three protein bands. Upon isoelectric focusing gel analysis, the purified antigen showed two bands but the two bands were antigenically identical by isoelectric focusing crossed immunoelectrophoresis.

Heat extract or potassium thiocyanate extract antigen of pasteurella strains was adsorbed on to gluteraldehyde fixed and stabilised sheep erythrocytes (GA-SRBC) or tanned GA-SRBC respectively and used in the indirect haemagglutination test for the detection of Pasteurella antibody (Sawada et al., 1982). The IHA test reaction was capsular group specific with heat extract antigen sensitized GA-SRBC but not KSCN extract antigen sensitized tanned GA-SRBC.

Kajikawa and Matsumoto (1984) purified a protective antigen from a saline extract of type 1 strain of P. multocida of turkey origin, by chromatographic methods and its chemical and immunological characteristics were studied. Chemically the crude saline extract contained protein of about 650 ug/ml and carbohydrate about 152 ug/ml.SDS-PAGE analysis revealed four identical components with the molecular weight of 44000, 31000, 25000 and 20000 Daltons (Da) respectively.

The lipopolysaccharides (LPS) of five isolates of P. multocida from rabbits were characterized by SDS-PAGE, immunoblots and ELISA (Manning et al., 1986). Silver-stained SDS-PAGE profiles consisted of one or two bands with a molecular size of 4.3 k Da. Rabbit antisera to P. multocida whole cells used in Western blots and ELISA of unabsorbed and LPS-absorbed antisera revealed that the LPS of these isolates contained non-serospecific and serospecific antigens.

Potassium thiocyanate extract antigen was prepared from P. multocida 3:A of rabbits and chemically analysed by Lu et al. (1987). The extract contained protein about 2 mg/ml, carbohydrate 462.5 ug/mg of protein, hyaluronic acid 1700 ug/mg of protein DNA,105 ug/mg of protein, RNA 100 ug/mg of protein and the LPS 2.51 mg/mg of protein. The SDS-PAGE analysis showed the protein profile similar to the membrane vesicles of this organism prepared by lithium chloride extraction and the predominant protein is 37000 Da outer membrane protein. The LPS was also analysed by SDS-PAGE and observed that the characteristics were similar to that of rough mutants of Salmonella minnesota.

Rahman et al. (1987) evaluated the use of sensitized RBC with sonicated antigen from P. multocida in single radial hemolysis test to measure serum antibody titre against P. multocida of bovine origin. They claimed that the test was simple, sensitive and reliable for estimation of the antibody level.

Johnson et al. (1991) demonstrated that protein profiles of 14 isolates of P. multocida from animals with haemorrhagic septicemia were relatively homogenous. They observed that H.S. isolates of P. multocida of Carter type B had a major protein band of 32 kDa which was unique to that type whereas type E of Carter had a major protein band of 37

kDa. The other major proteins were of 27, 45 and 47 kDa molecular weights which were shared by all strains studied. Further they showed that different isolates uniformly showed a low molecular weight lipopolysaccharide.

The soluble proteins contained in the sonicated antigen from chicken isolates of P. multocida serotype 1 were demonstrated to be similar by Ireland et al. (1991). They noted major difference between isolates in the position of the major proteins at the 34-38 kDa region detected by SDS-PAGE.

Zimmerman et al. (1992) separated the polypetides from whole cell preparations of P. multocida serotypes A:12 and A:3 of rabbit origin by SDS-PAGE and transferred to nitrocellulose paper for Western blot analysis. The three polypeptides with approximate molecular mass of 28, 30 and 37 kDa were detected when tested against sera from both experimentally and naturally infected rabbits.

Immunogenicity of P. multocida

Eversince the report of Louis Pasteur (1880), the use of live attenuated bacteria as vaccine to prevent fowl cholera, several other vaccines of various kinds of inactivated and live attenuated bacteria have been used to immunize farm animals against Pasteurellosis.

In rabbits, attempts to eliminate or control the disease have included use of antibiotics, establishment of pasteurella free colony, development of genetically resistant strain and vaccines. The development of effective vaccine is most attractive because of the emergence of antibiotic resistant strains, and infeasibility of establishing and maintaining pasteurella free rabbits. vaccines should be useful in preventing transmission between rabbitries and with long duration of immunity.

Hadley (1914) was successful in immunizing rabbits against P. multocida by subcutaneous inoculations of an avirulent culture followed by inoculation of a virulent culture. This procedure was not satisfactory when applied to chicken.

Mack and Records (1916) showed that bacteria could be successfully used to produce resistance against fowl cholera and they reported that there was no apparent difference in the results whether homologous or heterologous strains of P. multocida were used in preparation of bacterins.

Van Es and Martin (1920) reported that bacterin as vaccine could not prevent fowl cholera in a vaccinated flock.

DeKruif et al. (1921) studied the efficiency of

P. lepiseptica as vaccine in rabbits and showed variation in protection in different races of rabbits.

Immunization of cattle against haemorrhagic septicemia was attempted by several workers employing several immunizing agents such as attenuated organisms in glycerin broth (Hardenbergh 1916), aggressin obtained by candle filtered germ free inflammatory exudate (Gochenour, 1924) and 5 per cent saponin vaccine (Delpy, 1938).

Hilbert and Tax (1938) made autogenous bacterins from organisms isolated from ducks affected with fowl cholera by adding phenol at 0.5 per cent level. This bacterin provided solid immunity in ducks even after 2 years of vaccination. They further observed that the same vaccine was able to check fowl cholera in a flock of chicken. Bacterial suspensions in immune serum or toxin obtained by lysing the culture with distilled water at 48°C for 26 h were used by Jacotot (1940) in cattle. The vaccine containing endotoxin was also shown to confer protection in rabbits when challenged with virulent organisms.

Carter (1950) compared the immunity produced in mice by broth bacterins and chicken-embryo vaccine made from type 1 P. multocida and showed that chicken embryo vaccine was superior over the broth bacterins.

Three different immunogens such as formalinized broth vaccine, formalinized agar wash vaccine and heat treated agar wash vaccine were tried for vaccinating the buffalo calves and rabbits (Rau and Govil, 1950). Their results showed that agar wash vaccines when given in two doses with an interval of one month produced a high grade immunity when compared to broth vaccine. It was also observed that the higher the concentration of agar wash vaccine, the stronger the immunity it conferred.

Bolin et al. (1952) prepared bacterin of P. multocida by pooling several isolates from various sources. These pooled bacteria were maintained in broth at 37°C until all organisms were dead and formalin was added to this bacterin as a preservative. The bacterin was shown to be highly immunogenic but the immunity was of short duration.

The advantage of incorporation of oil-adjuvant in preparation of an emulsified haemorrhagic septicemia vaccine was reported by Bain and Jones (1955). Haemorrhagic septicemia vaccine prepared with the addition of mineral oil and lanolin was shown to be highly protective in vaccinated calves, when challenged with virulent strain of P. multocida (Iyer et al., 1955).

strong specific antibody responses but the degree of passive protection of mice by this antisera did not correlate with the amount of antibody.

Cameron and Smith (1970) immunized sheep with polyvalent vaccine which resulted in sound immunity to challenge with P. multocida strain A:14 and an increase in resistance to strain D:1. This vaccine also induced very good immunity in mice.

A polyvalent inactivated vaccine against P. multocida was effective in reducing the mortality in young and adult rabbits when inoculated subcutaneously (Galassi and Giulani, 1971). Rabbits vaccinated with pig pasteurella vaccine by intramuscular route, conferred about 57-90 per cent protection (Tacu, 1971).

Heddleston and Rebers (1972) reported that fowl cholera bacterins prepared with liver and blood from a turkey that died of acute fowl cholera induced immunity in turkeys against a different immunogenic types of P. multocida but not by the bacterins prepared from bacteria grown on laboratory media.

Penn and Nagy (1976) observed that a saline extract of type B capsular antigen in the presence of aluminium hydroxide gel adjuvant, was poorly immunogenic in rabbit but in cattle,

the same vaccine elicited a dose-dependent serological response as demonstrated by the mouse passive protection test.

An oil-adjuvant vaccine prepared from the pasteurella isolates, was shown to confer a high level of immunity in rabbits upto a period of one year under field conditions by Zaher et al. (1976).

Mittal et al. (1977) prepared a multiple emulsion vaccine by secondary emulsification of the oil-adjuvant vaccine with Tween 80 and noted that both the oil-adjuvant vaccine and multiple emulsion vaccine were equally immunogenic as assessed by direct challenge test and passive mouse protection test.

Potassium thiocyanate extract of P. multocida was used as immunogen by Gaunt et al. (1977) and showed that vaccinated chicken resisted infection on challenge with homologous as well as heterologous strains. Further they reported that KSCN extract obtained from P. multocida serotype 1 and 3 of avian origin presented antigenically identical components by gel diffusion tests.

Ribosomal fraction from P. multocida was shown to have intense protective antigenicity in mice and chickens when compared to lipopolysaccharide (endotoxin) and other bacterial cell fraction (Baba, 1977). Further he demonstrated that the

Protective effect of bacterins prepared by formalized suspension of P. multocida with or without adjuvants was studied in rabbits by Okerman and Spanoghe (1981). It was found that bacterins without adjuvant and bacterins combined with the adjuvant tested, provoked immunity against aerosol infection with homologous strain but not with heterologous strains.

Kodama et al. (1981) reported that the crude capsular antigen extracted from bacterial cells grown on membranes by heating at 56°C in a 2.5 per cent NaCl solution was immunogenic in young adult turkeys. The crude capsular antigen was shown not to loose the immunogenic property even after treatment with heat (100°C for 5 min), chloroform or trypsin but lost its immunogenicity completely on acid hydrolysis.

Baljer et al. (1982) compared the efficacy of vaccines prepared from different strains of P. multocida by formalin treatment and heat inactivation. The heat inactivated vaccine was also tried by different routes such as oral, intranasal and subcutaneous. The results obtained by them showed that the efficacy of vaccines were independent of the inactivation procedure, and differences were noted when challenged with heterologous strains. The routes of

administration of vaccine did not influence the immune response.

The use of a commercial formalinized, aluminium hydroxide adsorbed vaccine against P. multocida and P. hemolytica was reported by Bennewitz et al. (1983) to control an outbreak of pneumonia in pigs by vaccinating unweaned piglets with two doses of vaccine at 2-3 days interval.

Solano et al. (1983) used the ELISA and IHA test to measure humoral antibody responses of chickens against P. multocida. They compared the efficacy of oil-emulsified formalin killed bacteria and live P. multocida antigens using ELISA and IHA test and reported that both methods were efficient to detect antibody titre, though ELISA was atleast twice as sensitive as IHA. Antibodies detected against the two antigens measured by ELISA or IHA correlated significantly with protection against P. multocida challenge.

Philips and Rimler (1984) showed that ribosomal vaccines prepared from non-capsulated P. multocida offered protection against fowl cholera in chickens. They also observed that ribosomes-lipopolysaccharide vaccines produced serological responses to lipopolysaccharide which were atleast

five fold greater than those produced by Methylated Bovine Albumin lipopolysaccharide vaccine.

Kajikawa and Matsumoto (1984) purified protective antigen from a saline extract of P. multocida strains by chromatographic methods and its chemical and immunological characteristics were studied. Procedures such as gel diffusion, Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophoresis (SDS-PAGE) and DEAE cellulose affinity chromatography were used for purification and fractionation of protective antigens. The results obtained by them indicated that protective antigens obtained from different strains possessed similar, physico-chemical properties but they were immunologically distinct, from each other.

Potassium thiocyanate extract antigen from virulent P. multocida was shown to be protective in rabbit when challenged with virulent organisms except for that it could not prevent otitis media (Ringler et al., 1985). The vaccinated rabbits produced serum IgG and nasal mucosal IgA which were detected by ELISA.

Kim et al. (1986) compared the immunogenicity of antigens of P. multocida prepared by various inactivation methods using heat, formalin, phenol, sodium azide and merthiolate and reported that formalin treated antigen was

most immunogenic in mice. These antigens were also shown to confer cross-protection when mice were challenged with heterologous strains but offered comparatively low degree of protection.

Lu et al. (1987) described the use of KSCN extract of a virulent P. multocida 3:A rabbit isolate as a vaccine. They observed that intranasal route was superior over intramuscular route as there was a significant reduction in the severity of pneumonia produced and number of virulent P. multocida colonised in nasal cavities and lungs on challenge exposure with homologous strain.

Lu et al. (1988) identified several immunogenic outer membrane protein fractions of P. multocida isolates from rabbits as 27 kDa, 37.5 kDa, 49.5 kDa, 58.7 kDa and 64.4 kDa are the major one out of 18 proteins, by radio immuno-precipitation procedure and Western blot analysis. The outer membrane proteins were shown to be immunogenic and were able to detect antibody in lung lavages of rabbits immunized with the KSCN extract of P. multocida.

To demonstrate the antigenic similarity between endotoxins of Escherichia coli and P. multocida, Al-Lebban et al. (1988) vaccinated rabbits with boiled cells of P. multocida and a cross reactive uridine diphosphogalactose

epimerases deficient mutant of E. coli J5. Cross reactive IgG and IgM titres to P. multocida were demonstrated when rabbits were vaccinated with J5. They also observed that heat stable antigen from P. multocida were protective against homologous challenge while E. coli J5 mutant provided partial protection.

A combined vaccine for control of snuffles in rabbits was tried by Cho et al. (1989) incorporating formalin inactivated P. multocida and Bordetella bronchiseptica strains and showed that the vaccine produced a good antibody response in rabbits and even afforded protection against experimental challenge. With a booster dose of vaccine the protective level of antibodies persisted in vaccinated rabbits for five months.

Giridhar et al. (1990) tested the efficacy of subcutaneous vaccination of calves with different doses (3, 5 and 10 ml) of alum precipitated P. multocida vaccine by passive haemagglutination and complement fixation test. They observed that all the three doses of vaccine could confer satisfactory immunity. CFT using killed whole cell antigen was better than PHA using capsular antigen for measuring protective antibody titres.

While assessing the duration of immunity conferred by the alum precipitated haemorrhagic septicemia vaccine,

Venkatesh et al. (1991) observed that animals received one or more booster vaccination were solidly immune compared to animals received the vaccine for the first time. They have shown a positive correlation between the passive mouse protection test and CFT in the assay of immune response.

Piglets borne from gilts which were vaccinated with purified non-toxic derivative of formaldehyde treated native P. multocida toxin before farrowing were shown to be immune from atrophic rhinitis when experimentally infected with toxigenic P. multocida and B. bronchiseptica (Nielsen et al., 1991).

Monoclonal antibodies directed against the 37.5 kDa outer membrane protein was shown to be protective in mice and rabbit when antibodies are passively transferred (Lu et al., 1991). By passive transfer studies they observed that rabbit and mice were protected against homologous and heterologous challenges with P. multocida strains.

Azam et al. (1991) compared the immunogenicity of sonicated P. multocida vaccine with or without adjuvants and formalinized bacteria in rabbits by estimating the antibody using indirect haemagglutination test. The range of antibody titres observed in sonicated antigen with or without adjuvant was 8-64 while for formalin killed bacteria it was 4-16.

Rabbits inoculated with the above vaccine uniformly showed a dose-dependent immune response.

Live vaccines of P. multocida

A stable avirulent high temperature mutant strain of P. multocida was used as live vaccine in turkey by Maheswaran et al. (1973). When the vaccine was administered in drinking water or endotracheally it induced a high degree of protection against virulent homologous and heterologous strains when challenged by contact exposure or nasal cleft method. The results obtained by them suggested that the live vaccine induced a localized protection in the respiratory system and did not provide a systemic protection.

Wei and Carter (1978) mutagenised a wild strain of P. multocida into an avirulent streptomycin-dependent strain by treating the organisms with N-methyl-N-nitro-N-nitroso-quanidine. When this mutant strain was used as vaccine in mice and rabbits they were protected against homologous challenge with the wild strain.

The use of streptomycin-dependent vaccine strain of P. multocida as vaccine was further studied by Lu and Pakes (1981) and reported that vaccination protected rabbits against homologous challenge. They further observed that the vaccine prevented colonisation of the virulent challenge organisms in

lungs, liver, spleen, genital tract and blood but not in the nasal cavities.

Attenuation of the P. multocida vaccine strain was attempted by treating with 2, 8 (3,6) diamino-10-methyl acridinium chloride and/or 2, 8 (3,6) diamino acridine (Kucera et al., 1981). They claimed that the chemically altered strain afforded protection in mice, calves, swine and sheep against challenge exposure with virulent P. multocida organism.

A virulent attenuated P. multocida serotype 6:B was tried as a vaccine in mice and pigs by Chen et al. (1984) and it was shown to induce a high level of immunity against virulent strain of the homologous serotype but only low or no immunity against strains of heterologous serotypes. A similar work has been reported by Derieux (1984) where in a virulent P. multocida gave protection in chicken upto 80 weeks.

Kadel et al. (1985) conducted a field trial evaluation of streptomycin-dependent P. multocida and P. hemolytica vaccine for prevention of bovine pneumonia in calves. Vaccinal efficacy was defined in terms of great body weight gain, less severe clinical signs of pneumonia and lower death rates as compared with the same factors in non-vaccinated

calves. They have also observed that there was an economic advantage in administering a booster dose of the vaccine.

Percy et al. (1985) reported that rabbits immunized with streptomycin dependent mutant of P. multocida serotype 12:A, were protected against homologous and heterologous (3:A) challenges. The vaccinated rabbits challenged with homologous strain showed a more rapid nasal clearance of the organism than the vaccinated group challenged with the heterologous strain.

The safety and efficacy of a freeze-dried attenuated vaccine prepared from avian strain of P. multocida in rabbits was studied by Long et al. (1986). The vaccine was found to be effective in adult rabbits when subcutaneously injected with 10-40 hundred million bacteria, while the young rabbits were protected at comparatively lower dose, 5-20 hundred million bacteria.

Digiacomo et al. (1987) evaluated the safety and efficacy of a streptomycin dependent live P. multocida (12:A) vaccine in rabbits. This vaccine strain could colonise in rabbit nares and was genetically stable in vivo. The vaccinated rabbits could withstand the challenge with a homologous pasteurella serotype and not against heterologous serotype.

A live vaccine of non-replicable P. multocida FS_3 cells prepared by psoralen treatment and long wave U-V irradiation was shown to protect mice against challenge with homologous FS_3 and heterologous (T_3) strains by 100 per cent and 33 per cent respectively (Chai et al., 1988).

Sokolov (1988) reported that inactivated P. multocida culture which contained 2 x 10^{10} cells/ml by a final concentration of 0.1 per cent ethylene diamine dimer at 37°C for 8 h could be used as a vaccine. The vaccine was found to be more immunogenic than formaldehyde inactivated vaccine or emulsified vaccine which were used for cattle and sheep immunization.

Myint and Carter (1989) vaccinated young cattle and buffaloes subcutaneously and intradermally with a live freeze dried vaccine containing P. multocida serotype B:3,4 and showed protection on challenging with serotype B:2 even 13 months after the vaccination.

The efficacy of lyophilized live streptomycin-dependent P. multocida vaccine against experimentally induced pneumonia, pasteurellosis was assessed by Chengappa et al. (1989) and reported that the control calves had significantly higher clinical scores and more severe gross lesions than the

vaccinated calves although the vaccinated calves did not show a significant increase in the immunoglobulin M and G titres.

Experimental infection and immunity in mice

Mice were shown to be susceptible to P. multocida infection and are often used as experimental animals to determine the virulence (Roberts, 1947, Carter and Bigland, 1953, Collins, 1973 and Okerman et al., 1979).

Mouse models of P. multocida infection as a method to test the efficacy of vaccines have been recommended by Murata et al. (1964) and Heddleston and Rebers (1969).

Carter (1967) was of opinion that capsulated strains recovered from acute or moderately acute natural infections were only generally virulent. Collins (1973) used mice for testing pathogenicity of P. multocida and reported that the increase in the number of P. multocida was the cause of death in mice when experimentally inoculated.

Okerman et al. (1979) estimated LD_{50} of 20 strains of P. multocida by infecting mice with varying dose from 1×10^6 to 3×10^6 bacteria and observed the animals for 7 days. The results obtained by them showed marked difference in LD_{50} for different strains and the estimated LD_{50} ranged between 3×10^3 and 1×10^6 bacteria.

Mukkur (1979) determined the LD_{50} of P. multocida of bovine origin, in mice as $5.0 \pm 2.1 \times 10^4$ cfu by intranasal inoculation and 5.0 ± 2.8 cfu by intraperitoneal inoculation. He has also studied the immunogenicity of KSCN extract of P. multocida type A bovine strain by immunizing mice intraperitoneally with 1 ml extract containing either 31.25, 62.25 and 125 ug protein. The vaccinated mice were challenged 4 weeks post-immunization intraperitoneally with 10, 10^2 or 10^3 cfu and intranasally with 10^6 cfu. The result of the experiment showed that the antigen imparted complete protection in mice vaccinated with KSCN extract containing 62.25 ug protein when challenged with 1.6×10^3 cfu of homologous strain.

The virulence of fully encapsulated form of P. multocida of bovine origin could be maintained by passaging the organism in mice at four weeks interval (Mukkur, 1979, Mukkur and Pyliotis, 1981).

The KSCN extract of P. multocida was shown to protect mice from an intranasal challenge with upto 300 LD_{50} of virulent culture (Smith et al., 1981). The results obtained by them indicated that the protection was specific and superior over immunity produced by formalin killed bacteria.

The immunogenicity and cross protection of KSCN extract from type A P. multocida strain were evaluated in mice by Ryu and Kaeberle (1986). Their antigen induced protection against challenge with virulent homologous but not consistently with heterologous bacteria. Physico-chemical characterization of this antigen indicated that the different strains were with specific antigen along with common shared antigens.

Wijewardana and Sutherland (1990) used a challenge dose of P. multocida strain W-674 of bovine origin in mouse and suggested 3×10^8 cfu as optimum as it approximately contained 10 LD_{50} for mice.

The suitability of using mice to study the pathogenicity to haemorrhagic septicemia was assessed by Ramdani et al. (1990) and reported that as few as 20 cfu could produce an overwhelming mice septicemia in less than 30 h.

Lethality of 22 isolates of P. multocida from rabbits with or without rhinitis were tested for their pathogenicity in six weeks old female mice inoculated with 0.2 ml (10^8 organisms) of 18 h nutrient broth culture (Kawamoto et al., 1990). Out of the 22 isolates, eight isolates killed mice in two to seven days and the rest failed to do so, indicating that isolates varied in their virulence in mice.

Materials and Methods

MATERIALS AND METHODS

Pasteurella multocida strains and its maintenance

Three strains of *P. multocida* were employed in the present study. Out of these three, two strains (R_9S and $R_{23}S$) were originally isolated from the trachea of rabbits which died of respiratory infection and the other one was a vaccine strain (P_{52}) of bovine origin. The rabbit isolates were the one maintained in the Department of Microbiology and the vaccine strain was obtained from Veterinary Biological Institute, Palode. The isolates were maintained in paraffin sealed Trypticase Soy Agar slants containing 5 per cent defibrinated sheep blood (TSA) at 4°C, subcultured once in four weeks and passaged through mice once in four weeks to maintain it in the fully encapsulated virulent form.

Determination of LD_{50}

The LD_{50} of the three *P. multocida* strains were determined in mice by the intraperitoneal route of inoculation.

Phosphate buffer saline (PBS): pH 7.2

Stock solutions

Solution A:

Sodium dihydrogen phosphate ($2H_2O$)	-	1.78 g
Distilled water	-	1000 ml

Solution B:

di Sodium hydrogen phosphate ($2H_2O$)	-	1.56 g
Distilled water	-	1000 ml

Solution C:

Sodium chloride	-	0.58 g
Distilled water	-	1000 ml

Working solution

Solution A	-	28 ml
Solution B	-	72 ml
Solution C	-	100 ml

Bacterial suspension

P. multocida was grown on TSA plates for 18 h and washed twice with 0.01 M PBS (pH 7.2). Then cells were suspended in the same buffer to contain $3x10^8$ bacteria/ml.

Ten fold dilutions were made which ranged between $3x10^3$ to $3x10^7$ bacteria/ml from the above suspension. Four mice were used for each dilution, and 0.1 ml was inoculated into each mouse. Mortality were recorded for one week post-inoculation. Post-mortem examinations were performed on the dead animals and P. multocida was isolated and identified from lungs, heart and spleen.

Preparation of antigens

The procedure followed for the preparation of the antigens from different strains were same throughout the experiment.

Heat inactivated crude extract antigen

The antigen was prepared by certain modification of the procedure described by Syuto and Matsumoto (1982).

The bacterial colonies grown, for 18 h at 37°C were collected in 2.5% sodium chloride solution, and the population was adjusted to have a uniform suspension of $3x10^9$ bacteria/ml.

The bacterial suspension was then agitated for 1 h at 56°C in a shaker water bath. Following the heat treatment, the suspension was centrifuged at 17000 x g for 20 min. and the supernatant was dialyzed for 48 h (Dialysis sacks, 250-7 U) in 0.85% sodium chloride solution. The sterility of the dialyzate was tested by plating 0.2 ml on TSA and sterile preparations were stored at -60°C until further use.

Potassium thiocyanate (KSCN) extract antigen

KSCN solution: (pH 6.3)

KSCN	-	4.86 g
Sodium chloride	-	4.68 g
Distilled water	-	100 ml

The antigen extraction procedure described by Lu et al. (1987) was followed with modifications.

Bacterial colonies grown for 18 h at 37°C on TSA plates were collected in 0.01 M PBS (pH 7.2) and washed twice in the same buffer. The washed cells were suspended in KSCN solution to contain $3x10^9$ bacteria per ml. The bacterial suspension was then incubated in a rotary shaker at 37°C for 5 h. Afterwards cells were removed by centrifugation at 27,000 x g for 30 min. The supernatant was dialyzed extensively against 0.01 M PBS (pH 7.2). Sterility of the dialyzate was tested by plating 0.2 ml on TSA and was then stored at -60°C until further use.

Sonicated antigen

A modified method of the procedure described by Ireland et al. (1991) was followed for the preparation of sonicated antigen.

The colonies of P. multocida grown on TSA for 18 h at 37°C, were harvested in PBS and washed twice in the same buffer. The washed cells were resuspended in PBS to contain $3x10^9$ bacteria/ml. Bacterial cell suspension held on an ice bath was then disrupted by sonication at 250 V for a total of 5 min. with 10 x 30 sec bursts in a sonicator fitted with a

12 mm diameter titanium probe (SONITRON, IMECO Ultrasonics, Bombay).

The sonication was interrupted for 30 sec between each burst for cooling. The sonicate was centrifuged at 8000 x g for 30 min. The supernatant was collected and tested for its sterility by plating 0.2 ml on TSA plates and then stored at -60°C until further use.

Characterisation of antigens

Protein estimation

The procedure described by Bradford (1976) for the quantitation of protein utilizing the principle of protein-dye binding, was employed for estimation of protein in different antigenic preparations.

Standard protein

Bovine serum albumin was prepared in 0.15 M sodium chloride solution to contain 500 ug to 7000 ug per millilitre of solution.

Protein reagent

Hundred microgram of Coomassie brilliant blue G-250 was dissolved in 50 ml of ethanol. To this solution 100 ml

85% (wt/vol) phosphoric acid was added. The resulting solution was diluted to a final volume of one litre.

Protein assay

Standard bovine serum albumin solution in 0.1 ml quantity was pipetted into 12 x 100 mm test tube and 5 ml of protein reagent was added to it. After thorough mixing the color absorbance at 595 nm was measured in Spectronic-20 (Milton Roy Company) after 2 min but before one hour against a reagent blank prepared from 0.1 ml of saline and 5 ml of protein reagent. A standard curve was plotted with the amount of protein against the corresponding absorbance.

The amount of protein in the antigen was determined by referring the standard curve constructed as above. The estimation was done for three times and the average was taken.

Carbohydrate estimation

Carbohydrate content of the antigens was determined by using phenol-sulfuric acid method with glucose as standard (Dubois et al., 1956).

Two millilitre of sugar solution containing 20 to 260 ug of glucose were pipetted into 12 test tubes and 0.05 ml of 80% phenol was added to each tube. Then 5 ml of concentrated sulfuric acid was added rapidly in order to obtain good

mixing. The tubes were allowed to stand for 10 min., shaken well and then placed in water bath at 30°C for 10 to 20 min. Color absorbance was measured at 490 nm in Spectronic-20 (Milton Roy Company) using blanks prepared by substituting distilled water for sugar solution. A standard curve was plotted with the amount of sugar against the corresponding absorbance.

The amount of sugar content in the antigen was determined by reference to the standard curve prepared as above. The estimation was done for three times and the average was taken.

Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed by following the method of Laemmli (1970) for analysing the protein profiles of the different antigenic preparations.

Preparation of Gel for SDS-PAGE

Solution A:

Acrylamide .	-	30.0 g
Bis-acrylamide	-	0.8 g
Distilled water (made upto)	-	100 ml

The solution was filtered through cotton and stored in amber colored bottle at 4°C.

Solution B:

Tris-Hcl	-	1.97 g
Sodium dodecyl sulphate	-	1.00 g
Distilled water	-	100 ml

pH was adjusted to 6.8 with Tris solution and then filtered through whatman No.1 filter paper and stored at 4°C.

Solution C:

Tris-Hcl	-	5.91 g
Sodium dodecyl sulphate	-	1.00 g
Distilled water	-	100 ml

pH was adjusted to 8.8 with Tris solution and then filtered through whatman No.1 filter paper and stored at 4°C.

Solution D:

Acrylamide	-	10 g
Bis-acrylamide	-	2.5 g
Distilled water (made upto)	-	100 ml

Prepared similar to solution A.

Composition of stacking gel (4%)

Solution D	-	1.5 ml
Solution B	-	1.5 ml
Total		3.0 ml

Two drops of tetra methyl ethylene diamine (TEMED) and 2 drops of 10% ammonium persulphate were added to the 3 ml solution to induce polymerization and was used as stacking gel in vertical slab gel preparation.

Composition of resolving gel (10%)

Solution A	-	6.3 ml
Solution C	-	12.7 ml
Total		19.0 ml

Polymerization was induced by adding 0.1 ml of TEMED and 0.1 ml of 10% ammonium persulphate and was used as resolving gel in the vertical slab gel preparation.

Composition of resolving gel (12.5%)

Solution A	-	7.9 ml
Solution C	-	11.1 ml
Total		19.0 ml

Prepared similar to resolving gel 10%.

Tris-Glycine (pH 8.3)

Tris	-	3.03 g
Glycine	-	14.41 g
Sodium dodecyl sulphate	-	10.00 g
Distilled water	-	1000 ml

The pH was adjusted either with Tris or Glycine solution only. Buffer was filtered through whatman No.1 filter paper and stored at room temperature.

Sample buffer (pH 6.8)

Solution B	-	85 ml
Sodium dodecyl sulphate	-	2 g
Glycerol	-	10 ml
2-mercaptoethanol	-	5 ml
Bromophenol blue	-	1 mg
Total		100 ml

Preparation of sample

Equal volume of sample buffer and antigen were mixed and kept for a minute in a boiling water, then cooled and stored at 4°C.

Staining solution

Coomassie brilliant blue R	-	1.25 g
Methanol	-	227 ml
Glacial acetic acid	-	46 ml
Distilled water (made upto)	-	500 ml

The staining solution was filtered through Whatman No.1 filter paper and stored at room temperature.

Destaining solution

Methanol	-	50 ml
Acetic acid	-	75 ml
Distilled water (made upto)	-	1000 ml

Procedure for SDS-PAGE

Vertical anionic electrophoresis was carried out in 1.5 mm thick poly-acrylamide gel using a vertical slab gel electrophoretic apparatus (Manufactured by Genei & Co., Bangalore). A 10 per cent acrylamide resolving gel and 4 per cent stacking gel were employed for heat inactivated crude extract and KSCN extract antigens while 12.5 per cent resolving gel and 4 per cent stacking gel were used for sonicated antigenic preparation. In both cases, Tris-Glycine was used as electrode buffer.

The sample well were individually charged with 50 µl of different antigenic preparations. Bovine serum albumin was used as a marker.

SDS-PAGE was carried at 25°C with initial current maintained at 15 mA till the samples showed uniform movement to the top of resolving gel. At this stage, the current was increased to 25 mA and the electrophoretic run continued till the tracking Bromphenol blue dye reached the bottom of the gel.

Then, the gel was removed, fixed and stained as per the procedure described by Weber et al. (1972), in the staining solution for 3 h at room temperature. After staining, the gel was decolorized in the destaining solution by periodical changing of the solution until the band become clear.

Interpretation

The protein profiles of antigens obtained by SDS-PAGE, were compared between them after the staining procedures, by calculating the Relative mobility (R_f) value (Weber, 1972 and Chrambach et al., 1976).

$$R_f \text{ value} = \frac{\text{distrace migrated by protein} \times \text{length of gel before staining}}{\text{distance migrated tracking dye} \times \text{length of gel after staining}}$$

Antiserum

P. multocida free healthy rabbits, aged about 3-4 months were used for raising antiserum against the different antigens prepared from P. multocida strains.

Each antigenic preparation was emulsified with equal quantity of Freund's Complete Adjuvant and 2 ml of the emulsified antigen was injected subcutaneously into two rabbits. One millilitre of the plain antigenic preparation was subsequently inoculated intravenously to these rabbits, at five days intervals. Meanwhile the rabbits were monitored for the presence of specific antibodies at periodic intervals. When sufficient level of antibody was found in the serum, 20 ml blood was collected from each rabbit through cardiac puncture, separated the serum and stored at -60°C until used.

Serological tests

Agar coated glass slides/plates

Clean microscopic glass slides/plates were dipped in 1 per cent melted agarose in distilled water and dried in air by keeping the slides horizontally over glass rods. Dried slides were stored at room temperature until used.

Agarose preparation

Agarose	- 0.9 g
Sodium chloride	- 0.85 g
Phenol	- 0.2 ml
Distilled water	- 100 ml

Staining solution

Amido black 10 B	- 1 g
Sodium chloride	- 8.5 g
Distilled water	- 1000 ml

Decolorizer I:

Methanol	- 40 V
Acetic acid	- 10 V
Distilled water	- 10 V

Decolorizer II:

Absolute alcohol	- 35 V
Acetic acid	- 5 V
Distilled water	- 10 V

Agar Gel Precipitation test

Agar Gel Precipitation Test (AGPT) described by Heddleston et al. (1972) was employed with slight modifications.

The melted agarose was pipetted on to glass slides/ plates and wells were cut with a diameter of 4 mm and 6 mm

apart. The peripheral wells were charged with different antigens along with a blank and the central well with the antiserum against a particular antigen. The agar gel plates/ slides were incubated at 37°C in a humid chamber and the results of AGPT were first recorded after 24 h of incubation. The plates/slides were observed for period of 72 h before declaring the test as negative.

Then the slides/plates were washed by soaking in two changes of normal saline for 24 h and then with distilled water for a further 24 h to remove unreacted excess proteins. The slides/plates were dried by keeping at 37°C and then stained with amido black stain for 15 min. The stained plates later decolorized in decoloriser I and II for 20 min. each. The dried slides/plates were made permanent by mounting with DPX.

Immunoelectrophoresis

Tris-barbital buffer (pH 8.6)

Barbitone sodium	- 9.9 g
Tris (hydroxy methyl) amino methane	- 17.7 g
Sodium azide	- 0.3 g
Distilled water	- 2000 ml

pH was adjusted with IN hydrochloric acid.

Agarose preparation

Agarose	- 0.9 g
Phenol	- 0.2 ml
Tris-barbital buffer	- 100 ml

Melted 0.9 per cent agarose in Tris-barbital buffer was pipetted onto agar coated glass plates to get a thickness of 3 mm uniformly by keeping on a levelled surface. Allowed the agar to harden for 30 min at 4°C. Multiple wells were cut with a diameter of 4 mm and 6 mm apart, at one third distance of glass plate from cathode end and troughs were also cut between wells. After removing the agar, the wells were filled with the different antigenic preparations. A drop of bromophenol blue dye was added to the side of the well as indicator.

The glass plate was then placed in the electrophoresis chamber in such a way that wells were nearer to the cathode. Contact between plate and the buffer was effected by filter paper wicks, one on each end of the plate, so that each covered about ½ cm of the agarose on the plate. A current at the rate of 3 mA per 2.5 x 7.5 cm glass slide was given and the electrophoresis was continued till the indicator dye reached 1 cm away from the anode end of the plate. Then the plate was taken out, the agarose in the troughs were removed carefully and filled with antisera against a particular

antigen. The plates were then kept for 24-48 h at room temperature, in the electrophoretic chamber itself.

Then the glass plates were washed, stained, decolorised and mounted as it was done for AGPT tests.

Indirect haemagglutination test

The test was carried out according to the method described by Sawada et al. (1982).

Alsever's solution

Sodium chloride	-	4.2 g
Trisodium citrate	-	8.0 g
Citric acid	-	0.55 g
Glucose	-	20.5 g
Distilled water	-	1000 ml

Each ingredient in the above order was added to 500 ml of distilled water in a graduated cylinder and stirred until the chemicals dissolved completely. The volume was then made upto one litre with distilled water and finally steamed for 10 min. The solution was freshly prepared before use.

Fixation of sheep red blood cell (SRBC)

One part of fresh sheep blood was collected in five parts of Alsever's solution and the erythrocytes were washed

by centrifugation (650 x g for 20 min.) six times in 0.85 per cent sodium chloride solution. After the last wash, the packed cells were suspended in PBS (pH 7.2) to yield a 10 per cent suspension (vol/vol) and chilled to 4°C in an ice bath. A 25 per cent solution of gluteraldehyde was diluted to 1 per cent (vol/vol) with PBS and chilled to 4°C. The 10 per cent suspension of washed SRBC was mixed with an equal volume of the 1 per cent solution of gluteraldehyde, and the mixture was incubated at 4°C for 30 min. with gentle stirring. The mixture was then centrifuged at 650 x g for 10 min. at 25°C. The pelleted fixed cells were washed three times with PBS containing 0.1 per cent sodium azide, suspension. The gluteraldehyde fixed sheep RBC (GA-SRBC) was stored at 4°C.

Tanned - GA - SRBC

The GA-SRBC to be sensitized with protein antigen of KSCN extract were only treated with tannic acid. The 10 per cent suspension of GA-SRBC was mixed with an equal volume of PBS containing 0.005 per cent tannic acid (wt/vol), and the mixture was incubated at 37°C for 30 min. with occasional shaking. The tanned GA-SRBC (T-GA-SRBC) were pelleted by centrifugation and washed three times in PBS. After the last wash, the T-GA-SRBC were suspected in PBS to yield a 10 per cent suspension.

Sensitization of GA-SRBC with heat inactivated crude extract or sonicated antigens

The 10 per cent suspension of GA-SRBC was mixed with an equal volume of a two fold diluted antigens. The mixture was incubated at 37°C for 1 h with occasional shaking. The sensitized cells were washed three times in PBS containing 0.25 per cent bovine serum albumin (BSA-PBS) and 0.1 per cent sodium azide to yield a 0.5 per cent suspension (vol/vol).

Sensitization of T-GA-SRBC with KSCN extract antigen

The 10 per cent suspension of T-GA-SRBC was mixed with an equal volume of a two-fold dilution of KSCN extracted antigen. The mixture was incubated at 37°C for 30 min. with occasional shaking. The sensitized cells were washed three times with PBS containing 0.25 per cent BSA and 0.1 per cent sodium azide by centrifugation and resuspended in BSA-PBS to yield a 0.5 per cent suspension (vol/vol).

Procedure

IHA was carried out in a 'U' bottomed micro titre plates. Serial two fold dilutions of antiserum in BSA-PBS were taken in 25 ul quantity in 24 wells. 25 ul of the sensitized SRBC was added to each well and the plates were shaken and allowed to stand for 2 h at 25°C before SRBC

settling patterns were read. The IHA titre was expressed as the reciprocal of the highest dilution of serum showing a definite positive pattern (flat sediment), compared to the pattern in negative control well (smooth dot in the centre of the well).

Two control wells were set in the experiment, one as positive with 25 ul of sensitized GA-SRBC/T-GA-SRBC plus 25 ul of test serum and the other as negative with 25 ul of sensitized SRBC plus 25 ul of BSA-PBS.

Potency test in mice

The test was based on the method of Penn and Nagy (1974) with modifications.

Antigenic preparations viz., heat inactivated crude extract, KSCN extract, sonicated antigen and Freund's complete adjuvant emulsified sonicated antigen prepared from three P. multocida strains under study were individually used as immunogen in the potency tests. Swiss albino mice of 4-6 weeks old were immunized by administering two doses of antigen administered at 0 and 14th day. 0.1 ml of each antigenic preparation was injected intraperitoneally in 18 mice except the adjuvant emulsified sonicated antigen which was injected subcutaneously in 0.2 ml quantity.

On 21st day of post-vaccination, the mice were randomly grouped into three of six mice each and were challenged individually with three strains of P. multocida. The challenge dose was 100 LD_{50} of fully encapsulated virulent form of each strain.

Thirty mice which were divided into three groups of 10 each, were intraperitoneally injected individually with 100 LD_{50} of P. multocida formed the control experiment. The mice were observed for seven days after the challenge exposure for death/survived.

Results

RESULTS

The three strains of P. multocida viz., R_9S, $R_{23}S$ and P-52were maintained in its fully encapsulated virulent form by repeated passage in mice and subculturing on TSA containing 5% defibrinated sheep blood.

Determination of LD_{50}

The LD_{50} of R_9S, $R_{23}S$ and P-52strains in mice were determined to be $3x10^4$, $3x10^3$ and $3x10^5$ bacteria respectively.

Preparation of antigens

The antigens namely heat inactivated crude extract, KSCN extract and sonicated antigen were prepared from the three strains of P. multocida were found to be sterile and retained the antigenicity by storing them at -60°C through out the period of study.

Characterization of antigens

Protein content

Protein content of the antigens prepared from different strains of P. multocida could be quantitated by the Bradford method.

The average protein content in the heat inactivated crude extract, KSCN extract and sonicated antigens of R_9S were 1.2 mg/ml, 2.3 mg/ml and 2.8 mg/ml respectively, while the concentrations were 1.0 mg/ml, 2.3 mg/ml and 2.7 mg/ml for antigens from $R_{23}S$ and the respective values were 0.9 mg/ml, 2.1 mg/ml and 2.5 mg/ml for antigens obtained from strain P-52 (Table I).

Carbohydrate content

Carbohydrate content in the antigens prepared from different strains of P. multocida were estimated by phenol sulfuric acid method.

The average carbohydrate content in different antigens viz., heat inactivated crude extract, KSCN extract and sonicated were 650 ug, 920 ug and 970 ug per millilitre of antigen prepared from R_9S strain respectively, while the values were 610 ug, 950 ug and 960 ug per millilitre of antigen obtained from the strain $R_{23}S$ and it were 590 ug, 910 ug and 930 ug per millilitre of P-52 antigen (Table I).

Protein profiles by SDS-PAGE

The protein profiles discerned with each antigen preparation by SDS-PAGE were nearly same, irrespective of the source of antigen (Fig.i).

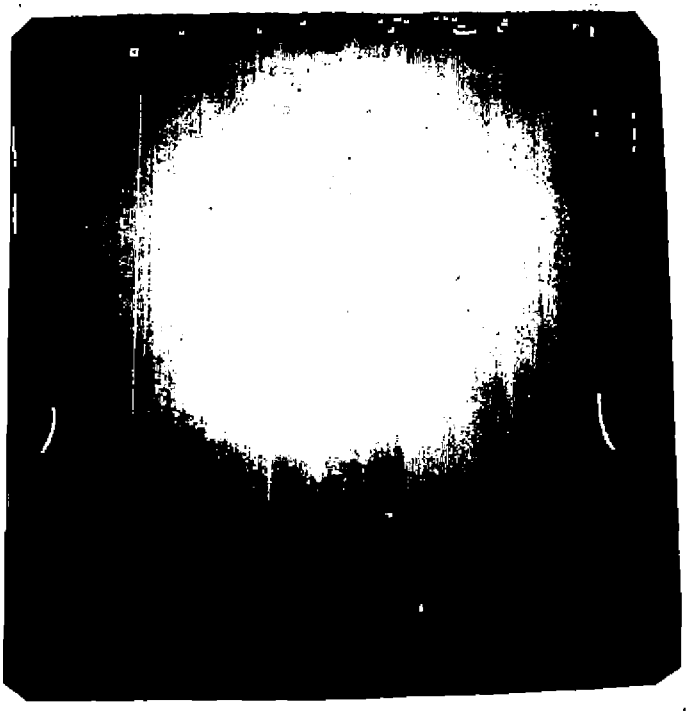
Table I. Protein and carbohydrate contents of the antigens from R_9S, $R_{23}S$ and P-52 strains of *P. multocida*

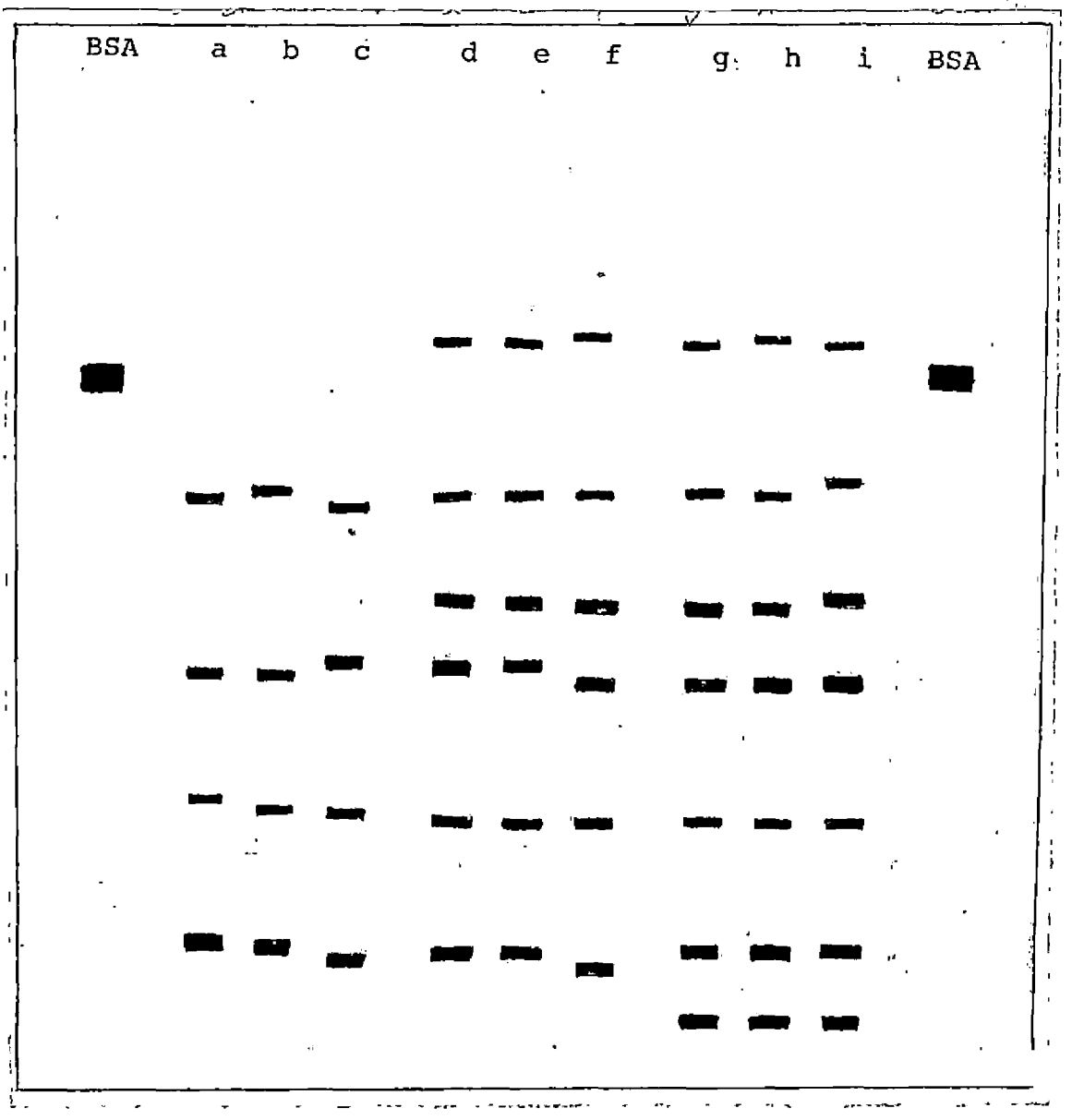
Source of antigen	Type of antigen	Protein mg/ml	Carbohydrate ug/ml
R_9S strain	HICE Ag	1.2	650
	KSCN Ag	2.3	920
	Son. Ag	2.8	970
$R_{23}S$ strain	HICE Ag	1.0	610
	KSCN Ag	2.3	950
	Son. Ag	2.7	960
P-52 strain	HICE Ag	0.900	590
	KSCN Ag	2.1	910
	Son. Ag	2.5	930

Note: HICE Ag - Heat inactivated crude extract antigen

KSCN Ag - Potassium thiocyanate extract antigen

Son. Ag - Sonicated antigen





The heat inactivated crude extract antigens from all the three strains of P. multocida presented four protein bands and their relative mobility (R_f) values were 0.6429, 0.7500, 0.7976, 0.8810 (Table II). All the four protein bands which were present for the heat inactivated crude extract antigen were also present for KSCN extract and sonicated antigens. The KSCN extract antigen from all the three strains presented two additional bands having the R_f values near to 0.5476, 0.7143. In case of sonicated antigen it presented an additional band compared to the KSCN extract antigen with a R_f value of 0.9226.

Antiserum

Specific antibody could be detected in the serum of rabbits even at 10th or 15th day of the antigen administration and the titre was found to be increasing as the injection continued. The antigen administration was continued till 50th day and by this time the level of antibody present in serum was sufficient to be detected by the serological tests employed in the present study.

Table II. Relative mobility values of each protein band from different antigens of R_9S, $R_{23}S$ and P-52 strains of P. multocida by SDS-PAGE

Heat inactivated crude extract			KSCN extract			Sonicated antigen		
R_9S	$R_{23}S$	P-52	R_9S	$R_{23}S$	P-52	R_9S	$R_{23}S$	P-52
			0.5476	0.5476	0.5416	0.5476	0.5416	0.5476
0.6429	0.6370	0.6548	0.6429	0.6429	0.6429	0.6429	0.6429	0.6370
			0.7143	0.7143	0.7143	0.7143	0.7143	0.7083
0.7500	0.7500	0.7441	0.7441	0.7441	0.7500	0.7500	0.7500	0.7500
0.7976	0.8036	0.8036	0.8036	0.8036	0.8036	0.8036	0.8036	0.8036
0.8810	0.8810	0.8869	0.8810	0.8810	0.8869	0.8810	0.8810	0.8810
						0.9226	0.9226	0.9226

Note: R_f value of Bovine Serum Albumin is 0.6012

Agar Gel Precipitation Test (AGPT)

Antiserum against the heat inactivated crude extract antigen of R_9S strain

This serum presented two identical precipitin lines when tested against heat inactivated crude extract antigen of homologous and two heterologous strains ($R_{23}S$ and P-52). The same antiserum when tested with homologous KSCN extract antigen gave two precipitin lines which were identical to the precipitin lines of the heat inactivated crude extract antigen. When reacted with sonicted antigen, there were two precipitin lines of which one was identical to the heat inactivated crude extract antigen. and other was non-identical and unique (Fig.ii-A).

Antiserum against the heat inactivated crude extract antigen of $R_{23}S$ strain

The results obtained in the AGPT employing this antiserum against the antigens were exactly same as that of R_9S strain (Fig.ii-B).

Antiserum raised against the heat inactivated crude extract antigen of P-52 strain

This serum presented multiple precipitin lines in AGPT. There was one precipitin line which was identical with

all the three antigenic preparations of the homologous P-52 strain, and the heat inactivated crude extract antigen of heterologous R_9S and $R_{23}S$ strains. The test presented another precipitin line which was identical for the three antigens of homologous strain. A third precipitin line which was present against the heat inactivated crude extract antigen from heterologous strains was identical. The sonicated antigen produced a unique precipitin line which was not present with any other antigens tested (Fig.ii-C).

Antiserum against KSCN extract antigen of R_9S strain

When this serum reacted against the three antigenic preparations from homologous strain and KSCN extract antigen from heterologous strains developed multiple precipitin lines (Fig.ii-D). The homologous KSCN extract antigen presented four precipitin lines, out of which two were identical with that of antigens from $R_{23}S$ and P-52 and the third precipitin line was identical only with $R_{23}S$ strain.

Antiserum against KSCN extract antigen of $R_{23}S$ strain

This serum also produced multiple precipitin lines against the three homologous antigens and KSCN extract antigen from heterologous strains. The homologous KSCN extract antigen presented four precipitin lines out of which three

were identical with that of R_9S and two were common to P-52 (Fig.ii-E).

Antiserum against KSCN extract antigen of P-52 strain

Multiple precipitin lines were developed when the serum was reacted against the antigens from homologous strains and KSCN extract antigen from heterologous strains (Fig.ii-F). The KSCN extract from the homologous strain presented three precipitin lines, out of which two were common with the lines developed by the KSCN extract antigen from heterologous strains R_9S and $R_{23}S$.

Antiserum against sonicated antigen of R_9S strain

This serum produced multiple precipitin lines when tested with the three antigens of homologous strain and sonicated antigen from $R_{23}S$ and P-52 strains. The homologous reaction of the sonicated antigen with the antiserum gave four precipitin lines of which two were identical to the lines developed with sonicated antigen from the heterologous strains and one precipitin line was identical with the sonicated antigen from $R_{23}S$ only (Fig.ii-G).

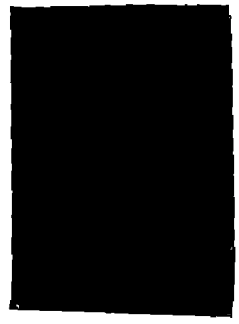
Antiserum against sonicated antigen of $R_{23}S$ strain

Multiple precipitin lines were obtained when this serum was tested against the three homologous antigens and two

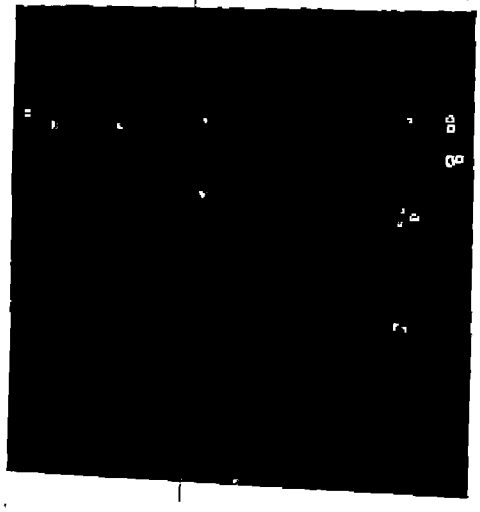
(ii)-A

(ii)-B

(ii)-C



(ii)-D



(ii)-F
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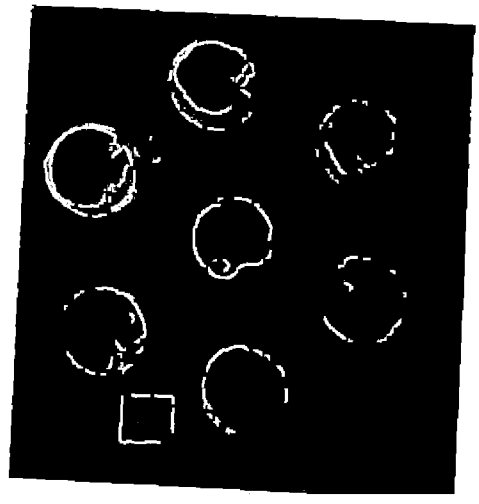
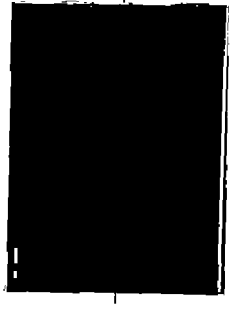


(ii)-E



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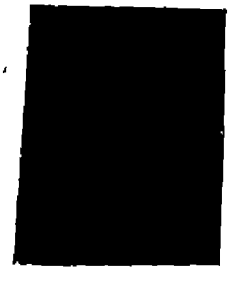


(ii)-H

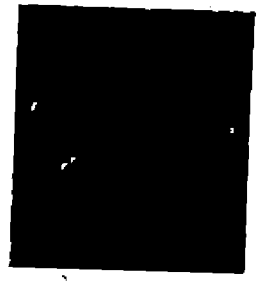


Fig.(i) Protein profiles of the different antigens from R_9S, $R_{23}S$ and P-52 strains of P. multocida discerned by SDS-PAGE with diagramatic illustration

a - HICE Ag - R^9S

b - HICE Ag - $R^{23}S$

c - HICE Ag - P-52

d - KSCN Ag - R^9S

e - KSCN Ag - $R^{23}S$

f - KSCN Ag - P-52

g - Son. Ag - R^9S

h - Son. Ag - $R^{23}S$

i - Son. Ag - P-52

HICE Ag - Heat Inactivated crude extract antigen

KSCN Ag - Potassium thiocyanate extract antigen

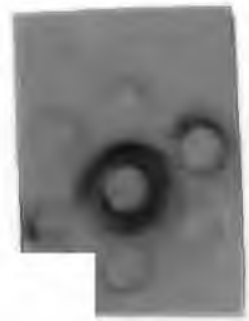
Son. Ag - Sonicated antigen

Fig.(ii) A-I Precipitin patterns of different antigens of *P. multocida* from R_9S, $R_{23}S$ and P-52 strains (representative figures only)

(ii)-A

(ii)-B

(ii)-C



ii)-F
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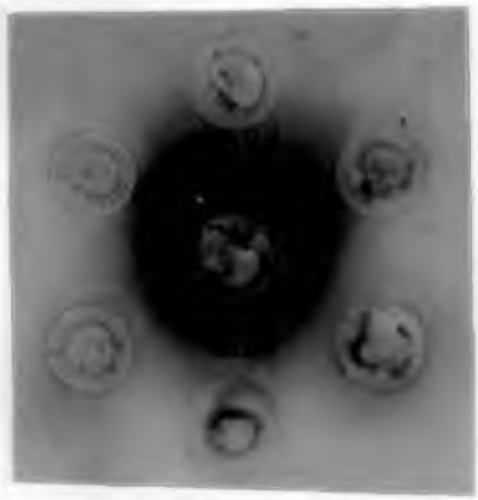


(ii)-E

(ii)-G

(ii)-H

Fig.(ii) A-I Diagramatic illustration of the precipitin patterns of different antigens of P. multocida from R_9S, $R_{23}S$ and P-52 strains

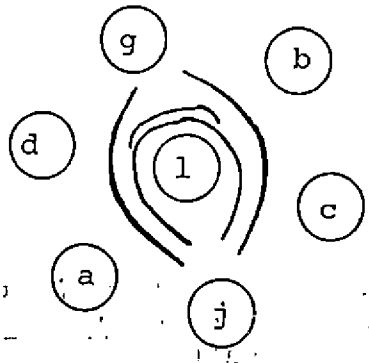
a - HICE Ag - R^9S	1. Antiserum against HICE Ag - R_9S
b - HICE Ag - $R^{23}S$	2. Antiserum against HICE Ag - $R_{23}S$
c - HICE Ag - P-52	3. Antiserum against HICE Ag - P-52
d - KSCN Ag - R^9S	4. Antiserum against KSCN Ag - R_9S
e - KSCN Ag - $R^{23}S$	5. Antiserum against KSCN Ag - $R_{23}S$
f - KSCN Ag - P-52	6. Antiserum against KSCN Ag - P-52
g - Son. Ag - R^9S	7. Antiserum against Son. Ag - R_9S
h - Son. Ag - $R^{23}S$	8. Antiserum against Son. Ag - $R_{23}S$
i - Son. Ag - P-52	9. Antiserum against Son. Ag - P-52
j - Blank	

HICE Ag - Heat Inactivated crude extract antigen

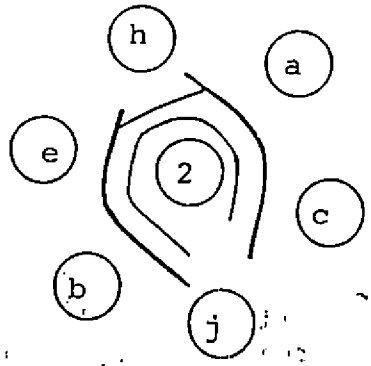
KSCN Ag - Potassium thiocyanate extract antigen

Son. Ag - Sonicated antigen

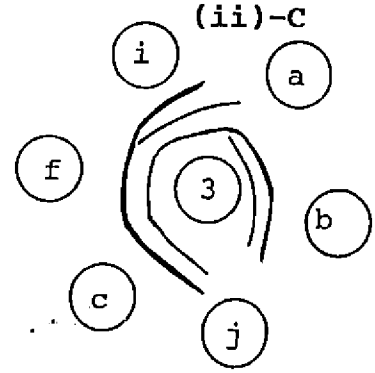
(ii)-A



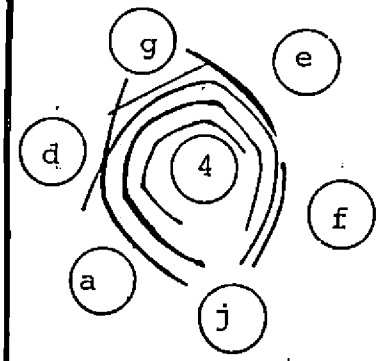
(ii)-B



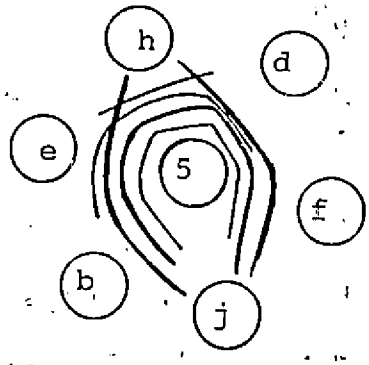
(ii)-C



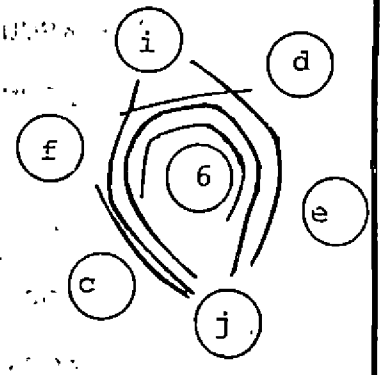
(ii)-D



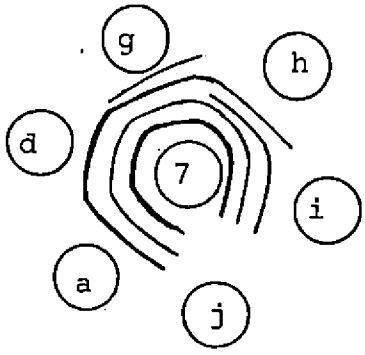
(ii)-E



(ii)-F



(ii)-G



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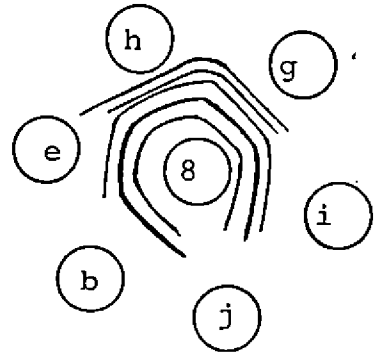
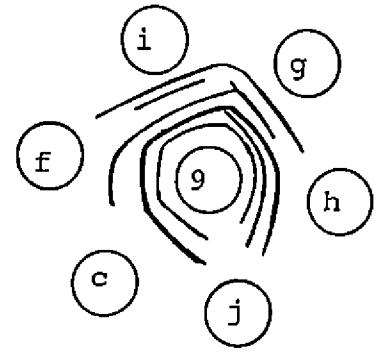


(ii)-I



heterologous sonicated antigens. The homologous sonicated antigen produced five precipitin lines, in that three were identical with the lines produced by sonicated antigen from heterologous strains, and the other two precipitin lines were identical only with R_9S antigen (Fig.ii-H).

Antiserum against sonicated antigen from P-52 strain

This serum also gave multiple precipitin lines on testing with the three antigens from homologous strain and with the sonicated antigen from heterologous strains. Out of the five precipitin lines given by the sonicated antigen from homologous strain, two were common for the sonicated antigens from heterologous strains R_9S and $R_{23}S$ and another two were identical to the sonicated antigen of R_9S strain only (Fig.ii-I).

Immuno electrophoresis

In the immunoelectrophoresis analysis, irrespective of the source or form of the antigen it presented multiple precipitin arcs when reacted against homologous/heterologous serum.

Antiserum against heat inactivated crude extract antigen from R_9S strain

The heat inactivated crude extract antigen from homologous and heterologous strains showed three precipitin arcs which were in similar position and comparable (Fig.iii-A).

Antiserum against heat inactivated crude extract antigen of $R_{23}S$ strain

Three precipitin arcs developed with the heat inactivated crude extract antigen from the homologous and heterologous strains, were nearly same (Fig.iii-B).

Antiserum against heat inactivated crude extract antigen from P-52 strain

The heat inactivated crude extract antigen of homologous and heterologous strains produced three arcs which were similar in position (Fig.iii-C).

Antiserum against KSCN extract antigen from R_9S strain

The KSCN extract antigen from R_9S and P-52 strains produced three precipitin arcs in which one precipitin arc developed for P-52, differed in its position on comparison. The KSCN extract antigen from $R_{23}S$ produced only two

precipitin arcs which were comparable to the precipitin arcs produced against the same type of antigen from R_9S and P-52 (Fig.iii-D).

Antiserum against KSCN extract antigen from $R_{23}S$ strain

Four precipitin arcs were developed by this serum against the homologous and two heterologous KSCN extract antigens which were comparable in their position (Fig.iii-E).

Antiserum against KSCN extract antigen from P-52 strain

The P-52 KSCN extract antigen developed four precipitin arcs, while the $R_{23}S$ three arcs and the R_9S two arcs indicating that two arcs were common for the strains P-52, R_9S and $R_{23}S$ (Fig.iii-F).

Antiserum against sonicated antigen from R_9S strain

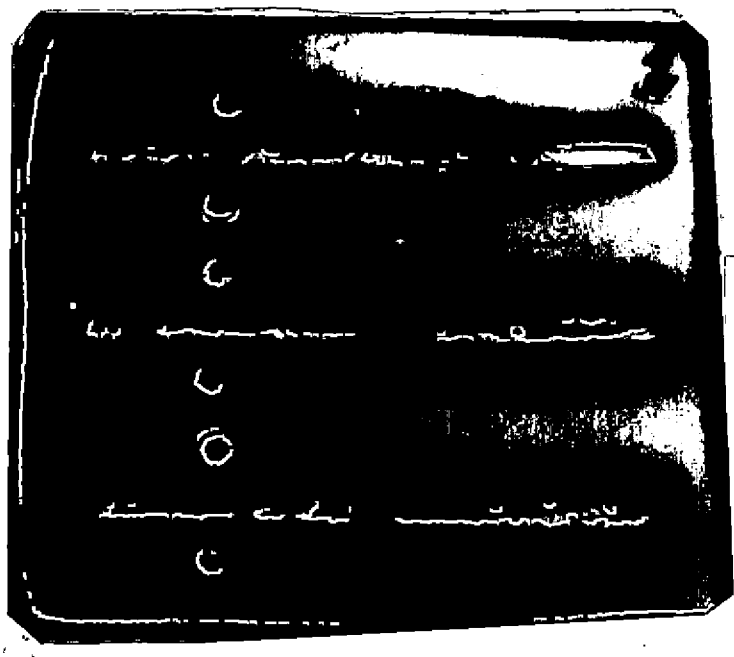
The sonicated antigen from the homologous strain and the heterologous strains $R_{23}S$ and P-52, developed six comparable precipitin arcs with this serum (Fig.iii-G).

Antiserum against sonicated antigen from $R_{23}S$ strain

The sonicated antigen from $R_{23}S$ and R_{9S} strains developed six precipitin arcs while the P-52 had presented only three arcs (Fig.iii-H).

Fig.(iii) A-I Immunoelectrophorogram of different antigens of *P. multocida* from R_9S, $R_{23}S$ and P-52 strains (Representative figures only)

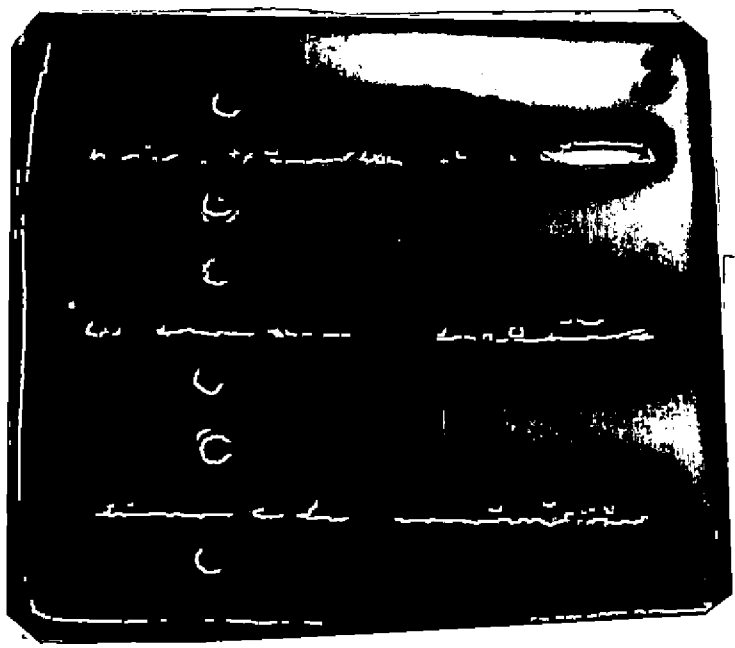
(iii)-A



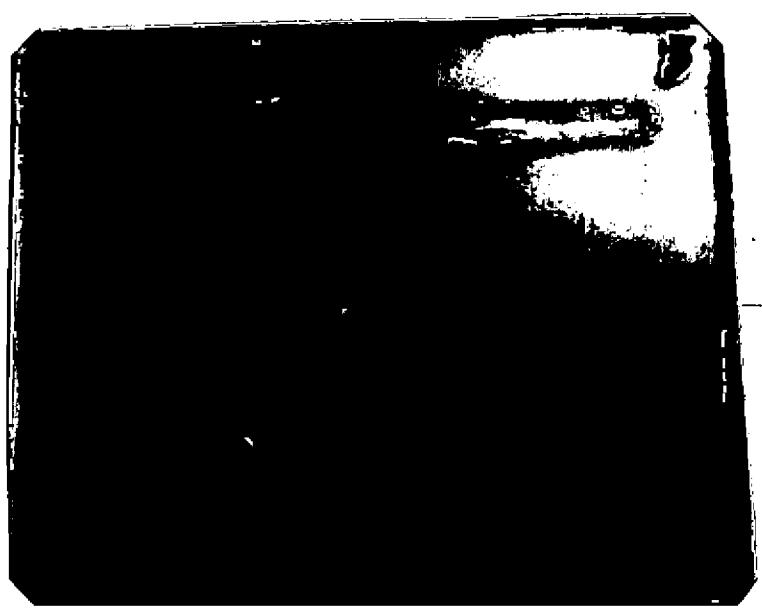
(iii)-D

(iii)-G

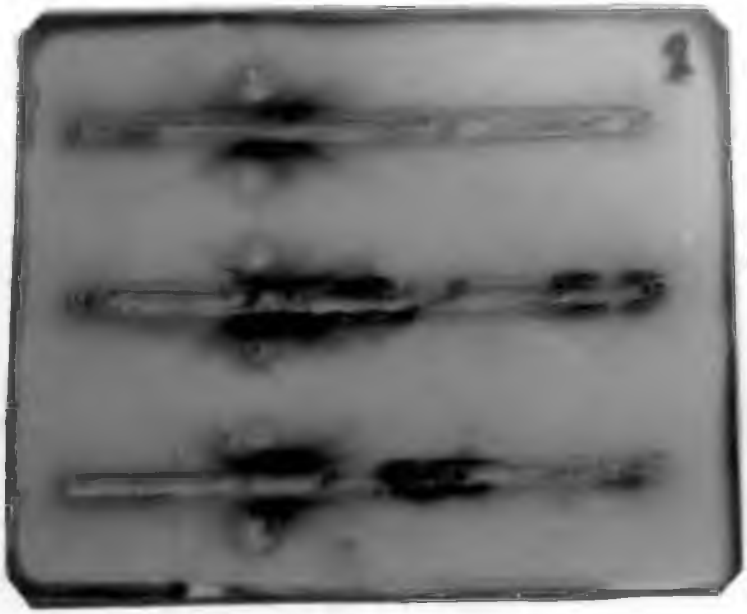
(iii)-A



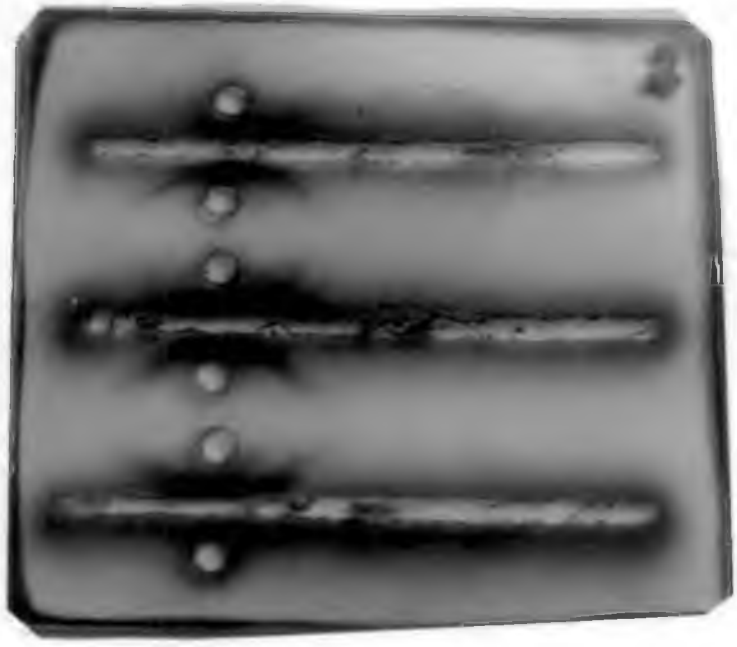
(iii)-D



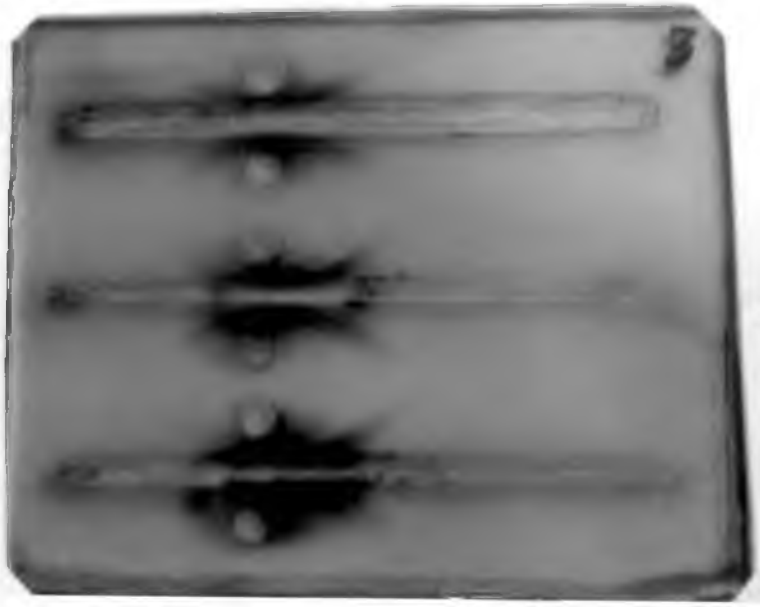
(iii)-G



(iii)-A



(iii)-D



(iii)-G

Fig. (iii) A-I Diagramatic illustration of immunoelectrophorogram of different antigens of P. multocida from R_9S, $R_{23}S$ and P-52 strains

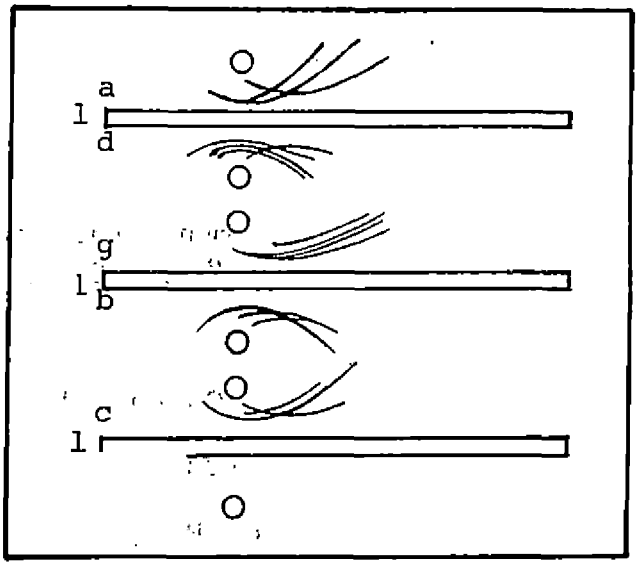
a - HICE Ag - R^9S	1. Antiserum against HICE Ag - R_9S
b - HICE Ag - $R^{23}S$	2. Antiserum against HICE Ag - $R_{23}S$
c - HICE Ag - P-52	3. Antiserum against HICE Ag - P-52
d - KSCN Ag - R^9S	4. Antiserum against KSCN Ag - R_9S
e - KSCN Ag - $R^{23}S$	5. Antiserum against KSCN Ag - $R_{23}S$
f - KSCN Ag - P-52	6. Antiserum against KSCN Ag - P-52
g - Son. Ag - R^9S	7. Antiserum against Son. Ag - R_9S
h - Son. Ag - $R^{23}S$	8. Antiserum against Son. Ag - $R_{23}S$
i - Son. Ag - P-52	9. Antiserum against Son. Ag - P-52
j - Blank	

HICE Ag - Heat Inactivated crude extract antigen

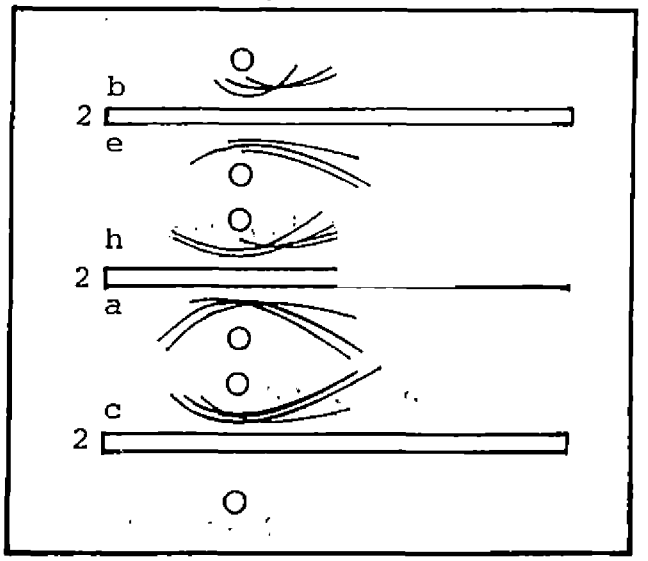
KSCN Ag - Potassium thiocyanate extract antigen

Son. Ag - Sonicated antigen

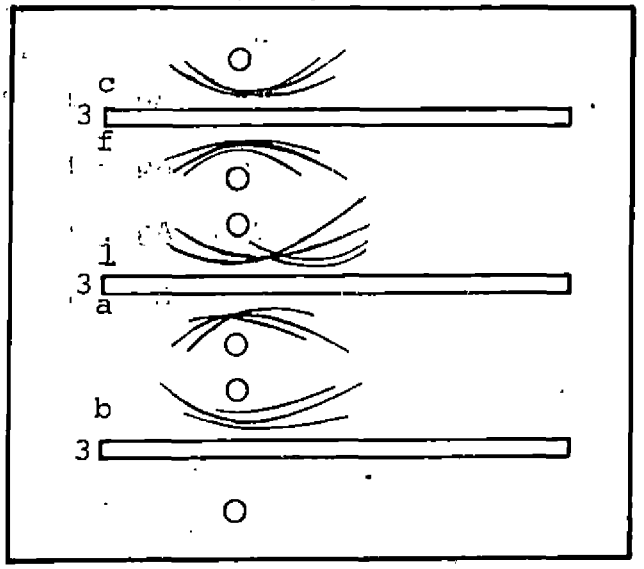
(iii)-A



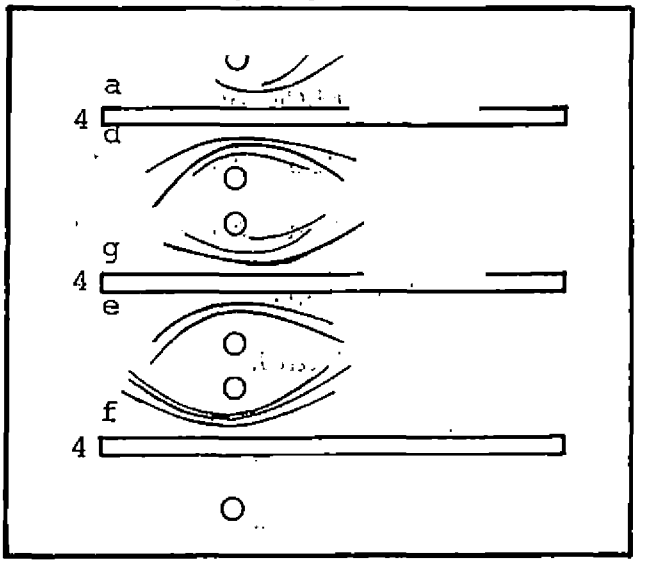
(III)-B



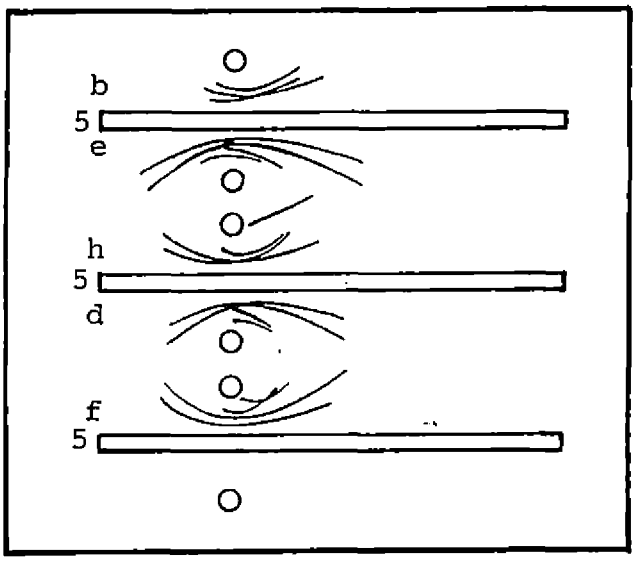
(iii)-C



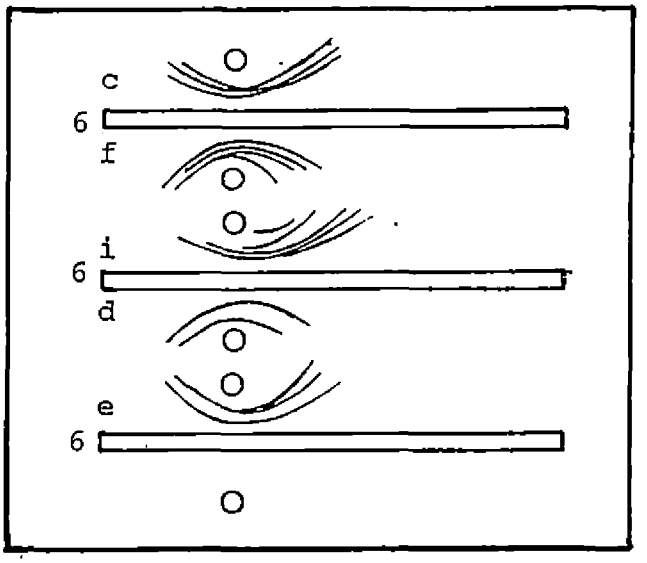
(iii)-D



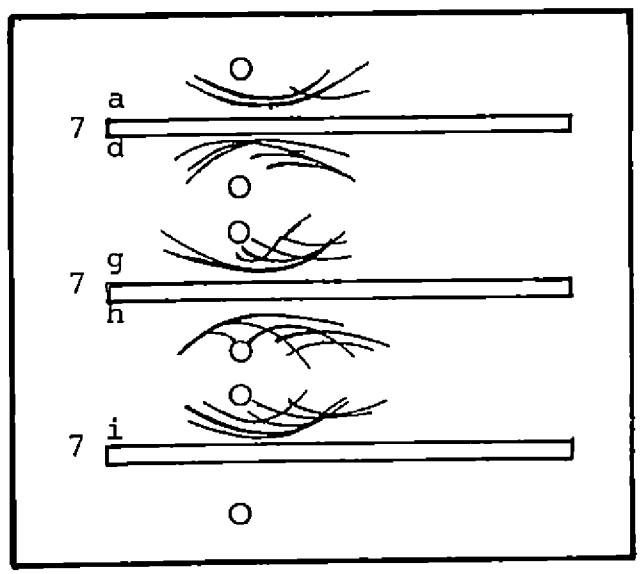
(iii)-E



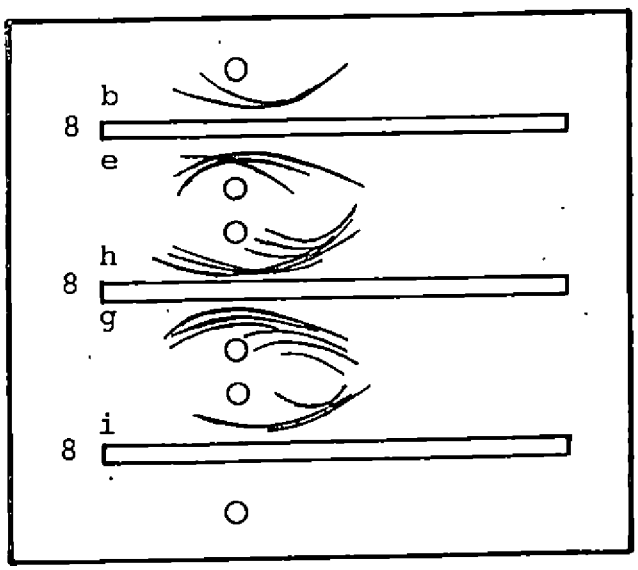
(iii)-F



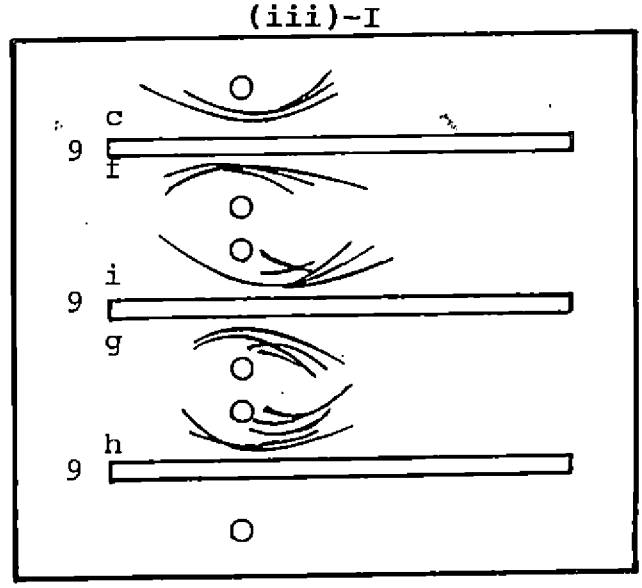
(iii)-G



(iii)-H



(iii)-I



Antiserum against sonicated antigen from P-52 strain

The sonicated antigen from both homologous strain P-52 and heterologous strain $R_{23}S$, presented five arcs each while only four precipitin arcs were found to be produced against sonicated antigen from R_9S strain (Fig.iii-I).

Indirect Haemagglutination Test

The three antigenic preparations viz., heat inactivated crude extract, KSCN extract and sonicated antigen from strains R_9S, $R_{23}S$ and P-52 were adsorbed on to Sheep Red Blood Cell (SRBC) and the sensitized SRBC were specifically agglutinated by rabbit antiserum. Controls of sensitized SRBC with diluent buffer (BSA-PBS) showed a definite negative reactions.

Results of antibody titration in antiserum employing the SRBC sensitized with homologous and heterologous antigenic preparations are given (Table III). The antiserum employed were raised against each antigenic preparation obtained from a particular strain of P. multocida.

The GA-SRBC sensitized with heat inactivated crude extract antigen from R_9S, $R_{23}S$ and P-52 showed the antibody titres of 2048, 4096, 2048 with their respective homologous antiserum. The KSCN extract from the strains R_9S, $R_{23}S$ and

Table III. Indirect haemagglutination titre of antisera raised against each antigen preparation from R_9S, $R_{23}S$ and P-52 strains of P. multocida

Type of Antigen	Source of Antigen	IHA titre in the antiserum against								
		Heat inactivated crude extract			KSCN extract			Sonicated antigen		
		R_9S	$R_{23}S$	P-52	R_9S	$R_{23}S$	P-52	R_9S	$R_{23}S$	P-52
Heat inactivated crude extract antigen	R_9S	2048	256	32						
	$R_{23}S$	128	4096	64						
	P-52	64	64	2048						
KSCN extract antigen	R_9S				4096	256	512			
	$R_{23}S$				512	8192	256			
	P-52				128	128	4096			
Sonicated antigen	R_9S							4096	1024	1024
	$R_{23}S$							512	8192	256
	P-52							256	512	4096

P-52, were sensitized with tanned GA-SRBC and gave antibody titres of 4096, 8192, 4096 with their homologous antiserum respectively (Fig.iv) while the GA-SRBC sensitized sonicated antigen from R_9S, $R_{23}S$ and P-52 strains gave a titre of 4096, 8192, 4096 in their respective homologous antiserum. But the heterologous antiserum from the three strains showed a cross titre ranging from 32 to 1024 on testing with the three sensitized antigens from the strains R_9S, $R_{23}S$ and P-52.

Potency test in mice

The immunogenic potential of heat inactivated crude extract antigen, KSCN extract antigen, sonicated antigen and adjuvanted sonicated antigen obtained from the three strains of *P. multocida*. R_9S, $R_{23}S$ and P-52 were studied by immunizing mice and challenged with virulent homologous/ heterologous strains. Mice immunized with the antigens did not show any depression or clinical signs or death during the period of 21 days of immunization. Control as well as some immunized mice died during the period between 16 h and 72 h after challenge exposure and few of the surviving mice were clinically ill upto 72 h but gradually recovered. The immunization protected a high percentage of mice against challenge with homologous strain when compared to the heterologous challenge.

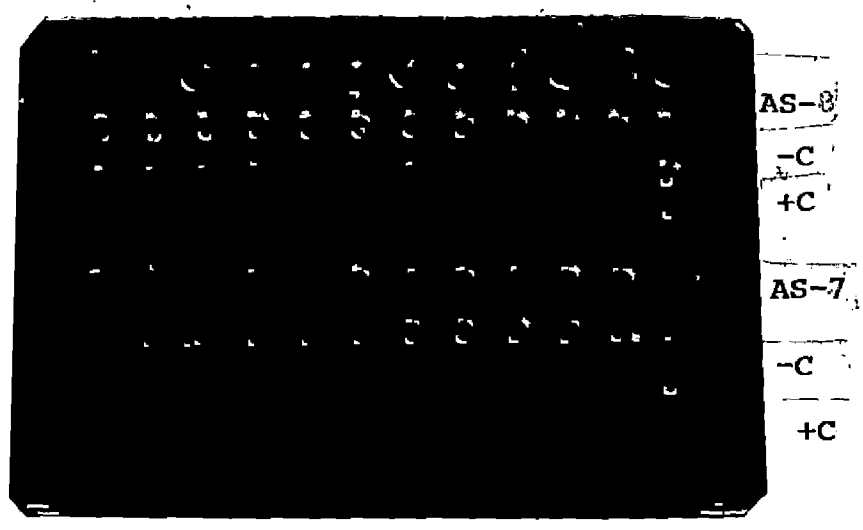
Fig.(iv) Indirect haemagglutination test

AS-8 - Antiserum against sonicated antigen of $R_{23}S$

AS-7 - Antiserum against sonicated antigen of R_9S

-C - Negative control

+C - Positive control


AS-8
-C
+C
AS-7
-C
+C

Potency test with antigens of R_9S strain

The above said four immunogens gave a homologous protection of 67, 100, 100, 83 per cent respectively on challenging but a cross protection about 17 per cent was observed with KSCN extract and sonicated antigen on challenging with $R_{23}S$ strain and on challenging with P-52 gave a 17 per cent protection with sonicated antigen only (Table IV).

Potency test with antigens of $R_{23}S$ strain

A homologous protection of about 50, 83, 100, 83 per cent was observed with the immunogens under test. A heterologous protection of 17, 33, 33, 17 per cent was observed with all the four immunogens respectively on challenging with R_9S strain while challenging with P-52 strain, a 17 per cent protection with KSCN extract and sonicated antigen (Table V).

Potency test with the antigens of P-52 strain

The immunogens from this strain gave a 50, 83, 100, 67 per cent homologous protection when challenged with P-52 strain but the challenge with heterologous strain R_9S or $R_{23}S$ gave 17 and 33 per cent with KSCN extract and sonicated antigen (Table VI).

Table V. Results of potency test of $R_{23}S$ antigens in mice

Type of Antigen	No.of mice immu-nized	No. of mice challen-ged	Challen-ging organism	No. of survi-ved	Percent-age of survived
Heat inactivated crude extract	18	6	$R_{23}S$	3/6	50
		6	R_9S	1/6	17
		6	P-52	0/6	0
KSCN extract	18	6	$R_{23}S$	5/6	83
		6	R_9S	2/6	33
		6	P-52	1/6	17
Cell sonicate	18	6	$R_{23}S$	6/6	100
		6	R_9S	2/6	33
		6	P-52	1/6	17
Cell sonicate with Freund's complete Adjuvant	18	6	$R_{23}S$	5/6	83
		6	R_9S	1/6	17
		6	P-52	1/6	17
Saline control	10	10	$R_{23}S$	0/10	0

Table VI. Results of potency test of P-52 antigens in mice

Type of Antigen	No.of mice immu-nized	No. of mice challen-ged	Challen-ging organism	No. of survi-ved	Percent-age of survived
Heat inactivated crude extract	18	6	P-52	3/6	50
		6	R_9S	0/6	0
		6	$R_{23}S$	0/6	0
KSCN extract	18	6	P-52	5/6	83
		6	R_9S	1/6	17
		6	$R_{23}S$	1/6	17
Cell sonicate	18	6	P-52	6/6	100
		6	R_9S	2/6	33
		6	$R_{23}S$	2/6	33
Cell sonicate with Freund's complete Adjuvant	18	6	P-52	4/6	67
		6	R_9S	0/6	0
		6	$R_{23}S$	0/6	0
Saline control	10	10	P-52	0/10	0

Discussion

DISCUSSION

P. multocida infection in animals is a world wide problem and is one of the major causes of economic loss to the animal industry. Rabbit and rabbitry are no exemption to this. P. multocida strains isolated from rabbits differ widely in virulence for rabbits and mice. The protective antigen of the P. multocida strains has not been conclusively identified so far and several of the antigenic preparations are on trial for immunization in rabbits. In the present work the three antigenic preparations viz., heat inactivated crude extract, KSCN extract and sonicated prepared from two rabbit isolates R_9S, $R_{23}S$ and a vaccine strain P-52 were studied for the physico-chemical character, serological behaviour and immunogenicity.

P. multocida which are virulent to experimental and natural hosts are generally capsulated (Carter, 1967). The P. multocida strains R_9S, $R_{23}S$ and P-52 were maintained in its virulent form during the period of this study by repeated passaging in mice and subculturing on TSA slants. When the stock culture was maintained at 4°C in air tight slants, the culture need to be subcultured once in four weeks. Mukkur and Pyliotis (1981) reported that virulence of P. multocida could

be maintained only when subcultured on horse blood agar plates and passaged in mice on every 4th week.

The LD_{50} of the three strains of P. multocida viz., R_9S, $R_{23}S$ and P-52 in mice by intraperitoneal route of inoculation were determined to be $3x10^4$, $3x10^3$ and $3x10^5$ bacteria respectively.

Okerman et al. (1979) estimated the LD_{50} of 20 strains of P. multocida from rabbits by infecting mice intraperitoneally and the number of organisms ranged between $3x10^3$ and $3x10^6$ bacteria. Comparatively a low LD_{50} 5.0 $\pm$ 2.8 colony forming units has been determined for P. multocida of bovine origin in mice by intraperitoneal route of inoculation (Mukkur, 1979).

The results obtained in this study indicated that the strains differ in their virulence and the rabbit strains of P. multocida are more virulent to mice when compared to P. multocida of cattle strain P-52. Between the two rabbit isolates $R_{23}S$ is more virulent than the R_9S.

The three antigens viz., heat inactivated crude extract, KSCN extract and sonicated antigen from the three strains of P. multocida, were found to be stable in their antigenicity. Each form of antigen irrespective of the source was homogenous in their chemical and physical characters.

The different antigenic preparations from the three strains were analysed for their protein and carbohydrate content. The protein content estimated by protein-dye binding principle. The average protein content in heat inactivated crude extract, KSCN extract and sonicated antigens of R_9S strain were 1.2, 2.3 and 2.8 mg/ml of antigen respectively. The concentration of protein in antigens from $R_{23}S$ were 1.0, 2.3 and 2.7 mg per millilitre and for the antigens from P-52 the values were 0.9, 2.1 and 2.5 mg/ml (Table I). Kajikawa and Matsumoto (1984) quantitated the protein content in antigen extracted with saline and purified by gel chromatography from P. multocida of turkey origin and reported that it contained 650 ug of protein per millilitre of antigen. Lu et al. (1987) estimated the different components of KSCN extract antigen of P. multocida type 3:A and it was reported to contain protein 2 mg/ml.

The average concentration of carbohydrate estimated by phenol-sulfuric acid method in the three antigens viz., heat inactivated crude extract, KSCN extract and sonicated antigens, were 650, 920, 970 ug per millilitre of antigen from R_9S strain, while the concentrations in the antigens from $R_{23}S$ were 610, 950 and 960 ug/ml and the quantities were 590, 910 and 930 ug per ml of antigen from P-52 strain respectively. Kajikawa and Matsumoto (1984) also quantitated the

carbohydrate content in antigen extracted with saline and purified by gel chromatography from P. multocida of turkey and reported to contain 152 ug of carbohydrate per millilitre of antigen. Lu et al. (1987) estimated the different components of KSCN extract antigen of P. multocida type 3:A and it contained 462.5 ug of carbohydrate per mg of protein. Lin et al. (1988) described the qualitative features of capsular material prepared from P. multocida and reported that it contained 55.5 $\pm$ 12 per cent protein, 4.7 $\pm$ 1.5 per cent carbohydrate, 10.9 $\pm$ 2.5 per cent phospholipids, trace of nucleic acids and O-acetyl group.

In the methods adopted for the preparation of different forms of antigen, the initial bacterial suspension were uniformly adjusted to contain $3x10^9$ bacteria/ml and at the end of the procedure the soluble preparation of the antigen was obtained as supernatant after centrifugation. As per the results of this study maximum concentration of protein and carbohydrate were highest in sonicated antigen irrespective of the source, followed by KSCN extract and then heat inactivated crude extract antigen. The reason for the high content of protein/carbohydrate in sonicated antigen is as it contains the cell lysates/disintegrates while the other two antigens are only the extracts which ought to contain only certain fractions of the organisms.

The protein profiles obtained by SDS-PAGE analysis with each form of antigen prepared from the three strains of P. multocida were almost similar (Fig.ii). The heat inactivated crude extract antigen from all the three strains revealed four protein bands with R_f values 0.6429, 0.7500, 0.7976 and 0.8810 (Table II). These four protein bands were common for the KSCN extract and sonicated antigens also. Further the KSCN extract antigen and the sonicated antigen presented two additional bands having R_f values 0.5476 and 0.7143. The sonicated antigen in addition to the above six bands had presented a unique protein band, having R_f value 0.9226.

Thus the number of protein bands revealed with heat inactivated crude extract, KSCN extract and sonicated antigen irrespective of the strain of P. multocida from which they were prepared, were four, six and seven respectively.

The R_f value of protein band from bovine serum albumin fraction V on SDS-PAGE when it was used as a marker, in the test, was 0.6012. From the results obtained in the test it is observed that all the protein bands except one protein band in cases of KSCN extract and sonicated antigen had R_f values higher than that of BSA.

The molecular weight of BSA on SDS-PAGE is supposed to be 68 kDa (Weber et al., 1972). Since no other protein marker with known molecular weight was incorporated in the test, the result obtained could be inferred only in comparison to BSA. The four protein fractions contained in the heat inactivated crude extract, five protein bands in KSCN extract antigen and six protein bands in somatic antigen were with molecular weight lower than BSA.

Several workers had demonstrated the protein profiles of different antigens of P. multocida by SDS-PAGE analysis. Syuto and Matsumoto (1982) reported that the saline extracted purified antigen of P. multocida from turkey, presented three protein bands with molecular weights of 44000, 31000 and 25000 Da respectively. Kajikawa and Matsumoto (1984) also analysed the purified saline extract antigen of P. multocida from Turkey by SDS-PAGE and revealed four protein components with the molecular weights of 44000, 31000, 25000 and 20000 Da respectively. KSCN extract antigen prepared from P. multocida of rabbits, was analysed by SDS-PAGE and showed the protein profile similar to the membrane vesicles of this organism prepared by lithium chloride extraction and a 37000 Da outer membrane protein was the predominant one (Lu et al., 1987).

The protein profiles of 14 isolates of P. multocida from H.S. animals were described by Johnson et al. (1991) in

SDS-PAGE. They observed unique and common protein bands for Carter types, which were 32 kDa in type B, 37 kDa for the type E and the common protein bands were 27, 45 and 47 kDa. The soluble proteins contained in the sonicated antigen from chicken isolates of P. multocida were demonstrated to be similar by Ireland et al. (1991). They observed the major difference between isolates in the position of the major proteins at the 34-38 kDa region as detected by SDS-PAGE. Zimmerman et al. (1992) separated the polypeptides from whole cell preparation of rabbit P. multocida isolates by SDS-PAGE and westernblot analysis. They could detect three polypeptides with the molecular weight of 28, 30 and 37 kDa on testing against sera from both experimentally and naturally infected rabbits. Thus indicating that protein fraction having molecular weight 28-37 kDa were immunologically reactive. In the present work, the molecular weight of the protein bands could not be determined. An alternative method was resorted to compare the protein bands in different antigens, calculating the R_f values based on mobility of protein band and the tracking dye.

There existed a positive correlation between the protein content and the number of protein fractions contained in the antigens. It was further elaborated in the results

obtained in the serological tests viz., agar gel precipitation test and immunoelectrophoresis.

Individually, antiserum was raised in rabbits against three antigenic preparations obtained from the three strains of P. multocida under study. In these cases, the first dose of antigen was injected subcutaneously after emulsifying with equal quantity of Freund's Complete Adjuvant. Subsequently plain antigen was administered intravenously without adjuvant at 5 days interval. Specific antibody activity could be detected in the serum of rabbits by 10th to 15th day of antigen administration but, immunization was continued with an intention to get higher antibody titre in the serum. Irrespective of the form of antigen, immunization was stopped at 50th day and animals were profusely bled for serum collection. The serum was used for AGPT, immunoelectrophoresis and indirect haemagglutination test.

For raising antiserum against whole cell antigen in rabbits Manning (1984), injected them intramuscularly with equal volume of the antigen and Freund's Complete Adjuvant and subsequent four injections were done similarly every 10 to 14 days with incomplete Freund's adjuvant.

Antiserum against heat inactivated crude extract antigen of R_9S strain presented two identical precipitin lines

when tested against antigen from homologous (R_9S) and heterologous ($R_{23}S$ and P-52) strains. The same serum gave two identical precipitin lines on testing with KSCN extract antigen of R_9S. Eventhough the sonicated antigen of R_9S produced two precipitin lines, only one was identical to the precipitin lines developed with heat inactivated crude extract antigen and KSCN extract antigen (Fig.ii-A).

Antiserum against the heat inactivated crude extract antigen of $R_{23}S$ strain gave similar reactions to that of R_9S, when tested against the antigens from homologous and two heterologous strains (Fig.ii-B). Thus the results obtained in AGPT indicates that R_9S and $R_{23}S$ are closely related as far as the antigens which are serologically active.

When the same type of antiserum, from P-52 strain was studied in AGPT, multiple precipitin lines were observed with antigens from homologous and heterologous heat inactivated crude extract antigen from R_9S and $R_{23}S$ strains (Fig.ii-C). Out of these multiple lines only one from homologous strain was shown to be identical with the two heterologous strains - R_9S and $R_{23}S$ indicating that heat inactivated crude extract antigen of P-52 strain are not so related to R_9S or $R_{23}S$.

The results obtained in the present study were comparable with observations made by previous workers. Syuto

and Matsumoto (1982) and Kajikawa and Matsumoto (1984) observed two distinct precipitin lines when the crude extract antigen of P. multocida of turkey origin was reacted with its antiserum on AGPT. Penn and Nagy (1974) could get two precipitin lines when antiserum against whole organisms was tested with saline/phenol water extract antigen of P. multocida of bovine origin (P-52).

Antiserum against KSCN extract antigen from R_9S strain developed multiple precipitin lines when reacted with the three homologous antigens/heterologous KSCN extract antigens. The KSCN extract from R_9S developed four precipitin lines, out of which two were identical with the homologous heat inactivated crude extract and sonicated antigen and KSCN extract antigen from two heterologous strains and a third line was identical to $R_{23}S$ only (Fig.ii-D). The antiserum raised against KSCN extract of strain $R_{23}S$ produced multiple precipitin lines against the three homologous antigens and the KSCN extract from R_9S and P-52. The KSCN extract from $R_{23}S$ produced four lines out of which three were identical to the lines produced by KSCN extract from R_9S and two were common to P-52 strain (Fig.ii-E). Antiserum against KSCN extract of P-52 strain gave multiple precipitin lines when reacted against the three antigens from P-52 and KSCN extract antigen from strains-R_9S and $R_{23}S$ (Fig.ii-F). The homologous KSCN

extract developed three lines and of which two were identical with the lines developed by the two heterologous strains. The results reveal that the rabbit strains are related to each other than the bovine strain.

The use of ultrasonic disintegrates in serum preparation and as antigen in immunodiffusion studies, resulted in detection of cross-reactions involving internal components (Prince and Smith, 1966). The heterogeneity of this antigen preparation was already shown in SDS-PAGE analysis. It is further confirmed by this method.

Antiserum against sonicated antigen from R_9S developed many precipitin lines when reacted against the three antigens from R_9S and sonicated antigens from $R_{23}S$ and P-52 (Fig.ii-G). The homologous reaction of the sonicated antigen gave four lines of which three were common to the sonicated antigen from $R_{23}S$ and two were common with P-52. The antiserum against the sonicated antigen of $R_{23}S$ strain when reacted against homologous sonicated antigen produced five precipitin lines which were all identical with the lines produced by the sonicated antigen from R_9S whereas only three lines from homologous antigen were identical to sonicated antigen from P-52 strain (Fig.ii-H).

Same type of antiserum from P-52 strain produced five precipitin lines with homologous sonicated antigen, in which two were common to the sonicated antigens from heterologous strains and another two lines were identical with the antigen from R_9S strain only (Fig.ii-I).

From this study it is seen that the sonicated antigen was more heterogenous as far as their antigens which are serologically active and the number of antigenic moiety present in the sonicated antigen irrespective of the source, were more compared to other forms of antigens obtained from the same strain. On the perusal of the available literature it is seen that similar work has not been reported earlier.

The different forms of antigen from the three strains of P. multocida when subjected to immunoelectrophoresis it too developed multiple precipitin arcs when reacted against homologous/heterologous antiserum.

Antiserum against heat inactivated crude extract antigen from R_9S strain developed three precipitin arcs with antigens from homologous/heterologous strains which were similar in their position and comparable (Fig.iii-A). The antiserum against the same type of antigen from $R_{23}S$ developed three precipitin arcs with homologous antigen which were comparable to the arcs developed with the heterologous heat

inactivated crude extract antigen from R_9S and P-52 (Fig.iii-B). The same type of serum from P-52 strain showed three precipitin arcs with the homologous and heterologous antigens which were also comparable (Fig.iii-C).

While typing the bovine strains of P. multocida, Penn and Nagy (1974) used somatic antigen obtained by phenol-water extraction and capsular antigen by saline extraction in immunoelectrophoresis, when these antigens were reacted against their respective antiserum, they too produced only three precipitin arcs which is comparable with the results obtained in the present work.

The antiserum against KSCN extract antigen from strain R_9S developed three precipitin arcs with KSCN extract antigen from R_9S and the same antigen from P-52 while only two precipitin arcs with strain $R_{23}S$. Out of these precipitin arcs except for one arc developed for P-52 others were comparable to each other in position (Fig.iii-D).

Antiserum raised against KSCN extract antigen of $R_{23}S$ produced four precipitin arcs with the KSCN extract from $R_{23}S$, R_9S and P-52 which were all comparable (Fig.iii-E). The same type of antiserum from P-52 strain produced four precipitin arcs with the homologous strain P-52, three with antigen from

lines obtained in AGPT which indicates the sensitivity of this test.

From the results it could be inferred that the strain R_9S and $R_{23}S$ were closely related when compared to the bovine strain P-52.

For IHA, Sheep Red Blood Cells (SRBC) fixed and stabilised by 1 per cent gluteraldehyde (GA-SRBC) were employed. This saves time spent in preparing SRBC suspension for every test. Moreover it increases the reproducibility of the test as the same RBC lot is used in the whole sero monitoring experiment. Another advantage noted while using GA-SRBC was that it could be stored at 4°C for more than seven months without deteriorating the quality of SRBC. Sawada et al. (1982) also reported the advantages of using GA-SRBC for IHA test and they could use such stabilized SRBC for at least seven months when stored at 4°C.

In this study, the heat inactivated crude extract antigen and sonicated antigen from R_9S, $R_{23}S$ and P-52 were adsorbed on to GA-SRBC for antibody assay in the serum. Whereas KSCN extract antigen from all the strains were adsorbed on to tanned GA-SRBC. This is because the initial experiments indicated that if we are employing GA-SRBC for adsorption of KSCN extracted antigen very low titres only

could be obtained compared to the crude extract antigen or sonicated antigen.

The antisera raised against each antigenic preparation from a particular strain of P. multocida were titrated with the homologous and heterologous antigens adsorbed either to GA-SRBC or to T-GA-SRBC (Fig.iv)

The GA-SRBC sensitized with heat inactivated crude extract antigen from R_9S, $R_{23}S$ and P-52 showed antibody titres of 2048, 4096 and 2048 with their specific homologous antiserum (Table III). The IHA titres indicated that $R_{23}S$ antigen is more immunogenic active compared to R_9S and P-52 antigens.

The T-GA-SRBC sensitized with KSCN extracted antigen $R_{23}S$ and P-52 showed titres of 4096, 8192 and 4096 respectively with the homologous antiserum (Table III). Here also it was proved beyond doubt that $R_{23}S$ antigen is superior to the other two types of antigen derived from R_9S and P-52 strains of P. multocida.

The GA-SRBC adsorbed with sonicated antigen from R_9S, $R_{23}S$ and P-52 strains gave titres of 4096, 8192 and 4096 respectively with their homologous antiserum (Table III) indicating the superiority of using $R_{23}S$ as antigen compared to the sonicated antigen obtained from R_9S and P-52 strains.

Irrespective of the methods of preparation of antigens i.e. either heat inactivated crude extract, KSCN extract or sonicated, the antigens obtained from $R_{23}S$ could produce more antibodies. Another conclusion obtained from this study is that heat inactivated crude extract of P. multocida is less immunogenic compared to the KSCN extracted antigen or sonicated antigen. The KSCN extracted and sonicated antigens showed same titres with the homologous serum indicating the same immunogenic potentialities for both methods of P. multocida antigen preparation.

Cross-titration was also carried out using antiserum raised against heat inactivated crude extract of R_9S strain with GA-SRBC sensitized with heat inactivated crude extract antigen from $R_{23}S$ and P-52 gave a cross-titres of 128 and 64 respectively. Same type of antiserum from $R_{23}S$ strain when titrated with GA-SRBC sensitized antigen from R_9S and P-52 showed titres of 256 and 64 while, antiserum raised against P-52 strain showed cross titres of 32 and 64 on testing with GA-SRBC sensitized with heat inactivated crude extracted antigen from R_9S and $R_{23}S$ strains respectively. The cross titres obtained by R_9S and $R_{23}S$ revealed a close association of the two rabbit strains compared to the strain P-52 of bovine origin.

The antiserum raised against the KSCN extracted antigen from R_9S showed heterologous reaction titres of 512 and 128 with KSCN extract antigen sensitized GA-SRBC from $R_{23}S$ and P-52 strains. The antiserum against $R_{23}S$ strain gave titres of 256 and 128 when reacted with KSCN extracted antigen of R_9S and P-52 strains and the cross titres with P-52 antiserum were 512 and 256 when tested with antigens from R_9S and $R_{23}S$.

As observed in the present study Sawada et al. (1982) also reported that the maximum IHA titre he could obtain was 8192 with homologous system whereas a maximum IHA titres of 512 only could be obtained with heterologous system KSCN extracted antigen from different cattle strains of P. multocida.

The heterologous reactivity were more prominent with antiserum raised against sonicated antigen. Because the antiserum of R_9S sonicated antigen gave IHA titres of 512 and 256 on cross-titration with GA-SRBC sensitized sonicated antigen from $R_{23}S$ and P-52 respectively. Whereas antiserum against $R_{23}S$ showed titres of 1024 and 512 with the sonicted antigens. The antiserum against P-52 strain also gave IHA titres of 1024 and 256 with the sonicated antigens from R_9S and $R_{23}S$ strains respectively. The results of cross-titration with sonicated antigen indicate that if a heterologous system

is employed in IHA test for seromonitoring against P. multocida, sonicated antigen must be preferred over heat inactivated crude extract or KSCN extracted antigen. But it is always advisable to use a homologous system for seromonitoring because high IHA titres could be obtained.

In a dose-dependent immune response studies, Azam et al. (1991) could obtain a maximum IHA titres of 64 only at the second week of inoculation in a single dose immunization in rabbit. In the present study employing even with a heterologous system using a sonicated antigen of $R_{23}S$ an IHA titre of 1024 could be obtained, indicating that IHA test with sonicated antigen from $R_{23}S$ strain can be recommended for serological screening of rabbit against P. multocida infection. The studies carried out by Manning (1984) indicate that four times immunized rabbits with heat killed whole cells of P. multocida in adjuvant could produce only an IHA titre of 640 whereas in the present study even in a heterologous system employing sonicated antigen from $R_{23}S$ strain could obtain an IHA titre of 1024.

The potency of the heat inactivated crude extract antigen, KSCN extract antigen, sonicated antigen and adjuvanted sonicated antigen obtained from the three strains of P. multocida R_9S, $R_{23}S$ and P-52 were studied by active

immunization of mice and challenging them with virulent homologous/heterologous strains.

The mice were immunized by giving two doses of the respective antigens on 0 and 14th day. The immunized mice were challenged on 21st day of post-vaccination and the protection rate was scored for a period of 7 days. Similar experiment was done to study the potency of formalinised and KSCN extract antigens of P. multocida by Smith et al. (1981) wherein they immunized mice on 0 and 14th day and the immunized mice were challenged on 28th day post-vaccination. In the present experiment, none of the vaccinated mice died nor showed any prolonged illness during the period of immunization. On challenge inoculation of immunized mice with virulent homologous/heterologous strains, the survival rate varied between zero to 100 per cent while in controls the survival rate was nil. Pasteurella vaccine has been reported to give optimal protection against heterologous challenge as there are many serotypes and most vaccines are serotype specific (Al-Lebban et al., 1988).

The rate of protection in mice vaccinated with heat inactivated crude extract antigen, KSCN extract, cell sonicate and sonicated antigen with Freund's Complete Adjuvant (FCA) of R_9S strain on challenging with homologous strain, were 67, 100, 100 and 83 per cent respectively. On the other hand,

when such vaccinated mice were challenged with the heterologous strains $R_{23}S$ and P-52, the survival rate ranged between zero to 17 per cent which indicated only insignificant cross-protection (Table IV). The survival rate of 50, 83, 100 and 83 per cent were observed in mice which were vaccinated with heat inactivated crude extract, KSCN extract, cell sonicates and sonicated antigen with FCA from strain $R_{23}S$, on challenging with the virulent homologous strain. Cross-protection experiment results with strains R_9S and P-52, presented a very low survival rate of 0 to 33 per cent (Table V). It may be further noted that the highest cross-protection was between R_9S and $R_{23}S$ and that too in mice which were vaccinated with KSCN extract and sonicated antigen.

The results of the potency test with the antigens of homologous P-52 strain indicated that the immunogens gave a 50, 83, 100 and 67 per cent homologous protection when challenged with P-52 strain. But the challenge with heterologous strain R_9S gave 17, and 33 per cent with the KSCN extract and sonicated antigen while a 17 per cent cross-protection was observed with KSCN extract and sonicated antigen on challenging with $R_{23}S$ strain (Table VI).

Mouse model of P. multocida infection as a method to test the efficacy of vaccines has been used by various workers. Mukkur (1979) employed 15 mice per antigen group

with either formalin killed vaccine or KSCN extract antigen for the potency test. Ryu and Kaeberle (1986) have employed 10 number of mice per KSCN extract antigen of a strain in the immunogenic potential study. Baljer et al. (1982) studied the efficacy of inactivated P. multocida vaccines in mice and reported that the vaccine was effective irrespective of the inactivation procedures adopted for the preparation of antigen and the route by which it was administered in animals.

In the present work the plain form of antigen was administered by intraperitoneal route and adjuvanted antigen by subcutaneous route. From the results obtained it is observed that a uniformly low protection was afforded with adjuvanted antigen. The reason for this may be the too short period of immunization (21 days) before the challenge exposure. With the virulent culture as the adjuvanted antigen require more time for absorption and release of antigen from the site of inoculation.

The immunizing efficiency of KSCN extract antigen from cattle strain of P. multocida in mice was reported by Mukkur (1979). The mice were immunized with a single dose of antigen intraperitoneally and on homologous challenge with P. multocida on 28th day post-immunization, a cent per cent protection was observed. He further reported that when

animals were challenged on 7th day of immunization, the protection rate was only 50 to 53 per cent. Ryu and Kaeberle (1986) evaluated the immunogenicity and cross-protectivity of KSCN extract antigen from bovine strains of P. multocida and according to them the vaccinated mice were well protected against homologous and heterologous challenge infections.

The results presented in the present work indicated that there was reasonably high degree of protection in mice vaccinated with KSCN extract and sonicated antigens against challenge infection with homologous strain but not with heterologous strains.

Penn and Nagy (1974) reported active immunization of mice with killed P. multocida of bovine origin when two doses of vaccine administered at three weeks interval and then challenged with homologous/heterologous strains after the 5th week of post-vaccination establishing the cross-protection.

The sonicated antigen which was used as immunogen in the potency test included more serologically active moieties as evidenced in the results obtained with AGPT, immunoelectrophoresis and IHA. The results of potency test also substantiated the above point as there were 100 per cent protection of vaccinated animals against challenge infection with homologous strains. But mice which were vaccinated with

sonicated antigen could not resist infection with heterologous rabbit strains or bovine strain of P. multocida. These observations further suggest that though the rabbit strains of P. multocida under study are found to be serologically comparable but they are immunologically distinct.

Summary

SUMMARY

Three strains of P. multocida comprising two local isolates from rabbits (R_9S and $R_{23}S$) and one vaccine strain P-52 of bovine origin were maintained in the laboratory in its virulent form by intermittent passage in mice and subculturing on TSA at four weeks interval.

LD_{50} of the above strains in mice by intraperitoneal route was determined and were 3×10^4, 3×10^3 and 3×10^5 bacteria respectively.

Three methods of antigen preparation viz. heat inactivated crude extract, KSCN extract and sonicated antigen were employed. The above antigenic preparations from the three strains were subjected to physico-chemical, serological and immunological studies.

Protein content of the antigens were quantitated by Bradford method. The average protein content in the heat inactivated crude extract, KSCN extract and sonicated antigens from R_9S were found to be 1.2 mg, 2.3 mg and 2.8 mg/ml antigen respectively as against the corresponding values of 1.0 mg, 2.3 mg and 2.7 mg antigen for $R_{23}S$ strain and 0.9 mg, 2.1 mg and 2.5 mg for P-52 strain. The sonicated preparation from

all the three strains invariably contained high amount of protein.

Carbohydrate content of the antigens was estimated by phenol-sulfuric acid method. The concentration of carbohydrate in heat inactivated crude extract, KSCN extract and sonicated antigens were respectively 650 ug, 920 ug and 970 ug per millilitre in the case of R_9S, 610 ug, 950 ug and 960 ug/ml in case of $R_{23}S$ and 590 ug, 910 ug and 930 ug/ml in the case of P-52.

The protein profiles obtained with each form of antigen from the three strains of P. multocida by Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophoresis were almost comparable in their position. The four protein bands demonstrated with heat inactivated crude extract antigen from the three strains were with molecular weights lesser than 68 kDa. These four bands were uniformly present with KSCN extract and sonicated antigens obtained from the three strains also. The KSCN extract antigen from different strains revealed two additional bands of which one was with molecular weight higher than 68 kDa. The sonicated antigen presented one additional protein band than that of KSCN extract with a molecular weight lesser than 68 kDa.

Antiserum against each antigenic preparation obtained from different P. multocida strains was raised in pasteurella free NewZealand white rabbits aged 3-4 months and used for further serological studies.

Agar Gel Precipitation Test was used to study the antigenic relationship between strains. Each antiserum was tested against the three homologous and two heterologous antigens and was found to produce multiple precipitin lines. The lines produced by the homologous system were shown to be identical with the lines produced by the heterologous antigens. The sonicated antigen invariably presented more number of precipitin lines when compared to the other two forms of antigen. The results obtained in AGPT indicated close serological relationship between the two rabbit strains than the bovine strain P-52.

On immunoelectrophoresis, the three forms of antigen from each strain of P. multocida when reacted with homologous/heterologous antiserum developed multiple precipitin arcs. The preciptin arcs presented by the homologous and heterologous system were comparable in their number and position. In this test also the sonicated antigen developed maximum precipitin arcs compared to the other forms of antigen.

For indirect haemagglutination test, three antigen preparations of the strains R_9S, $R_{23}S$ and P-52 viz. heat inactivated crude extract, KSCN extracted and sonicated antigen were adsorbed onto either GA-SRBC or T-GA-SRBC which were specifically agglutinated by rabbit antiserum against each antigen preparation. Antibody titration was done with the antiserum employing the SRBC sensitized with homologous and heterologous antigen preparations.

GA-SRBC sensitized with heat inactivated crude extract from R_9S, $R_{23}S$ and P-52 showed the antibody titres of 2048, 4096 and 2048 with their respective homologous antiserum. The KSCN extracts from the three strains were sensitized with T-GA-SRBC gave a titre of 4096, 8192 and 4096 with their homologous antiserum respectively and the GA-SRBC sensitized sonicated antigen from the three strains gave a titre of 4096, 8192 and 4096 with their respective antiserum. But the heterologous antiserum from the three strains showed a cross-titre ranging from 32 to 1024 on testing with the three sensitized antigens from the three strains. Of which highest cross-titre was given by the antiserum raised against sonicated antigen from $R_{23}S$ strain only.

Among the three strains, the $R_{23}S$ gave highest antibody titres against all types of antigen. Though the KSCN extract and sonicated antigen showed same antibody titre,

antiserum raised against sonicated antigens that too from $R_{23}S$ strain gave good cross-titre indicating superiority of this antigen for serological survey of P. multocida infection in rabbits.

Potency of the four antigens viz., heat inactivated crude extract, KSCN extract, sonicated antigen and sonicated antigen with adjuvant from three strains were tested in mice by injecting two doses of vaccine at 14 days interval and challenging on 21st day. Invariably the antigens conferred significant homologous protection and an insignificant cross-protection.

The rate of protection in mice vaccinated with heat inactivated crude extract, KSCN extract, cell sonicate and sonicated antigen with Freunds Complete Adjuvant (FCA) of R_9S strain on challenging with the homologous were 67, 100, 100 and 83 per cent respectively. On the other hand, when such vaccinated mice were challenged with the heterologous strains $R_{23}S$ and P-52, the survival rate ranged between zero to 17 per cent. The survival rate of 50, 83, 100 and 83 per cent were observed in mice which were vaccinated with heat inactivated crude extract, KSCN extract, cell sonicates, and sonicated antigen with FCA from strain $R_{23}S$ when challenged with the virulent homologous strain. The vaccinated mice when challenged with R_9S and P-52 strains, a very low survival rate

of 0 to 33 per cent was observed and such cross-protection was between rabbit strains, in the mice vaccinated with KSCN extract and sonicated antigen. The results of the potency test with the antigens of P-52 strain indicated that the immunogens gave a 50, 83, 100 and 67 per cent homologous protection when challenged with P-52 strain and on heterologous challenging conferred a meagre protection of 0 to 33 per cent in the vaccinated mice.

The sonicated antigen which was used as immunogen in the potency test included more serologically active moieties as evidenced in the results obtained with AGPT, immunoelectrophoresis and IHA. The results of potency test also substantiated the above point as there were 100 per cent protection of vaccinated animals against challenge infection with homologous strains. But mice which were vaccinated with sonicated antigen could not resist infection with heterologous rabbit strains or bovine strain of P. multocida. These observations further suggest that the rabbit strains of P. multocida under study, though found to be immunologically distinct are serologically comparable and apparently related.

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ANTIGENS OF *Pasteurella multocida* ISOLATES FROM RABBIT AND THEIR IMMUNOGENICITY

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ABSTRACT

Two rabbit strains viz. R_9S and $R_{23}S$ and a bovine vaccine strain P-52 which were maintained in virulent form, were used for the preparation of three forms of antigen viz., heat inactivated crude extract, KSCN extract and sonicated antigen. These antigens were chemically analysed for protein and carbohydrate contents and were found to be higher in the sonicated antigen preparation irrespective of the source. In SDS-PAGE analysis, the protein profiles discerned by heat inactivated crude extract, KSCN extract and sonicated antigens were four, five and six protein bands with molecular weights lesser than 68 kDa while the KSCN extract and sonicated antigen presented an additional protein band with molecular weight higher than 68kDa.

Three types of antigen of *P. multocida* were characterized and analysed for the inter relationship and the immunogenic potential in mice.

Antiserum was raised against each antigenic preparation from the three strains in rabbits and used for serological study. In AGPT and immunoelectrophoresis the serum developed multiple precipitin lines and arcs respectively when reacted against the three homologous and two

heterologous antigens in which a few were identical to the heterologous antigens. The results revealed stronger serological relationship between the two rabbit strains than with the cattle strain and the heterogeneity of the sonicated antigen. The antibody titre in each antiserum was measured by IHA using the sensitized GA-SRBC/T-GA-SRBC and the titres were more in the homologous antiserum and high titre for the heterologous serum was seen with the sonicated antigen.

The LD_{50} determined for the three strains R_9S, $R_{23}S$ and P-52 was found to be 3×10^4, 3×10^3 and 3×10^5 bacteria. Immunogenic potential of the three antigens and an adjuvanted sonicated antigen were tested in mice by giving two doses of vaccine at 14 days interval and challenging on 21st day with homologous and heterologous strains. A higher percentage of protection was conferred by homologous strains and it was cent per cent (100%) with sonicated antigen. The percentage of protection against challenge with heterologous strains was low. An elaborated study on immunity trials with these immunogens is needed before recommending the $R_{23}S$ as a candidate vaccine strain.