CHAPTER II

REVIEW OF THE RELEVANT LITERATURES

1. Description of Pasteurella multocida

1.1 Taxonomy

P.multocida is synonymous with P.gallicida which has been given many names corresponding to animal from which it was isolated, such as P.avicid, P.aviseptica, P.muricida and P.muriseptica etc. However, P.multocida, proposed by Rosenbusch and Merchant (1939), has been accepted as the official name in Bergey's Manual.

P.multocida is classified as belonging to the family Pasteurellaceae, genus Pasteurella which includes : P.multocida, P.haemolytica, P.pneumotropica, P.ureae, P.aerogenes and P.gallinarum. The principal differential characteristics were shown in Table 1 (9).

1.2 Microscopic and Colonial Morphology

P.multocida is a gram-negative, bipolar staining, nonmotile, nonsporeforming coccobacilli or short rods with a bipolar appearance occuring singly, in pairs, and occasionally as chains or filaments. It measures $0.2-0.4 \times 0.6-2.5$ μm but tends towards pleomorphicity after repeated subculture. Virulent strains are in encapsulated form, which is lost upon subculturing (10).

The organism could be grown in beef infusion media but better growth was obtained from blood or chocolate agar where it produced small, translucent, convex nonhemolytic dot (approximately 1-2 mm in diameter after 18-24 hr) but most strains produced a

brownish discolorization in region of confluent growth with a characteristic musty odor, possibly related to indole (11, 12, 13).

Colonial morphology observed with obliquely transmitted light could distinguish three colonial forms which related to virulence in animal, ie. a) smooth or fluorescent (irredescent) colonies, produced by capsulated and highly virulent strains, b) blue or rough colonies from non - capsulated ones of low virulence and c) mucoid or gray colonies from large capsulated organisms Noncapsulated culture and mucoid strains were generally associated with carrier-states and chronic diseases (14, 15). Furthermore, there is a relationship between acriflavin reaction and colonial morphology. Flocculation with acriflarin is associated with rough colonies but suspension is mucoid or smooth colony (13, 16). The principle of differential characteristics of these three variants are summarized in table 2.

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

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

[a] Data from Carter (1981). For symbols see standard definitions.
[b] Strains from dogs and cats may be negative for mannitol.

Table 2 Some Characteristics of principal variants of Pasterella multocida

Variants	Colonies on Agar	Growth in Broth	Mouse Virulence
Mucoid	Large sized, flowing	Mucoid deposit	Moderate
Smooth (Fluorescent, Irridescent)	Medium sized, discrete	Diffusion	High
Rough (blue)	Small sized, discrete	Autoagglutination	Low

1.3 Growth Characteristic

P.multocida is an aerobe or facultative anaerobe. Growth occurs between 25 - 40° c. Most avain strains and a few others grow at 42° c (9). The optimal growth temperature is 37° c and its optimal pH range is 7.2-7.8, but it will also grow in the range of 6.2-9.0 depending on composition of the medium. In liquid media maximum growth is obtained in 16-24 hr. The broth becomes cloudy, and in a few days a sticky sediment can be collected at the bottom. With some isolants, a flocculent precipitate occurs (17).

The bacterium will grow on meat infusion media; growth is enhanced by enriching the media with emzymatic digest of peptone, casein hydrolysate, or avian serum (17). It has been reported that the high concentration of sodium chloride inhibited growth, while that of fumaric acid promoted growth (18). Studies of Wess Man found that six strains of P.multocida grew well with normal amount of thiamine (19).

The compositions of media affect to the presence or absence of capsule. Studies of Lipipun (20) have shown that the organism produced capsule on tryptose agar, yeast extract proteose peptone-cystine agar and dextrose strach agar but did not produce on tryptic soy agar and nutrient agar.

1.4 Biochemical Reaction

Biochemical reactions for P.multocida are listed in table 3 (II). The major biochemical characteristics of this organism include : nitrate reduction, indole production. oxidase positive, fermentation of glucose and sucrose without gas formation, usually negative fermentation reactions in lactose and maltose, absence of growth on

Mac Conkey agar, a negative urease reaction and a positive ornithine decarboxylase reaction. All of the above informations could be used for key test of P.multocida (21).

There is strain-to-strain variability in the fermentation of xylose, manitol, lactose and maltose. The various patterns of fermentation observed at CDC are presented in table 4 (11).

1.5 Serotyping System

Since 1900, several schemes have been developed for serological classification and epidemiologic study of the organism (22, 23, 24, 25, 26, 27, 28). These schemes have included specific agglutination and precipitation (22, 23, 24, 25, 26), passive protection of mice (27), passive heamagglutination (PHA) (16,29,30), gel diffusion precipitin test (31, 32). However, only the two latter are currently in prominent use. At the present time, the system of designating serotypes is based on the identification of capsular or K antigens and somatic or "O" antigens which associated with endotoxin (33).

Carter has used capsular antigens by means of 56°c, 30 minutes saline-extracting cells as serologic indicator in the passive hemagglutination test to group P.multocida to devide into four different capsular types designated A, B, C, and D (16). Type C was subsequently discarded because of difficulties in recognition (34) and additional capsular variety, type E was added (35). And recently, Rimler and Rhoades found out a new serotype F isolated from turkeys in 5 states of U.S.A. (30). So up to the present time, there are 5 capsular serotypes ie., A, B, D, E and F in Carter's typing system.

Table 3 Biochemical characteristics of species of Pasteurella

Characteristic	P. multocida (n = 306)		Pasteurella sp. new species 1 (n = 91)		P. pneumotropica (n = 107)		P. ureae (n = 97)		P. haemolytica (n = 67)		P. aerogenes (n = 16)	
	Sign[a]	%+[b]	Sign	%+	Sign	%+	Sign	%+	Sign	%+	Sign	%+
Hemolysis (clear zone)	−	0	−	0	−	0	−	0	v	72	−	0
Motility	−	0	−	0	−	0	−	0	−	0	−	0
Gas from glucose	−	0	v	16	−	0	−	0	−	0	+	100
Acid from:												
Glucose	+	100	+	100	+	97 (3)	+	100	+	96 (4)	+	100
Xylose	v	67	−	0	+ or (+)	76 (19)	−	0	v	66 (3)	v	81
Mannitol	x	78	−	0	−	2 (1)	+	99 (1)	v	30 (9)	−	6
Lactose	−	8	−	3	v	14 (39)	−	0	v	7 (34)	v	19 (38)
Sucrose	+	100	+	99	+	97 (3)	+	99 (1)	+	97 (3)	+	94
Maltose	−	2	+	100	+	97 (3)	+	91 (5)	+ or (+)	85 (13)	+	100
Catalase	+	98	+	96	+	100	v	63	v	84 (2)	+	100
Oxidase	+	97	+	98	+	99	+	99	+	91	+	100
Growth on MacConkey agar	−	2 (1)	−	1	v	36 (17)	−	0	x	79 (6)	+	100
Simmons citrate	−	0	−	0	−	0	−	0	−	0	−	0
Urease	−	0	v	78 (3)	+	95 (1)	+	100	−	0	+	100
Nitrate reduction	+	99	+	100	+	100	+	99	+	100	+	100
Gas from nitrate	−	0	−	0	−	0	−	0	−	0	−	0
Indole	+	99	+	100	+	90	−	0	−	0	−	0
Gelatin hydrolysis	−	0	v	13	−	0	−	0	v	5 (7)	−	0
TSI[c] slant, acid	+	98 (1)	+	100	+	100	+	100	+	100	+	94
TSI butt, acid	+	99	+	100	+	97	+	99	+	100	+	100
Esculin hydrolysis	−	0	−	0	−	0	−	0	v	23	−	0
Lysine decarboxylase	−	0	−	2	v	33	−	0	−	3	−	0
Arginine dihydrolase	−	0	−	0	−	0	−	0	−	0	−	0
Ornithine decarboxylase	+	94	−	0	+	100	−	0	−	10	v	88

[a] Sign: +, 90% or more positive in 1 or 2 days; −, no reaction, 90% or more; + or (+), 90% or more positive, some strains positive after 3 or more days; v, more than 10% and less than 90% positive.
[b] Numbers in parentheses indicate percentage of delayed reactions (3 days or more).
[c] TSI, Triple sugar iron.

Table 4 Characteristics of the biotypes of Pasteurella multocida

Acid from	Biotype				
	n = 168	n = 66	n = 32	n = 24	n = 7
Glucose	+	+	+	+	+
Xylose	+	−	−	+	+
Mannitol	+	−	+	+	+
Lactose	−	−	−	+	−
Sucrose	+	+	+	+	+
Maltose	−	−	−	−	+

Namioka and Murata have showed that strains of P.multocida possessed several different O or somatic types, based on the use of acid treated cells and agglutinin-absorption procedures (36). For capsular types, they have performed a slide agglutination of whole cell. The result is closely related to Carter's system (28). There are 11 O groups in Namioka's system and 15 serotypes when combined with Carter serotyping (37). They proposed that serotype was identified by a number which indicated the O type, followed by a letter indicating the capsular type. For example, the important fowl cholera serotype was designated 8:A (38).

Heddleston et al. have identified different serotypes by means of a gel diffusion precipitin test which has employed an extracted antigen obtained by heating cell at 100°c for 1 hour. And they ultimately identified 16 somatic groups, because the variety of antigenic determinants more than one serotype have been found for one strain (31, 32).

Carter and Chengappa (39) have recommended the system developed by Carter and Heddleston should be combined and used as a standard way to designate serotypes by first listing its capsular type followed by an arabic number indicating the somatic type, such as B: 2 or A: 3.

1.6 Antigenic Structures and Immunogenicity

At preliminary study, Carter and Annaul (40) have identified his capsular type specific and found them to be a polysaccharide. Type A organisms also consist largely of hyaluronic acid. Later, they based on immunoelectrophoretic antigen to analyse the capsular type B and E and other variety of P.multocida. Prince and

Smith (41, 42, 43) have characterised three components as follows :

1) β - Antigen : type-specific polysaccharide; adsorbed cells in the PHA procedure.

2) $\propto$ - Complex : probably a polysaccharide-protein complex; closely adherent to the cell wall ; immunogenic ; probably somewhat labile.

3) γ - Antigen : lipopolysaccharide found in organisms from all variants ; to make up cell wall ; each has one or more antigenic determinants responsible for different O or somatic serological varieties.

Many researchers have believed that there is a good correlation between serological grouping and protection. It seems likely that most of the important immunological types have now been identified as a result of serotyping. Thus over the last several years the concentration on serological study has diverted efforts to detect new immunological (ie. protection) types (44). Various extraction procedures have been used to obtain crude extracts of somatic or capsule antigen for imnunizing animal or detecting antibody titer. Such immunogenic preparations is as follow :

KSCN - extracted antigen has been used as a vaccine against homologous challenge by several researches (45, 46, 47, 48) and has also been shown to confer cross-protection (49, 50). It was reported that there are two components in KSCN- extracts of both serotype 3 and 1 which were antigenically identical by Ouchterlony's gel diffusion analysis. Further study by Mckinney et al. (51, 52) found that there

are 2 different components - 40 p (particulate fraction) and 40s (soluble fraction) separated by ultracentrifuge. The 40 p fractions contained LPS and protein ; whereas, the 40s fractions contained protein and polysaccharide, but no LPS. The 40p antigens have stronger immunogenicity and toxicity than 40s antigens. Since the 40s fractions have no LPS thus it is likely that there is another one toxin over and above LPS. Similarly to the recent study by Ryce and Kaeberle (50) they found that mice given KSCN - extracts showed sign of depression and study by crossed immunoelectrophoresis showed a precipitation line similar to LPS.

Capsule antigen was extracted by many methods which were claimed to be immunogenic effect, for example, multiple freezing and thawing (53), solvent fractionation (54), sodium salicylate extraction (55), saline extraction (40). The most popular method is crude capsular antigen (CCA) of Carter and Annaul (40) by heating and shaking at 56°c for 1 hr in 2.5 % sodium chloride solution. Purified polysaccharide antigen (PPA) was prepared from such CCA by 3-step procedure utilizing cetylpyridinium chloride (56). However, it has been reported that only CCA, instead of PPA, can induce protective immunity in turkeys. It is likely that polysaccharide protein complex in CCA conferred the protection. Moreover, it has been showed that such CCA obtained by this method contaminated less endotoxin (57). Further study of Syuto and Matsumoto (58) found that there were four protein peaks obtained by gel filtration and only the first peak fraction which contained carbohydrate / protein ratio of 1.5, induced immunity in turkeys. It seemed that there was a close relationship between this fraction and glycoprotein fraction of the same strain P-1059 (Heddleston type 3 and Namioka 8: A) which was antigenically

distinct from LPS in previous study of Srivastava and Foster (59).

LPS-protein complex obtained from a formalinized saline extraction has previously been shown to be immunogenic in mice, chickens and turkeys (60). Apparently, protein integrated in the basic LPS structure was essential for immunogenicity. Since LPS purified from the complex by Westphal method, using aqueous phenol extract at 70° c (61), dominantly showed passive-immunity only in chicken (62). When compared with gel filtration study by Ganfield et. al. (63) showing 3 components of LPS-protein complex, but only 2 of the 3 components were immunogenic in mice and turkeys.

Kodama et al. (64) have evaluated various antigens prepared from a single source. CCA and LPS-protein complex showed more protective effect in turkeys than potassium thiocyanate and sodium salicylate-extracted antigens. And the greatest amount of endotoxin are LPS-protein complex, KSCN, sodium salicylate extrcated antigens and CCA in that order.

In summary, P.multocida has a numerous and complex of antigenic structures. Antigenic analysis by crossed immunoelectrophoresis of a serotype 1 organism showed at least 55 cytoplasmic and 19 cell-envelope antigens (65). And the antigenic components identified in each of KSCN extract antigen, CCA, LPS, could have diversed cellular origins. Hence, none of the extraction procedures of fluids was selective in removing antigens specifically from the cell surface, the cell envelope or the cytoplasm of the organism (66).

1.7 Epidemiology

P.multocida is a pathogen of several species of animals but rarely pathogenic for humans. It is responsible for outbreaks of cholera of domestic or wild fowl ; hemorrhagic septicemia of cattle and buffaloes ; also primary and secondary pneumonias. Infection in human is commonly transmitted by direct contact, usually through a bite and scratch wounds. Generally can be included in one of three categories (i) localized infection consisting of cellulitis, abscesses (ii) superinfection of a chronically diseased lung and (iii) other foci of disease that are secondary to septicemia (13).

In addition to these principal diseases, P.multocida is a commensal in the mouth, throats and noses of a variety of wild mamal and birds as well as humans in the absence of apparent infection (13).

There is a broad relationship between serologic type and host distribution. Type A and D are widely distributed in nature and most respiratory tract disease in humans is caused by type A (67). Type D strains are associated with a wide range of infections in cattle, sheep, pig and poultry (68). From early studies, type B and E strains have not been reported from avian host. It has been recovered from cattle and buffalo (69, 70). However, it was recently reported that a single (71) and further 4 type B strain have been isolated from chicken, turkey and swan (72).

The ability of P.multocida to invade and reproduce in the host is related to its capsule. However, many isolants from cases of fowl cholera have large capsules but are of low virulence. Of interest is the variation in virulence among capsular groups, since

severe outbreak of fowl cholera generally results from infection with group A organisms, and those group organisms that have been examined posses little pathogenicity for chickens or turkeys (73). Moreover, there is an evidence of with-group variation, because not all group A strains are highly virulent (32) and comparison of group F strains indicated differences in virulence (30). Therefore, the ability to invade and grow in the host is apparently related to some chemical substances associated with the capsule rather than with the physical presence and the virulent factor is questionable (17).

Endotoxins are produced by both virulent and nonvirulent P.multocida. The difference between virulent encapsulated and nonvirulent unencapsulated strain is not their ability to form endotoxin but the ability of virulent strains to invade and multiply to the extent that sufficient endotoxin is produced in vivo, which gives rise to pathologic processes (17).

2. Fowl Cholera (avian cholera, avian pasteurellosis, avian hemorrhagic septicemia)

2.1 Sign of Infection (73)

Acute form : sudden death, live birds sick with mucous discharge from mouth and nostrils, fever, anorexia, increased respiratory rate, diarrhea with watery and whitish initially and later mucus, greenish fecal.

Chronic form : swollen wattles in chickens, joint infections, chronic fowl cholera may follow an acute stage of the disease or result from infection with low virulent strains. Birds may succumb, remain infected for long period or recover.

2.2 Pathogenesis and Epizootiology

Usually routes of infection is the pharynx and the upper respiratory tract (74). The organisms multiply in the lungs and then spread to the liver and spleen where they cause pathological changes (necroti foci). The terminal manifestations of the disease are similar to those observed during endotoxic shock (17).

The susceptibility to the organism depends on age, host species and contact conditions. Fowl cholera usually occurs in young mature turkeys (over 8 weeks of age), but all ages are highly susceptible, occasionally appeared in broilers from 3 weeks of age. Both growing and adult duck succumbing to the disease usually occur over 4 wk of age. Chicken under 16 weeks of age are quite resistant, and it losses from fowl cholera in chicken commonly seen in laying flocks (17).

2.3 Diagnosis is best effected by culturing the organism, recognizing its bipolar staining and identifying it biochemically (75).

2.4 Treatment : Sulfaguinoxaline is one of the most effective treatments. It has been used widely in amount of 0.01-0.05 % in drinking water. Other parenteral medications such as penicillin, chloramphinol,chlortetracycline, oxytetracycline and streptomycin were effective in treating fowl cholera (76).

2.5 Prevention & Control : Prevention of fowl cholera can be effected by eleminating reservoirs of P.multocida or by preventing the access to poultry flocks. Good management practices and sanitation are the best means of prevention. Vaccination should be considered in area where fowl cholera is prevalent but it should not be substituted

for good sanitary practice (17).

3. Fowl Cholera Vaccines

The first vaccine produced by Pasteur was an avirulent culture attenuated by prolonged growth on artificial medium. In field, using this vaccine did not prove practical because uniform attenuation could not be obtained and heavy losses sometimes occured in vaccination flocks. This method is used occasionally in Europe (17).

Since Pasteur's classic work, there have been numerous attempts to produce efficient vaccines against fowl cholera. Oral immunization of chicken with a killed suspension of strain X-73 was reported by Heddleston and Reber (77). At the same time, Bierer and Eleazer (78) described the use of an attenuated strain of P.multocida given through the drinking water; however, the used strain had lost its immunogenicity through laboratory passages and never be seen widespread use. At present, only 2 types of vaccines are available for prophylaxis.

3.1 Killed Vaccines : They are usually prepared by growing selected immunogenic strains on a suitable medium and suspending in formalinized saline solution. The killed organisms are usually incorporated with an adjuvant and injected subcutaneously or intramuscularly. It may combine with a few serotypes. Nevertheless immunity induced by these vaccines is type specific (79).

Occasional outbreaks occuring in vaccinated groups are caused by a difference in antigenic structures between vaccinal strains and field strains or by a low degree of bacterin-induced immunity in animals under stress conditions. Evidence does not exist

to support the possibility that a minor antigenic difference other than major type specificity as defined by Heddleston et al,constitutes a marked difference in immunogenicity. Hence, the use of autogenous bacterin has no scientific basis (56).

3.2 Live Vaccines : There are many live attenuated vaccines reported. A mutant strain P-1059 was used by Heddleston et al (80) to immunize turkeys through the drinking water, Maheswaran et al (81) reported the efficacy of another strain M-2283 which is given either endotracheally or in the drinking water. And an avirulent strain (Heddleston serotype 3) was presented by Singer and Malkinson (82). The widest spread used live vaccine in U.S.A now is the CU strain described by Bierer and Derieux (4). It is now available as a federally licensed, lyophilized vaccine.

Many reseachers have showed that live vaccine and killed vaccine prepared from tissues of infected turkeys can induce immunity in turkeys against a different immunogenic type. A baterin (killed vaccine) prepared with bateria grown on conventional agar media did not induce cross-immunity. These studies indicate that P.multocida produces a wider spectrum of immunogens in vivo than in vitro (83, 84, 85, 86).

3.3 CU Vaccine

The formal name of CU strain is CS-148. It was originally isolated from a field outbreak of fowl cholera. There was no sign of increasing in virulence and impairment of immunization after 5-10 turkey passages (87). It produced irresdescent colonies on dextrose starch agar with the encapsulated cells and has been classified by Heddleston as a P-1059 strain. Its serotype is 3x4 in Heddleston's

system. First report published by Bierer and Derieux (4) showed that the CU vaccine induced higher immunity in turkeys than the oil base bacterin, but only 2.5 % infection. Study of Coates et al. (88) about the responses of turkeys in 3 doses of CU vaccine (4.5×10^9, 6.0×10^7 4.0×10^5 cells per average turkey) found that only high dose induces cholera death, and the percentage of survival after challenge was 96, 77 and 0 in heavy, medium and light dose respectively. Bierer has stated that 1.13×10^8 of viable bacilli consumed per average turkey was the lowest concentration of bacilli resulting in a favorable immune response (89) and the earliest age that turkeys could be vaccinated was 5 to 6 weeks of age (30). The protection in turkeys started 4 days and remained highly 1 to 4 week after vaccination (91). Response of each avian species to CU strain was so different. It was moderately virulent for coturnix quail by oral route and highly virulent for bobwhites by stick-wing. This vaccine was avirulent for guinea fowl and provided protective immunity by oral or stick-wing routes (8). Chickens had failed to respond to the Cu vaccine in the drinking water but the subcutanous routes produced the great degree of protection (86, 87, 88). A Commercial CU vaccine recommend 4×10^8 cells in 200 ml of drinking water per turkey. Turkeys are usually vaccinated at 6-8 weeks of age and booster at 3-4 weeks after vaccination (91,5).

Little was known about the mechanism of immunity. Dua and Maheswarau (94) have demonstrated by indirect immunofluorescence technique that the CU vaccine induced local antibodies by the 10th day and persisted up to 42 days post vaccination whereas oil adjuvant bacterin did not.

There have been some reports that the CU vaccine could

induce synovitis, osteomyelitis, wing lesion, and mortality (4, 88, 92, 95). However, Schlink and Olson proposed that stress and age of vaccination are involving factors (95, 96). Hens and toms in production are though to be more susceptible to P.multocida than younger turkeys because of the immunosuppressive effect of their higher level of estrogens and androgens (97, 98). Recent study has suggested the vaccination via wing-web or subcutanous for flocks with a history of a high mortality after vaccination with the CU vaccine in drinking water (7). A recent serotypic survey of P.multocida isolated from poultry has showed the increased incidence of fowl cholera associated with strains of 3x4 serotype which may be due to the increased use of the CU vaccine (99). However, the result of comparison of nine isolates of P.multocida serotype 3x4 from turkeys with fowl cholera and CU strain has shown the difference between those (100).