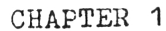

CHAPTER 1

INTRODUCTION

In Thailand, anthrax, hemorrhagic septicemia, black leg and brucellosis constitute the major bacterial diseases of livestock, and cause the death of several thousand of animals each year. The protection by vaccination against the causative agents of these diseases are the most important to prophylaxis and control them.

The specific cause of anthrax is *Bacillus anthracis* whereas the specific cause of hemorrhagic septicemia is *Pasteurella multocida*. Anthrax and hemorrhagic septicemia are the acute infectious diseases that have a rapidly fatal course and wide spread through the herd. The prophylactic vaccines are administered seperately every year, especially in the outbreaks. According to time, labour and operational cost, a suitable combined vaccine which confers the effective immunity against these diseases is needed.

For this aspect of prophylaxis, the main objective of this research project is to study the bivalent vaccine of *Bacillus anthracis* and *Pasteurella multocida* prepared from combination of the monovalent vaccine of each organism in experimental animals. This is only a preliminary research with anticipation of applying the combined vaccine against anthrax and hemorrhagic septicemia in the future.

1. Description of Bacillus anthracis

1.1 Taxonomy

B. anthracis belonged to the family Bacillaceae, genus Bacillus; it was one of a group of several species of aerobic, endospore-forming rods and saprophytic.[1]

Several workers and bacterial taxonomists had shown a close relationship between B. anthracis and B. cereus. Smith et al (1952)[2] concluded that B. anthracis was a pathogenic variety of B. cereus. They also stated that strains of B. anthracis which had lost their virulence could not be differentiated from B. cereus. Recently Brown et al (1958)[3] had supported this appearance.

By the use of specific gamma anthrax phage, string-of-pearls reaction, growth on bicarbonate medium under carbon dioxide, pathogenicity in lower animals and other differential procedures, Leise (1959)[4] and Burdon (1960)[5] showed that pure strain of B. anthracis which had partially or wholly lost their virulence remained identifiable as anthrax bacilli and could be differentiated from B. cereus and other sporeformers.

1.2 Morphology, growth characteristics and biochemical reactions

B. anthracis was a large, gram-positive, nonmotile, rod shaped organism, 3 to 8 μ long by 1 to 1.2 μ broad. The bacilli were straight or slightly curved; the ends were sharply cut off, or truncate. In blood of infected animal the bacilli occurred in pairs or in short chains of three or four, and their capsules were readily

demonstrable by suitable stain, such as Wright's or Giemsa's stain. The spores, which were not form in the animal body, were ellipsoidal and situated centrally in the cell. They were stained with difficulty.[6,7,8]

B. anthracis grew readily on customary laboratory media under aerobic conditions and also in partial anaerobic atmosphere. The optimum temperature for growth of bacilli was 35-37°C although growth could occurred within a range of 12-44°C. A slightly alkaline medium, pH 7.5 to 7.8 was most conductive to good growth.[9,10]

Capsules were not found in culture except on medium contained animal protein, such as serum. High partial pressure of carbon dioxide or addition of bicarbonate to solid media enriched with serum also caused B. anthracis to encapsulate.[8,11]

Spores were formed most abundantly at 32°to 35°C and only under aerobic condition.[6] Sporulation might be reduced or eliminated under certain conditions. A reduction in the partial pressure of oxygen did not reduce the ability of a culture to sporulate, whereas an increase in the carbon dioxide partial pressure did diminish sporulation.[8,11]

On nutrient agar the 'wild', rough or virulent type produced a characteristic colony after 24 hours' incubation, which was ground glass appearance with an irregular border from which long strands of cells were seen in parallel arrangement, giving the typical "medusa head" characteristic.[9,10] On blood agar B. anthracis produced non-hemolysis or only slight hemolysis. In gelatin stab

cultures the growth pattern was often referred to as resembling an inverted fir tree.[9]

The biochemical reactions and other tests on *B. anthracis* were listed in Table 1 page 5 and compared with the reactions of *B. cereus*.[12]

Table 1 Characteristics of Bacillus anthracis and Bacillus cereus

Characteristic	B. anthracis	B. cereus
Blood agar colony	Rough, flat, usually many comma-shaped outgrowths	Rough, flat, no or few comma-shaped outgrowths
Hemolysis	None or very weak	Usually beta-hemolytic
Penicillin	Sensitive	Not sensitive
Bicarbonate medium	White, round, raised glistening, mucoid	Flat, dull
Flourescent-antibody test	Positive	Negative
Gamma phage	Susceptible	Resistant
Animal pathogenicity	Positive	Negative
Litmus milk	Not reduced or slowly reduced and peptonized	Usually reduced in 2 to 3 days
Methylene blue	Not reduced or slightly reduced in 24 hr.	Usually reduced in 24 hr.
Motility	Negative	Usually positive
Voges-Proskauer test	Positive	Positive
Gelatin liquefaction (7 days)	Negative or partial	Usually complete
Fermentation tests:		
Glucose	Acid, no gas	Acid, no gas
Maltose	Acid, no gas	Acid, no gas
Sucrose	Acid, no gas or negative	Acid, no gas or negative
Arabinose	Negative	Negative
Mannitol	Negative	Negative
Xylose	Negative	Negative
Salicin	Usually negative or late	Usually positive in 24 hr.

1.3 Variation

Variation occurred with respect to virulence, spore formation, and colony form.[13]

The rough variant was the virulent and naturally occurring form of B. anthracis. A number of different types of variants were produced by aging and other treatment of stock laboratory cultures.[8,10]

Pasteur was first noted that prolonged cultivation of a virulent rough culture of B. anthracis at higher than optimum temperature, 42.5°C, resulted in a loss of virulence and the appearance of asporogenous variants.[8] It was by this method that he prepared his famous attenuated vaccine. The colonies of these attenuated smooth strains differed in appearance from virulent strains, being smaller, more convex and consisting of bacteria in bundles instead of in chains. Thus, the anthrax organism was an exception to the general rule because rough (R) strains of B. anthracis were virulent and smooth (S) strains were avirulent. Many attenuated strains still maintained their capacity to produce capsules and variants of virulent strains sometimes developed which were both rough and uncapsulated. It was evident that capsules alone were not responsible for virulence but that a strain which did not produce capsules could not be virulent.[9]

Further studies revealed that the bacilli of virulent R type appeared rough in the conventional culture, but they produced capsules in vivo and in vitro when the cultures were incubated under increased carbon dioxide tension in bicarbonate containing medium,

and the colonies were mucoid in appearance. Avirulent R variants resembled the virulent form in culture in air, but the colonies remained rough and capsules were not produced on culture under increased carbon dioxide tension.[14,15] Avirulent strains might also be encapsulated producing mucoid colonies in air culture, but did not form toxin.[8] In as much as virulence was appearently due to both capsule formation and toxin production.[6]

Paradoxically, virulence was not related to the ability to form spores, for both asporogenous virulent strains and spore-forming avirulent strain might be produced.[8]

1.4 Antigenic structure and toxin

The complex antigenic structure of B. anthracis was a capsular polypeptide, a somatic polysaccharide, and a somatic protein antigen.[6,9]

The capsular substance was composed of a γ-polypeptide of D-glutamic acid. It had aggressin activity, antiphagocyte and appeared to play a major role in virulence of the organism. Although only a single antigen type of capsular polypeptide had been described, antibodies to the capsule alone were usually not protective, because the organism also produced a lethal toxin.[6]

The somatic polysaccharide antigen contained equimolar quantities of D-glucosamine, D-galactose and acetic acid.[6] A small peptide moiety containing $\alpha\epsilon$-diaminopimelic acid was closely associated with the polysaccharide, and the complex appeared to form part of the cell wall.[16] The polysaccharide evidently played no

important role in virulence, since antibodies to it were not protective.[6]

The somatic antigen was protective and found in the oedema fluid of lesions in infected animals or in culture filtrates and elaborated during both aerobic and anaerobic growth. Although it was clearly contained in the lethal toxin of the organisms, its relation to the various toxin fraction had not been clearly defined.[6,9]

B. anthracis produced an extracellular toxin which was composed of three components.[17] The English workers called these components Factors I, II and III, whereas the American workers called the same factors edema factor (EF), protective antigen (PA), and lethal factor (LF), respectively. These three factors had now been isolated and examined individually. Factor I consisted of a chelating compound containing phosphorus with protein and carbohydrate moieties; Factor II and III consisted of proteins. It had been shown that both the toxic and immunogenic properties of the toxin were produced by combinations of these component factors,[18,19] and the most reliable anthrax vaccine should contain a mixture of all three components.[9] (Table 2 page 9)

Table 2 Showing the properties of the toxin component of *Bacillus anthracis*

Toxin components	Toxicity		Immunogenicity
	Edema	Lethality Mouse	Guinea pig
I (oedema factor)	-	-	-
II (protective antigen)	-	-	++
III (lethal factor)	-	-	-
I + II	++++	+	+++
I + III	-	-	+
II + III	-	++	++
I + II + III	++	+++	++

++++, +++, ++, + = Decreasing degrees of reaction

\- = No reaction

1.5 Disease in animal and man.

Cattle, horses, sheep, goats, and wild herbivores were most commonly affected and in these animals the disease was often fatal. Omnivora (man and swine) possessed the greatest natural resistance to the disease. Mice, guinea pigs, and rabbits, which

were used in laboratory diagnosis of anthrax, were very susceptible, while rats showed condiderable resistance.[7,20]

Infection in livestock usually was the result of grazing on infected pasture rather than by contact. Infection might also be caused by contaminated fodder or artificial feed-stuffs, such as bonemeal, fish meal, oil cake, and tankage; by drinking from contaminated pools; or by bites of contaminated flies.[20]

The disease in animals might occur in at least three different forms:

1) The peracute (apoplectic) form was most common in cattle, sheep and goats and occurred at the beginning of an outbreak. It was characterized by a sudden onset and rapidly fatal course. Victims were frequently found dead without showing any previous evidence of disease.[20] Following death, a bloody discharge from the natural body openings. Due to a toxin released by the organism, the blood clotting mechanism was inhibited and the blood failed to clot.[10,11]

2) The acute and subacute form. The disease developed more slowly than the peracute form. The acute form usually terminated in death in a day or two. The subacute might result in death in three to five days or longer, of in complete recovery after several days. These types of disease were common in cattle, horses and sheep.[20]

3) Chronic anthrax occurred mostly in swine, affected the mesenteric and submaxillary lymph glands, and was usually recognized

only on post mortem examination.[20]

In man, the disease was characterized by three major forms depending upon mode of entry and location of the site of infection. They were 1) cutaneous anthrax or malignant carbuncle, 2) pulmonary anthrax or wool-sorter's disease, and 3) intestinal anthrax. A fourth form, believed to be a sequela of cutaneous anthrax, was a meningeal form. Both miningitis and septicemia might be complications of any of the three major forms.[11]

1.5.1 Pathogenesis

When anthrax spores were ingested or inoculated into a susceptible animal they germinated within a few hours.[21] If the strain of organisms was highly virulent, capsules would be well formed and protected the organisms from phagocytosis and lysis. After the leison developing for only 3-4 hours it would consist of a mass of well capsulated bacilli contained in an oedematous area of tissue. This zone of oedema would continue to spread outwards from the center of the lesion. If the host showed sufficient resistance to infection, the borders of the lesion would be demarcated by large numbers of polymorphs and mononuclear cells which would inhibit the further encroachment of the lesion into the surrounding normal tissue. Following this stage, bacterial capsules would begin to disintegrate in the area of cellular infiltration, and then the bacterial bodies and some leucocytes would become destroyed. In the resistant animal these processes would continue until the cellular defences finally overcomed the infection and the lesion healed with fibrosis.[7,9,22]

Primary lesions which had develop in susceptible animals continued to over come the local cellular defences. Bacilli became distribured, initially, via the lymphatics where secondary lesions developed in lymph nodes. The multiplication of bacteria continued at a rapid rate. Many passed into the blood stream, became distributed throughout the body and continued to multiply until the animal died.[9,22]

Earlier concepts on the cause of death from anthrax were associated with the large number of bacilli present in the tissues throughout the body. It was supposed that this high rate of multiplication resulted in the blockage of blood capillaries by bacteria, and that the oxygen requirements of so large a bacterial population in the tissue resulted in the animal suffering from a fatal anoxia. The results of more recent experiments had disproved these theories and revealed the information on the pathogenesis of this disease related to the development of the three components of the toxin.[9,10]

Lincohn _et al_. (1964)[23] postulated that damaged to the CNS by the toxin was the primary cause of death. Beall and Dalldorf (1966)[24] found that anthrax toxin could increase vascular permeability, resulting in fluid loss. Smith and Stoner (1967)[19] concluded that this was the mechanism by which anthrax toxin caused death.

It seemed to be generally accepted now that death in anthrax was due to toxin.

1.5.2 <u>Treatment, prevention and control</u>

In the past antianthrax serum was most commonly used for the treatment of anthrax in animals. In recent years, certain chemotherapeutic agents, which had proved to be effective in the treatment of the disease in animals, were being used widely instead of specific antiserum.[25]

Sulfonamide had been successfully used for treatment and the most widely recommended was sulfathiazole, 3 to 4 gm. by mouth initially, with 1 to 1.5 gm. every three to four hours up to seven days in indicated.[20]

Antibiotics such as penicillin, tetracyclines, erythromycin, terramycin, chloramphenicol were effective.[20] Penicillin was the most active and was the drug of choice.[26] Antibiotics rapidly halted the extension of the disease and sterilized the tissues, but they did not reverse the toxic processes initiated by the infection.

It was suggested for the treatment of external anthrax that it should include the bactericidal antibiotics, penicillin and streptomycin, to control the bactericidal as rapidly as possible, with specific antiserum to neutralize the free toxins.[27]

When anthrax was suspected, however treatment should be begun at once and not delayed pending positive diagnosis.[20]

The spore forms of <u>B. anthracis</u> were very resistant to chemical and environmental influences. They remained viable in

buried carcasses or on surface of dry soil for decades and caused death from anthrax in grazing animal year after year.[26] The control of anthrax in animals was difficult, but was being accomplished by vaccination and strict sanitary police measures. Vaccination of livestock in infected areas well in advance of anthrax season was the most effective measure known for preventing the disease.[20]

In outbreaks of anthrax in livestock, the control measures, if properly carried out, would materially assist in checking the disease and preventing its spread to other areas: prompt disposal of dead animal by complete cremation or deep burial under a layer of quicklime without opening the carcasses or removing the skin; isolation of visibly sick animals and immidiate treatment; vaccination of the apparently well but exposed animals; destruction of manure, bedding, and other contaminated material by burning; disinfection of contaminated stables; and a strict quarantine of the infected premises, rigidly enforced to prevent the movement of livestock from or into the infected area.[20,25]

The prevention and control of anthrax in man depended on three factors: eradiation of the disease in animal, elimination of industrial infection from animal products such as hides and hair that originated in areas in which the disease was widespread and finally, earlier diagnosis and more prompt and energetic treatment of infected cases.[20]

1.6 Vaccination

Anthrax was one of the first disease for which a definite immunity was produced by the use of bacteria.[9,10,20,28]

In 1879, Chauveau made the observation that animals having survived experimental injection with anthrax were more resistant to subsequent challenge.[11,29]

Toussaint, in 1880, successfully vaccinated with defibrinated anthrax blood that had been heated for 10 minutes at 55°C.[26,30] In 1881, Pasteur introduced a method of vaccination, founded as the same principle as that which had been succeeded in diminishing the virulence of the fowl cholera organism. He found that it did not succeed with the anthrax organism because of its habit of forming spore, which resisted attenuation. Success was finally achieved when it was discovered that incubation at 42 to 43°C inhibited sporulation and attenuated the vegetative form. Pasteur prepared the anthrax vaccine and vaccination of animals had been successfully carried out since that time.[26]

1.6.1 Live vaccines

1.6.1.1 Pasteur vaccine

Pasteur prepared two vaccines; the premier vaccine or vaccine no. 1 was a subculture in broth from a strain that had been kept at 42-43°C for 15 to 20 days; its virulence was such that could killed mice and young guinea pigs, but not in adult guinea pigs or rabbits. The deuxième vaccine or vaccine no. 2 was subcultured after 10 to 12 days; it was more virulence, being able

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to kill mice, adult guinea pigs and a certain proportion of rabbits. Vaccine no.2 was given about 10 to 12 days after no.1.[11,32,33] The degree of immunity obtained appeared to be directly related to the virulence of the vaccine strains used; consequently the first and weaker vaccine was to prepare the animal for the more virulent second.[32]

The vaccine prepared by Pasteur had been extremely successful in many part of the world since the celebrated field trial on a farm at Pouilly-le-Fort.[31,32,33]

Attenuated vaccines; however, had been little used in later work, chiefly owing to the variations in virulence of attenuated cultures. Animals sometimes did not receive sufficient protection, and in some other case the vaccine proved too powerful and animals died from the treatment.[26,32]

According to Pasteur, the virulence of cultures at 42-43°C decreased gradully, so that at a given moment, a vaccine of the correct virulence could be selected. This view had not been established. Moreover, in cultures maintained at 42-43°C a variety of bacillary variants appeared, some of which were non-capsulated and virulent, some capsulated and fully virulent, and some of varying virulence that were either spore bearing or in capable of sporulation.[26]

A distinct disadvantage of this vaccine was that it did not keep well. The organism were supplied in the form of a bouillon culture to which preservations could not be added and in which sporulation did not occur readily; hence the organism

tended to die out rather rapidly.[32]

Vaccines of attenuated cultures had been replaced by the more stable and more consistently reproducible spore vaccine.[32]

1.6.1.2 Spore vaccine

In order to avoid the uncertainly occasioned by deterioration of the Pasteur vaccine, spore vaccines became popular. The anthrax cultures were attenuated. They were then grown on a peptone-free agar for 4 to 7 days at 37°C, at the end of which time the majority of the bacilli had sporulated. The cultures were washed down with sterile physiological salt solution. The resultant suspension was heated at 60°C for 30 minutes to destroy all vegetative form. The number of spores in the suspension was determined by plating measured quantities. The suspensions were now usually diluted until each ml contained one million spores. One ml of such a suspension constituted a normal dose for cattle and horse, about 0.25 ml for sheep. Each lot of spore vaccine should be tested for pathogenicity on guinea pigs and rabbits.[32]

1.6.1.3 Special vaccines

Sobernheim (1904), improved on Pasteur's method by using Pasteur vaccine no.2 simultaneously with immune serum. It had the merit of requiring injection on only one occasion instead of two; of conferring a passive immunity which protected the animal while an active immunity was developing; and of being attended by very little danger. It could be employed after an

epidermic had broken out, without fear of rendering the animals more susceptible to infection.[10,26]

Eichhorn (1916,1925)[10,20] demonstrated that a high degree of immunity could be produced by simultaneous vaccination with a potent anti-anthrax serum and an attenuated spore vaccine that prepared from a potent strain of bacillus.

Spore vaccines standardized to compare with Pasteur vaccine no.2, along with hyperimmune anti-anthrax serum, had been used in the United States for prophylactic immunization.[10]

Besides the vaccines of standard degree of virulence, as described under the heading Pasteur vaccine, a number of vaccines of other grades of virulence were avaiable and were used for special conditions. In some districts especially virulent strains of anthrax organism abounded and the ordinary vaccines would not fully protect. A no.3 and even no.4 vaccine, consisting of cultures less attenuated than the no.2 Pasteur , were used. Obviously the danger of vaccination troubles was greater when these more virulent vaccines were used; hence they should not be used unless it was known that the weaker vaccine would not protect.[32]

A special vaccine that apparently had advantages over the ordinary ones was that of Mazzucchi (1931)[34] ; it had been named "Carbozoo". This was no.2 spore vaccine suspended in a solution of saponin. The saponin acted as a local irritant inducing a rapidly forming gelatinous infiltration at the point of injection which walled off the spores from the lymph vessels and delayed their absorption. It was claimed that this vaccine immunized more

solidly and more safety than the usual ones.[32] However, postvaccinal death in sheep and goats were severe at time.[35]

1.6.1.4 Avirulent anthrax vaccine

This was the most significant breakthrough for animal vaccines discovered by Sterne (1939).[36,37]

When virulent B. anthracis strains were grown on 50 percent serum agar in an atmosphere of 30-50 percent carbon dioxide, the mucoid colonies which were smooth, clearly defined edges and quite different to the classical 'Medusa head' colonies usually associated with the virulent form developed. The bacilli forming these mucoid colonies were capsulated and in short chain. After incubation for 3-4 days, examination under a low-power objective would show that some of these colonies had distinct wedge or fan shaped outgrowths form their edges, these outgrowth having a flate, filamentous appearance; the bacilli forming these were in long chains and showed no trace of capsulation. These variants thus produced could be readily picked off and when re-seeded on fresh medium, either ordinary or enriched nutrient agar, would give rise to typical anthrax colonies with the 'frosted glass' appearance and hair-like structure, irrespective of whether they were grown in 30-50 percent carbon dioxide or not. On repeated culture or passage through animals, these characteristics did not alter and the organism showed no tendency to revert to the capsulated, virulent form. When tested in mice or guinea pigs, many of these variant strains would be found to be harmless though these animals might be killed if given relatively massive doses of culture but

even then no trace of capsulation was observed.[36,37]

By using organisms produced by this technique, an avirulent noncapsulated spore vaccine was developed and success--fully used in South Africa.[38] The spores were suspended in 50 percent glycerol saline that proved to be safer and producing less reaction than saponin.[37]

The Sterne strain vaccine had been shown to be efficacious in preventive medicine programs for farm animals. (35,39,40)

The first large field trial of this vaccine in the United States during the 1957 Oklahoma-Kansas epizootic supported its value in the control of an epizootic.[41]

Kaufmann _et al_[42] reaffirmed the usefulness of the Sterne strain noncapsulated avirulent spore vaccine in the Louisiana outbreak of 1971. The death rate decreased markly in the period 8 or more days after vaccination in vaccinated animals.

The efficacy and nonpathogenicity of the Sterne vaccine that eliminated the problem of vaccine deaths and vaccine-contaminated premises, had outmoded the previously popular virulent spore vaccine to the point that most vaccine manufacture had discontinued production of the latter products.[11]

1.6.2 Killed vaccine

This was prepared by using young, nonsporulated cultures attenuated with formalin.[10] Anthrax bacterin appeared to have very

little immunizing ability but longer duration than anthrax antiserum. It was seldom used in infected districts.[25,32]

However, because of fear of the introduction of living strains, eventhough attenuated, and the fact that some worker had found a little value in them, this product was the only permitted in some areas which had not been contaminated by successive outbreak of anthrax.[32]

1.6.3 <u>Non-living vaccines</u>

1.6.3.1 <u>Anthrax aggressin</u>

Bail (1904) showed that rabbits and sheep might be protected aganist otherwise fatal doses of anthrax bacilli by injecting them with filter-sterized fluid from the edematous tissue of local anthrax lesions. The immunizing substance in this material was termed 'aggressin' because when mixed with virulent anthrax culture it had the effect of increasing their virulent.

Anthrax aggressin had been made on a commercial scale, but the immunity produced was not always sufficiently strong to protect and the cost of production was fairly high. The product was seldom used at the present time.[32]

1.6.3.2 <u>Culture filtrate</u>

The basic for immunization with culture filtrated antigens was laid by Gladstone (1946)[23,43] who produced an antigen capable of protecting rabbits, sheep and monkeys against challenge doses in the filtrate of <u>B</u>. <u>anthracis</u> cultures grown in

serum of various species.

Many groups of investigators had been produced the protective antigen described by Gladstone in amounts by growth of B. anthracis in various modified mediums[44,45,46] and form suitable for limited test in man and domestic animals.[23,26]

The antigen was present in soluble form in the culture filtrates, and could be stabilized, concentrated, and partially purified by precipitation with alum or sedimentated with acids and alcohol.[45,46,47]

In two separate tests conducted by Schlingman et al (1956)[48] and Jackson et al (1957)[49] using the alum precipitated antigen of Wright et al (1954)[44,47] it was found that partial protection of cattle was afforded but that protection dropped off rapidly after 3 months.

Klien et al (1963)[45] compared the antigens of various mediums on the basis of resistance to challenge and on titer and suggested that the Belton and Strange antigen was a superior immunizing antigen. It was noted that this antigen contained all of the components of anthrax toxins.[50]

Surely, in the near future a chemical vaccine containing optimum proportion of protective antigen, edema factor, and lethal factor, with either part or all of the active factors in toxoid form, would be made avaiable.[23]

In man vaccine made with protective antigen was shown to be an effective immunizing agent and safety for those

exposed to the risk of industrial anthrax, certain laboratory personnel and some veterinarians.[51]

2. Description of Pasteurella multocida

2.1 Taxonomy

P. multocida belonged to the family Brucellaceae, genus Pasteurella; it was composed of four species: P. multocida, P. haemolytica, P. pneumotropica and P. ureae.[52] (Table 3)

Table 3 Characters of the species of genus Pasteurella.

Characteristic	1. P. multocida	2. P. pneumotropica	3. P. haemolytica	4. P. ureae
Hemolysis on blood agar	-[a]	-	+	+
Growth on Mac Conkey's agar	-	-	+	-
Indole	+	+	-	-
Urease	-	+	-	+
H_2S	+	+	d	-
Ornithine decarboxylase	+	d	d	-
Mannitol	+	-	+	+
Sorbitol	+	-	d	+
Trehalose	d	+	d	-
Xylose	d	d	+	-
Lactose	-	d	d	-

a- = most (90% or more) strains negative

\+ = most (90% or more) strains postive

d = some (less than 90%) strains positive, some negative.

2.2 Morphology, growth characteristics and biochemical reactions

P. multocida was a small coccoid rod 0.25 μ to 0.4 μ by 0.6 μ to 2.6 μ in size. After repeated culture on agar, the organism tended to from longer rod and to become more pleomorphic, forming chains, filaments and rods of various sizes. It was gram-negative, non-motile, non-sporeforming and often showed bipolar staining. When isolated from infected animal, it generally was encapsulated. However, after continuous subculture on antificial media the organisms tended to lose their capsule and was followed autolysis of some strains. Virulent stock culture could be preserved on blood agar in sealed tubes or by lyophilization.(52,53,54)

The organism was aerobic to facultatively anaerobic. Its optimum temperature for growth was 37°C, although it would grow from 20°-44°C. The pH growth range was from 6 to 8.5 with an optimum of 7.2 to 7.4.(53,54)

The use of digested protein media or proteose peptone stimulated the growth of the organism. The organism could be grown in beef infusion media, but better growth was obtained when blood or blood serum was added to the media. Cultures on blood agar had a faint but distinctive smell of value in recognition. Non-hemolytic, but most strains produced a brownish discoloration in regions of confluent growth.(52,53)

The colonial morphology was influenced by the presence or lack of capsule and the capsule's size and composition.(54,55)

According to many investigators, the organism had three

main colonial types, smooth(S), mucoid(M) and rough(R). The smooth was also called the fluorescent type. To this type belonged almost all freshly isolated cultures which possessed well-developed capsules. A culture of the mucoid type possessed the mucoid antigen with or without the capsular antigen. In cultures of the rough type, also called the blue type, no capsular nor mucoid antigen had been demonstrated.[56]

Carter(1967)[57] stated that virulence was related to the colonial morphology of P. multocida, eg. smooth colonies were almost always pathogenic for mice, mucoid vary, and rough colonies had a low virulence for mice.

Bain(1954)[58] divided the colonial form of P. multocida into several phase, I and other. It was considered that phase I corresponded to the culture of Carter's type B which possessed an unknown masked antigen other than the known capsule or, in other words, that the culture of phase I was the fully immunogenic form of type B. He suggested that the phase might be a mucoprotein surface antigen.

Some biochemical reactions had shown in Table 3 page 24

2.3 Typing of strains

The early bacteriologists named strains of virulent Pasteurella according to the species of origin. It was soon realized, however, that such system of classification had no scientific basis. Strains isolated from one species could not distinguished in the laboratory from those isolated from other and there was

reports of strains with no serological relation in strains from the same species.[59]

Most of orthodox biochemical and serological methods had been tried for several decades. The earlier systems of serological classification, such as Tanaka(1926) and of Cornelius(1929), had fallen into disuse.[57,60]

Rosenbusch and Merchant(1939)[61] divided their strains in three principle groups designated I, II and III, on the basis of action on xylose, arabinose, and dulcitol, and by means of a conventional agglutination procedure. Considerable cross agglutination was observed among members of the groups.[60]

Simple serological tests such as slide-agglutination, used by Little and Lyon(1943)[62], were of limited use because of the infrequency of inagglutinationable strains.

From the practical viewpoint, the most valuable basis of classification must be the immunological. Robert(1947)[63] demonstrated four types, designated I, II, III and IV,based on serum protection tests in mice. It was shown that immunological types were correlated with the fermentation of xylose, arabinose and ducitol.[60]

Hudson(1954)[59] had followed up Roberts' work and examined 58 strains to fall into 5 groups. He created type V for an addition group of strains.

Carter(1952,1955)[64,65] employed precipitin tests and indirect hemagglutination tests to identified four types: A, B, C

and D, on the basis of difference in capsular substances(K antigen). It was tentative concluded that the protective substance of Pasteurella organism obtained by Robert was the same as the K antigens.[57]

In 1955 Carter[65] claimed that types B, A, C and D were identical with Roberts' types I, II, III and IV, respectively, and demonstrated a strong correlation between serological types and host species[60]: type B strains included most of those causing epizootic pasteurellosis or hemorrhagic septicemia of cattle[66]; type A strains were isolated frequently from cases of fowl cholera[65]; type C cultures occurred infrequently, and at first they had been believed to come from dog and cats, but this was not confirmed and the category was subsequently abandoned[67,68]; type D strains were associated with a wide range of infections in cattle, sheep, pigs and poultry, including for example, shipping fever of cattle, virus pneumonia of pigs, fowl cholera, and bovine infections were of type A and D.[69]

Later, a further type E was recognized by Carter(1961)[70] and type C was shown to be not true capsular types. There were therefore only four capsular types, namely A, B, D and E.[71]

Table 4 page 29 showed serotypes established and equivalent serotypes in various methods of typing. Carter's serological classification of the Pasteurella organism was widely used in international as the most reliable system and it was included in Burgy's Manual 7th ed. (1957).[72]

Table 4 Types of Pasteurella multocida

Author	Methods	Equivalent serotypes				Other serotypes not equated
Robert(1947)	Mouse protec- -tion	I	II	III	IV	V(Hudson,1954)
Little and Lyon(1943)	Slide aggluti- -nation	2	1		3	
Rosenbusch and Merchant(1937)	Fermentation and agglutination	II	I			III
Carter(1955)	Hemagglutination precipitation	B	A	C	D	E(Carter,1961)

It was clear from a member of investigation that there was a sharing of somatic antigens among many strains of diverse origin. To reveal the somatic (O) antigen Namioka and Murata (1961)[57] removed the capsular material by treatment with N/HCl, suspended the washed organisms in saline at pH 7.0 and tested them against antisera prepared in rabbits by injection with similarly decapsulated organisms. By the use of agglutination and absorption tests, they established six O groups on the basis of somatic antigen; and later 12 O groups were found.[73]

Bain considered that the substance remained as the somatic antigen after the HCl treatment consisted of a part of the cell wall substance. It was assumed that the substance was a lipo-polysaccharide combined with a protein.[72]

According to the most widely used system, P. multocida was typed serologically by the indirect hemagglutination method of Carter(1955)[65], which determined capsular type, and the direct agglutination test of Namioka and Murata(1961)[57], which identified somatic antigens of acid-extracted cells.

Namioka and Murata(1961)[74] proposed that serotypes be identified by listing first the number standing for the specific somatic, or O component, followed by the capital letter standing for the specific capsular, or K antigen. This was the most widely used.

Relation between Pasteurella serotypes of capsule and somatic and host animals was shown in Table 5 page 31.

Table 5 Relationship between serotype and pasteurellosis[75]

Carter's capsular group	O group	Sero-type		Process of disease	Animals
A	5	5:A	Aviseptica group	Fowl cholera	Chicken
	8	8:A			Duck
	9	9:A			Turkey
B	6*	6:B	Boviseptica group	Hemorrhagic	Cattle
E	6	6:E		septicemia	
A	1**	1:A			
	3	3:A			
	7	7:A			
B	11	11:B		Pneumonia, Local	Various
D	1	1:D		Wound, Secondary	animals
	2	2:D		infection	and men
	3	3:D			
	4	4:D			
	10	10:D			
	12	12:D	(Provisional)		

* Subgroup presented in O group 6

** Subgroup presented in O group 1

2.4 Antigenic structure and toxin

There had been a number of investigations of the antigenic make up of strains of *P. multocida*. The results often had been difficult to interpret because different serologic varieties had been studied by different methods. The presence of serologically active capsular and somatic antigens had been referred to previously.

The antigenic structure of *P. multocida* was complex. For purpose of summary of this complex subject, the major antigenic components were following. Most of the information was based on the studied of Bain(1955)[76] and Dhanda(1960)[77] and their associates on type 1 strains.[78]

2.4.1 Polysaccharides

These soluble substances were produced by and readily recoverable from capsulated strains and were probably also produced by some noncapsulated strains. They behaved as haptens and acted as precipitating and complement-fixing substances. Their capacity to elicit some protection in mice was thought to be due to the presence of protein.[78]

2.4.2 Hyaluronic acid

This mucopolysaccharide was produced in large quantities by some type A strains, and was largely concentrated in the capsule. It was nonantigenic in the purified state.[78]

2.4.3 Lipopolysaccharides

These substances were employed in the indirect hemagglutination procedure and were responsible for "so-call" capsular specificity, although they were not necessarily confined to the capsule. They resembled similar substances often referred to misleadingly as endotoxin. They were toxic and pyogenic for rabbits and mice. Lethal dose ranged from 20 to 500 micrograms with different preparations. They were antigenic and some preparations elicited immunity. The immunologic behavior probably depended on their purity. Preparations had stimulated protective antibodies in rabbits which passively protected mice.[78]

2.4.4 Proteins

A single homologous protein had not yet been isolated that would account for protection. Purified fractions yielding 14% nitrogen with an amino acid composition indicating their protein nature had served to actively immunize mice, cattle and buffaloes.[78]

The organism did not produce exotoxin but autolysed cultures contained endotoxin. Endotoxins were produced by both virulent and non virulent P. multocida. They were not type specific antigen.[53]

2.5 Disease caused by P. multocida

The most important disease caused by P. multocida were hemorrhagic septicemia of cattle, sheep, horses and several species of wild animals, enzootic pneumonia of calves, swine plague and fowl cholera. Man was an occasional victim. Of the experimental animal, rabbit and mice were highly susceptible.[73,79]

There was evidence that _P. multocida_ existed as a commensal in respiratory tracts of animals, and that certain predisposing condition altered this balance between host and parasite, which resulted in the develop of disease.[80]

In tropical Asia generally, hemorrhagic septicemia denoted an acute infection, mostly of cattle and buffaloes, with high mortality.[80] The serotype involved was consistently 6:B in Asia.[72]

The great majority of cases were acute or even hyperacute, with death occurring 6 to 24 hours after the appearance of symtoms. The disease was characterized by a sudden onset of fever (41-42°C), signs of abdominal pain, severe diarrhoea and dysentery. Respiration became rapid and shortly before death the mucous membrane appeared cyanotic.[80]

In subacute case there was a rise in body temperature and oedema developed subcutaneously in the region of the head, neck and brisket. Most animal suffered from severe diarrhoea and sometimes dysentery, and some showed signs of respiratory distress and a nasal discharge which might be blood-stained or purulent. Death occurred within 2-4 days.[80]

In countries other than the tropics, _P. multocida_ infection might be associated with _P. haemolytica_ and established bronchopneu--monia and condition referred to as transit or shopping fever.[80]

Pasteurellosis in man followed two principle patterns: (1) a wound infection most commonly originated with an animal bite or scratch and (2) non traumatic infections, often more or less

obviously related to the respiratory tract but with no demonstrable extraneous source of the agent. In both cases, infection might reach other organ systems but were usually traceable to one of these two kinds of entry portals.[81]

2.5.1 Pathogenesis

A bacterimia, detectable culturally but not microsco--pically, was established with in 12 hours of experimental infection and commonly, but not invariable, the saliva contained Pasteurellae. Blood fibrinogen rose from the onset of symptoms. As the disease progressed, the animal lied down, was reluctant to move and showed some respiratory distress. Venous pressure falled. Pasteurella appeared in the fecus, urine and milk. The terminal condition was reminiscent of the endotoxin shock described in laboratory animals but not specifically studied so far in bovine.[80,82]

The majority cases had an oedematous swelling in the head-throat-brisket region. On incision, this was seen to be due to clear straw-coloured serous fluid distending the tissue spaces. The muscle were similary affected. There were scattered petechiae. Pharyngeal and cervical lymph nodes were swollen and sometimes hemorrhage. Slightly blood-tinged exess fluid was present in the thoracic, abdominal and pericadial cavities.[83]

There might be some early pneumonia, the extent depending on the duration of the case. Petechiae appeared under the serous membrances throughout the body. Apart from an occasional small hemorrhage the spleen was unchanged. Lesions in the intestinal

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tract were viable. Some animals, particularly calves, might show hemorrhagic gastritis or gastroenteritis.[83]

2.5.2 Treatment, prevention and control

A number of sulfonamide compounds, sulfamethazine, sulfadimidine, sulfadiazine, sulfapyridine and sulfathiazole, had been found effective in the treatment of infection. Sulfadimidine exerted a favour effect on hemorrhagic septicemia of cattle in Asia if it was administered sufficiently early.[54,78]

Of many antibiotics that were effective against *P. multocida* according to *in vitro* tests, only a small number were used widely. Penicillin had been found of value in the treatment of Pasteurella infection in animal and human.[78]

To be effective, treatment must be initiated in the early stages of the disease or before bacteremia became well established. For this reason the treatment of animal suffering from acute disease would be commend too late to be effective.[80,83]

Hyperimmune serum had been used for the control of hemorrhagic septicemia when immidiate protection of cattle was required to certain an outbreak or prevent developing during transport or other conditions of sudden stress. In practice, the injection of hyperimmune serum conferred immidiate protection, although the passive immunity was of short duration and limit value.[80]

It had already been stated that the only practical approach in the control of hemorrhagic septicemia appeared to be carry out timely prophylactic vaccination of all the cattle and

buffaloes in endemic areas and to treat the sporadic cases with the well-known sulfa preperation.[84]

2.6 Vaccination of hemorrhagic septicemia

The vaccination against hemorrhagic septicemia was one of the most important and entensively conducted. The vaccine was applied when the disease was confirmed as well during routine vaccination in certain area where the disease was constantly surfacing.

2.6.1 Production of vaccine

2.6.1.1 Selection of strains

Although Robert's type I strains were the only ones worth including in vaccines against hemorrhagic septicemia, little was known of the relative merits of various individual type I strains under standardized conditions. There were indications that some strains were more effective than others in active and passive immunity tests in mice: some were more effective against homologous that against heterologous challenge; others were uniformly effective against several challenging strains. The Australian, Asia and central Africa strains showed quite clear differences from each other, and it was probable that further differences exist within these groups. Until strains of particularly high and broad immunizing effectiveness were found, it was considered wise for countries at present to use indigeneous type I strains for making vaccine.[82]

2.6.1.2 Preservation of seed

Pasteurellae could change in two ways during

subcultivation: they altered phenotypically in response to the environment, giving varying yields of their different antigens, or they might alter genotypically and lost some antigens altogether. It was reasonable to assume that the ideal Pasteurella was the one which grew in the animal body in the course of infection. This was the kind of bacterium that the animal had to combat and hence its antigenic pattern should be maintained as nearly as possible in any vaccine.[82]

2.6.1.3 Killing culture

Formalin was used universally. It was cheap and effective and was a good preservative as well as bactericidal agent. The concentration in vaccine might have to be as high as 0.5% to inhibit the growth of molds. Formalin was probably effective because of its fixative action on the important protein antigens. It was difficult to dissociate the antigen complex in extracts from formalin-treated Pasteurellae.[82]

Killing cultures by heat was detrimental to the vaccine. Merthiolate was effective and did not harm the immunizing properties. It was used regularly in Delpy's vaccine.[82] Merthiolate and antibiotics produced satisfactory killed suspensions for short term use but they had no advantage over formalin.[85]

2.6.2 Type of vaccine

For practical purpose, a vaccine was considered worth while only if it could be easily made and distributed and produced lasting immunity from a single injection of a small volume without

undesirable reaction in the recipient animals. Freedom from adverse reactions such as shock, pain or swelling were most desirable in a vaccine, as these disabilities were not tolerated well by population which were just being convinced of the value of prophylactic vaccination.[82]

Effectiveness, stability, safety and blandness, therefore, were essential in a vaccine, along with simplicity and economy in its production. The FAO International Meeting on Hemorrhagic Septicemia (1962) recommended that killed cultures with adjuvants were the vaccines of choice.[82]

2.6.2.1 Killed vaccine

2.6.2.1.1 Plain bacterin

This might be prepared either from broth or agar culture. Ordinary broth bacterins were produced by growing Pasteurellae for 24 to 48 hours in simple meat infusion peptone broth. The density of the cultures could be increased by using improved media but aeration was necessary for really heavy growth. Formalin was used universally as the bactericidal agent.[82]

In challenge experiments in cattle, immunity from bacterin was rarely demonstrable for more than 6 weeks, as Holmes discovered in 1912. The onset of immunity was about the 5th day after injection, which made these vaccines popular for use in the face of outbreaks. The duration of immunity could be improved by increasing the dose, but this was accompanied by greater risk of a shock reaction which might even be fatal.[82]

2.6.2.1.2 Delpy's vaccine

This was introduced by Delpy in 1948. A type I strain was selected for its ability to lyse when incubated in distilled water and merthiolate (1:4,000). A suitable strain should show 60 percent clearing in 14 days at 37°C. When the clearing passed 50 percent, 0.5 percent saponin was added as an adjuvant.[82]

The vaccine was an effective vaccine and very satisfactory results had been claimed from its use in Iran but it had not been adopted in other countries as its production required the tedious process of cultivation on agar and the unpopular swelling produced by saponin.[78,82,85]

2.6.2.1.3 Alum-precipitated vaccine

This was apparently tried by American commercial manufacturers prior to 1950 and also in the Philippines.[82]

The vaccine was prepared quite simply from broth bacterin or aerated cultures. The methld was to add enough of a 10 percent potash alum solution to a formalinized suspension of bacteria to give a concentration of 1 percent alum in the vaccine. It was often necessary to add some alkali to bring the pH to about 6.5 so that flocculation could occur. The vaccine was injected subcutaneously in the same dosage as plain bacterin and some tissue reaction occurred at the site of inoculation. Immunity to challenge for up to 5 months had been demonstrated.[82]

2.6.2.1.4 Oil-adjuvant vaccine

This was designed to retard absorption of the vaccine and prolonged the antigenic stimulus to the antibody forming cells.

The vaccine prepared from bacterial suspension, killed by formalin, was emulsified in light mineral oil using a stabilizing agent suitable to a water-in-oil emulsion. Vegetable oils were less effective than mineral oils. The emulsion must be of the water-in-oil type so that the droplets of bacterial suspension were distributed throughout the oil. A reverse emulsion would be useless as the bacteria would be absorbed quickly leaving only inert oil at the site. The oil adjuvant vaccine was stated that the emulsion was stable if it showed no sign of cracking after 14 days at 37°C.[82] Emulsions must be made with care so that the individual droplets of suspension in the disperse phase were small enough and sufficiently stabilized to prevent coalescence. Coarse emulsion were absorbed too rapidly with loss of prolonged stimulating effects.[85]

Simple oil adjuvant vaccines caused some local reaction at the site of injection in muscles but the reaction was not excessive.[85]

Though this vaccine was known to impart high degree of immunity, it had not gained much favor in the field because it was not easily injectable due to its thick consistency. A multiple emulsion adjuvant of thin consistency was developed. It was found to be quite stable on storage, easily

injectable and merit field trial in cattle to give earlier onset of immunity with prolong protection than oil adjuvant vaccine.[86]

Several countries were now producing oil-adjuvant vaccine in increasing quantities, with the aim of making in their basic prophylactic against hemorrhagic septicemia.[82]

2.6.2.2 <u>Attenuated living vaccine</u>

Many workers had tried avirulent and attenuated living vaccines, but none of these vaccines had become established in practice because of the difficulties in preparation and distribution.

Hudson (1954) isolated blue variants from old aerated cultures and selected strain for use in cattle on the basis of reduced pathogenicity for mice. In some small trial in Thailand in buffaloes, these strains gave immunity to challenge for several months but no proper tests for duration of immunity had been done.[82]

It was possible that with improvement in technology in tropical countries, especially in freeze-drying, the use of living attenuated vaccine might become the main method of prophylaxis in the future.[82]

2.6.2.3 <u>Aggressin</u>

In 1924 Gouchenour isolated a Pasteurella from an American bison that was usually virulent for most of the domestic animals. By injecting a calf intrapleurally with this

isolate, he produced an aggressin that was quite popular for a time. It was no longer available. With regard to aggressins, it should be noted that they were antigenic, and temporarily then enhanced susceptibility rather than immunity; hence they should always be given to animals that were normal and not under the stresses that seemed to induce outbreaks.[87]