Chapter I

Introduction

Pasteurella multocida is a gram-negative bacteria. The name Pasteurella septica has been suggested by Topley and Wilson but Pasteurella multocida is now universally employed and has been given by Rosenbusch and Merchant since 1939. The "multocida" comes from the Latin meaning many-killing,i.e.pathogenic for many species of animals(1). The organism is the causative agent of an infectious disease "pasteurellosis" which is found world-wide in human, animals and birds. Not many cases of human infections have been reported throughout the world. It is apparently often overlooked as a pathogen (2). The failure to diagnose P. multocida infection correctly is most commonly due to misidentification on gram stained smears or inadequate laboratory identification techniques (3,4). On the contrary, animal infections such as fowl cholera in turkeys and poultry, and hemorrhagic septicemia in cattle have been more thoroughly investigated because of their economic and veterinary importance (5). The diseases in animals may be sporadic or occur in an outbreak leads to economic losses and the difficulty in controlling and prevention.

1. Bacterial classification

According to the first edition of Bergey's manual of systemic bacteriology 1984 (6), P. multocida has been classified in section 5 of facultatively anaerobic gram-negative rods, family III Pasteurellaceae.There are 3 genera in this family : Pasteurella, Haemophilus and Actinobacillus. Several characteristics distinguish them from each other. All Pasteurella species are usually oxidase positive. Therefore, differentiation among genera is easily performed by this reaction (7).

Genus Pasteurella is divided into 6 species : P. multocida, P. pneumotropica, P. haemolytica, P. ureae, P. aerogenes, and P. gallinarum. The differential characteristics among species are shown in table 1.

2. General characteristics

P. multocida (7) occurs most frequently as coccobacilli or short rods. Bipolar staining is common especially in preparations made from infected animal tissues. Strains from healthy animals are often pleomorphic with longer bacilliary forms and occasional short filaments.

The basic structure of the surface layers of P. multocida resembles other gram negative bacteria (Figure 1)

Table 1 Differential characteristics of the species of the genus *Pasteurella*[a]

Characteristics	1. *P. multocida*	2. *P. pneumotropica*	3. *P. haemolytica*	4. *P. ureae*	5. *P. aerogenes*	6. *P. gallinarum*
Hemolysis ( )	-	-	+	-	-	-
Growth on						
MacConkey's agar	-	-	+	-	+	-
Indole production	+	+	-	-	-	-
Urease activity	-	+	-	+	+	-
Gas from carbohydrates	-	-	-	-	+	-
Acid production from:						
Lactose	-	d	d	-	-	-
Mannitol	+[b]	-	+	+	-	-

a = Data from Carter (7). For symbols see standard definitions.

b = Strains from dogs and cats may be negative for mannitol.

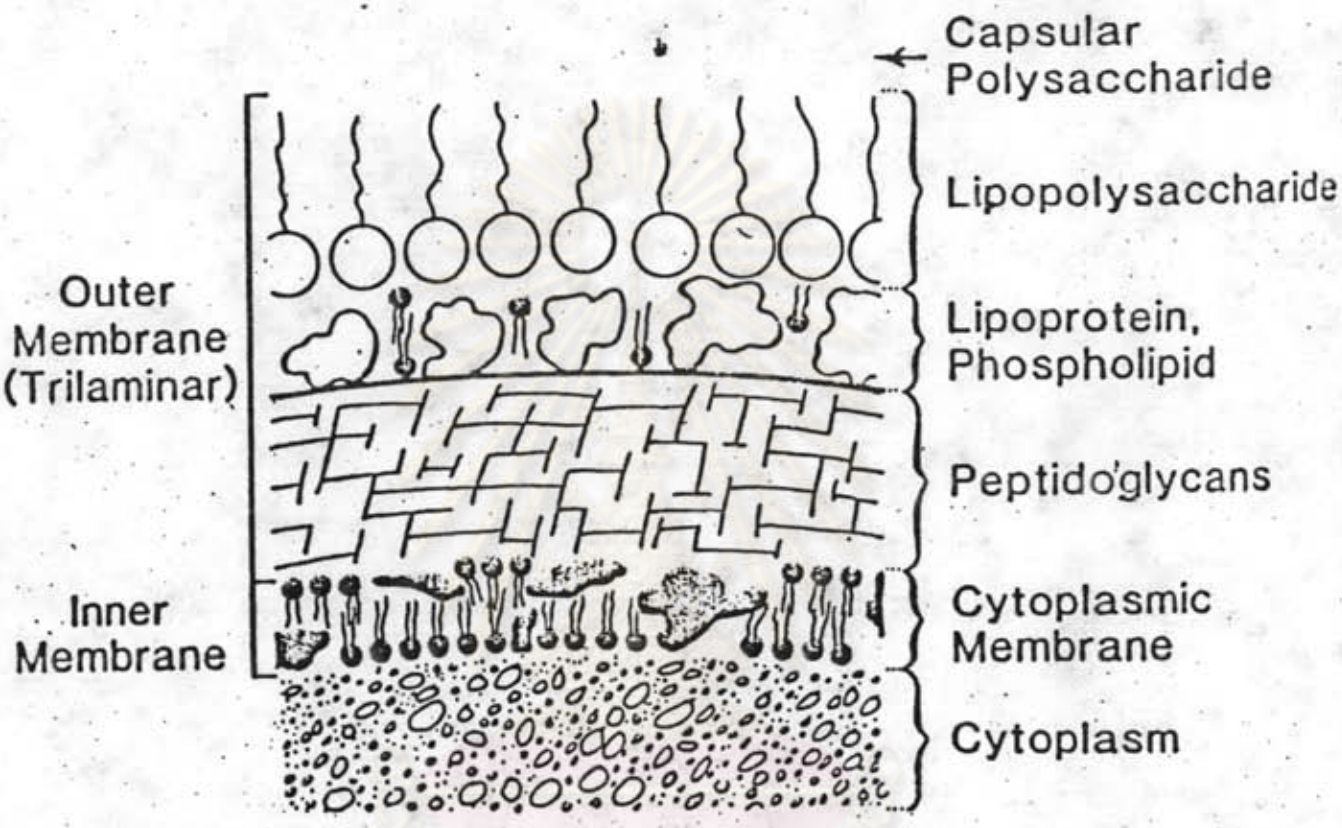

Figure 1 Schematic representation of cell membrane of gram negative bacteria (8)

It possesses a capsule of polysaccharide or, less often, a capsular matrix of glycoprotein that overlays the bacterial cell membrane or cell wall (8). The cell membrane is composed of an inner and outer portion. The former consists of the cell cytoplasm and cytoplasmic membrane, and the latter is made up of a complex trilaminar structure of peptidoglycans, lipoproteins and phospholipids and lipopolysaccharides. The capsular material is antigenic and LPS acts as a component of somatic antigen. They both are type specific when appropriate methods are used for serotyping.

P. multocida type A produces capsular hyaluronic acid which can be depolymerized by testicular and bacterial hyaluronidase (9,10). Capsule of type D contains N.acetyl glucosaminuronic acid which aggregates with 0.1% acriflavine (11). The capsules of type B and E are small. They contain protein, polysaccharide and LPS but no hyaluronic acid (12).

Most virulent strains of P. multocida produce capsules of varying sizes which can be demonstrated by Jasmin's method (13) or India ink procedure (14). They are frequently lost after several subcultures (7). The capsules of type A are thicker than those of type D, B and E. The average thickness of the type A capsular layer is 65 nm and that of the type D capsule is 20 nm (15). Encapsulated strains of P. multocida are virulent for mice,

rabbits and some other animals but a mutant uncapsulated strains are almost avirulent (16).

Colonies on blood agar (7) are non-hemolytic but produce a brownish discoloration in regions of confluent growth. They also produce a distinctive smell of value in recognition when grown on blood agar. Colonies on media without blood are divided into four variants : mucoid, smooth irridescent, smooth nonirridescent (grey or blue) or infrequently rough (16). They can not grow on MacConkey or SS agar (17) which is the routine media for primary isolation of gram negative bacteria.

P. multocida is not acid-fast, non-spore forming and non-motile (17). Growth occurs between 25 C and 40 C but optimum temperature is 37 C. Most avian strains and a few others grow at 42 C. Catalase is positive and almost always oxidase-positive. Nitrates are reduced to nitrites. Gelatinase is negative. MR-VP are negative. Lysine decarboxylase and arginine dihydrolase are not produced. Ornithine decarboxylase is always positive. They are facultatively anaerobic. Glucose and other fermentable compounds are fermented with the production of acid but usually no gas. P. multocida biotypes can therefore be classified by means of their fermentation reactions (18,19,20). Other bacteriological characteristics are shown in table 2.

Table 2 characteristics of Pasteurella multocida (17)

Test performed	Signs	% +
Morphology	short rods	
Motility	non-motile	
Gas from glucose	[-]	0
Action on blood	v	
Fermentative or oxidative	[F]	
Carbohydrate base	F	
Acid from		
Glucose	+	100
Xylose	+	67
Mannitol	+	78
Lactose	[-]	8
Sucrose	+	100
Maltose	[-]	2
10% Lactose	[-]	8
Catalase	+	98
Oxidase	+	97
Growth on :		
MacConkey	[-]	2
SS	-	0
Simmon Citrate	-	0
Urea Christensen's	-	0
Nitrate reduction	+	99
Gas from nitrate	-	0
Indole	+	99
TSI slant, acid	+	98
TSI butt, acid	+	99
H_2S (TSI butt)	-	0
H_2S (Pb ac paper)	v	76
MR	-	1
VP	-	0
Gelatin hydrolysis [a]	-	0
Litmus milk	-	3A
Growth at :		
25 c	+	91
35 c	+	99
42 c	v	32
Esculin hydrolysis	-	0
Lysine decarboxylase	-	0
Arginine dihydrolase	-	0
Ornithine decarboxylase	[+]	94
Nutrient broth without NaCl	v	68
Nutrient broth with 6% NaCl	-	2

P. multocida is susceptible to penicillin and other antimicrobial drugs for gram-negative bacteria such as Ampicillin,tetracycline,chloramphenicol,and cepholosporins (3). However, susceptibility testing of isolates should always be performed since strains of P. multocida vary in susceptibility to chemotherapeutic agents (21). Penicillin-resistant strains have also been described in a few human cases (22,23) and about 20% of animal strains (24).

3. Epidemiology

P. multocida can remain viable in water for 7 to 25 days and in soil for up to 21 days (25). In carcasses of fowl, the organism can persist for up to 60 days (25). It is killed by exposure to direct sunlight for 10 min (3). It has been found as a commensal in the upper respiratory tract of people professionally exposed to animals (26). and in patients with chronic pulmonary disease (27). Occasionally,it has been found as a commensal in sputum of well patients without underlying chronic pulmonary or sinus disease (3). P. multocida has been isolated from the digestive system or respiratory tract of domestic cats and dogs, rats, mice, rabbits, cattles, sheep, swine, reindeer, horses, monkeys, buffaloes and lions (3). In the oral cavities of 70-90% of cats and 50-60% of dogs are found to harbour this organism (28,29).

Most human infections result from direct inoculation via bites or scratches,however animal exposure in the absence of bites or scratches may cause of infections if P. multocida is inhaled through indirect contact with animal secretions (30). Deposits of the organism on skin injury or licking on a mucosal surface may also account for pathogenesis in some cases. Males and females are equally infected. The very young (0 to 4 years) and the older age group (over 55 years) have higher attack rates than the other age groups (2). Human to human spread of infection has not been documented, nor has contaminated food or water been implicated as a source of infection (3).

P. multocida from the upper respiratory tract of human being is not pathogenic for turkey but P. multocida from infected human may infect poultry via excretions from the nose or mouth (31). P. multocida from birds with fowl cholera will usually kill rabbits and mice (5). Colonization on the mucous membranes of the upper respiratory tract of carrier animals plays an important role in the maintenance and spread of infection (32). Secretions or discharges from infected animals are usually transmitted to other susceptible animals (31,32,33)

4. Pasteurella infection

4.1 Human pasteurellosis

P. multocida is a common cause of infection following animal bites or scratches which are mostly caused by cats or dogs (34). It causes the typical clinical manifestations of a developing cellulitis at the site of injury. The infection is potentially dangerous and can cause a chronic local infection of deep tissues and septicemia (2,35). P. multocida can be isolated as a commensal organism from the respiratory tract of patients with underlying pulmonary disease, but serious respiratory tract infections including pneumonia, empyema and lung abscesses may develop (3). P. multocida often acts as an opportunistic pathogen and causes bacteremia in patients with liver dysfunction, septic arthritis in damaged joints, meningitis in the very young and elderly and pulmonary colonization or invasion in patients with underlying respiratory tract abnormalities. Domestic animals are a potential threat to patients with cirrhosis especially if close contact with the animal occurs (34,36). The incubation period is uncertain but a short incubation period is suggested by the patients who developed peritonitis after endoscopy and a prolonged incubation period has been suggested in a patient who was bitten by a dog 5 months before developing peritonitis (37).

Some patients develop peritonitis with septicemia subsequent to infection. However, cases with septicemia without peritonitis have also been described (38,39,40). Isolation of identical biotypes from both animal and patient in the case of animal exposure suggests that the animal is the source of infection (34).

P. multocida is also a pathogen of systemic infections including meningitis, brain abscess, spontaneous bacterial peritonitis, intra-abdominal abscess (3), urinary tract infection (41,42) and renal abscess (43,44). Intestinal infection with P. multocida is very rare, only two cases from stool specimens have been isolated (3,44). Neonatal infections have also been reported and the patients died of sepsis due to P. multocida within the first 72 hrs of life (45,46).

4.2 Animal infections

P. multocida is pathogenic in many species of animals and is capable of causing serious epizootic infections (3).

Fowl cholera(avian cholera,avian pasteurellosis, avian hemorrhagic septicemia)

Toussant has been isolated and proved that P. multocida was the sole cause of fowl cholera since 1879(47). The disease usually appears as a septicemic disease associated with high morbidity and mortality, however chronic or benign conditions occur often. It occurs sporadically and

rapidly spreads through flocks of chicken,turkeys, pigeons, sparrows, ducks, other domesticated and wild fowl (31,48). Turkeys are more susceptible to infection than chickens and adult chickens are more susceptible than young (49). There is no evidence of transmission of fowl cholera through the eggs of chickens (50). Serotype A:1 and A:3 are important causes of fowl cholera (7). Capsular group D organism has proved to exhibit little pathogenicity (16) and group F strains vary in pathogenicity in mice and turkeys (51). Group B organism which has previously been described as non-pathogenic in birds is now proved that serotype B:4 isolated from a turkey in Texas can cause fowl cholera in turkeys and serotype B:1 isolated from a swan in California can develop fowl cholera in some turkeys (52).

Hemorrhagic septicemia (septicemic pasteurellosis of cattle)

The disease occurs mainly in tropical and subtropical countries (53). Serotypes 6:B (Asian strain) and 6:E (African strain) are the causative pathogens of this disease while serotype 11:B (Australian strain) is not (54,55). Serotype 11:B produces fatal septicemia following infection. Hemorrhagic septicemia is recorded chiefly in cattle, yaks, camels and water buffaloes. Hemorrhagic septicemia in pigs is identical with that in cattle (56) but is now very rare throughout the world

(57). The disease occurs in outbreak and spread occurs by the ingestion of contaminated food-stuffs, the infection originating from clinical normal carrier or clinical cases, or possible by tick (58) and biting insects. The organism can be isolated from saliva and blood stream during the early and late stage of the disease (59).

P. multocida (7) is also a common and important secondary invader of pneumonic lesions causing many cases of chronic to severe pneumonias. Shipment, transport and crowding of animals particularly during inclement weather are predisposing factors to the diseases.

The organism also causes pneumonia in goats and sheep which is usually associated with infection by P. hemolytica (59).

Laboratory rabbits can be infected by P. multocida both direct contact and airborne means (60). It is normal microbial flora of the rabbits' mucosal surfaces (61). and stress plays a major role in the infection (60). It is generally acknowledged as the primary etiologic agent of disease in rabbits (62,63). Chronic form of pasteurellosis is usually troublesome to researchers because affected rabbits are often being used in long-term studies which are at high risk of being terminated by infection (60).

5. Prevention and control

Pasteurellosis in animals is usually a major cause of economic losses because of death and the costs of treatment. Vaccinations are of considerable value for prevention and control.

Hemorrhagic septicemia vaccine and fowl cholera vaccine are two types of vaccines available in Thailand. They are the products of Division of Biological Products, Department of Livestock Development which comes under the jurisdiction of the ministry of Agriculture and Cooperative (64).

Hemorrhagic septicemia vaccine

It is formalin-killed vaccine prepared from 6:B local strain with aluminium hydroxide gel as adjuvant. Its indication is for preventing hemorrhagic septicemia in cattle, buffaloes and pasteurellosis in sheep and goats. Immunity persists 4 to 6 months dependent on the individual and the age of animals.

Fowl cholera vaccine

It is formalin-killed vaccine prepared from 8:A local strain. The vaccine is used for prevention of fowl cholera in birds such as chicken and geese. Immunity persists for three months, so that vaccination every three months is recommended. The efficacy of this vaccine is 70 per cent dependent on the virulence of an outbreak.

CU strain is another kind of fowl cholera vaccine. It is a live vaccine prepared from the Clemson University strain of an attenuated P. multocida (65). CU strain has antigenic characteristics of Heddleston serotype 3 and 4 (66). This vaccine is available in the United States of America but is not being used in Thailand.

Besides vaccination with specific serotyped strain, good sanitation and avoidance of overcrowding should be taken into account.

6. Serology of P.multocida

Serological studies of P. multocida have evolved over the years. Lignieres has studied the serotypes in order to correlate them with the host specificities (57) and many investigators have modified the serological methods for classification and epizootiological studies since then. The history of earlier serological studies on P. multocida is summarized in table 3.

Up to the present time, the serological classification uses both the results of capsular and somatic serotypes. The two systems (67) most commonly used to serotype isolates of P. multocida are :

a. Carter-Heddleston system

b. Namioka-Carter system

TABLE 3 Early serological studies on pasteurella multocida (16)

Investigators	procedures	Results
Cornelius (1929)	Agglutination absorption	Group I,II,III and IV
Ochi (1933)	Agglutination	Type A,B,C and D
Yusef (1935)	Precipitation	Group I,II,III and IV
Rosenbusch and Merchant (1939)	Agglutination, fermentation	Group I,II and III
Little and Lyon (1943)	Slide agglutination	Type 1, 2, and 3
Robert (1947)	Serum protection test in mice	Type I,II,III,and IV

Carter system is the procedure for capsular typing and the result is designated with a capital letter A,B,D and E.

Heddleston or Namioka system is the procedure for somatic typing. Although their methods and serotypes are different, the results of both methods are still designated with the same Arabic number; type 1-11 for Namioka's and type 1-16 for Heddleston's. One capsular serotype may be found among isolates of different somatic serotypes, for examples, A:1 and A:3 (Carter-Heddleston system) or 5:A and 8:A (Namioka-Carter system).

Carter(68)has introduced the IHA test for Capsular serotyping.The organisms are serotyped on the basis of the substance known as capsular antigen. These antigens which deposited on the surface of the cells (67), are extracted from the cells by heating for 30 min at 56 C. They are also believed to be specific and chiefly polysaccharides in nature (69). Using the IHA test, the capsular serotypes are classified into four types : A,B,C and D (68). which are Robert's type II,I,III and IV respectively (16). Type C has lately been excluded because the strains of type C are not of a capsular nature in the same sense that the others are and the titers of the type C sera are always low (16). Subsequently, serotype E is added. This serotype has been isolated from bovine cases of hemorrhagic septicemia in Central Africa (53). Rimler and Rhoades (51)

has recently found the serogroup F which was isolated from turkeys in various parts of the United States. Now-a-days capsular serotypes A,B,D,E and F are known. Cross reactions appear to be rare by this procedure in that isolates fall into only one capsular type (8). The relationship of P. multocida capsular serotypes to diseases is shown in table 4.

Namioka and Murata(70) demonstrate the slide agglutination test for capsular serotyping with similar results to Carter's when using irridescent colonies. However, their method is not well known and is not being used by any researchers.

Furthermore, non-serological test for capsular type A and D are described (71,11) but there are no non-serological tests for capsular type B and E.

Carter and Rundell (71) describe the method for identification of P. multocida type A by using a strain of Staphylococcus aureus producing hyaluronidase enzyme. The principle of this method is based on the depolymerization of hyaluronic acid in the capsule by the action of enzyme hyaluronidase resulting in the diminution of P. multocida growth at the intersection of the two organisms on culture media.

TABLE 4 Relationship of the capsular type of *Pasteurella multocida* and diseases

Capsular type	Diseases
A	Fowl cholera. Primary and secondary infections in a wide range of animal species.
B	Hemorrhagic septicemia of cattle and buffaloes principally. (Strains of this type but with different somatic composition have been found)
D	Primary and secondary infections in a wide range of animal species.
E	Hemorrhagic septicemia in cattle in Central Africa
F	Fowl cholera in turkeys in the United States of America.

Carter and Subronto (11) use acriflavine neutral in a dilution of 1:1,000 to identify P. multocida type D. Capsule of type D has N-acetyl galactosaminuronic acid that its physical characteristic resembles the Vi antigen of Salmonella typhi. This substance when it reacts with acriflavine will produce a heavy flocculant precipitate which becomes evident within 5 min. After 30 min, it settles and leaves a distinct supernatant.

Namioka and Murata (72) and Heddleston et al (73) have studied the somatic serotype of P. multocida. Both groups extract the antigens in different ways, i.e. their antigens are not exactly the same. Namioka's antigen is the residue left after hydrochloric acid treatment, which is proposed to be a stable entity of the O somatic antigen (72). By using the tube agglutination test, these O somatic antigens are classified into 11 groups. More than 15 serotypes have been established when the Namioka-Carter system is performed (69).

Heddleston's antigen is a heat-stable antigen (73). It is a LPS-protein complex. Its serological specificity is associated with an endotoxin which is the LPS component. (74). Further study of Brodgen and Reber (75) reveals that LPS is a major component of the heat-stable antigen responsible for the type specific. This antigen is extracted from whole cells by heating for 1 hr at 100 C. Using the GDPT, the antigens are classified

into 16 different types designated as type 1-16 (73,76). One strain may possess more than one serotype dependent on its antigenic complexity.

Prince and Smith(77,78,79)have studied the serotype of P. multocida on the basis of Ouchterlony's gel precipitation and immunoelectrophoresis. They state that β-antigen is type-specific polysaccharide and adsorbs to red cells in the Carter's IHA test. α-Complex is probably a polysaccharide-protein complex and closely adherent to the cell wall. It is immunogenic. γ-Antigen is the LPS found in cell wall and each has one or more antigenic determinants responsible for different Namioka's O or somatic serological varieties.

There are a lot of reports throughout the world on P. multocida serotypes isolated from animals. Several researchers use the prevalent serotype for preparing the vaccine in order to prevent the same disease in the animals.However,there are not many reports on P. multocida serotypes in human. Choudat et al (80) have used Namioka-Carter system to determine the serotype of patients. They have determined the antibodies in these patients and found that antisera against capsular antigen was 20 to 2560 and 5 to 640 against somatic antigen within 2 weeks after the first clinical sign. Carter (81) state that capsular serotypes of human are type A and D, however, untypable strains are reported as well.In addition, Blackburn et al

(82) state that human somatic serotypes are serotype 1;3; 3,4; 4; 6; 4,12; 12 and 13. Only one case of P. multocida infection in human has been reported in Thailand. The serotype of P. multocida from this isolate has not been performed (4). In addition, only two reports of capsular serotypes have been published in many animals tested (83,84).

7. Protein analysis by SDS-PAGE

Over the years, biochemical analyses of cell surface proteins from gram-negative bacilli have been studied. The protein analyses reveal that nearly half of the mass of the outer membrane is protein (85). Most proteins are thought to be located in the outer membrane, although some are found in the inner membranes and the cytoplasm (86,87).

SDS-PAGE is one of the most widely used techniques for the analytical separation of proteins and peptides (88). This technique has the advantages of high resolution and sensitivity. It is also simple to carry out. The pore size of the gel can be controlled by the percentage of the acrylamide and polymerizing agents used to make up the gel. The lower the percentage polyacrylamide gels are, the larger the pore sizes will be. The lower percentage gels are suitable for the separation of the compounds with higher molecular weights or vice versa (89).

The principle of protein analysis by SDS-PAGE is based on the interaction between SDS and proteins. In the present of 2-mercaptoethanol and SDS, proteins are dissociated into polypeptide chains and bound with a constant ratio (W/V) of SDS. The overwhelming negative charge provided by the SDS coating makes any charge contributed by the protein negligible, and seperation is therefore dependent on the molecular size of the protein. Thus, the size of the polypeptide chains of a given protein can be determined by comparing their eletrophoretic mobilities on SDS gels to the mobilities of marker proteins after the gels are fixed and stained with Coomassie blue (90,91).

SDS-PAGE with discontinuous buffer is used by Laemmli for study the structural protein of bacteriophage T4(92). His technique is now used by many investigators to determine the membrane proteins and LPS of gram-negative bacilli, such as study on E. coli outer membrane protein (93) and protein composition of E. coli cell envelope (94). The membrane proteins of P. multocida have also been studied by many researchers. Lugtenberg et al have studied the cell surface protein (95) and membrane protein and LPS patterns (96) of P. multocida strains that caused atrophic rhinitis in swine. According to these studies, they state that the protein profiles of cell envelope or cell membrane show distinct protein patterns with specific

protein band that may respond for protective antigen when further study has been done.Lee et al(97)have investigated the membrane protein of CU strain and other P. multocida serotype 3,4 strains by using the lysozyme treated cells for sonication. This study reveals that different protein patterns of the membrane protein are possibly found among the P. multocida serotype 3,4 strains.

8. Objective

As mentioned earlier, the identification of human pasteurellosis is still a problem. Most bacteriologists are unfamiliar with this organism and many identify it something else. For this reason,a study of the bacteriological characteristics will be an improvement in the identification of P. multocida.

It is well known that serotyping is the usefulness in epizootic infections. In some instances, it seems to correlate with host specificities. In the United States A:1 and A:3 are prevalent in fowl cholera of turkeys and chicken but they do not cause the same disease in cattle or pigs (31). In Asian countries, serotype 6:B (Asian strain) causes hemorrhagic septicemia in cattle but serotype 11:B (Australian strain) does not, even though they possess the same capsular serotype. If there is any study on P. multocida serotypes in human and animals in

Thailand, it should be the documentation of human and animal serotypes.

Furthermore, SDS-PAGE is a valuable technique in the study of the protein profiles of cell envelope or cell membrane proteins of microorganisms. Earlier studies reveals that different protein patterns among strains may be obtained if suitable methods are used to extract the proteins. For this reason, if the whole cell sonicated extracts of P. multocida isolated from human and animals are studied, they may reveal some additional information about these strains.

The purpose of this study is therefore focused on the characterization of P. multocida isolated from human and animals in Thailand on the basis of :

1. Bacteriological characteristics
2. Serological typing by the Carter-Heddleston system
3. The protein patterns of whole cell sonicated extracts by SDS-PAGE