CHARACTERISATION OF *Pasteurella multocida* ISOLATES FROM RABBITS

By

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THESIS

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Dedicated to

My loving Parents

&

My dear Brothers

DECLARATION

I hereby declare that this thesis entitled **"Characterisation of Pasteurella multocida isolates from rabbits"** 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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CERTIFICATE

Certified that this thesis entitled **"Characterisation of Pasteurella multocida isolates from rabbits"** is a record of research work done independently by **Miss Sheela Yohannan** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship, or associateship to her.

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Introduction

INTRODUCTION

In India, as in the case of any other developing country, the animal produce to meet the protein requirement of human population is far below the actual need. Hence, many thrust areas for augmenting the quantum of animal produce have been explored by farmers and scientists. Rabbit farming is one such field which is considered as more remunerative supplementary occupation for betterment of the economic status of marginal and small scale farmers. The attributes of rabbit are:

1. High fecundity and prolific nature
2. Relatively high feed conversion efficiency
3. Good demand and better price of meat/skin

Indeed, the rabbit production could be boosted by introducing exotic germplasm of fast growing broiler breeds like Grey Giant, Soviet Chinchilla, New Zealand White etc. But this enhanced production potential on account of improved genetic make up could be maximally exploited only if optimum health coverage is provided.

In general, animals with high productivity when shifted from its natural habitat to which they have been acclimatised for generations, are found to be more prone to

various diseases, causing heavy economic loss due to mortality and poor growth rate especially when reared under intensive farming system. Rabbit, is no exception to the above.

Of the various afflictions of rabbit, Pasteurellosis is a major bacterial infection which takes a heavy toll, sometimes depopulating the entire farm, if the infection happens to be of fulminating type. The clinical manifestation of P. multocida infection in rabbits are suppurative rhinitis, otitis media, enzootic pneumonia, conjunctivitis, pyometra, orchitis, subcutaneous abscess and septicaemia of which suppurative rhinitis and enzootic pneumonia are the most prevalent form of infection.

Eventhough high incidence of Pasteurellosis in rabbits has been on record, the epidemiological informations pertaining to the factors predisposing an outbreak, different strains increminated in outbreaks at various localities, the mode of perpetuation of infection and the possibility of evolving a vaccine strain from locally prevalent strains for prophylaxis are scanty.

Our knowledge on serology and immunology of P. multocida is largely from the investigation of Pasteurellosis in other species particularly cattle and birds.

Though this infection is amenable to antibiotic treatment, the prophylaxis, or the adage, "prevention is

better than cure", is the promising and feasible approach to curtail the economic loss due to Pasteurellosis.

On perusal of available literature, it is evident that the killed or attenuated vaccine prepared in the line of haemorrhagic septicaemia or fowl cholera could not confer absolute protection to rabbits against homologous or heterologous serotypes of P. multocida.

Though P. multocida infection have been reported to be prevalent in many rabbitries in the state, there has been no documentation of the possible differences in locally prevalent strains with respect to their virulence, pathogenicity, antigenic configuration and other characters.

A better understanding of the aforesaid aspects will facilitate chalking out control measures to curb Pasteurellosis in rabbits.

In view of the above circumstances, the present work was undertaken giving emphasis on:

(i) Isolation of local strains of P. multocida from rabbitries.

(ii) Characterisation of isolates to elucidate their identity.

(iii) Pathogenicity studies in order to understand the differences, if any, in the degree of virulence between the various isolates.

(iv) Serological test to find the differences in the antigenic make up of various strains isolated.

(v) Antibiogram of the isolates to select the suitable antibiotics for treatment.

Review of Literature

REVIEW OF LITERATURE

History and nomenclature

Perroncito (1878) was the first to isolate and describe an organism named bacillus of fowl cholera which is an acute to chronic disease of chicken, turkey, goose, duck and wild fowl. The most complete description of fowl cholera and the organism was made by Pasteur in 1880. Subsequently, several others have reported the isolation of similar organisms from cattle (Bollinger, 1878), rabbit (Davaine, 1880; Gaffky, 1881) and pig (Loeffler and Schutz, 1882).

Kitt (1885) made a comparative study of the organisms producing fowl cholera, rabbit septicaemia, swine septicaemia and septicaemia of cattle and wild animals and found that they were similar in many respects and referred to them as bacterium bipolare multocida. These organisms were later redesignated with the generic name Pasteurella in recognition of the voluminous work carried out by Louis Pasteur on the causative agent of fowl cholera.

Trevisan (1887) assigned the name Pasteurella for the bipolar stained organisms from septicaemia in animals. Flugge (1896) named the Haemorrhagic septicaemia organisms of bovine

and swine origin as Bacillus boviseptica and Bacillus suiseptica respectively. Since different isolates from divergent species had common characters, Lignieres (1900) assigned the generic name Pasteurella to these organisms with an indication of species of animals from which they were isolated.

According to Topley and Wilson (1936), Pasteurella septica was the most suitable name to indicate bacteria of septicaemia. Rosenbusch and Merchant (1939) named the typical Haemorrhagic septicaemia organisms as Pasteurella multocida (P. multocida) after comparing all the earlier names assigned to this bacteria.

Isolation and media

Pasteurella grow well on blood agar (Carter, 1967) and this medium is routinely used for the isolation of P. multocida from clinical specimens. But blood agar is not satisfactory for the identification of colonial variants. Carter (1967) found Brucella agar containing two per cent hemolysed rabbit serum satisfactory. Other medias used were Tryptic Soy blood agar and Tryptose blood agar containing five per cent sheep or bovine blood (Carter, 1967). When clinical specimens are contaminated with other organisms such as proteus species, the

swarming by proteus can be avoided by using blood agar containing four per cent agar.

Namioka and Murata (1961a) described a solid medium called Yeast Proteose Cystine (YPC) agar which they found well suited for demonstrating colonial morphology. This medium is composed of yeast extract, proteose peptone, L-cystine, glucose, sucrose, sodium sulphite, agar and potassium diphosphate.

Heddleston et al. (1964) reported that Dextrose starch agar can be used as a solid medium for the study of colonial morphology of P. multocida.

It was suggested by Neter and Dryja (1981) that the antibiotic nafcillin might be useful in the selective media for P. multocida, as it is resistant to nafcillin.

A selective medium for the isolation of P. multocida and Bordetella bronchiseptica has been described by De Jong and Borst (1985) which contains Tryptose Soy Agar, defibrinated sheep blood five per cent, Gentamycin sulphate, potassium tellurite, amphotericin-B and bacitracin. This medium when compared with mice inoculation and with the modified MacConkey's medium was found to be more efficient in the isolation of toxigenic strains of P. multocida from nasal swab.

According to Smith and Phillips (1990) P. multocida grows at a temperature between 12 and 43°C with an optimal temperature of 37°C, but Burrows and Gillett (1966) has suggested that the nutritive requirement of P. multocida is more exacting at 37°C than at a lower temperature. Targowski and Targowski (1979) isolated a Pasteurella strain from a rabbit abscess with an unusual growth requirement. This grew copiously only on media supplemented with serum and incubated in the candle jar. Under anaerobic condition growth was moderate on enriched media but it did not grow on unsupplemented media under these conditions or in an aerobic environment on enriched media.

Identification

Morphology, growth and colony characters

According to Mannheim (1984), as given in the first edition of Bergey's Manual of Systematic Bacteriology, Volume 2, the Genus Pasteurella comprises the species Pasteurella multocida, Pasteurella pneumotropica, Pasteurella haemolytica, Pasteurella urae, Pasteurella aerogenes and Pasteurella gallinarum. It is described as coccoid to rod shaped cells, gram negative, nonmotile, aerobic, facultative anaerobic, nitrate reducing, oxidase and catalase positive. According to Smith and Phillips (1990), P. multocida shows

bipolar staining when taken from animal tissues or smooth colonies and tend to become bacillary in nature when taken from rough colonies.

Dekruif (1921, 1922, 1923) and Webster and Burn (1926) demonstrated the existence of three colonial variants viz., (1) the smooth form which grows diffusely in broth and forms smooth and iridescent colonies on serum agar and is virulent for rabbits, (2) the rough form which gives a granular deposit in broth and form transluscent bluish colonies on serum agar is completely avirulent for rabbits, and (3) the mucoid form, which is of intermediate virulence.

Carter (1955), Smith (1958) and Namioka and Murata (1961b) reported that the highly virulent smooth form contains a type specific polysaccharide capsular antigen. The mucoid form is rich in hyaluronic acid, and may or may not possess a polysaccharide capsular antigen in addition. The rough form has neither a capsular nor a mucoid antigen.

Carter (1957) and Biberstein et al. (1958) have reported that P. multocida dissociates readily producing different colonial variants. Carter (1957) has reported that P. multocida commonly occurs as mucoid, smooth and blue variants. Smith (1958) found that the colonies of capsulated smooth strains were iridescent in obliquely transmitted light.

The variations in colony morphology and biochemical properties of P. multocida isolates from single animal facility were also reported by Lu et al. (1978) and Brogden (1980).

Carter (1967) recognised two forms of smooth colony - an S and SR. The S form is capsulated whereas SR form was like the rough form.

Webster and Baudisch (1925) found that the smooth variant of P. multocida would not grow aerobically in plain broth unless large number of organisms were inoculated but it could grow from a small inoculum, if a trace of rabbit blood or an iron compound with strongly catalytic properties was added to the medium and lower oxygen tension was provided.

According to Jordan (1952) aerobic growth of P. multocida was improved with supplementation of haematin or casein.

Priestley (1936) stated that an envelope like substance or an inconspicuous capsule was present only in virulent strains and Carter (1958) further proved this by experiments. The chemical composition of the envelope like substance was reported to be either hyaluronic acid or polysaccharide by Smith (1958) and it was shown to be distinct from the somatic substance.

Biochemical properties

Cowan (1974) reported that the primary biochemical tests for the identification of P. multocida are the test for catalase, oxidase, production of acid from glucose, and oxidative/fermentative utilisation of glucose.

The primary biochemical tests for identification of P. multocida as described by Buxton and Fraser (1977) are the growth on MacConkey's Agar, Hemolysis on blood agar, production of Indole, Hydrogen sulphide and the Urease activity.

According to Mannheim (1984), in the first edition of Bergey's Manual of Systematic Bacteriology Volume 4, the first stage table for the identification of P. multocida includes test for beta-hemolysis, growth on MacConkey's Agar, Indole production, Urease activity, gas production from carbohydrate and acid production from lactose and mannitol.

The second stage table for the identification of P. multocida as described by Cowan (1974) are the growth on MacConkey's Agar, growth in potassium cyanide, acid production from carbohydrate such as arabinose, lactose, maltose, mannitol, raffinose, salicin, sorbitol, sucrose, trehalose and xylose, beta-galactosidase activity, ornithine decarboxylase activity, nitrate reduction, nitrite reduction, production of indole and hydrogen sulphide.

Heddleston (1976) studied the physiologic characteristics of 1,268 cultures of P. multocida from various parts over a period of 10 year. He reported that 97-100 per cent of his isolates fermented lactose, glucose, mannitol, mannose, fructose and sucrose, produced hydrogen sulphide and indole, reduced nitrate and 6-91 per cent fermented arabinose, glycerol, sorbitol, trehalose and xylose. Fermentation of dextrin, inositol, inulin, lactose, maltose, raffinose, rhamnose and salicin, growth on MacConkey's agar, change of litmus milk, production of urease, hemolysis, liquefaction of gelatin, and motility were negative for 97-100 per cent of cultures. All were catalase and oxidase positive.

Smith and Phillips (1990) reported that all strains of P. multocida produced acid but no gas in glucose and sucrose. Most strains fermented galactose, mannitol, mannose, sorbitol and xylose. Strains of P. multocida were without action on litmus milk and gelatin. They produced indole, reduced nitrate and formed a small quantity of hydrogen sulphide as detected by lead acetate paper. The MR and VP reactions were both negative. The catalase and oxidase reactions were both positive though rather weakly so. Methylene blue was reduced by P. multocida. This did not utilise citrate as the sole source of carbon. Urea is not decomposed by it. Most strains were positive for ornithine decarboxylase but negative for

lysine, glutamic acid decarboxylase and arginine dihydrolase and were resistant to potassium cyanide.

The biochemical properties of 32 P. multocida isolates obtained from nares of healthy rabbits and rabbits with pneumonia were reported by Lu et al. (1978) in which majority of the isolates produced acids from glucose, mannitol, sucrose, sorbitol, arabinose, raffinose, maltose, salicin and trehalose but none from lactose. Only 15 isolates produced indole. There was no hydrogen sulphide production, urease activity or gelatin liquefaction. MR test was negative. Oxidase and catalase reactions were positive.

Brogden (1980) studying P. multocida cultures from rabbits reported that all cultures of P. multocida fermented glucose, sucrose, mannose, mannitol, galactose, levulose but not arabinose, raffinose, rhamnose, dextrin, inulin, salicin and inositol. A few cultures fermented lactose, maltose, trehalose, dulcitol, xylose, sorbitol and glycerol. Carbohydrate fermentation occurred within 18-24 h. However, some cultures slowly fermented the carbohydrates requiring the reactions to be read after three days of incubation. Forty one of 48 cultures produced indole and seven cultures did not, even when retested daily for five days. Forty of the 44 cultures produced detectable amount of hydrogen sulphide within 14 days. No liquefaction of gelatin, production of

urease or change in litmus milk was noted in any culture. None of the cultures grew on MacConkey's agar or exhibited motility in semi solid media. All isolates reduced nitrate.

While characterising rabbit isolates of P. multocida, Kawamoto et al. (1990) reported that all their 40 isolates were negative for urease and gelatinase activity while all reduced nitrate. Thirty four of the 40 isolates produced indole. Glucose, sucrose, mannose, xylose, mannitol, sorbitol, galactose, glycerol, and levulose were fermented by all isolates, but not lactose, maltose, trehalose, arabinose, dulcitol, rhammose, dextrin, inulin, salicin and inositol. Only eight of the 40 isolates fermented raffinose.

Antigens and serological characterisation

Pirosky (1938) extracted polysaccharide antigen from smooth cultures of P. multocida and demonstrated their serologic specificity by means of a precipitation test. He suggested that the specificity of the antigens might help in the serologic grouping of the species. Rosenbusch and Merchant (1939) divided their strains into three principal groups and designated I, II and III, on the basis of fermentation of xylose, arabinose and dulcitol and by means of a conventional agglutination procedure. Considerable cross agglutination reaction limited further use of this technique.

The first serologic classification to gain any acceptance was that of Little and Lyon (1943). By slide agglutination test, they divided P. multocida strain into three serologic types 1, 2 and 3. There was sufficient evidence to show that strains, could not be reliably categorised by simple agglutination procedure (Rosenbusch and Merchant, 1939; Carter, 1958; Namioka and Bruner, 1963).

Roberts (1947) employed serum protection test in mice to classify strains of P. multocida into four principal groups which he called Types I, II, III and IV. He observed that the strains of P. multocida causing haemorrhagic septicaemia in cattle and water buffaloes belonged to Type I. In addition to the above four types he also noted the prevalence of antigenic variants.

After attempting unsuccessfully to group the strains by a conventional agglutination procedure and the slide agglutination method of Little and Lyon (1943), Carter (1952) identified three groups or types of P. multocida by means of a precipitin test employing saline extracted capsular substances as antigen. The precipitation test which he described required considerable amounts of serum and antigen, and more over cross reaction were often recorded.

Carter (1955) demonstrated Indirect Haemagglutination

test (IHA) as to be reliable and superior over precipitation test for serological characterisation of P. multocida, as IHA overcame all the shortcomings of the precipitation test. In this procedure, he adsorbed capsular soluble antigen onto the human O erythrocytes and this sensitised erythrocytes were used for haemagglutination test with specific antiserum. Initially by IHA, he could determine four capsular types of P. multocida viz. A, B, C and D. He further observed that strains of type C were not a capsular type as the titers of type C were always low and thus the type was dropped.

Carter (1961) had further identified nine strains of P. multocida recovered from cases of bovine haemorrhagic septicaemia in Central Africa, as members of a new capsular type and designated as type E.

Namioka and Murata (1961a) described a simplified slide agglutination procedure for capsular typing of P. multocida and compared with that of haemagglutination procedure. They reported that the results of slide agglutination studies paralleled those of haemagglutination. Norrung (1963) had also adopted the slide agglutination procedure for typing 45 strains of P. multocida. Out of the 45 strains typed, 25 strains were type A, nine were type D and the rest were not typable.

Characterisation of somatic antigen

The serologic feature of the somatic antigens of P. multocida was not known prior to the studies of Namioka and associates. Namioka and Murata (1961b) have used agglutination tests for characteristion of somatic antigen of P. multocida employing formalinised, heated and alcohol treated antigens. They obtained reliable and constant results when smooth and mucoid cultures were treated overnight with 1N Hcl. The treated cells were readily agglutinable and were employed in a conventional tube agglutination procedure.

Namioka and Murata (1961c) proposed that serotypes of P. multocida could be identified by the numericals, representing the specific somatic or O component, followed by the english alphabet representing the specific capsular or K substance. They had further showed that a single capsular type of P. multocida may have multiple somatic antigens. In all, 11 somatic varieties of P. multocida were identified for different capsular types by Namioka and Murata (1964).

Heddleston et al. (1966) showed for the first time that the gel diffusion precipitin test could be used to demonstrate specific somatic antigens from two strains of P. multocida associated with fowl cholera when specific antisera was raised in chickens.

Carter (1972) treated P. multocida of avian origin with hyaluronidase to make them agglutinable for the identification of their somatic antigen.

Bapat and Sawhney (1972) and Manning (1984) reported that rabbit could be used for raising specific antisera against somatic antigen of P. multocida and they observed that sufficient level of antibody was produced only after 70 to 75 days of antigen administration by parenteral route.

Heddleston et al. (1972) had further used heat stable antigen extracted with formolinised saline in gel diffusion precipitin test to group 258 field isolates of P. multocida from fowl cholera. The isolates could be grouped into five serotypes viz., Type 1, 3, 4, 5 and 6. Further they have compared the behaviour of heat stable antigens by gel diffusion and immuno electrophoresis with partially purified lipopolysaccharide protein complex (endotoxin). The successful application of the above gel diffusion precipitin test for serotyping cultures of P. multocida regardless of the host species from which they were isolated were reported by Blackburn et al. (1975) and Heddleston and Weissman (1975).

Antigenic make up

The antigenic make up of strains of P. multocida have been studied by a number of investigators but the results

often have been difficult to interpret because different serologic varieties have been studied by different methods (Carter, 1967).

Carter and Annau (1953) analysed the type specific capsular polysaccharide of P. multocida and have shown it to contain hyaluronic acid by depolymerisation studies. At the same time Bain (1953) recovered a viscous polysaccharide comprising equal amounts of hexosamine and uronic acid from Type II and Type IV (Roberts).

The capsules of mucoid type A strains which could be greatly disintegrated by staphylococcal hyaluronidase has been reported by Carter (1958) and Mannheim (1961). They have observed that the presence of capsular hyaluronic acid could be the reason for the inagglutinability of many freshly isolated strains of P. multocida in serological tests. More evidence for the presence of copious amounts of hyaluronic acid and several other acidic capsular polysaccharides were reported by Cifonelli et al. (1970) and Maheswaran et al. (1973).

The chemical nature of the antigenic moiety of the capsular substances of P. multocida strains were studied by several investigators and reported to be monosaccharides (Bain, 1955), protein - polysaccharide complex (Briefman and

Yaw, 1958), protein - polysaccharide-lipopolysaccharide complex (Knox and Bain, 1960) and glycolipid (Perreau and Petit, 1963).

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

The principle antigenic components which are involved in IHA capsular serotyping and gel diffusion precipitin somatic typing methods were identified to be one and the same lipopolysaccharide (Knox and Bain, 1960; Carter and Rappay, 1963 and Brogden and Rebers, 1978).

Antigenic complexity of P. multocida was elucidated by immunoelectrophoretic analysis by Bhasin and Shaw (1980) and they demonstrated at least 55 cytoplasmic and 19 cell envelope associated antigens. These antigens were also shown to share

common antigen with gram negative bacteria (Prince and Smith, 1966).

Occurrence of capsular types in relation to host and disease

When several hundred strains of P. multocida from a number of animals were capsular typed, it was observed that type A and D were widely distributed in divergent host species, while type B and E were mainly from cattle, bison and water buffaloes (Carter, 1967). Avian, swine and human strains of P. multocida were either type A or D when capsular typed by IHA test (Perreau et al., 1962; Carter, 1962; Carter, 1967).

Somatic antigens

Somatic antigens of P. multocida were identified by agglutination test/Gel diffusion precipitin test and found that different capsular types possessed any one or more somatic antigens belonging to 11 major O antigens (Namioka, 1978; Manning, 1982).

Rabbit isolates of P. multocida

Carter (1967) has reported that of the 27 rabbit isolates, 11 were type A; 3 type D and 13 were not typable by IHA. While Lu et al. (1978) recovered a high percentage

(67 per cent) of type A strain and five per cent type D strain of P. multocida, when they examined 42 isolates of P. multocida from rabbits. However, 29 per cent of the isolates were not typable. In another study, Chengappa et al. (1982) reported that of 79 isolates, 74 were type A and five type D.

Forty-eight cultures of P. multocida from rabbits were typed by gel diffusion precipitin test for their somatic types and the result revealed that serotypes 3 and 12 were the most prevalent accounting for 25 per cent and 67 per cent of the isolates, respectively (Blackburn et al., 1975).

Mushin and Schoenbaum (1980) has reported that out of 51 isolates of P. multocida from rabbits, 94 per cent failed to form major type specific precipitin reactions with any of the 16 P. multocida reference serotypes. Three isolates were identified as serotype 3, whereas eight isolates reacted slightly with antisera from other serotypes.

Brogden (1980) have reported the serological characteristics of 48 rabbit cultures of P. multocida collected from a variety of geographic locations over a 56 year period by using the gel diffusion precipitin test. Heat stable antigens from these 48 cultures reacted with the antisera prepared from P. multocida type cultures representing serotypes 1, 3, 4, 12 and 15. Antigens from 15 cultures

reacted with antisera from more than one serotype. Gel diffusion precipitin reactions involving serotypes 3 and 12 were the most prevalent.

Chengappa et al. (1982) found that 84 per cent of the 79 isolates of P. multocida from rabbits were of serotype 12. DiGiacomo et al. (1983) reported that of 29 isolates of P. multocida from rabbits, 93 per cent were type 12 while antigens from 17 cultures reacted slightly with antisera from other serotypes.

Infection in animals

Pasteurellosis, an important disease due to P. multocida is fowl cholera in domestic and wild fowl, haemorrhagic septicaemia in cattle and water buffaloes, primary or secondary pneumonia in a number of species and sporadic infections of a wide range of animals (Carter, 1967).

The severity of the disease was reported to vary from inapparent carrier state to septicaemic death, chronic abscessation and suppuration to acute fatal epizootic infection (Iliev et al., 1963; Krishnamurthy and Kaushik, 1965; Young, 1965) depending on the species of animals affected and the virulence of the serotype involved.

Members of the genus Pasteurella with the exception of a few are commensals on the mucous membrane of vertebrate animals (Smith, 1955; Carter, 1967; Biberstein, 1990).

Biberstein (1990) observed that many of the pasteurella have pathogenic potential which may produce disease under condition of immunodeficiency and stress.

Pasteurellosis in Rabbit

The clinical forms of Pasteurellosis in rabbit include rhinitis (snuffles), conjunctivitis, abscess, otitis media, pneumonia, pyometra, orchitis and septicaemia (Hagen, 1958 and Flatt, 1974).

The primary locus of infection can occur in almost any tissue and spread to any other tissue although the nasal passage is the most common atrium of infection. The infection ranges from inapparent carrier animals, per acute to septicaemic death, chronic abscessation and suppuration in adult rabbits. Acute enzootic pneumonia is the common clinical syndrome in young rabbits. Neonates was reported to die from septicaemia (Harkness and Wagner, 1989).

The mortality rate in rabbits has been reported to range from 25 to 50 per cent by Ward (1973).

Smith (1927) reported that snuffles was one of the most commonly observed disease in domestic rabbits and the morbidity ranges from 20 to 70 per cent. Webster (1924) reported seasonal incidence of snuffles with peaks in the fall and spring and the lowest being 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 observation of Hagen (1958) on the transmission of P. multocida was that the offsprings gets infection from the dam via the respiratory route shortly after birth. He also stated that the etiological agent spreads by direct contact as well as by air borne means.

Hagen (1966) reported the isolation of P. multocida from 69 per cent of rabbits maintained in an environment where enzootic pasteurellosis was prevalent, while Lu et al. (1978) could isolate P. multocida from only 30 per cent of rabbits, when their respiratory tracts were culturally screened.

Kluger and Rothenburg (1979) observed that when the rabbits are infected with P. multocida, the concentration of iron in the plasma decreases and rectal temperature increases.

The natural mode of infection with P. multocida in rabbits was studied by DiGiacomo et al. (1983). This study revealed that nares of rabbits were not colonised before weaning at eight weeks of age, regardless whether the does were infected or not. The earliest nasal colonisation was detected at 12 weeks of age and 23 per cent of the rabbits were reported to be colonised at 22 weeks of age. They further reported that 72 per cent of the breeding colony of adult rabbits had P. multocida colonisation in their nares and the majority of isolates belonged to somatic type 12.

P. multocida was isolated by Kunstyr and Naumann (1985) from pus and nasal mucous membrane of 18 New Zealand White rabbits having torticollis, otitis and empyema of one or both ears.

Nakagawa et al. (1986) observed the prevalence of P. multocida in rabbits belonging to breeding colonies and that the isolation rate 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.

When upper respiratory tract disease of rabbits were investigated by DiGiacomo et al. (1987b), P. multocida was found to be invariably involved in atrophic rhinitis and turbinate atrophy and the serotype involved was identified to be 12:A.

Pasteurellosis and coccidiosis were indentified and reported by Nair et al. (1987) as two important problems responsible for mortality in rabbits reared in Kerala state. Devi et al. (1990) further reported pasteurellosis as the most important single disease which caused highest mortality in rabbits over six weeks of age.

The natural infection with P. multocida and B. bronchiseptica in domestic rabbits was studied by Deeb et al. (1990). They observed that at weaning stage, 25 per cent of the rabbits had nasal colonisation with P. multocida and 75 per cent had colonisation with B. bronchiseptica. At two to four months of age about 50 per cent of rabbits had dual population of both the residents P. multocida and B. bronchiseptica.

The aetiology and pathology of pulmonary abscesses in rabbits slaughtered at an abattoir in Northern Italy was reported by Guarda et al. (1991). They observed the association of P. multocida in several instances.

Pathogenicity

Though the pathogenicity of P. multocida vary for different animal species, mice and rabbits were frequently used to isolate P. multocida from overtly contaminated materials as the species were highly susceptible to experimental infection (Carter, 1967).

Rabbit

Flatt and Dungworth (1971) investigated the development and regression of lesions in lungs of rabbits intratracheally instilled with P. multocida. They reported that on experimental infection, P. multocida could produce pneumonia, identical to the naturally occurring disease. Experimental infection could produce death of five rabbits out of 18 intratracheally instilled with P. multocida. The experimental infection began with acute catarrhal broncho-pneumonia which spread very rapidly to produce fibrinopurulent and necrotising pneumonia and finally resulting in fatality.

According to Watson et al. (1975) intra-nasal instillation of P. multocida into specific pathogen free rabbits produced only a mild mucopurulent nasal discharge, even after four to six days post inoculation. Out of the nine rabbits inoculated, four rabbits failed to develop clinical signs. The gross and microscopic lesions did not differ in

character or distribution among the inoculated rabbits. The infection was characterised by an acute upper respiratory syndrome accompanied by mild broncho-pneumonia.

Chengappa et al. (1980) observed that intra-nasal instillation of rabbits with P. multocida serotype 12:A could neither develop gross evidence of pneumonia nor death.

The pathogenic potential of P. multocida serotype 3:A and serotype 12:A were compared in rabbits after experimental inoculation by Lu and Pakes (1981). They observed that serotype 3:A P. multocida was more virulent than serotype 12:A which indicated that the pathogenicity and virulence of rabbit isolates of P. multocida were associated with serotypes and other unknown factors of the organism.

Lu et al. (1982) studied the pathogenic effect of P. multocida serotype 12:A in normal rabbits and rabbits stressed with administration of hydrocortisone. Rabbit stressed with hydrocortisone developed extensive pneumonia than rabbits not treated with hydrocortisone.

The histopathological changes caused by P. multocida at the upper respiratory tract including the nasal bones were described by DiGiacomo et al. (1991). The changes detected were mainly in the epithelial cells, lamina propria and the periosteum.

Mice

Smith (1958) observed that canine strains of P. multocida possessed low virulence for mice while cat strains, possessed somewhat higher virulence which indicated that the strains of P. multocida differ considerably in their pathogenic potential to mouse depending on the species of origin.

The pathogenicity of two serotypes 1:A and 5:A for chickens and mice were tested by Murata et al. (1964) and reported that serotype 1:A was not pathogenic for chicken while serotype 5:A was, and that both serotypes were pathogenic for mice.

Collins (1976) employed mice as a choice of animal for testing pathogenicity of P. multocida, and reported that an overwhelming increase in the number of P. multocida in visceral organs was the cause of death of mice when experimentally inoculated.

Twenty strains of P. multocida isolated from rabbits were investigated for their pathogenicity in mice by Okerman et al. (1979). They observed marked differences in subcutaneous LD 50 for mouse and also differences in virulence according to the different colony types. P. multocida isolated from septicaemic form of disease was shown to be highly virulent when compared to isolates from typical snuffles.

Mushin and Schoenbaum (1980) observed that strains of Heddleston's serotype 3:A were lethal for mice within 24 h while untypable strain differed in virulence.

Lethality of 22 isolates of P. multocida for 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 many of the isolates were usually virulent for mice. The bacteria were recovered from liver and spleen of dead mice.

Immunity

Eversince, the report of Louis Pasteur (1881), the use of live attenuated bacteria as vaccine to prevent fowl cholera, several other vaccines of various kinds have been used to immunise farm animals against Pasteurellosis.

Rau and Govil (1950) used suspension of P. multocida organisms lysed by saponin as vaccine in cattle and buffaloes. They observed that a heavy concentration of organism was required for a good immune response.

Bain and his associates were instrumental for developing a killed bacterial oil adjuvant vaccine which was

able to confer solid immunity in cattle and buffaloes upto 12 months (Bain and Jones, 1958; Knox and Bain, 1960, 1963). Even now oil adjuvant vaccine is considered as the most effective immunising agent for prevention of haemorrhagic septicaemia in animals. Formalin killed bacteria was widely used for the prevention of fowl cholera. Heddleston and Hall (1958) compared the immunising efficacy of oil adjuvant aqueous suspension, alum precipitated and chicken embryo vaccine and found that adjuvant vaccine was superior to others. Bierer and Scott (1969) reported the use of attenuated P. multocida as a commercial vaccine for the prevention of fowl cholera.

Heddleston et al. (1970) reported that turkeys vaccinated with killed P. multocida could be protected against homologous but not heterologous challenge.

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

The success in preventing fowl cholera with avirulent, high temperature dependent mutant was reported by Maheswaran et al. (1973).

A polyvalent inactivated vaccine against P. multocida was reported to be effective in reducing the mortality in young and adult rabbits when inoculated subcutaneously (Galassi and Giulani, 1971).

A protection rate of 57-90 per cent was observed in rabbits vaccinated with pig Pasteurellosis vaccine by the intra-muscular route (Tacu, 1971).

Oldenburg et al. (1975) prepared a formolised vaccine from cultures of Bordetella bronchiseptica and P. multocida isolated from the lungs of rabbits in two commercial farms in Berlin.

An oil adjuvant vaccine prepared from Pasteurella isolates by Zaher et al. (1976) protected rabbits against challenge with a highly virulent strain one month later and conferred a high level of immunity for upto a year under field conditions.

Morisse (1979) compared the efficacy of three vaccines, namely (1) a preparation of ribosomes and cell wall extracts of Klebsiella pnuemoniae, (2) an emulsified inactivated fowl cholera bacteria and (3) inactivated trivalent, P. multocida, Bordetella bronchiseptica and Haemophilus influenzae vaccine. The inactivated fowl cholera

bacteria and inactivated Haemophilus influenzae were found effective.

A streptomycin dependent live P. multocida vaccine for the prevention of rabbit pasteurellosis was reported by Chengappa et al. (1980). The vaccine given either by the intra-nasal or subcutaneous route provided complete protection against homologous challenge as evidenced by the absence of clinical signs or gross lesions and failure to isolate the organism from mucous secretions or tissues from vaccinated individuals.

Lu and Pakes (1981) have also reported about the use of streptomycin dependent P. multocida serotype 3:A live mutant vaccine to protect rabbits against experimental pasteurellosis. The vaccine did not cause clinical disease, gross or microscopic lesions. The vaccine prevented colonisation of the virulent challenge organisms in lungs, liver, spleen, genital tract and blood but not the nasal cavities.

Okerman and Spanoghe (1981) studied the protective effects of inactivated pasteurella vaccines in specific pathogen free rabbits. They observed that bacterins with or without adjuvant produced varying degrees of protection against air borne infection with homologous virulent strain of P. multocida but not to heterologous strain.

A vaccine produced by Ringler et al. (1985) from a potassium thiocyanate extract of virulent P. multocida serotypes 3, 12, 15:D protected the challenged rabbits against clinical disease but did not prevent the colonisation of bacteria in the middle ear. They also demonstrated serum immunoglobulins of IgG class and nasal mucosal immunoglobullins of IgA class. Lu et al. (1987) have also reported the efficacy of potassium thiocyanate extract of P. multocida as a vaccine employing the serotype 3:A which conferred protection to rabbits.

Long et al. (1986) studied the safety and efficacy of a freeze dried attenuated vaccine prepared from avian strain of P. multocida in rabbits. 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. A similar study was reported by Long et al. (1988) but with shorter period of immunity.

The safety and efficacy of a streptomycin dependent live P. multocida (serotype 12:A) vaccine in rabbits were evaluated by DiGiacomo et al. (1987a). 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.

Alebban et al. (1988) reported that when rabbits were vaccinated with P. multocida bovine strain (1062) and turkey strain (1059), they were protected against fatal bacteremia on homologous challenge. They further observed that mucosal routes were superior to intravenous route for conferring protection to rabbits against pasteurellosis.

Sensitivity to Chemotherapeutic agents

According to Flatt (1975) and Holmes (1984) the therapy of Pasteurellosis with antibiotics was relatively ineffective. The antibiotic treatment could bring out remission of clinical signs and the removal of antibiotics was accompanied by a recurrence of the disease.

Lu et al. (1978) determined the sensitivity to chemotherapeutic agents of 42 isolates of P. multocida. In this study, they employed seven antibiotics, two nitrofurans and five sulphonamides. All the isolates were sensitive to the nitrofurans and the antibiotics, except streptomycin and clindamycin. Most of the isolates were resistant to sulphonamides with multiple resistance.

Thirty-eight P. multocida isolates from divergent species of animals were tested for their drug sensitivity by Sharma et al. (1979). All the isolates were sensitive to nitrofuran and resistant to ampicillin, dihydrostreptomycin, oxytetracycline and tetracycline.

Volkolupova (1979) reported that in a rabbit farm where pasteurellosis was a problem, administration of anti-infective agents viz., oxytetracycline, intramuscularly or by aerosol, neomycin intramuscularly, sulfamethoxypyridazine or furazolidone by mouth did not interfere with the agglutinin titer or resistance to challenge induced by formaldehyde inactivated P. multocida vaccines.

In vitro antibiogram studies employing a wide range of chemotherapeutic agents was reported by Mushin and Schoenbaum (1980) and they reported that the P. multocida isolates from rabbits were susceptible to several chemotherapeutic agents in contrast to the results based on in vivo studies published by earlier workers.

Jaslow et al. (1981) tried the efficiency of penicillin therapy to control chronic rhinitis caused by P. multocida and observed a negative result.

Pasteurella species were reported to be susceptible to sulphadiazine, penicillin, streptomycin, chloromphenecol and numerous other commonly used antibiotics (Escande, 1986). Multiple drug resistance in P. multocida and P. haemolytica was reported by Chang and Carter (1976) and small nonconjugal plasmids encoding resistance to tetracycline, streptomycin and the sulphonamides have been described by Hirsh et al. (1985).

Kawamoto et al. (1990) reported that when 40 isolates of P. multocida from rabbits were tested for their sensitivity to 11 antibiotics, all the isolates were susceptible to all the antibiotics tested except clindamycin.

Materials and Methods

MATERIALS AND METHODS

Specimens for bacterial isolates

The biomaterials employed for the purpose of isolation of P. multocida comprised the samples collected from rabbits maintained in the Small Animal Breeding Station (SABS) and Rabbit Research Station (RRS), College of Veterinary and Animal Sciences, Mannuthy and also from rabbits maintained by local farmers. The animals subjected to cultural screening were of varying age and different breeds viz., New Zealand White, Grey Giant, Soviet Chinchilla and cross bred rabbits.

Collection of clinical materials

The biosamples were collected from 36 ailing/dead and 76 apparently healthy animals. The details are presented in Tables 1 and 2. From healthy/ailing rabbits deep nasal swabs were collected for bacterial isolation, while from necropsied/ dead animals, nasal swab, tracheal swab, liver, lung and heart blood were collected for further processing. Cotton swabs soaked in peptone water were used for nasal and tracheal swab collection. Heart blood was obtained by using sterile pasteur pipette/platinum loop and was directly plated on culture media for bacterial isolation and also for smear preparation.

Table 1. Details of ailing/dead animals and nature of samples screened

Source	Breed	Age	Number of animals	Ailing/ dead	Materials collected
1. Kuttanelloor	New Zealand White	Adult*	1	0/1	Nasal swab, Tracheal swab, Lung, Heart blood and Liver
2. Palghat	New Zealand White	Adult	2	0/2	"
3. Attapadi	New Zealand White	Adult	1	0/1	"
	New Zealand White	Young**	1	0/1	"
4. Small Animal Breeding Station (Mannuthy)	New Zealand White	Adult	5	1/4	"
	New Zealand White	Young	9	6/3	"
	Soviet Chinchilla	Young	3	3/0	"
5. Rabbit Research Station (Mannuthy)	New Zealand White	Adult	5	0/5	"
	New Zealand White	Young	2	2/0	"
	Soviet Chinchilla	Young	3	3/0	"
	Grey Giant	Young	2	0/2	"
	Grey Giant	Adult	2	0/2	"
		Total	36		

* Adult > 22 weeks

** Young < 22 weeks

Table 2. Details of healthy animals and nature of samples screened

Source	Breed	Age	Number of animals	Materials collected
1. Rabbit Research Station (Mannuthy)	Grey Giant	Young**	4	Nasal swab
	Grey Giant	Adult*	10	"
	New Zealand White	Adult	12	"
	New Zealand White	Young	4	"
	Soviet Chinchilla	Adult	2	"
2. Small Animal Breeding Station (Mannuthy)	Soviet Chinchilla	Young	5	"
	Cross bred	Adult	18	"
	Cross bred	Young	3	"
	New Zealand White	Adult	12	"
	New Zealand White	Young	4	"
	Grey Giant	Adult	2	"
		Total	76	

* Adult > 22 weeks

** Young < 22 weeks

Lung and liver tissues were cut from suspected sites of infection and were used for direct inoculation of culture media.

Isolation

Isolation of P. multocida was made by the procedure described by Kawamoto et al. (1990).

Briefly, nasal swabs/clinical materials collected were directly and separately inoculated on to blood agar containing five per cent sheep/bovine blood and incubated aerobically at 37°C upto 96 h and the negative plates were then discarded.

The bacterial colonies which grew on the plates were then subjected to Gram's staining. The organisms of gram negative colonies were tested for motility by the dark field microscopy. Capsule staining of the organisms were conducted by Indian ink method of negative staining. Non motile bacterial isolates were then inoculated on MacConkey's agar to test their growth on this medium. The gram negative, non motile bacteria which failed to grow on MacConkey's agar was taken in stock culture on Dextrose Starch Agar (DSA) slants and were identified following the methods described by Cowan (1974). The tests included activity of catalase and oxidase, anaerobic growth, fermentation of glucose, sucrose, mannitol, raffinose, salicin, arabinose, maltose, xylose,

trehalose, dulcitol, inositol, adonitol and lactose, reduction of nitrate and nitrite, indole production, hydrolysis of urea and gelatin, hydrogen sulphide production, growth in potassium cyanide broth and orthonitrophenyl-beta-D-galactopyranoside test (ONPG).

Catalase test

A loopful of culture from a DSA plate was inoculated into a test tube containing one millilitre of three per cent hydrogen peroxide and closely watched for the formation of air bubbles.

Oxidase test

Filter paper strips to which the reagent was adsorbed were utilised for this purpose. To test, the bacterial colony was taken with the tip of a sterile glass rod and smeared on to the filter paper strip. Absence of any blue colouration at the site of smearing within five to ten seconds was taken as negative.

Anaerobic growth

The isolates were inoculated into Robertson's cooked meat medium and incubated at 37°C for 24 to 48 h and examined for any growth.

Carbohydrate fermentation

Ten per cent stock solution of each carbohydrate was prepared in distilled water. One per cent of each carbohydrate was then made from the stock solution in phenol red broth and used for fermentation test. The tubes after inoculation were incubated at 37°C for a minimum of seven days and examined daily for any acid production as indicated by a colour change in the phenol red indicator.

Nitrate reduction test

Nitrate broth was inoculated with the culture under test and incubated for five days. On the fifth day nitrate test was performed by adding three drops each of sulphanilic acid and alpha naphthylamine. Development of red colour following the addition of above reagents indicated that the isolate could reduce nitrate to nitrite. In negative cases absence of nitrate reduction was confirmed by the addition of a few zinc granules.

Indole production test

Peptone water was inoculated with the culture under test and incubated and examined for upto seven days. The development of a red colour ring in the reagent layer on adding 0.5 ml of Kovach's reagent is considered to be positive.

Urease activity

The test for urease activity was performed by inoculating heavily the slope of Christensen's urea medium with 24 h dense culture and incubating it at 37°C. Urease positive cultures change the colour of the indicator to purple pink. The tubes are declared negative only after four days of incubation.

Test for hydrogen sulphide production

The test was performed by inoculating the test organisms into triple sugar iron agar slants and incubating it at 37°C which was then observed daily upto seven days for blackening due to hydrogen sulphide production.

Gelatin liquefaction

A stab culture was made on nutrient gelatin using an inoculum from an overnight culture. Liquefaction is tested for at intervals by removing the nutrient gelatin cultures from the incubator and holding them at 4°C for 30 min before reading the result.

Potassium cyanide test

One loopful of culture was inoculated from a 24 h nutrient broth culture into potassium cyanide broth and

incubated at 37°C with the cap tightly screwed down to prevent air exchange. The broth was observed upto 48 h for evidence of growth.

ONPG test

The isolates were inoculated into tubes of ONPG broth and incubated for 24 h. Positive cases of beta galactosidase activity was indicated by the appearance of a yellow colour due to ortho-nitro-phenol.

Nitrite test

The nitrite broth was inoculated and incubated at 37°C for seven to 14 days. To this nitrite reagent was added. Negative test was indicated by the absence of red colour.

The isolates identified as P. multocida were then subcultured at weekly intervals initially and then at 72-96 h interval as the isolates were loosing viability on culturing.

The isolates were further grown on Dextrose Starch Agar to determine the morphology of the colony. The colony morphology was described by examining the colonies using obliquely transmitted light. The colony character was grouped and described based on the colour, consistency and appearance.

Pathogenicity studies

The isolates of P. multocida were tested for their pathogenicity in mice and rabbits.

Mice

P. multocida free swiss albino mice of six to eight weeks of age received from the SABS were kept under observation for a minimum period of three days before they were utilised for this purpose.

Experimental infection

Eighteen hour nutrient broth culture of each of the six P. multocida isolates were prepared which were adjusted to 1 x 10^8 organims/ml before inoculation. The method described by Kawamoto et al. (1990) was followed. Four mice per isolate were used for this purpose. Each animal was injected intra-peritoneally with 0.2 ml of inoculum containing 1 x 10^8 organisms/ml. The control mice were injected with 0.2 ml sterile saline. All the injected animals were observed for seven days for the clinical symptoms of infection/death. Blood smears were prepared from the experimentally inoculated animals at 24 h interval to demonstrate the bacteriemic phase.

The re-isolation of P. multocida from dead mice were attempted from liver, lung, heart blood and trachea. The

animals which did not show any signs of illness at the end of the observation period were necropsied and screened for bacteria.

Rabbit

P. multocida free rabbit aged two to three months were employed for the pathogenicity studies. These animals were received from SABS and kept under observation for a minimum period of seven days.

Experimental infection

The method described by Lu et al. (1982) was followed.

Overnight growth of each isolate on blood agar was suspended in 0.01 M phosphate buffered saline. After mixing it thoroughly over a cyclomixer to avoid bacterial clumps, the concentration of the organism was adjusted to 6.4×10^9 organisms/ml before inoculation.

The rabbits were infected by intranasal instillation at the rate of 0.5 ml of the above bacterial suspension using three rabbits per isolate. All the infected rabbits were housed separately so as to eliminate chances of cross infection and were observed for the development of clinical signs/infection for varying periods upto 21 days. Rectal

temperature of these animals were recorded daily. Deep nasal swabs were collected from each of the inoculated rabbits at 48 h interval and re-isolation of the organism was attempted.

Out of the three animals, one rabbit from each group was necropsied at seven days interval after inoculation and detailed post-mortem examination was conducted for gross and microscopic lesions. From the necropsied animals, nasal septa, nasal turbinates, trachea, lung and liver were collected for histopathological studies. Re-isolation of the organisms from such necropsied animals were tried from tracheal swab, lung, liver and heart blood.

Histopathological studies

The tissue specimens collected for histopathological studies from lung, liver, trachea and nasal septa were fixed in 10 per cent formalin. These were then washed in running tap water and processed by routine paraffin embedding technique as detailed in the Armed Forces Institute of Pathology (1968). Paraffin blocks were cut to get sections with a thickness of four micron. The sections were deparaffinised and stained with hematoxylin and eosin methods of Harris as described by Disbrey and Rack (1970).

Sensitivity to chemotherapeutic agents

Disc diffusion method described by Bauer et al. (1966) was used for studying the sensitivity of different isolates of P. multocida to a number of commonly employed chemotherapeutic agents and antibiotics. Sensitivity tests were carried out on nutrient agar containing five per cent sheep blood.

Five colonies from each pure culture were picked up with sterile platinum loop and incubated in five millilitre of nutrient broth at 37°C for two hours. The bacterial suspension thus obtained was adsorbed into a sterile cotton swab and uniformly inoculated on blood agar and allowed to dry for ten minutes. The different antibiotic disc's were then placed on the medium suitably spaced with the help of a flamed and cooled forceps. These plates after 18-24 h of incubation at 37°C were examined for the sensitivity pattern.

The diameter of the inhibition zones were measured and recorded for interpretation according to the guidelines of Bauer et al. (1966). Commercial antibiotic discs from Span Diagnostics, Udhna (Surat) India were used for this study. They included streptomycin (10 mcg), penicillin G (10 U), ampicillin (10 mcg), gentamycin (10 mcg), colistin (10 mcg), kanamycin (30 mcg), sissomycin (10 mcg), erythromycin (15 mcg), oxytetracycline (30 mcg), polymyxin (300 U), cloxacillin (5 mcg), chloramphenicol (30 mcg), novobiocin (30 mcg),

cefazolin (30 mcg), furazolidone (100 mcg), nitrofurantoin (300 mcg) and sulphadiazine (300 mcg).

Antigenic relationship studies

Antigenic relationship of the new isolates was studied by comparing their somatic antigens by gel diffusion precipitin test (GDPT).

Antiserum preparation

Animals

Apparently healthy rabbits of three months of age free from P. multocida infection were employed for raising antiserum against somatic antigens of bacterial isolates. All the animals were pre-tested for antibodies to all the isolates and the vaccine strain P_{52}.

Antigen

Antigen for raising antibody in rabbits were prepared separately from three isolates obtained during this study and also from P. multocida vaccine strain P_{52}, supplied by Veterinary Biological Institute, Palode, Thiruvananthapuram as per the procedure described by Lu et al. (1982). Briefly, overnight growth of each isolate and vaccine strain P_{52} was suspended separately in 0.01 M phosphate buffered saline and

washed by centrifugation three times with the same buffer. The supernatant was discarded and 0.3 per cent formol saline was added to the packed bacteria and resuspended to get a population density of 1 x 10^9 cells/ml. The suspension was then heated in a boiling water bath for one hour and checked for sterility by plating on blood agar.

Immunisation

The rabbits were initially injected by the subcutaneous route at the flank region with one millilitre of 1:1 mixture of bacterial antigen and Freund's complete adjuvant. Subsequent injections were given without adjuvant and were given intraveinously at the rate of one millilitre on every sixth day. The animals were periodically bled at weekly intervals from the ear vein for the detection of antibody. Antigen administration was discontinued when sufficient antibody titer was detected by GDPT.

GDPT

Gel diffusion precipitin test described by Heddleston et al. (1972) was employed with slight modification.

The gel was prepared with 0.9 per cent agarose in 0.85 per cent sodium chloride and 0.2 per cent phenol in distilled water. The molten agar was pipetted on to glass

plates/microscopic slides and wells were cut with a diameter of four millimetre and six millimetre apart. The peripheral wells were charged with antisera raised in rabbits against various isolates and the central well with one of the antigen preparation. The agar gel plates/microscopic slides were incubated at 37°C in a humid chamber and the results of GDPT were first recorded after 24 h of incubation. The plates were observed for a period of 72 h before declaring the test negative.

RESULT

Antemortem and post mortem findings of animals screened

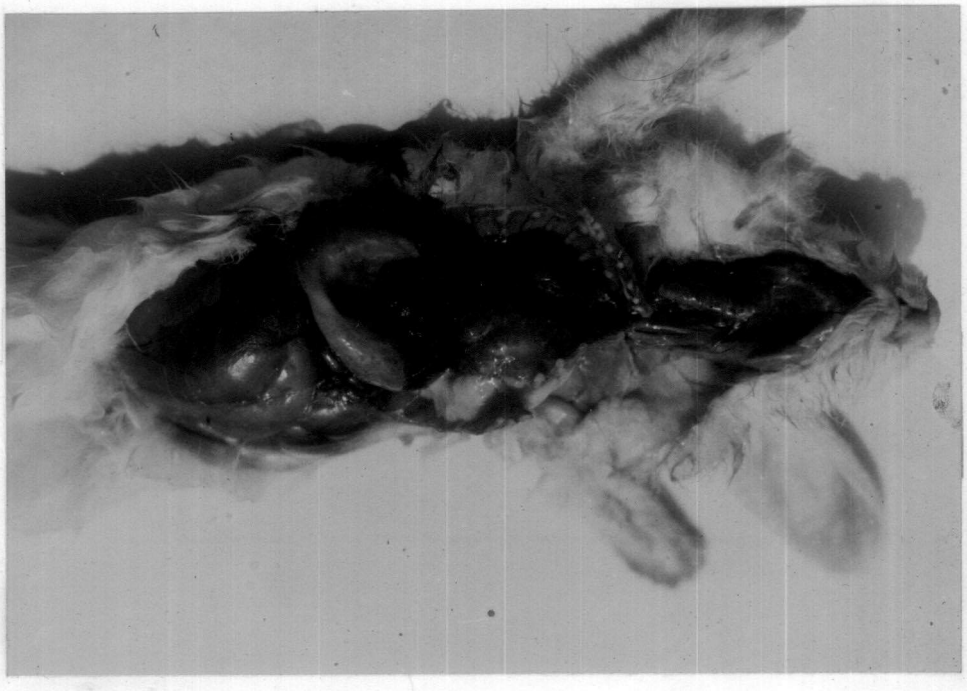
The 112 rabbits examined during this study included both live and dead rabbits. The live animals were either apparently healthy or sick. The sick animals were showing anorexia and depression and torticollis in few cases, particularly young rabbits.

Out of the 36 dead animals examined, 17 were from the Small Animal Breeding Station, Mannuthy, 14 from the Rabbit Research Station, Mannuthy, while the remaining five were from the local rabbit breeders. Twenty six of the dead rabbits belonged to the New Zealand White breed, six were of Soviet Chinchilla and four were of the Grey Giant breed (Table 1).

Post mortem examination of the dead rabbits were invariably conducted within six hours of death and bacterial isolation was tried from biomaterials collected from such animals. Gross pathological lesions observed were typical haemorrhages in lungs and trachea, necrotic foci in liver and abscessation in lungs (Fig.1 and 2). No lesions were detected in the middle ear or nares.

Fig.1 Rabbit - Natural infection - Post mortem lesions - Haemorrhagic tracheitis and pulmonary congestion

Fig.2 Rabbit - Natural infection - Post mortem lesions - Lung gross enlargement and induration with micro abscesses



Screening for bacterial agents

Microscopical examination of blood smears prepared from ear vein of the sick animals were negative for bacteria. Deep nasal swabs collected from 76 rabbits (20 young and 56 adult) did not reveal any *P. multocida*.

Isolation of *P. multocida* was successful only from dead adult animals which belonged to Rabbit Research Station. Of the various biosamples screened, tracheal swab was the most successful as tracheal swab collected from all the six rabbits, which gave positive isolations revealed organism while only one each of lung and heart blood did so. All the other samples were negative. The results of various biosamples screened for *P. multocida* are given in Table 3.

The six rabbits from which the organism was isolated belonged to New Zealand White (5) and Grey Giant (1). The six isolates were designated as R_9S, $R_{10}S$, $R_{17}S$, $R_{18}S$, $R_{22}S$ and $R_{23}S$. The breed-wise distribution of the isolates of *P. multocida* are given in Table 4.

Properties of the isolates

Colony characters, Morphology and Growth

Based on the colonial characters, the six isolates

Table 3. Results of various biosamples screened for *P. multocida*

Age group	Number of animals screened	Live/ dead	*P. multocida* isolation					Total (animals positive)
			Nasal swab	Tracheal swab	Lung	Heart blood	Liver	
Young (below 22 weeks)	20	Live	0/20	ND	ND	ND	ND	Nil
	20	Dead	0/20	0/20	0/20	0/20	0/20	Nil
Adult (above 22 weeks)	56	Live	0/16	ND	ND	ND	ND	Nil
	16	Dead	0/16	6/16	1/16	1/16	0/16	6
Total	112			6	1	1	--	6

ND - Not done

Table 4. Isolation of P. multocida - Breed-wise distribution

Breed	Biosamples - No. positive/No. screened					Total number of rabbits positive
	N.S.	T.S.	Lung	H.B.	Liver	
New Zealand White	0/26	5/26	1/26	1/26	0/26	5/26
Soviet Chinchilla	0/6	0/6	0/6	0/6	0/6	0/6
Grey Giant	0/4	1/4	0/4	0/4	0/4	1/4

could be grouped into two. Isolates R_9S, $R_{10}S$ and $R_{23}S$ produced small, smooth and grey coloured colonies (Fig.3). On the other hand the colonies of $R_{17}S$, $R_{18}S$ and $R_{22}S$ were medium sized, grey in colour and were mucoid in nature. Iridescence was not seen in any of the above two types of colonies when they were examined for the same.

Though all the six isolates were capsulated as evidenced by Indian ink method of negative staining, the capsule of isolate $R_{23}S$ was larger compared to others.

Biochemical characters

The primary biochemical tests for the identification of P. multocida were based on the following criteria. Grams staining reaction, motility, growth on MacConkey, catalase activity, oxidase activity, growth under anaerobic conditions, carbohydrate break down (O/F) and Hemolysis on blood agar. The results of these tests are given in Table 5.

The isolates were uniformly Gram negative, non motile, cocobacilli. Of the six isolates, two were oxidase negative, namely R_9S and $R_{10}S$ and the remaining four isolates were oxidase positive. All of them were catalase positive, grew anaerobically, utilised glucose fermentatively and none produced hemolysis on blood agar.

Fig.3 Dextrose Starch Agar plate showing colonies of Pasteurella multocida isolate

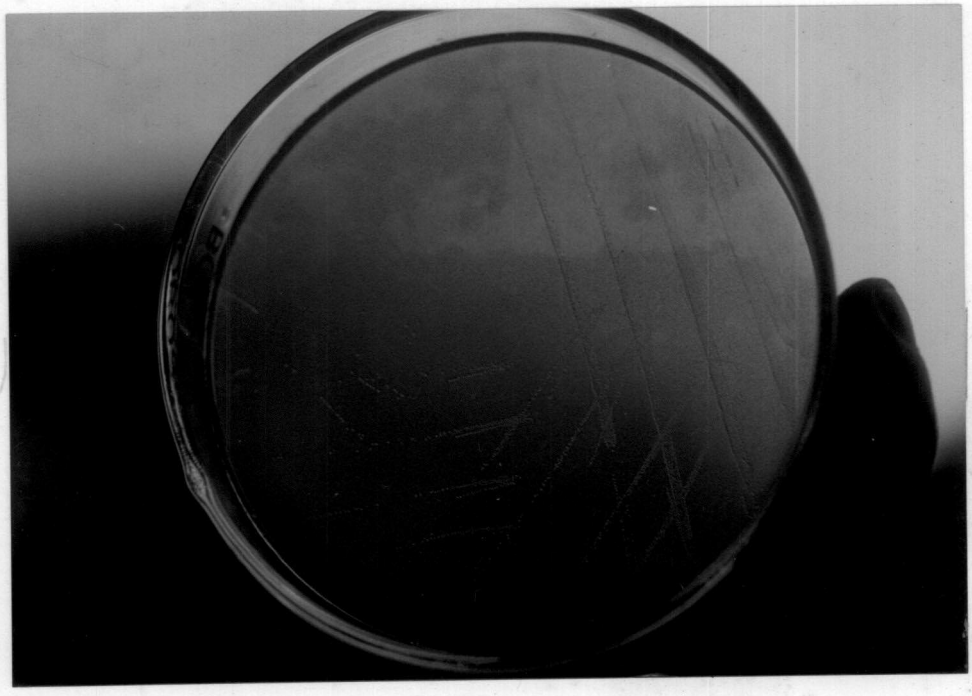


Table 5. Results of the primary tests of the P. multocida isolates and the vaccine strain P_{52}

Test	P_{52}	Isolates					
		R_9S	$R_{10}S$	$R_{17}S$	$R_{18}S$	$R_{22}S$	$R_{23}S$
Grams reaction	-	-	-	-	-	-	-
Cell morphology	cocco bacilli	cocco bacilli	cocco bacilli	cocco bacilli	cocco bacilli	cocco bacilli	cocco bacilli
Motility	-	-	-	-	-	-	-
Oxidase	-	-	-	+	+	+	+
Catalase	+	+	+	+	+	+	+
Growth in MacConkey	-	-	-	-	-	-	-
Growth anaerobically	+	+	+	+	+	+	+
O/F	F+	F+	F+	F+	F+	F+	F+
Hemolysis	-	-	-	-	-	-	-

The results of the second stage biochemical tests are presented in Table 6. The test results of all the six isolates were uniform and comparable to that of P. multocida strain, P_{52} except for ONPG in R_9S, indole production, ONPG, acid production from raffinose, salicin and inositol in case of $R_{17}S$, nitrate reduction, ONPG and acid production from arabinose, xylose and sorbitol in case of $R_{18}S$, indole production, ONPG, and acid from lactose, sorbitol and adonitol in case of $R_{22}S$, and acid production from trehalose in case of $R_{23}S$.

Pathogenicity trials

Pathogenicity in mice

Three isolates of P. multocida viz., R_9S, $R_{10}S$ and $R_{23}S$ were tested for their pathogenicity in mice. Each culture was injected into a group of four mice by intraperitoneal route. Out of the four mice, two died within 24 h, when inoculated with the isolates R_9S and $R_{23}S$ and the remaining two died within 48 h (Table 7). The peripheral blood smear prepared from live mice at 24 h post-inoculation did not reveal any bacteria.

No gross lesions could be observed in the internal organs in any of the dead animals. However, P. multocida

Table 6. Results of the biochemical tests of the P. multocida isolates and the vaccine strain P_{52}

Tests	P_{52}	Isolates					
		R_9S	$R_{10}S$	$R_{17}S$	$R_{18}S$	$R_{22}S$	$R_{23}S$
Nitrate	+	+	+	+	-	+	+
Nitrite	-	-	-	-	-	-	-
Indole	+	+	+	-	+	-	+
Growth in KCN	+	+	+	+	+	+	+
H_2S from TSI	-	-	-	-	-	-	-
Urease	-	-	-	-	-	-	-
ONPG	+	-	+	-	-	-	+
Gelatin hydrolysis	-	-	-	-	-	-	-
Arabinose	+	+	+	+	-	+	+
Mannitol	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+
Salicin	+	+	+	-	+	+	+
Xylose	+	+	+	+	-	+	+
Raffinose	+	+	+	-	+	+	+
Lactose	+	+	+	+	+	-	+
Trehalose	-	-	-	-	-	-	+
Inositol	+	+	+	-	+	+	+
Dulcitol	+	+	+	+	+	+	+
Sorbitol	-	-	-	-	+	+	-
Adonitol	+	+	+	+	+	-	+

Table 7. Results of the experimental infection of mice with *P. multocida* isolates

Bacterial isolate	Number of animals injected	Mortality		
		24 h	48 h	72 h
R_9S	4	2/4	4/4	--
$R_{10}S$	4	0/4	1/4	4/4
$R_{23}S$	4	2/4	4/4	--

could be recovered in pure culture from the heart blood, lung liver and spleen.

Out of the four mice injected with isolate $R_{10}S$, one mouse died on the second day of post-inoculation and the remaining on the third day (Table 7). The peripheral blood smear prepared at 24 h interval from these animals did not reveal any bacteria. Post-mortem examination of the dead mice revealed haemorrhagic tracheitis. No other lesions were observed in any of the internal organs. The bacteria could be isolated from heart blood, lung, liver and spleen.

Pathogenicity in rabbit

The rabbits which were inoculated intra-nasally with 6.4×10^9 organisms per millilitre of P. multocida isolates developed symptoms like general weakness, lethargy and impaired appetite within 12 h. All the rabbits had exhibited rise in body temperature which lasted for 48 h post inoculation. No further clinical signs of pneumonia, rhinitis or other form of pasteurellosis were noted in any of the inoculated animals.

Re-isolation of P. multocida from the inoculated animals were tried by culturing the nasal swabs collected at every 24 h interval. The bacteria could be re-isolated from

all the rabbits inoculated with R_9S upto seven days, while with isolate $R_{10}S$, it was possible only upto three days. The re-isolation of bacteria from rabbits inoculated with the remaining isolates namely, $R_{17}S$, $R_{18}S$, $R_{22}S$ and $R_{23}S$ were possible only upto 48 h.

Necropsy findings

On the seventh day post inoculation, one rabbit from each group was necropsied and detailed post mortem examination was conducted. All the rabbits necropsied were negative for any gross lesions.

On the 14th day of inoculation, rabbits necropsied showed haemorrhagic tracheitis and consolidation of lung irrespective of the cultures used. In addition to this rabbit inoculated with R_9S, the right upper lobe of lung had an abscess. The organism could be isolated from the abscess.

The rabbits necropsied on the 21st day, no gross lesions were seen in any of the organs except mild haemorrhagic tracheitis.

Histopathology

The nasal septa, trachea, lung and liver collected from the experimentally infected animals revealed various pathological alterations at various intervals.

Nasal septa

The lesions in the septa and its mucosa were observed in only those rabbits inoculated with isolates R_9S and $R_{10}S$. Congestion, hyperplasia of goblet cells and mild to moderate infiltration of mononuclear cells in the septal mucosa and sinuses were the predominant lesion. Mild degenerative changes were also observed in the pseudostratified septal epithelium. The lesions were mild on the seventh day but predominant on the 14th day post inoculation.

Trachea

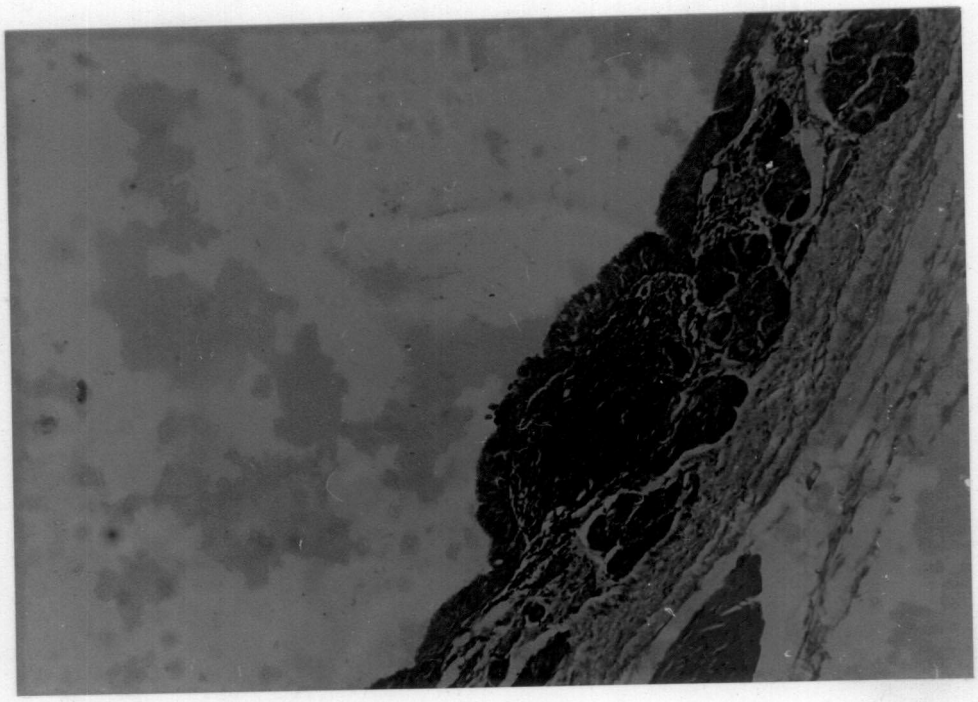
Focal congestion and mild to severe haemorrhages were observed in the mucosa and lamina propria. Infiltration of mononuclear cells were observed in the mucosa and lamina propria either in a diffuse pattern or as nodular aggregates (Fig.4).

Lung

Moderate to severe congestion, oedema, frank haemorrhages (Fig.5 and 6) and severe suppurative bronchopneumonia (Fig.7) were the predominant lesions. The lesions were evident by 14th day and persisted throughout the experimental period. The severity of the lesion aggravated as the infection advanced. All the isolates produced lung lesions.

Fig.4 Rabbit trachea - Experimental infection manifesting haemorrhagic tracheitis - H&E x 250

Fig.5 Rabbit lung - Experimental infection manifesting congestion and haemorrhage - H&E x 250



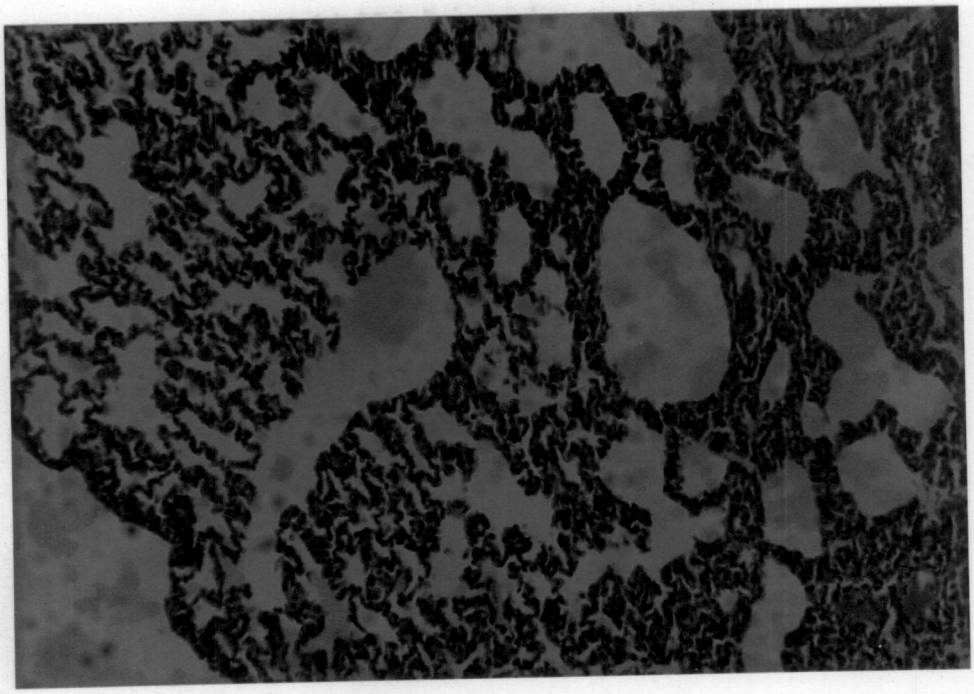
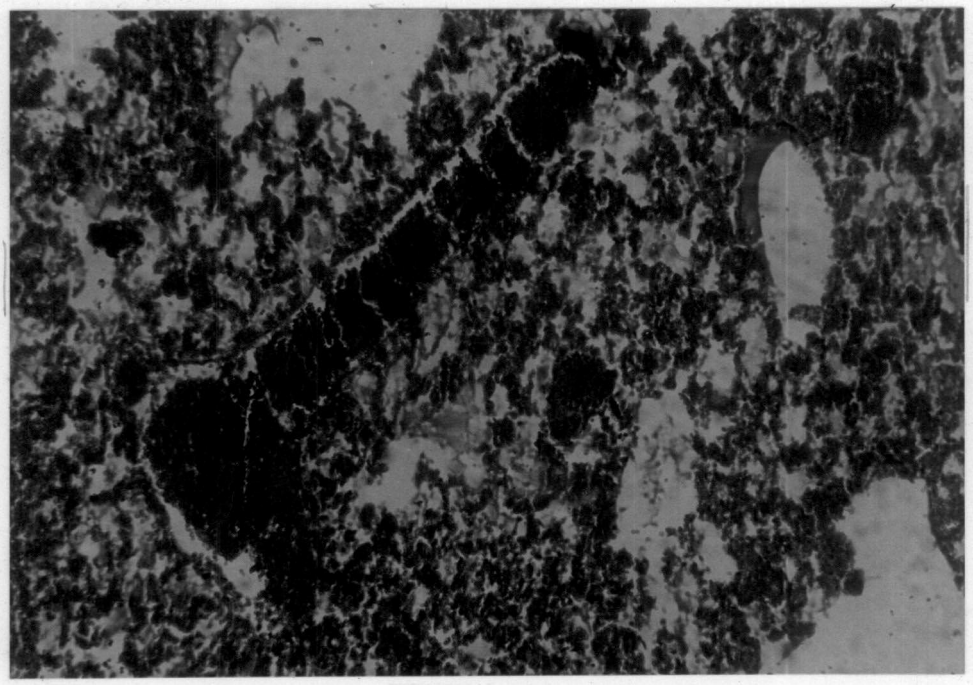


Fig.6 Rabbit lung - Experimental infection manifesting congestion, oedema and haemorrhage - H&E x 250

Fig.7 Rabbit lung - Experimental infection manifesting suppurative bronchopneumonia - H&E x 250



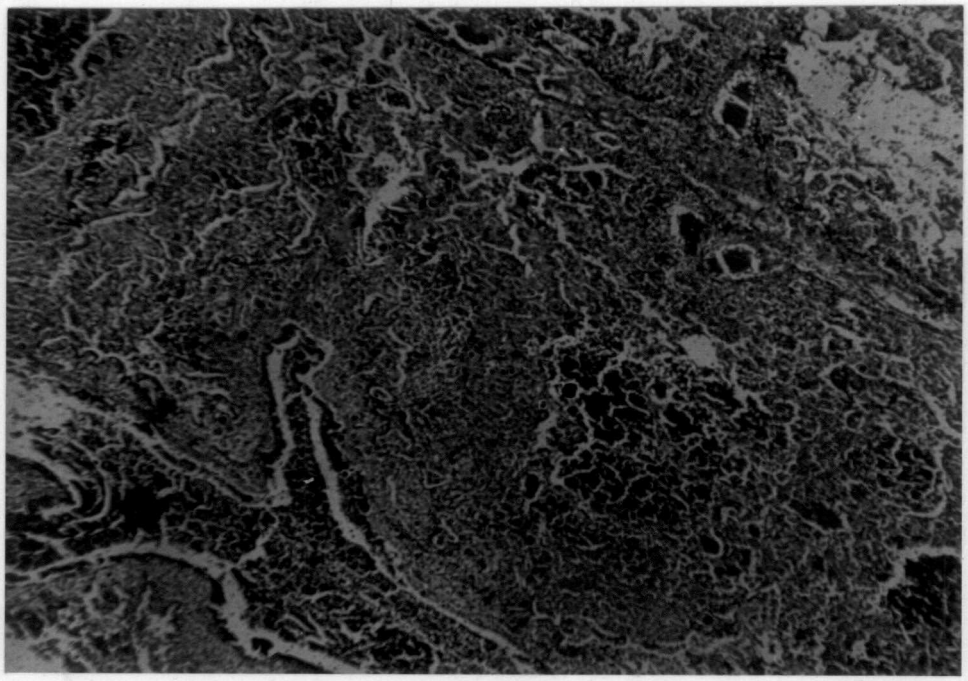
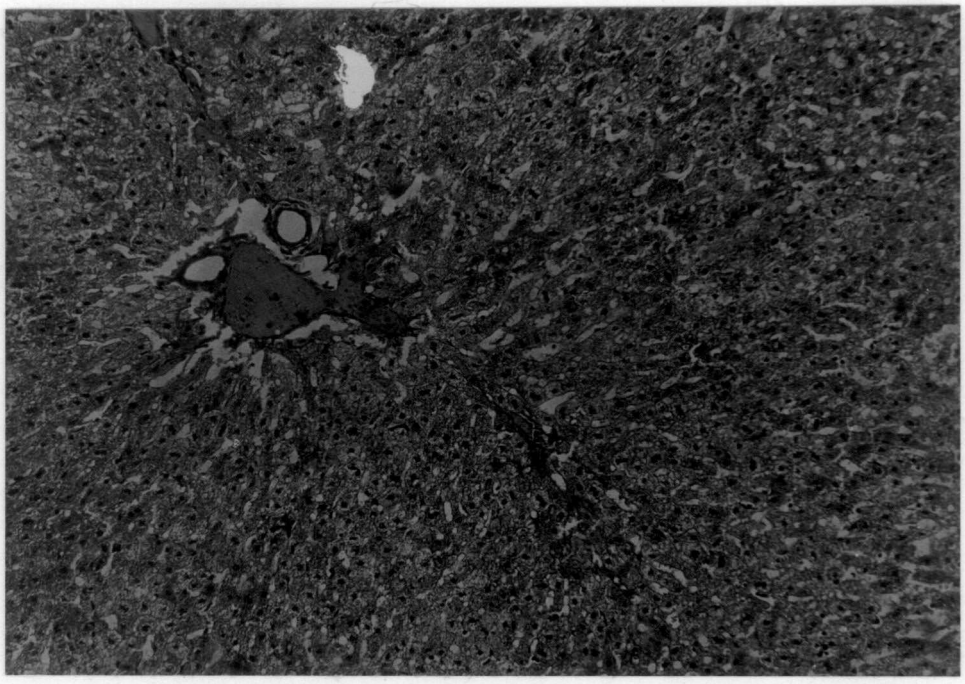


Fig.8 Rabbit liver - Experimental infection manifesting centrilobar necrosis and moderate fatty change - H&E x 250



Liver

Centrilobar necrosis, moderate to severe fatty change (Fig.8) and congestion were evident on the 14th day post inoculation in those rabbits inoculated with the isolates R_9S, $R_{10}S$, $R_{17}S$ and $R_{23}S$.

Sensitivity to chemotherapeutic agents

The results of the antibiogram studies of the six isolates to various drugs and chemotherapeutic agents are presented in Table 8.

All the isolates were uniformly sensitive to streptomycin, ampicillin, gentamycin, sissomycin, polymyxin, chloramphenicol, novobiocin, cefazolin, furazolidone, nitrofurantoin and sulphadiazine, while all the isolates were resistant to erythromycin (Fig.9).

R_9S was sensitive to all the chemotherapeutic agents except erythromycin, $R_{10}S$ was resistant to Penicillin, Kanamycin, Oxytetracycline and Cloxacillin; $R_{17}S$ to Colistin and Cloxacillin; $R_{18}S$ to Penicillin, Kanamycin and Cloxacillin; $R_{22}S$ to Colistin and Kanamycin and $R_{23}S$ to Penicillin and Colistin.

Fig.9 Drug sensitivity pattern of the Pasteurella multocida isolate

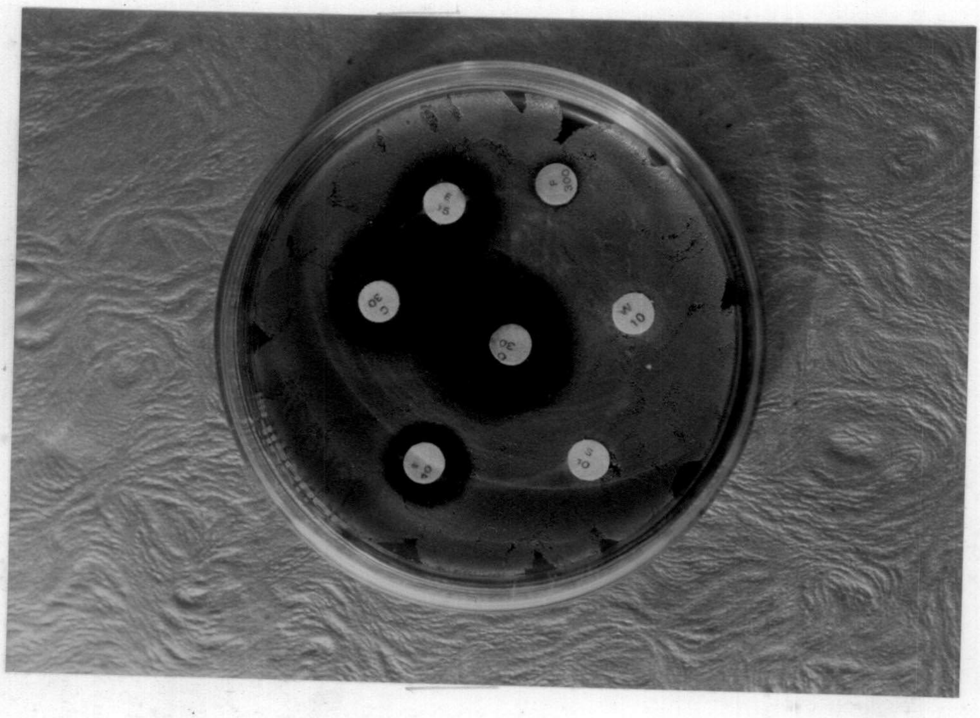

C 30
W 10

Table 8. Results of the drug sensitivity studies of the P. multocida isolates and the vaccine strain P_{52}

Chemotherapeutic agent	P_{52}	Isolates					
		R_9S	$R_{10}S$	$R_{17}S$	$R_{18}S$	$R_{22}S$	$R_{23}S$
Streptomycin	-	+	+	+	+	+	+
Penicillin	-	+	-	+	-	+	-
Ampicillin	+	+	+	+	+	+	+
Gentamycin	+	+	+	+	+	+	+
Colistin	-	+	+	-	+	-	-
Kanamycin	-	+	-	+	-	-	+
Sissomycin	+	+	+	+	+	+	+
Erythromycin	+	-	-	-	-	-	-
Oxytetracycline	+	+	-	+	+	+	+
Polymyxin	+	+	+	+	+	+	+
Cloxacillin	+	+	-	-	-	+	+
Chloramphenicol	+	+	+	+	+	+	+
Novobiocin	+	+	+	+	+	+	+
Cefazolin	+	+	+	+	+	+	+
Furazolidone	-	+	+	+	+	+	+
Nitrofurantoin	+	+	+	+	+	+	+
Sulphadiazine	-	+	+	+	+	+	+

+ Sign indicates sensitivity

- Sign indicates resistance

Serological studies

Pre-immunisation serum collected from all the six rabbits were negative for antibodies to both homologous and heterologous heat stable antigens of P. multocida.

With all the isolates used for antibody production along with the vaccine strain P_{52}, line(s) of precipitation was observed only after eight intravenous injections in addition to the first subcutaneous injection with antigen.

Gel diffusion precipitin test

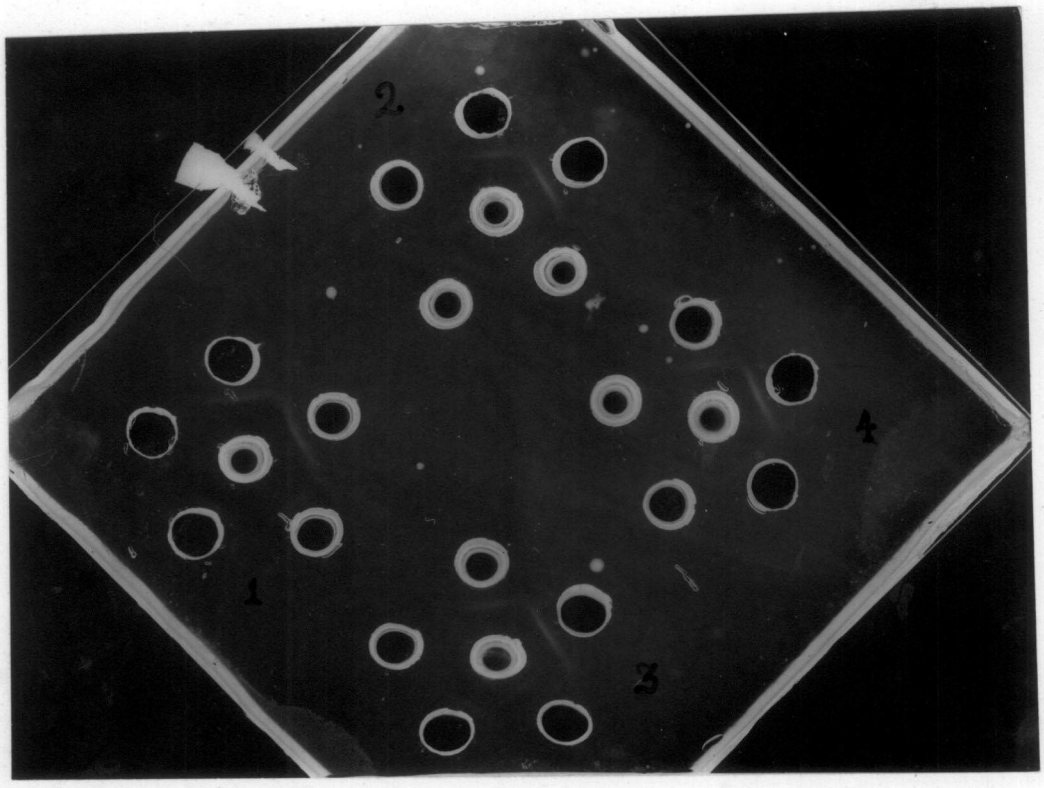
When the antigens were tested against the homologous antisera, the strains P_{52} and $R_{23}S$ produced an additional precipitin line, when compared to the solitary line produced by R_9S and $R_{10}S$. When the antigens from the isolates R_9S was tested against homologous and heterologous antisera, it produced a strong homologous precipitin and a strong heterologous precipitin line against $R_{10}S$. It further showed weak cross reaction with $R_{23}S$ and P_{52}.

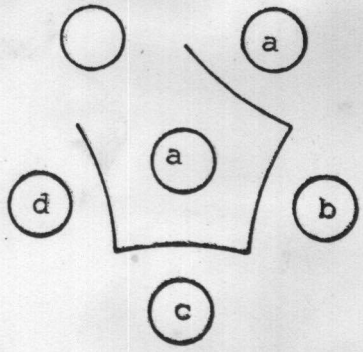
The antigen from the isolates $R_{10}S$ was also found to produce strong homologous precipitin line against R_9S. This isolate was also found to produce cross reaction with $R_{23}S$ and P_{52}.

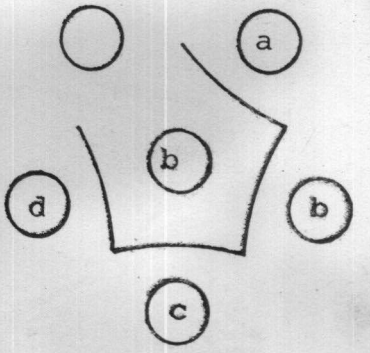
The line of identity was observed when antigen from $R_{23}S$ and P_{52} were tested against homologous and heterologous antisera (Fig.10). The precipitin patterns of the Pasteurella multocida heat stable antigens R_9S, $R_{10}S$, $R_{23}S$ and vaccine strain P_{52} has been diagramatically illustrated (Fig.11).

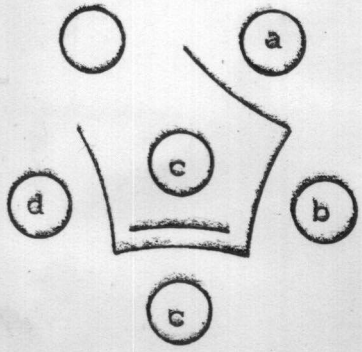
Fig. 10 Precipitin patterns of Pasteurella multocida heat stable antigens - R_9S, $R_{10}S$, $R_{23}S$ vaccine strain P_{52} antigen in the central well and antisera in the outer wells

Fig. 11 Diagramatic illustration of the precipitin patterns of Pasteurella multocida heat stable antigens R_9S (a) $R_{10}S$ (b), $R_{23}S$ (c) and vaccine strain P_{52} (d) - antigen in the central well and anitsera in the outer wells


2
4
1
3


a
a
d
b
c


a
b
d
b
c


a
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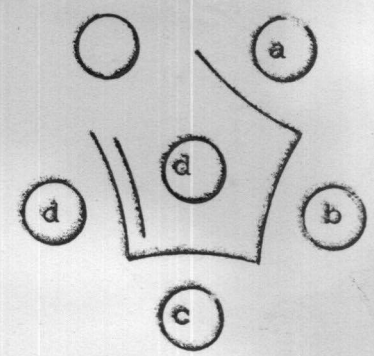
b
c


a
d
d
b
c

DISCUSSION

In Kerala, the broiler rabbit rearing has become more popular among the small scale farmers and has attained an important place in the rural economy as a supplementary occupation to increase the income from farm operations. Rabbits are found to be more prone to various diseases causing often heavy economic loss due to mortality and poor growth especially when reared under intensive farming system.

Of the various afflictions of rabbits, Pasteurellosis caused by P. multocida has been reported to be one of the common and serious bacterial infection in virtually all conventionally housed colonies of rabbits including colonies maintained by animal suppliers and research laboratory. A variety of clinical signs and lesions have been associated with P. multocida in domestic rabbits. However, the organism has also been generally considered to be a relatively non pathogenic resident of the respiratory tract of the domestic rabbits. It has also been demonstrated that rabbits can harbour P. multocida as either apparent on inapparent carrier (Flatt, 1974; Lu et al., 1978).

Very few systematic studies to understand the prevalence and the nature of this disease in rabbits in Kerala

have been reported barring the documentation by Nair et al. (1987) and Devi et al. (1990).

During the present study a total of 112 rabbits consisting of young and adult apparently healthy and sick/dead were screened for P. multocida and the organism could be isolated from six cases giving an isolation rate of 5.36 per cent. A higher rate of isolation of P. multocida was recorded by previous workers irrespective of whether samples were from healthy (Hagen, 1967; Lu et al., 1978) or clinically infected rabbits (DiGiacomo et al., 1983).

When the data was analysed on the basis of age-wise distribution, it was seen that none of the animals below 22 weeks of age, be dead or live were positive for this bacteria. The same was also true when nasal swabs collected from 76 rabbits were examined. On the other hand, when 16 adult rabbits with clinical infection were bacteriologically characterised, six isolates of P. multocida were obtained giving an isolation rate of 37.5 per cent. This observation indicated the persistent infection with P. multocida in rabbits and the usefulness of older animals for monitoring the presence of bacteria in rabbit. In earlier studies, Nakagawa et al. (1986) observed that in breeding colonies, isolation rate of P. multocida increased with the age of rabbits.

Tracheal swabs were positive for P. multocida from all the six rabbits from which the isolations were obtained while only one each of lung and heart blood could give positive isolations. This shows that tracheal swabs are the most suitable specimens of choice for P. multocida isolation. This could probably be due to the localisation and persistence of the organism in this region. The low isolation rate obtained in the present study may be due to the fact that nasal swab was used for bacterial isolation in most of the cases particularly from 76 of the total 112 animals screened. If the percentage of isolation was calculated on the basis of rabbits with clinical disease and examination of tracheal swabs it was 16.67 per cent. The low percentage of isolation of P. multocida can also be attributed to the lower percentage of infection rate in the flock from which the specimens were collected thus indicating a better health status of the animals maintained in these flocks.

Low isolation rate of 0.9 per cent of P. multocida was also reported by Kawamoto et al. (1990) from infected rabbits in rabbitries with monitoring system. Moreover, Lukas et al. (1987) reported that 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.

Blood agar containing five per cent sheep/bovine blood is one of the recommended media for primary isolation of P. multocida while it is described to be unsuitable for determining the colony morphology (Carter, 1967). Several other media have been reported to be useful for the primary isolation of P. multocida either alone or in combination. They included Trypticase soy blood agar, Tryptose agar, Tryptose blood agar (Carter, 1967) and YPC agar (Namioka and Murata, 1961). Blood agar containing five per cent sheep/bovine blood was the only media employed for the isolation of P. multocida from rabbit in this study. This medium was found to support the growth of P. multocida well even after several subculturing. Since no other medium has been tried for isolation, the efficiency of this medium could not be compared or commented.

YPC agar (Namioka and Murata, 1961a), Dextrose starch agar (Heddleston et al., 1964) and Brucella agar containing two per cent hemolysed rabbit serum (Carter, 1967) were reported to be highly suitable for discerning the colony morphology of P. multocida and in these media, the colonial variants viz., smooth, mucoid and rough were demonstrated.

All the six isolates of P. multocida grew on Dextrose starch agar and three of them (R_9S, $R_{10}S$ and $R_{23}S$) formed smooth, small greyish colonies and the rest formed greyish but

mucoid and medium sized colonies. The observation indicates the presence of different variants of P. multocida when isolated from one and the same rabbit colony. Similar observations were also reported earlier by Lu et al. (1978) and Brogden (1980) as there were variants in colonial morphology and biochemical properties, when P. multocida isolates from rabbits of a single animal facility were studied.

Capsulation is one of the distinguishing feature of P. multocida and the amount and type of capsular material is reported to be closely associated with the virulence (Carter, 1958). In this study, out of the six, one isolate $R_{23}S$ was with a large distinct capsule, while the rest with moderate and inconspicuous capsule. The Pasteurella isolates were obtained from rabbits with a history of clinical infection simulating pasteurellosis which might indicate the virulence in rabbits.

The present isolates grew well aerobically and anaerobically at 37°C which is the normal growth requirement for P. multocida. In one solitary report Targowski and Targowski (1979) described the isolation of a strain of P. multocida from rabbit which grew only when media was supplemented with serum and incubated in a candle jar or under anaerobic condition.

The results of the primary tests for the characterisation of P. multocida were identical and typical for the six isolates except for the oxidase test. The isolates $R_{17}S$, $R_{18}S$, $R_{22}S$ and $R_{23}S$ were oxidase positive, while the rest were oxidase negative.

Mannheim (1984) observed that most strains of P. multocida are only oxidase positive barring a few negative strains which was in agreement with our findings.

The results of the second stage biological and biochemical properties of the six isolates of P. multocida were in conformity with the normal characters assigned to the species barring few variable reactions (Table 6).

The atypical variations that were observed in this study were for nitrate reduction, indole and ONPG hydrolysis, fermentation of salicin, xylose, raffinose, lactose and trehalose. Biological and biochemical properties of P. multocida isolates from rabbits reported by several workers indicate several atypical variations. Heddleston (1976) observed that rabbit isolates of P. multocida were slow indole producers and the cultures were to deviate considerably from the typical fermentation reaction of sugars.

When 32 isolates of P. multocida from rabbits were biochemically characterised, Lu et al. (1978) observed that

indole production and L-ornithine decarboxylase were not consistent. Their isolates differed considerably in carbohydrate fermentation specifically with arabinose, raffinose, maltose, salicin and trehalose. They suggested that these differences in the biochemical characters might be associated with virulence of the isolates.

Atypical variations in the physiological characteristics of 48 cultures of P. multocida from rabbits have been reported by Brogden (1980). He observed that variations in the definitive characteristics such as indole and hydrogen sulphide production, as well as rare variations of lactose and maltose fermentation, make the few atypical isolates of P. multocida from rabbit difficult to identify, often necessiating serological confirmation. While characterising P. multocida in rabbits Kawamoto et al. (1990) observed variations in indole production and the fermentation of raffinose, when 40 isolates of P. multocida were biochemically characterised.

When the three isolates of P. multocida (R_9S, $R_{10}S$ and $R_{23}S$) were tested for their pathogenicity in mice, two isolates R_9S and $R_{23}S$ were found to be comparatively more virulent since two of the inoculated mice from each group were dead within 24 h. However, all the inoculated mice were dead

within 72 h post inoculation indicating the varying degree of their virulence to this species of animal.

Okerman et al. (1979) reported that P. multocida originated from rabbitries with a septicaemic form of pasteurellosis were highly virulent while the isolates from typical snuffles have moderate or low virulence. Mushin and Schoenbaum (1980) observed that strains of Heddleston's serotype 3 were lethal for mice within 24 h while untypable strains differed in their virulence. P. multocida isolates from rabbits and from their environment were reported to be only moderately virulent for mice by Kawamoto et al. (1990).

Though the isolates under study were having the same colony morphology, they differed in their degree of virulence in mice, which indicates that colony morphology alone may not be used as a marker for virulence. This observation was in close agreement with the findings of Okerman et al. (1979). However, Webster and Burn (1926) were of the opinion that organism producing smooth type colonies were more virulent compared to mucoid or rough types.

None of the mice revealed any bacteraemic phase as evidenced from periodical peripheral blood smear examination. The internal organs of the dead mice were without any gross lesions. However, the bacteria could be recovered in pure

cultures from lungs, liver and heart blood which indicated the cause of the death. Collins (1976) observed that an overwhelming increase in numbers of P. multocida in visceral organs is associated with death in mice when experimentally inoculated.

Pathogenicity of various serotypes of P. multocida in rabbits differ considerably with respect to inoculum, route of inoculation, methods employed for evaluating the pathogenicity, period of observation and the age of experimental animals employed (Flatt and Dungworth (1971); Lu et al. (1987). In the present study, rabbits of 2-3 months of age were intranasally inoculated into both nares with 3.2×10^9 bacteria and the animals were observed for a period of 21 days. The earliest symptoms were exhibited within 12 h which included general weakness, lethargy and impaired appetite. The animals also exhibited a transient temperature rise, but it was not consistent with the development of any clinical infection.

Earlier workers were seen to have used varying dose of inoculum ranging from 2.75×10^6 cells to 3.2×10^9 cells per animal when intranasally inoculated (Flatt and Dungworth, 1971; DiGiacomo et al., 1987a; DiGiacomo et al., 1987b; Lu et al., 1987).

None of the experimentally inoculated rabbit did produce specific clinical sign/gross lesions of pneumonia or rhinitis at antemortem/necropsy examination. DiGiacomo et al. (1987a) was of the opinion that neither the wild type of P. multocida strain nor the mutant streptomycin dependent strain of P. multocida can produce a uniform onset of clinical signs of pasteurellosis in rabbits on experimental inoculation.

Rabbits inoculated intranasally with the isolate R_9S developed no clinical evidence of pneumonia but bacteria was isolated from nasal swab for a period of seven days. Recovery of the bacteria from rabbits inoculated with $R_{10}S$ was successful upto 72 h while for the rest upto 48 h. Though the biochemical characteristics of isolates, routes of exposure and the dose of inoculum were similar, the isolates differed in the degree and period of colonisation in the nares of inoculated rabbits. Similar observations were made when Lu et al. (1982) compared the pathogenicity and immunising property of different isolates belonging to the same serotype, suggesting that isolates of the same serotype of P. multocida may exhibit differences in pathogenicity and virulence in rabbits.

The colonisation of P. multocida in nasal cavities and the lack of clinical disease indicate that carrier state can be established in rabbits when they are naturally infected.

The necropsy findings of rabbits were negative for any gross lesions on the seventh day post inoculation while it showed haemorrhagic tracheitis and consolidation of lung on the 14th day. A pulmonary abscess was seen in one of the rabbits inoculated with R_9S isolate, from which bacteria could be re-isolated. The lesions observed on 14-21st day post inoculation were only haemorrhagic tracheitis and all other organs were normal.

Flatt and Dungworth (1971) inoculated rabbit with P. multocida by intra-tracheal route and they observed that clinical signs produced were surprisingly mild considering the severity of pneumonia detected at necropsy. They have further reported that the lesions were limited to the respiratory system and were seen in rabbits on sixth day post inoculation, carried upto 18 days. Fibrinopurulent and necrotising pneumonia were the marked gross lesions in the experimentally infected rabbits. Pulmonary abscess was reported by Guarda et al. (1991) in 0.3 per cent of rabbits slaughtered in Northern Italy and P. multocida was isolated from 62 abscesses. Abscess formation was described as one of the many clinical forms of pasteurellosis. Fibrinous pneumonia and abscesses in many sites were reported to be produced in rabbits on intra-tracheal or intranasal inoculation with P. multocida serotype (Flatt, 1974).

The histopathological changes in nasal septa, trachea, lungs and liver collected from experimentally infected animals which were necropsied at varying intervals differed depending on the degree of infection caused by P. multocida isolates.

The observation made in the present study demonstrated that there was no absolute relationship between the clinical infection and the extent of histopathological changes in the above tissues.

Congestion, hyperplasia of goblet cells and mild to moderate infiltration of mononuclear cells were the predominant lesion in the septal mucosa and sinuses when rabbits were inoculated with R_9S and $R_{10}S$ isolates. Pseudostratified septal epithelium presented mild degenerative changes and the lesions were predominant on the 14th day post inoculation.

DiGiacomo et al. (1991) described the histopathological changes caused by P. multocida at the upper respiratory tract including the nasal bones. The epithelium was hyperplastic, composed of columnar cells with loss of cilia and increased number of goblet cells. In many areas epithelial cells were necrotised and heterophils infiltrated the epithelium. The lamina propria was increased in depth with oedema and inflammatory cells. The turbinate bones had irregular outlines with proliferation of fibroblasts in the periosteum.

Focal congestion and mild to severe haemorrhages were the main lesios observed in the mucosa and lamina propria of the trachea. Diffused or nodular aggregates of infiltrated mononuclear cells were also observed in mucosa and lamina propria. The lungs showed moderate to severe congestion, oedema, haemorrhages and severe suppurative bronchopneumonia which were predominant on the 14th day post inoculation which persisted throughout the experimental period. These lung lesions were invariably present in all the rabbits inoculated with each of the six isolates. The above changes in the trachea and lungs of rabbits inoculated are the reflection of bacterial infection induced in the animals. The histopathological changes observed in the present study were similar to those reported earlier as characteristic lesions of Pasteurellosis in rabbits (Hagen, 1958; Flatt and Dungworth, 1971; Lu and Pakes, 1981; DiGiacomo et al., 1991). The main lesions in the lungs were bronchopneumonia with consolidation and necrosis. The trachea was inflamed and the bronchi were filled with thick purulent exudate. In severe cases, fibrinopurulent and necrotising pneumonia were described with pulmonic parenchyma containing degenerating cellular debris centrally and surrounded by fibrous tissue.

The liver showed centri-lobular necrosis, congestion and fatty changes on the 14th day post inoculation in rabbits inoculated with four out of the six isolates. The significance

of these lesions has not been properly understood as none of the previous workers have reported about the lesions in liver. However, the above lesions could be attributed to the toxic effects of the bacteria, since P. multocida is a known potent endotoxin producer.

Antibiotics such as penicillin, streptomycin or tetracycline are used for the treatment of pasteurellosis in rabbit. Flatt (1974) and Holmes (1984) have described that the therapy with antibiotics were relatively ineffective. In contrast to the results based on in vivo studies, P. multocida isolates from rabbits were reported to be susceptible to a wide range of chemotherapeutic agents (Lu et al., 1978; Mushin and Schoenbaum, 1980, Kawamoto et al., 1990).

The six isolates of P. multocida tested for their antibiogram in this study were uniformly sensitive to streptomycin, ampicillin, gentamycin, sissomycin, furazolidone, polymyxin, sulfadiazine, nitrofurantoin, chloramphenicol, novobiocin and cefazolin but resistant to erythromycin.

Lu et al. (1978) reported that all his 42 isolates of P. multocida from rabbits were sensitive to all the seven antibiotics employed in the study except streptomycin and clindamycin. But in this study all the isolates were sensitive to streptomycin. Clindamycin was not used in this

study. All the isolates were sensitive to sulphadiazine unlike most of the isolates of Lu et al. (1978) which were resistant to sulphonamides with multiple resistance. Similarly Kawamoto et al. (1990) also stated that all of his 40 isolates were sensitive to 12 antibiotics except clindamycin. Unlike the present observation wherein all the six isolates were resistant to erythromycin, all the isolates of Kawamoto et al. (1990) were sensitive to this antibiotic.

Heat stable antigens extracted with formolsaline prepared from the three isolates R_9S, $R_{10}S$ and $R_{23}S$ and the P. multocida vaccine strain P_{52} produced specific antiserum in rabbits.

The first dose of the antigen preparation was with Freund's adjuvant by subcutaneous injection and subsequently by repeated intravenous injections without adjuvant. A positive test for the specific antibody was obtained only after 54 days post injection, when tested by GDPT. With all the antigen, antibody could be detected only after administering eight doses. The serum level of antibody had further improved when the antigen administration was repeated and sufficient level had reached by 72 days when tested by GDPT.

For raising antiserum against somatic antigens of P. multocida, Heddleston et al. (1972) had used chickens,

while Namioka and Murata (1961) and Manning (1984) have used rabbit. Both the species of animals were reported to be efficient in the production of specific antibody when injected with bacteria, purified lipopolysaccharide and lipopoly-saccharide extracted by phenol water method. The rabbits which were employed for antiserum raising produced sufficient level of antibody irrespective of the form of the antigen used, only after 45 days of injection. Bapat and Sawhney (1972) and Manning (1984) reported that sufficient level of antibody was produced in rabbit only after 70 to 75 days of somatic antigen injections. The result obtained in the present study are in close agreement with the findings of the previous workers.

The results of the somatic antigen reaction with its homologous/heterologous antiserum in the GDPT indicated that the formal saline extract of the whole cells of all the isolates and the vaccine strain P_{52} produced atleast one precipitin line of identity. Somatic antigens from the isolates $R_{23}S$ and vaccine strain P_{52} produced an additional precipitin line when they were reacted against their homologous antiserum. However, antigenic differences were noticed for $R_{23}S$ from that of R_9S and $R_{10}S$ as evidenced from the partial line of precipitation formed against the respective antigens.

The present results suggests the prevalence of P. multocida as one of the causative agent of respiratory tract infections of rabbits kept at laboratory animal facilities. The biological and biochemical characterisation of the rabbit strains of P. multocida were similar to those characters which were reported by earlier workers. These isolates were pathogenic to mice while it was not for rabbits, though it colonised in the nares of rabbits. The antibiogram of the isolates were studied and all the strains were sensitive to several of the chemotherapeutic agents. Serologically, the identity of these isolates could be establishd by GDPT. Further work to evaluate the antigenic make up of these isolates and to utilise them for vaccine preparation can be taken up.

Summary

SUMMARY

One hundred and twelve rabbits were examined during this study which included 76 apparently healthy and 36 ailing/dead rabbits. The live animals comprised 20 young and 56 adult animals, of which, 32 were of New Zealand White, 16 Grey Giant, seven Soviet Chinchilla and 21 cross bred. This included both healthy and sick animals. Of the 36 ailing/dead rabbits, 26 of them were of New Zealand White, six of Soviet Chinchilla and four of Grey Giant. This included 20 young and 16 adult animals. Pasteurella multocida could be isolated from five adult New Zealand White and one adult Grey Giant which died of respiratory infection. Gross pathological lesions observed in post mortem examination were typical haemorrhages in the trachea, haemorrhages and abscessation in lung and necrotic foci in liver.

All the six isolates were gram negative coccobacilli, non motile and produced catalase. Two isolates were oxidase negative and four oxidase positive, grew anaerobically, utilised glucose fermentatively and none produced hemolysis of sheep red blood cells.

The isolates were positive for nitrate, indole and potassium cyanide except for one isolate which was

nitrate negative and two were indole negative. All were negative for hydrogen sulphide production, urease and gelatin hydrolysis. Only one isolate was positive for growth on ONPG. Majority of the sugars were fermented by these isolates.

These isolates were tested for their pathogenicity in mice and rabbits. Intra peritoneal injection of one millilitre of an overnight culture containing 10^8 bacteria/ml, killed mice between 24-72 h post inoculation and the organism could be re-isolated from the dead animals. When rabbits were intra-nasally inoculated, with 0.5 ml of overnight culture containing 3.2×10^9 bacteria, none of the isolates could establish clinical infection. Though the inoculated rabbits were apparently normal, one isolate colonised within the nares of the rabbit and was shedder for a period of seven days, while the other rabbits inoculated with the remaining five isolates were shedders only upto 48 h.

Histopathological studies revealed haemorrhagic tracheitis, extensive haemorrhage in lung and bronchopneumonia catarrhal inflammation in the nasal septa and fatty degeneration in the liver.

All the isolates were uniformly sensitive to streptomycin, ampicillin, gentamycin, sissomycin, polymyxin,

chloramphenicol, novobiocin, cefazolin, furazolidone, nitrofurantoin and sulphadiazine but were resistant to erythromycin.

Antiserum raised in rabbits against heat stable antigens prepared from three of the isolates and the vaccine strain P_{52} were used in the gel diffusion precipitin test to compare and contrast the antigenic relationship. The results indicated that the formal saline extract of the whole cells of the three isolates tested and the vaccine strain P_{52} produced at least one precipitin line of identity. Of the three isolates, one isolate and the vaccine strain P_{52} produced an additional precipitin line, when they were reacted against the homologous antisera.

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CHARACTERISATION OF *Pasteurella multocida* ISOLATES FROM RABBITS

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ABSTRACT OF A THESIS

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ABSTRACT

The prevalence of Pasteurella multocida in rabbits in and around Thrissur was probed by cultural isolation and confirmed by the elucidation of the identity of the isolates by their physiological, biological and serological characteristics.

A total of 112 rabbits comprising 76 apparently healthy and 36 ailing/dead animals of various age groups and breed were subjected to cultural screening. This attempt fructified in the isolation of P. multocida from five rabbits of New Zealand White breed and one of the Grey Giant breed which died of respiratory infection. Post mortem examination of these animals revealed typical haemorrhages in the trachea, haemorrhages and abscessation in lungs and necrotic foci in liver.

The present study suggests the prevalence of P. multocida as one of the causative agent of respiratory tract infection of rabbits kept at laboratory animals facilities.

The biological and biochemical characters of all the six isolates were similar to those characters of rabbit strains of P. multocida which were reported by earlier workers.

These isolates were pathogenic to mice when intraperitoneally inoculated as all the mice were killed within 72 h. In rabbits, these isolates could not establish clinical infection on experimental intranasal inoculation, while at necropsy the animals revealed macroscopic and microscopic lesions suggestive of pasteurellosis.

The antibiogram of the isolates were studied and all the isolates were sensitive to several of the chemotherapeutic agents but uniformly resistant to erythromycin.

Serologically, a common somatic antigen in all the three rabbit isolates tested and vaccine strain P_{52} could be established by gel diffusion precipitin test. However, additional somatic antigen for one isolate and the vaccine strain P_{52} could also be detected when they were reacted against their homologous antisera.