Prevalence, epidemiology, and virulence of *Pasteurella multocida* and related organisms obtained from poultry and their animal contacts

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Preface

This thesis is made up of field investigations performed in Denmark and Tanzania followed by laboratory and challenge experiments conducted in Denmark from September 1997 to June 2000. The field study in Tanzania was conducted in Morogoro rural district, under the Faculty of Veterinary Medicine, Sokoine University of Agriculture. The studies in Denmark were performed at the Department of Veterinary Microbiology, The Royal Veterinary and Agricultural University, Copenhagen.

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Appendix I-IV

I) Muhairwa, A.P., Christensen, J.P., Bisgaard M. (2000). Investigations on the carrier rate of *Pasteurella multocida* in healthy commercial poultry and flocks affected by fowl cholera. *Avian Pathology.* 29:133-142.

II) Muhairwa, A.P., Mtambo, M.M.A., Christensen, J.P., Bisgaard, M. Occurrence of *Pasteurella multocida* and related species in free ranging village poultry and their animal contacts. In press. Veterinary Microbiology.

III) Muhairwa, A.P., Christensen, J.P., Bisgaard, M. Relationships among *Pasteurellaceae* isolated from free ranging chickens and their animal contacts as determined by quantitative evaluation of phenotypic data, ribotyping and REA-typing. In press. Veterinary Microbiology.

IV) Muhairwa, A. P., Christensen, J.P., Bisgaard, M. Investigations on the correlation between serum resistance, outer-membrane proteins and virulence in *Pasteurella multocida.* Draft for submission to *Avian Pathology.*

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Summary

This thesis is divided into two sections. The first section presents six reviewing chapters in relation to work performed and a chapter detailing conclusions from the study. The second part contains four papers referred to as Appendix I-IV, which detail the work in the thesis. The articles I and II present prevalence and epidemiological investigations of *P. multocida* in commercial poultry flocks in Denmark, and free-range village chickens and their contact animals in Tanzania. Article III discusses phenotypic, molecular and epidemiological investigations of *Pasteurella* species obtained from Tanzania. The last article presents the study on correlation between serum resistance, outer-membrane proteins and virulence of *P. multocida.*

The prevalence of *P. multocida* carriers in the flocks with a history of fowl cholera was shown to be significantly higher than that of the flocks without a history of fowl cholera in commercial flocks in Denmark (Appendix I). These findings underline the importance of surviving birds in the epidemiology of fowl cholera. The carriers in flocks without history of fowl cholera were also demonstrated, but their role in fowl cholera is still uncertain. All three subspecies of *P. multocida*, namely ssp. *multocida*, ssp. *septica* and ssp. *gallicida* were demonstrated, but the subspecies *gallicida* was obtained exclusively from Pekin ducks from a farm with a history of fowl cholera. A single clone affected each of the investigated flocks, but some clones appeared in more than a single flock. Investigations into strains of *P. multocida* from one of the farms in four consecutive outbreaks demonstrated a different clone in each outbreak. This indicates that outbreaks are clonal, and the disease can be eliminated from the infected farms. Isolation of *P. multocida* from the cloacal mucosa of carriers was reported for the first time, however, the significance of cloacal carriers in spreading infection is not known.

Occurrence of *P. multocida* and related species in free ranging chickens and ducks, dogs and pigs (Appendix II) was investigated in three subclimatic zones; hot, warm

and cool, in rural Morogoro district, Tanzania. The strains obtained included *P. multocida* ssp. *multocida* from chickens, ducks, cats and dogs, *P. multocida* ssp. *septica* from dogs and cats, *P. gallinarum* from a duck, *P. canis* and *P. dagmatis* from dogs, and *P. stomatis* from dogs and cats. Other strains included organisms with uncertain taxonomic affiliation named taxon 16, and unclassified strains with *Pasteurella* like features. *P. multocida* was obtained from two chickens (2%) and 11 ducks (22%) of warm zone. No isolates were obtained from poultry in the remaining zones. Cats had the highest prevalence of *P. multocida* ssp. *multocida*, while *P. canis* and taxon 16 were predominant in dogs. Mouse inoculation was more sensitive in detecting *P. multocida* ssp. *multocida* than blood agar and selective medium. Direct culture on blood agar recovered most of the other *Pasteurella* spp.. These findings demonstrated the existence of *P. multocida* carriers in the free ranging village poultry, cats and dogs, and underline the potential of exchange of strains between the different animal species. Surveillance of diseases in free ranging village chickens to gauge the clinical importance of fowl cholera and other diseases was recommended.

One hundred and forty-three *Pasteurella* spp. strains and ten unclassified strains obtained from free ranging poultry, dogs and cats were investigated by extended phenotypic characterization (Appendix III). One hundred and forty-nine of these strains were selected for further studies using ribotyping and REA-typing to evaluate the role of dogs and cats in *P. multocida* transmission to poultry. Seven and six-type strains were included for comparison in phenotyping and genotyping, respectively. Eleven clusters and six unclustered strains were revealed by phenotyping. Ribotyping outlined twelve clusters and six unclustered strains. A correlation between clusters obtained by phenotyping and ribotyping was demonstrated which indicated that a genetic basis exists for clusters outlined by quantitative evaluation of phenotypic data. Similarities and differences in hosts, phenotype, ribotype, and zone of isolation were demonstrated among *Pasteurella* strains investigated. Isolates of *P. multocida* from ducks were shown to be clonal by both phenotyping and ribotyping. These strains were identical to one of the chicken strains. REA-typing, however, showed that the chicken strain was different underlining that exchange of clones of *P. multocida*

between avian species seems to be rarely happening under village conditions. Management practices in the villages suggest the potential for exchange of *P. multocida* between poultry and animals kept in contact. The present findings, however, did not indicate that clones of *P. multocida* are widely exchanged between poultry and other animal species even though close contact exists. In the present investigation, exchange of clones of *P. multocida* was only demonstrated among animals belonging to the same species. Caution is drawn to the use of ribotyping alone for epidemiological typing and tracing of *P. multocida*. The present results also underline the importance of proper phenotyping in the identification of *P. multocida* and related species.

Serum resistance of *P. multocida* in the sera from chickens, turkeys, ducks and pigs was determined and correlated with *in-vitro* and *in-vivo* outer-membrane proteins expression and virulence in chickens. Eighty-seven field strains of *Pasteurella* and nine reference strains representing different clones were grown in sera from chickens, ducks, turkeys and pigs. Serum activity of each strain was measured by changes in the optical density of the serum after inoculation and incubation at 41 °C for chicken, turkey and duck serum and 39 °C for pig serum. The strains were classified into High serum resistant (R), moderate serum resistance (M), and serum sensitive (S) by comparing with strains of known serum activity. Strains of identical genotype by Restriction endonuclease analysis were found to have identical growth curves and the same maximum OD values, when cultured in serum from the same host species. Turkey serum was shown to be less inhibitory to a wide range of *P. multocida* strains than chicken, duck and pig sera. Serum resistant strains were demonstrated among avian as well as mammalian strains, with the proportion of serum resistant strains being higher in fowl cholera outbreak strains than in non-outbreak avian strains. A range of minor and major outer-membrane proteins were common among the selected serum resistant and serum sensitive strains, when cultured in BHI, *in-vivo* and in chicken serum. However, no specific OMP expressed *in vitro* or *in vivo* was consistent with serum resistance or sensitivity among the strains investigated. Although most severe lesions in experimentally infected chickens were produced by a serum resistant

strain, lesions were also found in chickens infected by serum sensitive strains, indicating the involvement of multiple factors in the virulence of *P. multocida*. Further investigations on serum resistance should also relate other host and bacterial factors responsible development of fowl cholera.

Introduction

FAOSTAT database indicate the presence of about 15 billion poultry in the world, with domestic chickens, ducks, turkeys and geese as the most common species (FAO, 2000). Industrial poultry production is the major form of poultry keeping in the developed countries as represented by Denmark in this study. In this system adequate nutrition, biosecurity and disease prevention and monitoring are optimum, leading to high productivity and control of diseases and nutritional disorders. Free-range management is the most common poultry raising method in developing countries (Minga et al., 1989; Sonaiya, 1989), in Tanzania it comprises more than 95% of poultry (MOA, 1995). The birds are enclosed at night in simple houses made of straws, woods or old roofing sheets or in the kitchen (Mwalusanya, 1998). During the day, feed may be provided, but the birds depend largely on scavenging around the houses and in the fields (Minga et al., 1989; Kabatange and Katule, 1990). Infectious diseases exist in both commercial and free-range poultry management systems, however, the epidemiology and extent is different. Industrial poultry production has resulted into significant reduction of diseases (Anon, 1996), whereas in free-range poultry disease is still a major problem (Minga et al., 1989; Dipeolu et al., 1996). In this study, comparison of the two production systems was done to obtain a possible explanation to *P. multocida* epidemiology in relation to management system.

Fowl cholera belongs to the important disease conditions, which are reportable to OIE and causing problems in industrial production (Rimler and Glisson, 1997; Appendix I) despite the advancement in methods of disease control. In Tanzania, like other developing countries the epidemiology of fowl cholera is poorly known. With inadequate disease surveillance programmes presence of fowl cholera in village free ranging poultry cannot be ruled out. *P. multocida* and fowl cholera have been sparsely reported in free ranging chickens in Africa (Sa'idu et al., 1994). However, due to low availability of birds for post-mortem examination (Muhairwa et al., 1999) most investigations have relied on the demonstration of carriers by serology (Kelly et al.,

1994) and isolation of *P. multocida* (Karmy et al., 1983). Since seroconversion cannot explain the significance and epidemiology of the strains involved in the flocks, isolation by culture was employed in the present study. In the free-range management, chickens and ducks have maximum contact with other domestic animals, which suggests high risk of exchange of *P. multocida* among these animals. However, this has not been investigated. Reports elsewhere have indicated the potential of animal contacts in transmitting pathogenic *P. multocida* to domestic poultry (Snipes et al., 1988; Van Sambeek et al., 1995). Epidemiological studies were conducted in commercial poultry flocks in Denmark and in free ranging village chickens in Tanzania. In the study conducted in Tanzania, dogs, cats and pigs kept in contact with poultry were screened for the presence of *P. multocida*.

Reinvestigation of the genus *Pasteurella* Trevisan 1887 during the last two decades has resulted into major changes in the taxonomy of these organisms (Mutters et al., 1985a and b; Angen et al., 1999). Although significant changes have been made, indications still exist that the genus is complex and heterogeneous (Dewhirst et al., 1993; Bisgaard, 1995b). The reorganization by Mutters et al. (1985b), which included outlining of new species and new criteria for identification have significant impact on previous studies which often do not allow a precise identification of the organisms reported. Consequently, the prevalence, epidemiology and pathogenesis of these organisms in poultry and other animals are poorly understood and need reinvestigation (Christensen and Bisgaard, 1997).

The objectives of the present work were to investigate the epidemiology of *P. multocida* in different poultry production systems, and the role of in contact animals in the epidemiology of *P. multocida* in domestic poultry. Prevalence of *Pasteurella* spp. was investigated in commercial production flocks in Denmark (Appendix I) and free ranging village chickens and their contact animals in Tanzania (Appendix II). The phenotypic and genotypic relatedness of the strains from poultry and their animal contacts was subsequently, investigated (Appendix III) followed by *in- vitro* and *in-vivo* virulence studies (Appendix IV) in chickens.

1. The Genus *Pasteurella* as defined by Mutters et al. (1985)

1.1 Taxonomy of the genus *Pasteurella*

The original description of the genus *Pasteurella* was given by Rivolta 1877 and Revolee 1879 (Henderson, 1963;Hussaini, 1975) after outbreaks of a disease in fowl designated fowl cholera by Mailet 1836 (cited by Rhoades and Rimler, 1989). Rosenbusch and Merchant (1939) designated the name *Pasteurella multocida* after years of nomenclature changes. Based on phenotypic features the genus *Pasteurella* was subsequently conceived to have the following other species; *P. haemolytica* (Newsom and Cross, 1931), *P. pneumotropica* (Jawetz, 1950), *P. gallinarum* (Hall et al., 1955) *P. ureae* (Jones, 1962) and *P. aerogenes* (McAllister and Carter, 1974). The genus was later expanded to include more taxa including *Pasteurella* 'gas' or *Pasteurella* new species 1 (Gump and Holden, 1972), SP group (Frederiksen, 1973), bovine lymphangitis group (Jayaraman and Sethumandhavan, 1985), *Pasteurella testudinis* (Snipes and Biberstein, 1982), as well as some new taxa isolated from poultry (Bisgaard, 1982), guinea pigs (Bisgaard et al., 1983) and calves (Madsen et al., 1985). With many of these new organisms, however, problems of discriminating between members of the genus *Pasteurella* and the genus *Actinobacillus* Brumpt 1910 were significant.

Difficulties of phenotypic differentiation between these genera lead into early proposal for reclassification of these organisms. Mraz (1969) proposed the reclassification of *P. haemolytica* as *Actinobacillus haemolyticus* because of the phenotypic similarities of this organism with the genus *Actinobacillus*. Subsequently Frederiksen (1973) suggested that *P. ureae* should be regarded as a species of the genus *Actinobacilus* because of the similarities to this group, a proposal that was supporting Jones (1962) observations. Classification of *Pasteurella* became even more complicated when phenotypic delineation of the genus *Haemophilus* Winslow 1971 was considered since V- factor dependency was no longer a criterion for separating genera (Pohl et al. 1983). In attempt to resolve these problems Mutters et al. (1985a) used DNA-DNA

hybridization to establish genetic relationships within the genus *Pasteurella*, the type species of the family *Pasteurellceae* Pohl 1979. The most common species of the genus *Pasteurella*, *P. multocida* was divided into three subspecies namely *P. multocida* subspecies *multocida*, *P. multocida* subspecies *septica* and *P. multocida* subspecies *gallicida*. Other species in the genus are, *P. canis*, *P. stomatis*, *P. dagmatis*, *P. anatis*, *P. langaa*, *P. gallinarum* *P. volantium*, *P. avium* and two unnamed species *Pasteurella* sp. A and *Pasteurella* sp. B. These taxa can be identified according to a standard set of biochemical reactions defined by Mutters et al. (1985b) (Table 1).

After the genus reclassification, six new taxa have been proposed to belong to the genus *Pasteurella*. These include *[P]. granulomatis* (Ribeiro et al., 1990), *[P]. caballi* (Schlater et al., 1989), *[P]. betti*, *[P]. lymphangitis*, *[P]. mairii* and *[P]. trehalosi* (Sneath and Stevens, 1990). Subsequent investigations have shown lack of close affiliation of these strains to genus *Pasteurella*. DNA-DNA hybridization studies indicated that *[P.] trehalosi* might belong to a new genus within *Pasteurellaceae* family while *[P]. granulomatis* has now been reclassified under the genus *Mannheimia* (Angen et al., 1999). Other unclassified taxa with features related to *Pasteurella* genus have also been reported (Bisgaard and Mutters, 1986; Bisgaard et al., 1999; Appendix III), however, their taxonomic destination is not yet available.

Despite the remarkable progress in the reorganization of the genus *Pasteurella* Trevisan 1887, the results obtained by rRNA:DNA hybridization and 16S rRNA sequencing have indicated that the genus *Pasteurella sensu stricto* is still heterogeneous. According to rRNA: DNA hybridizations *P. multocida* NCTC 10322^T rRNA branch contained only three out of five accepted species investigated of the genus *Pasteurella sensu stricto* (De Ley et al., 1990). *P. multocida* ssp. *gallicida* SSI P426^T, and *P.* sp. *B* SSI P683^T were located outside the *Pasteurella* branch. Thus, these findings do not agree with the criterion that strains belonging to a well-defined genus should fall on the same rRNA branch (De Ley et al., 1990). Phylogenetic studies of Dewhirst et al. (1992) separated the genus *Pasteurella* into two subclusters, Cluster 3A and 3B. Cluster 3A included *P. gallinarum*. *P. volantium*, *P. avium*, *P.* sp. *A*, *P. anatis* and *P. langaa* in

addition to *[H]. paragallinarum* and *[H]. taxon C*. Cluster 3B contained *P. multocida* ssp. *multocida*, *P. dagmatis*, *P. stomatis*, *P. canis* and *P.* sp. *B*. Subsequent studies which included 16 new taxa further separated clusters 3A and 3B (Dewhirst et al., 1993). A new subcluster 3D included *P. langaa*, Bisgaards taxon 2 and taxon 3, *P. anatis* and *[A]. salpingitidis*.

Further investigations on 16S rRNA based on more strains are recommended, this will alleviate the weaknesses caused by limited number of strains in the previous studies. Heterogeneity among the strains like that shown among *P. multocida* strains (Appendix III) should also be addressed by these methods so as to reach a safe conclusion on the taxonomy of these organisms.

1.2 Ecology of avian taxa of *Pasteurella*

Species belonging to *Pasteurella sensu stricto* (Mutters et al., 1985a) demonstrate a considerable ecological diversity on the mucosal membranes of domestic and wild animals. They may be commensal or opportunistic secondary invaders of the oral cavity mucosa, upper respiratory tracts, and lower genital tracts (Bisgaard, 1993). With the exception of *P. multocida*, other members of the genus are regarded as opportunistic pathogens only, causing secondary infections in animals and man (Bisgaard, 1993; Frederiksen, 1993). Fowl cholera is the major disease condition caused by *P. multocida* in avian hosts (Rhoades and Rimler, 1989), but localized infections have also been reported (Glass and Panigrahy, 1990). Other members of *Pasteurella* which have been found in avian hosts are *P. gallinarum, P. volantium, P. avium. P. anatis, P. langaa* and unnamed species *P.* sp. A.

P. multocida ssp. *multocida* and *P. multocida* ssp. *septica* are the most common isolates of *Pasteurella* from wild birds, turkeys, chickens and ducks (Hirsh et al., 1990; Blackall et al., 1995; Appendix I and II), while *P. multocida* ssp. *gallicida* seems more prevalent in web-footed birds (Appendix I). Avian strains of *P. multocida* have been routinely isolated from turkeys and chickens with fowl cholera (Carpenter et al., 1989; Fegan et al., 1995; Christensen et al., 1998), and from oropharynx of surviving birds from

diseased flocks (Mraz et al., 1980; Curtis and Ollerhead, 1981). The present study has documented carriers in apparently healthy commercial flocks and free ranging village chickens and ducks (Appendix I and II). In healthy carriers and birds surviving clinical disease *P. multocida* has been cultured from upper respiratory tract and oral cavity. However, the present study (Appendix I) documented isolation of *P. multocida* from the cloaca of chickens and ducks.

P. multocida is a commensal in the upper respiratory tract and oral mucosa of dogs and cats (Saphir and Carter, 1976; Baldrias et al., 1988; Ganierre et al., 1993). Carrier rates of *P. multocida* in cats have been shown to range from 20% to 100% while the prevalence in dogs is usually below 40% (Baldrias et al., 1988; Ganierre et al., 1993; Loubinoux et al., 1999). Actual proportions of the *P. multocida* ssp. *multocida* and *P. multocida* ssp. *septica* in the individual animals are not well known, however, these subspecies seem to in equal proportions in cats (Ganierre et al., 1993; Appendix II). Loubinoux et al. (1999) demonstrated 3:1 ratio of *P. multocida* ssp. *multocida* to *P. multocida* ssp. *septica* among the dogs, however, the present results (Appendix II) found very low prevalence of *P. multocida* ssp. *multocida*, and no *P. multocida* ssp. *septica* was recovered from dogs.

Apart from being a normal flora, *P. multocida* has been associated with bite wounds, abscesses and secondary infections in dogs and cats (Manning et al., 1989). Batamuzi et al. (1996) found *P. multocida* from vaginal mucosa and prepuce of dogs suffering from transmissible venereal tumours. Although most publications before reorganization of the genus *Pasteurella* do not allow precise identification of the taxa involved, recent works show that *P. multocida* ssp. *multocida* and *P. multocida* ssp. *septica* are involved in pathological processes in dogs (Mohan et al., 1997). These include bite wounds, respiratory disease and genitourinary infections. *P. multocida* strains from cats have been shown to cause systemic infection in other animals including birds (Snipes et al., 1988; Korbel et al., 1992) and human beings (Fajfar-Whetstone et al., 1995).

P. multocida has been co-isolated consistently with *Bordetella bronchiseptica* in pigs

suffering from progressive atrophic rhinitis (Chanter and Rutter, 1989). Blackall et al. (1997) demonstrated *P. multocida* ssp. *multocida* to be the predominant isolate from pigs with various clinical conditions. However, the subspecies *gallicida* has also been reported to cause haemorrhagic septicaemia in pigs (Cameron et al., 1996). Strains of *P. multocida* from cattle, sheep and goats have been associated with pneumonic pasteurellosis (Frank, 1989; Gilmour and Gilmour, 1989) and haemorrhagic septicaemia of cattle and water buffalo in Asia and Africa (Carter and de Alwis, 1989). Dabo et al. (1999b) reported isolation of *P. multocida* in the digestive tract and upper respiratory tract mucosa of healthy cattle. Although healthy carriers of *P. multocida* are known among the tropical cattle (De Alwis et al., 1990), cattle were not included in the present investigations as they were not regularly in contact with poultry in the areas investigated. Investigations into strains of *P. multocida* from healthy and diseased cattle as well as wild animals and birds are recommended to assess their epidemiological relationship to poultry isolates.

The remaining species of *Pasteurella* are less heterogeneous in terms of host than *P. multocida* (Mutters et al., 1985b; Bisgaard, 1993), this has also been supported in the present investigations (Appendix I and II). Since the first isolation of *P. gallinarum* from chickens with atypical fowl cholera (Hall et al., 1955), subsequent isolations have been done from pathological lesions (Yadav et al., 1977; Droual et al., 1992b) and in upper respiratory mucosa of carrier chickens only (Mraz et al., 1980). However, the present study showed *P. gallinarum* in an apparently healthy duck (Appendix II). Baldrias et al. (1988) recorded an isolate of *P. gallinarum* from the tonsils of dogs, but supporting data on the occurrence of this species in dogs have not appeared since then. Recently, *P. gallinarum* has been reported from human patient with septicaemia and endocarditis (Fajfar-Whetstone et al., 1995). However, caution is needed in the interpretation of reports of *P. gallinarum* outside avian hosts. This is because of limited number of tests available for distinguishing *P. gallinarum* from other members of *Pasteurella sensu stricto* and unclassified *Pasteurella*-like organisms like taxon 16 (Bisgaard and Mutters, 1986; Mutters et al., 1985b).

Members of *P. avium* are generally regarded as harmless commensals in poultry, however, non-V factor requiring strains have been isolated from pneumonic lesions in cattle (Bisgaard et al., 1991a, 1991b). The original isolation of *P. avium* (biovar 1) from chickens was followed by isolation from turkeys, pigeons and wild birds (Bisgaard 1993). Recently, Bragg et al. (1997) isolated *P. avium* from the sinuses of layers and broilers with clinical signs of infectious coryza. Strains classified as *P. volantium* have been obtained from chickens and turkeys (Mutters et al., 1985b; Peters, 1989). Mutters et al. (1985a) also reported the isolates from a peacock and human tongue, whereas Bragg et al. (1997) isolated the organism from chickens with respiratory disease. Other isolates have been reported from the respiratory tract and internal organs of web-footed birds, and wild birds (Peters, 1989) however, pathological potential of these organisms seems unclear. *P.* sp. A have been isolated from chickens and turkeys (Peters, 1989), and pigeons (Mutters et al., 1985a). Although the significance of these organisms remains uncertain, they have been isolated in pure culture from backyard chickens with sinusitis and conjunctivitis (Bisgaard, 1993) and chickens with respiratory symptoms (Bragg et al., 1997).

P. anatis and *P. langaa* (Mutters et al., 1985b) have been obtained from the intestinal and respiratory tracts of chickens, respectively (Bisgaard, 1982). *P. anatis* was originally isolated from sinus infraorbitalis of ducks. Subsequently Beichel (1986) obtained the *P. anatis* from the airsac of a duck and the lung of a goose.

2. Fowl cholera and other diseases caused by *P. multocida*

2.1 Signs and lesions

A wide range of signs may be observed in infections caused by *P. multocida*, the signs varying with the nature of infection. Few signs may be observed in peracute, and acute infections (Rhoades and Rimler, 1989) and sudden death may be only sign of disease in the flock. In more protracted cases, sero-mucous discharges from the mouth, nose and eyes, cyanosis, general depression, ruffled feathers and diarrhoea may be observed. In chronic infections, signs are mainly due to localized infections of leg or wing joints, comb, wattles and subcutaneous tissue of the head (Gustafson et al., 1998; Rimler and Glisson, 1997), oviduct (Bisgaard, 1995a), and the respiratory tract (Rimler and Glisson, 1997). Severe forms of cellulitis have been reported in turkeys (Glass and Panigrahy, 1990;Frame et al., 1994).

Fowl cholera occurs sporadically or enzootically in most countries (Rimler and Glisson, 1997). Morbidity varies with host factors such as age, species of birds (Rhoades and Rimler 1989), and mortality may range from only a few percent to 100% under natural conditions (Rimler and Glisson, 1997). Experimental infection of three-weeks-old turkeys resulted in 100% mortality within 24 hours, while in pheasants it was approximately 50% after 24 hours (Petersen et al., 2000). However, none of the chickens died after infection of chickens with the same strain (appendix IV).

Lesions

In the case of peracute forms of fowl cholera, general septicaemic lesions including vascular disturbances, as reflected by general passive hyperemia and congestion throughout the carcass dominate the postmortem findings. Petechial and ecchymotic haemorrhages are often present in the subserosal abdominal and coronary fat, and haemorrhages may be observed in the intestinal mucosae and on subserosal surfaces in the thoracic and abdominal cavities. The liver and spleen are often swollen and contain multiple small focal areas of coagulative necrosis or organs may undergo more

generalized necrosis. The lungs are often involved especially in turkeys, where the lesions may be very characteristic. In the most acute forms of infection, the lung lesions are dominated by haemorrhages, but this is soon followed by necrosis and fibrinous pleuro-pneumonia where affected areas are clearly marked from unaffected tissue. A unilateral or bilateral productive inflammation of pleura and lungs with extensive exudation of fibrin is common in chickens (appendix IV).

Acute general passive hyperemia indicative of shock is one of significant histologic changes in the infected birds (Rhoades, 1964). Other lesions include heterophilic infiltrations in the liver, lungs, adrenal, kidney and thyroid while in the bone marrow heterophilic depletion and haemopoietic cell necrosis are prominent (Rhoades, 1964). In the chronic form heterophilic infiltrations are consistently observed in the middle ear and meninges (Olson et al., 1966). Multinuclear giant cells are often associated with necrotic masses of heterophils in the air spaces (Olson et al., 1966; Olson and McCune, 1968).

In chronic forms of *P. multocida* infections, purulent lesions may be widely distributed often involving the respiratory tract, the conjunctival and adjacent tissues of the head (Rimler and Glisson, 1997). Caseous arthritis and productive inflammation of the peritoneal cavity and the oviduct are also common in this form of fowl cholera. A fibrinopurulent dermatitis including caudal parts of dorsum, the abdomen and breast, and involving cutis, subcutis and the underlying muscle has been observed in turkeys and broilers (Glass and Panigrahy, 1990; Frame et al., 1994).

2.2 Diagnosis

A conclusive diagnosis of the disease should be based on history, clinical signs, necropsy findings and isolation of *P. multocida* (Rimler and Glisson, 1997). Liver imprints stained by Wright's stain can be used for presumptive diagnosis where bipolar organisms will be seen under a light microscope. *P. multocida* can be isolated directly from viscera of affected birds and from lesions in chronic cases; bone marrow, heart blood, liver, meninges or localized lesions are preferred for culturing (Rimler and

Glisson, 1997). In living birds mucus from the nostrils or swabbing into the nasal cleft (Rimler and Glisson, 1997) or cloacal mucosa should be cultured (Appendix I).

2.2.1 Isolation methods for *P. multocida* and related species

Growth requirements

Members of *Pasteurella sensu stricto* grow best at temperatures of 35-37° C (Mutters et al., 1985b). Growth on nutrient agar is supported by addition of 5% bovine, horse or sheep blood (Rhoades and Rimler, 1989; Appendix I and II). Ryu (1959) observed inhibition of growth of *P. multocida* by addition of sheep, bovine, or horse blood, however, this inhibition does not appear to affect isolation under field conditions as observed in the present studies (Appendix I and II). Growth of certain strains of *P. multocida* (Mutters et al., 1985a) *P. avium* biotype 1, *Pasteurella volantium* and *Pasteurella* species A depend on the presence of V-factor (Mutters et al., 1985a, 1985b).

Blood agar

Blood agar supports growth of most known taxa within the genus *Pasteurella* (Mutters et al., 1985b; Appendix II). Colonies of *P. multocida* on blood agar are round, grayish in colour and can grow to about 2 mm in 48 hrs; rough colonies of about 1 mm in diameter have also been observed (Mutters et al., 1985b). Pure colonies of *P. multocida* are generally regarded as non-haemolytic on blood agar, but hemolytic strains have been observed (Bisgaard, personal communication). *P. multocida* produce mucoid or non-mucoid colonies (Bisgaard et al., 1991b) with a characteristic sweetish smell (Baldrias et al., 1988).

Comparison of isolation techniques showed that blood agar is more efficient for isolation of *Pasteurella* species other than *P. multocida* (Baldrias et al., 1988; Appendix II). Direct isolation from contaminated materials using blood agar, frequently may result into heavily contaminated cultures, thus dilution of the initial streak into multiple plates before incubation is needed to facilitate identification of *Pasteurella* colonies

(Appendix II). However, for strains that are sensitive to drugs used in selective media, blood agar should remain the first choice for their isolation (Baldrias et al., 1988; Appendix II). Isolates obtained from the selective medium and mouse inoculations are usually propagated on blood agar for purification and characterization of colonial morphology (Baldrias et al., 1988; Appendix I and II).

Selective media for *Pasteurella* isolation

A variety of selective media have been designed for isolation of *P. multocida* from contaminated animal sources (Morris, 1958; Knight et al., 1983; Avril et al., 1990). A selective medium CGT designed by Knight et al. (1983) readily detected *P. multocida* from dogs and cats, but not in human beings. Avril et al. (1990) found that a selective medium made by incorporating 2mg amikacin, 4mg vancomycin in 1 liter of Mueller-Hinton agar to be efficient in isolating *P. multocida* from pig breeders. Seemingly, efficiency of a selective medium might vary with the host animal being investigated, however, no comparison have been done on the efficiency of selective media. Apart from the selective enrichment procedure that was developed by Moore et al. (1994) for isolating *P. multocida* from the environment no attempts have been made to develop a selective medium specific for avian species. Neither has any medium been shown to be useful for all species of animals. Consequently, the present study (Appendix II) employed 8HPG medium (Smith and Baskerville, 1983), which was previously shown to have potential for isolating *P. multocida* from different animal species. A selective medium incorporating 0.02% bacitracin and 1% neomycin was used in the investigation of carriers of *P. multocida* in flocks with a history of fowl cholera and apparently healthy flocks in Denmark.

Selective media have been found to cause a decrease in colony size (Morris, 1958; Smith and Baskerville, 1983) and colony count of *P. multocida* (Avril et al., 1990) just as it lowers the isolation rates of non-*P. multocida* strains (Appendix II). Furthermore, growth on selective medium has been shown to require longer incubation time (48 or 72) hours, which prolongs the time required for identification (Appendix II). However, the weaknesses of selective media are unlikely to outweigh the practical and economic

advantages of the technique (Rhoades and Rimler, 1989). The selective medium adopted in a particular study should represent a compromise between inhibition of the maximum number of other organisms and the quantitative growth of the maximum number of *Pasteurella* strains (Morris, 1958). This balance should be adjusted to suit particular circumstances and for detection of required strains.

Mouse inoculation

Virulent strains of *P. multocida* can be recovered from specimens containing other flora by mouse inoculation (Rhoades and Rimler, 1989). *Pasteurella*-free mice are inoculated with a specimen containing *P. multocida* by subcutaneous, intraperitoneal or intramuscular injection. Virulent strains of *P. multocida* often kills susceptible mice within 48 hrs (Ganiere et al., 1993; Appendix I and II), subsequently *P. multocida* can be recovered from the spleen, liver, or heart blood (Chandrasekaran and Yeap, 1982; Appendix I and II). Chandrasekaran and Yeap (1982) showed that as small as 13 colony-forming units of some virulent strains can kill a susceptible mouse within 24 hours. Despite these strengths, several drawbacks of mouse for isolation of *P. multocida* have been observed.

First of all, the obtained isolates represent strains that are pathogenic to mice (Ganiere et al., 1993; Appendix I). However, not all strains of *P. multocida* are pathogenic to mice (Ganiere et al., 1993) and non-*P. multocida* strains are likely to be excluded by mouse inoculation (Appendix II). Isolation based on mouse inoculation alone might consequently represent a lower than the actual prevalence of *P. multocida* in a population. The cost of mice and ethical values regarding the use of experimental animals should also be considered. The use of animals for experimental studies is discouraged by animals' rights organisations, and the cost of mice might be too high for large-scale epidemiological studies. Despite the above disadvantages, mouse passage, has been shown to be a more reliable method of isolating *P. multocida* from poultry than other methods (Appendix I; Appendix II).

In summary, none of the isolation methods mentioned above can be considered as the most accurate way of detecting organisms under *Pasteurella* species. Choice of method of isolation should largely depend on the purpose of the study and the animal host investigated. However, applying more than one method of isolation can yield better results (Appendix II). More attempts using selective enrichment procedure (Moore et al., 1994) are needed to validate this method, which seems promising in the isolation of *P. multocida* from contaminated environmental samples.

2.2.2 Identification of *P. multocida* and related species

Phenotypic methods for identifying *Pasteurella* species

After isolation, identification is normally based on the results of biochemical tests. The most valuable tests for differentiation of *P. multocida* from other organisms are shown in Table 1. However, the clinical diagnostician is always faced with difficulties in the diagnosis of members within family *Pasteurellaceae*. Consequently extended characterization, including the use of reference strains (Bisgaard, 1993) was employed in this study (Appendix I and II).

The members of the genus *Pasteurella* are described as small, non-motile, Gram-negative rods or coccobacilli, which are facultative aerobic or microaerophilic bacteria, and produce acid from glucose. They metabolise glucose by fermentation in Hugh & Leifson medium, and have positive oxidase reaction (Mutters et al., 1985b). They measure 0.2-0.4 μm, and repeated subcultures may lead into pleomorphism (Rimler and Glisson, 1997). Organisms in tissues, blood and recently isolated cultures stain bipolar (Rimler and Glisson, 1997).

Colonies of *P. multocida* on blood agar are normally non-hemolytic, grey in colour and translucent with characteristic sweet smell (Mutters et al., 1985b; 1989). Mucoid colonies have been described for isolates obtained from cats (Baldrias et al., 1988) rabbits and pneumonic calves (Mohan et al., 1994). Hughes (1930) distinguished three different types of colonial morphology; the iridescent type, blue and intermediate type

by using stereo microscope and obliquely transmitted light on a transparent agar. Although these colonial types were associated with different forms of fowl cholera, variations in the morphology of colonies are known to exist (Heddleston et al., 1964), making colonial morphology unreliable for identification of *Pasteurella* species. Variations in the physical appearance of colonies belonging to the same species were observed in the present study. Although predominantly non-mucoid colonies represented *P. multocida*, colonies of *P. multocida* of different texture were present too. On this basis, physical appearance of colonies should only be regarded as a presumptive feature for the identification of *P. multocida*.

Identification of *Pasteurella* into species and subspecies is done according to phenotypic schemes of identification, and several identification tables have been published (Mutters et al., 1985b; Mutters et al., 1989). The most common biochemical reactions used for separation of *Pasteurella* include, decarboxylation of ornithine, production of indole, catalase reaction, breakdown of urea, and production of acid from maltose, mannitol, dulcitol, sorbitol, arabinose, and trehalose (Mutters et al., 1985b; Mutters et al., 1989). Table 1 summarizes the most common biochemical reaction patterns used to identify members of *Pasteurella sensu stricto*. Despite the availability of various identification tables, confusions still exist in the identification of *Pasteurella* species for a clinical bacteriologist. This is mainly due to significant variations in the physiologic properties of these organisms (Heddleston, 1976; Petersen et al., 1998; Appendix III). In the present study, extended phenotypic typing was found useful in identifying strains that could not be precisely identified with precision by a limited set of biochemical reactions.

The present investigations have outlined phenotypic variations among different taxa investigated (Appendix III), which included, fermentation of maltose and glycerol for strains of *P. multocida* ssp. *multocida* and *P. multocida* ssp. *septica*. Other reactions which varied among the strains of *Pasteurella* were D(+) xylose, L(+) arabinose, D(-) arabinose, L(-) fucose, trehalose, raffinose, PNPG (α-galactosidase), and PGUA (β-glucouronidase). The strain of *P. gallinarum* obtained differed from the Type strain

(ATCC 13361^T) of Hall et al. (1955) in ONPG reaction and D(-) arabinose fermentation (Appendix III). This biovar which has previously been isolated from chickens (Mraz et al., 1980; Droual et al., 1992b) represents the first isolate from ducks.

Genotypic methods for identification of *P. multocida*

Although DNA based methods seems to offer an attractive option for identification of bacteria, only two PCR tests that claim species specificity have been described for *P. multocida* (Kasten et al., 1997; Townsend et al., 1998b). Kasten et al. (1997) described a PCR that is based on the P6-like (PSL) protein. However this PSL is known to share similarities with the P6 protein of *Haemophilus influenzae* (Kasten et al., 1995). This PCR was shown to detect each reference strain of the 16 Heddleston somatic serovars, a field isolate, and live vaccine strain (Kasten et al., 1997). PSL-PCR gives a positive reaction with *H. Influenzae* (Kasten et al., 1997), but this false positive reaction seems not to be a practical concern as *H. Influenzae* has not been found in avian strains. However, more strains should be tested with PSL-PCR to ensure its specificity as it has not been checked for specificity with any other *Pasteurella* other than the type strain of *P. avium* which gave a negative reaction.

Townsend et al. (1998b) developed a PCR assay for species identification of *P. multocida* isolates. This PCR was able to give a positive signal with 13 field strains (representing capsule serotypes A, B, D and F), and the type strains of the three subspecies of *P. multocida*. Negative reaction to type strains of *P. anatis*, *P. langaa*, *P. canis* biovar 1, *P. dagmatis*, *P. stomatis*, and *Pasteurella* sp. B was observed, however, positive reaction was found when tested with the reference strain of *P. canis* biovar 2 (Townsend et al., 1998b). Like PSL-PCR, the PCR developed by Townsend et al. (1998b) has not been extensively evaluated with a collection of diverse field isolates of known avian *Pasteurella* species and un-named *Pasteurella*-like taxa. More investigations are needed with the field isolates of *P. canis* biotype 2, to determine if all strains will give a uniformly positive reaction. Overall, more evaluations are needed with both types of PCR as they seem to offer potential advantages to the diagnostic laboratories. With the wide range of avian *Pasteurella* species many routine diagnostic

laboratories might be willing to adopt PCR identification rather than the use of extended phenotypic characterization.

Table 1. Characters used for separation of species belonging to *Pasteurella sensu stricto* (Mutters et al., 1985b)

Taxon	Ornit hine	In- dole	Urea -se	Catal -ase	Acid produced within 24 to 48 hrs from					
					Malt ose	Man nitol	Dulc itol	Sorb itol	Glucose /gas	Treha lose
P. m. ssp. *multocida*	+	+	-	+	-	+	-	+	-	d
P. m. ssp. *septica*	+	+	-	+	-	+	-	-	-	+
P. m. ssp. *gallicida*	+	+	-	+	-	+	+	+	+	-
P. dagmatis	-	+	+	+	+	-	-	-	-	+
P. gallinarum	-	-	-	+	+	-	-	-	-	+
P. canis	+	d	-	+	-	-	-	-	-	d
P. anatis	-	-	-	+	-	+	-	-	-	+
Pasteurella sp. A	-	-	-	+	-	d	-	-	d	+
Pasteurella sp. B	+	+	-	+	+	-	+	-	-	+
P. langaa	-	-	-	-	-	-	-	-	-	-
P. avium	d	-		+	-	-	-	-	-	+
P. volantium	-	-		+	+	+	d	d	-	+

+ ≥ 90% of strains are positive; - ≥ 90% of the strains are negative; d, positive and negative strains have been observed

2.3 *P. gallinarum* infection in poultry

P. gallinarum was originally reported in association with upper respiratory disease in chickens (Hall et al., 1955; Mushin et al., 1977), however, septicaemic infection (Yadav et al., 1977) and isolation in mixed infections (Droual et al., 1992b) have also been reported. Mushin et al. (1977) found *P. gallinarum* infection in a flock of 5-months old chickens, which was clinically featured by inflamed and swollen wattles. Extension to the respiratory tract was shown in severely affected chickens. Ojo (1971) reported keratoconjunctivitis in adult chicken that was solely due to *P. gallinarum*. Systemic *P. gallinarum* infections have been found in one-month-old chickens (Yadav et al., 1977). The predominant lesions in these chickens included petechiae in the liver, heart, gizzard and ecchymoses on the intestinal and subcutaneous mucosa. These lesions were reproduced in experimentally infected chickens. Droual et al. (1992b) reported a mixed infection of *P. gallinarum* and *Mycoplasma synoviae* in-six weeks old chicken flock, which was featured by pericarditis and perihepatitis. *P. gallinarum* has also been isolated from chickens with salpingitis (Bisgaard and Dam, 1981).

3. Typing methods for *P. multocida* with reference to avian isolates

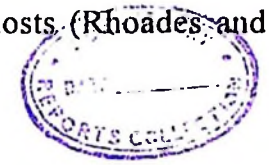
Classical typing methods include biotyping and serotyping, which are based on expression of phenotypic traits (Wachsmuth, 1986). These methods, which were of much use prior to the advent of DNA-based methods, are of limited value in epidemiological typing of *P. multocida* (Christensen et al., 1998). Genotypic typing methods are based on characterization of DNA of the organism by either analysis of the plasmid DNA or chromosomal DNA content. Analysis of chromosomal DNA has been used for typing *P. multocida* with considerable success, and at present it is the most reliable approach to epidemiological investigations of *P. multocida* (Snipes et al., 1990a; Christensen et al., 1998; Blackall et al., 2000). Various molecular typing schemes have been applied in different studies of *P. multocida* such as identifying sources of infection in poultry (Snipes et al., 1990a; Christensen et al., 1998; Appendix I and II) and correlating outbreak and vaccinal strains (Kim and Nagaraja, 1990). It is assumed that with ideal typing method it should be possible to state whether two or more isolates are related or not (Olsen et al., 1993). Typing methods and their significance in *P. multocida* will be discussed in the following subsections.

3.1 Phenotyping methods for *P. multocida* and related species

3.1.1 Serotyping

Capsular and somatic serotyping systems are the first documented methods developed by early workers for epidemiological typing of *P. multocida*. Serological typing methods mostly used are capsule typing and somatic serotyping. Indirect haemagglutination test distinguishes *P. multocida* into 5 capsular serotypes namely A, B, D, E and F (Carter, 1955; Brogden et al., 1978). Somatic antigen typing includes two methods, tube agglutination test (Namioka, 1978) and gel diffusion precipitin test (Heddleston et al., 1972; Brogden et al., 1978). Eleven serotypes are recognized by Namioka system (1-11) and 16 serotypes (1-16) by Heddleston system.

Capsular serotype A predominates among avian strains (Carter, 1967; Rhoades and Rimler, 1987b) and is recognized as the primary cause of fowl cholera (Rhoades and Rimler, 1989). Limited information is available on the virulence of capsular serotypes B, D, and F organisms which infect avian hosts. Capsular serotype B strains did not exhibit virulence for chickens (Chandrasekaran et al., 1985) while capsular serogroup D exhibit slight to moderate virulence (Carter, 1967; Rhoades and Rimler, 1990a). Capsular serotype F strains vary in pathogenicity for turkeys (Rhoades and Rimler, 1987b) and a strain of serogroup F exhibited slight virulence for chickens (Aye et al., 1999) and Pekin ducks (Pehlivanoglu et al., 1999). Capsular serotype E which have been isolated from haemorraghic septicemia cases in cattle and buffalo in Africa (Carter and De Alwis, 1989) have not been reported from avian hosts (Rhoades and Rimler, 1987b).



Most of the 16 somatic serotypes have been isolated from birds (Blackburn et al., 1975; Rhoades and Rimler, 1987b; Blackall et al., 1998). The capsular serotypes contain a variety of somatic serotypes, however, most of the somatic serotypes have been demonstrated within capsular serotype A strains of avian *P. multocida* (Blackburn et al., 1975; Rhoades and Rimler, 1987b). Somatic serotypes 1,3 and 4 within capsular serotype A are considered the most common causes of fowl cholera (Bhasin, 1982; Brogden and Rhoades, 1983; Hirsh et al., 1990). Relatively little information is available as to the virulence of somatic serotypes to allow a safe conclusion on their role in the aetiology of fowl cholera.

Lack of heterogeneity among the capsular types of *P. multocida* have limited the use of serotyping in epidemiological investigations of *P. multocida* (Snipes et al., 1990b). It was shown that capsular serotype A comprises 87.1% of isolates of turkeys dying from fowl cholera, 95% of isolates from live turkeys, and 83.8% of wildlife isolates. Rhoades and Rimler (1987b) found that 23% of avian strains in their study were non-capsulated and they could not assign these to any of the existing capsular groups. Thus, the low discrimination power and low typeability of strains does not qualify capsular serotyping as a typing method for *P. multocida*.

Somatic serotyping has also been used with limited success in typing of *P. multocida* isolates from avian hosts (Heddleston et al., 1972; Wilson et al., 1993). Heddleston et al. (1972) demonstrated correlation between different fowl cholera epidemics in domestic and free flying birds by somatic serotyping. However, subsequent investigations showed that genotypic differences exist among isolates of the same serotype (Kim and Nagaraja, 1990; Carpenter et al., 1991; Wilson et al., 1993). Somatic serotyping was also shown to compare unfavorably with multilocus enzyme electrophoresis and ribotyping in distinguishing avian *P. multocida* isolates (Blackall et al., 1998). Serotypes obtained were not correlated with ribotypes, and could not delineate relationships among the isolates obtained from poultry with fowl cholera. These findings provide evidence for inefficiency of serotyping in epidemiological studies of *P. multocida.*

In addition to poor discrimination power of serotyping, numerous bacterial factors and antiserum-donor host problems have been described for serological typing methods. These include inagglutinability of isolates, presence of common antigens other than specific capsule or somatic antigens and antigen variations with growth conditions of the organism (Rhoades and Rimler, 1989). Animals used for antiserum production may have naturally-acquired antibodies resulting from *P. multocida* epitopes shared with other bacterial species belonging to normal flora (Rhoades and Rimler, 1989). Thus, it seems reasonable to conclude that both capsular and somatic serotyping are of limited use as epidemiological markers of *P. multocida* in poultry.

3.1.2 Protein Profiling

Modern phenotypic typing methods that are based upon separation of proteins have been of limited use within the genus *Pasteurella,* mainly due to their low discrimination potential. Protein typing methods that have been applied in typing *P. multocida* include outer membrane protein profiling (OMP) (Knights et al., 1990) whole cell protein analysis (WCP) and multilocus enzyme electrophoresis (MLEE) (Blackall et al. 1998). In addition to epidemiological typing, MLEE appears to be useful for studying population structure and diversity of *P. multocida* (Blackall et al., 1999).

With exception of few publications where OMP has been used for typing *P. multocida* (Dabo et al., 1997, 1999b), much of the interest on OMP's of avian strains has been due to their immunogenic properties which make them potential vaccine candidates (Ikeda and Hirsh, 1988; Choi-Kim et al., 1991). In epidemiological typing of bovine and rabbit *P. multocida* isolates OMP typing could not compare favourably with other typing methods (Dabo et al. 1997, 1999a). A comparison of different methods of typing showed OMP profiling to have a lower discrimination index compared with WCP and genotyping methods for rabbit isolates of *P. multocida* (Dabo et al., 1999a). In another study, it was shown that REA and WCP could subtype strain with identical ribotype while OMP profiling could not (Dabo et al., 1999a). Although OMP has not been applied for typing avian isolates, the results of typing *P. multocida* isolates from other animals do not warrant the use of this technique for studying the epidemiology of fowl cholera.

Whole cell protein profiling (WCP) demonstrated a relatively high degree of homogeneity among bovine haemorrhagic septicemia isolates obtained from a wide geographic distribution and a range of animal host species (Johnson et al., 1991). Serotype B isolates of Asian and North American origin had a major protein band of approximately 32 kDa, whereas serotype E from Africa contained a major band of 37 kDa molecular mass. However, the technique could not separate the strains belonging to the same serotype. More recently, Dabo et al. (1997, 1999b) used WCP in combination with other techniques to characterize *P. multocida* isolates from bovine haemorrhagic septicemia and rabbits. Haemorrhagic septicaemia strains obtained from different geographical locations had different WCP profiles (Dabo et al., 1999a), however, the method was not found to have the similar precision for *P. multocida* strains from rabbits. It was conceived that rabbit isolates were obtained from different sources, as WCP results compared well with PCR and REA in same isolates. Potential benefits of WCP for epidemiological typing of *P. multocida* is still debatable, as the results of isolates from different animals have not been consistent.

MLEE of *P. multocida* strains proved to be a valuable tool for population diversity

studies (Blackall et al., 1998) and investigating the relationship among fowl cholera outbreaks (Blackall et al., 1999). The results of MLEE correlated with DNA-based techniques in showing that five out of 8 fowl cholera outbreaks were caused by distinct isolates of *P. multocida*, which were different from the other outbreaks, although in the other three outbreaks the results were different (Blackall et al., 1998). One outbreak, which was caused by a single clone according to REA and ribotyping, appeared to be caused by two distinct, but closely related strains by MLEE. The remaining two outbreaks were linked by both REA and ribotyping, but were shown to be caused by two distantly related strains by MLEE. The reasons for discrepancies between MLEE and DNA typing results could not be established. Although the authors suggested that MLEE might be of use as a tool for typing *P. multocida*, it should be considered that expression of phenotypic properties as measured by MLEE may not be as stable as DNA.

3.2 Genotyping methods for *P. multocida* and related species

Genotypic typing is based on characterization of DNA of the organism by analysis of the chromosomal DNA content or plasmid DNA. Methods which have been applied for chromosomal typing of *P. multocida* include restriction endonuclease analysis (REA), ribotyping, Pulse field gel electrophoresis (PFGE) and polymerase chain reaction (PCR)-based methods. Only chromosomal DNA analysis has been used with considerable success for typing for *P. multocida*.

3.2.1 Plasmid profiling

Strain differentiation based on plasmid profiles can only be used in strains that harbour several plasmids of different molecular weights. As plasmids are extrachromosomal DNA molecules presumably of the same origin of replication, members of the same clonal lines are expected to similar plasmids. Plasmid profiling has been reported to be of limited use as an epidemiological marker of avian strains of *P. multocida* due to a low number of plasmid carrying strains. Plasmid carriage in avian isolates from two studies ranged from 20% (Christensen et al., 1998) to 70% (Hirsh et al., 1985) of the

isolates investigated. However, Ikeda and Hirsh (1990) showed that possession of plasmids in *P. multocida* does not necessarily imply clonality. Isolates possessing similar non-conjugative R-plasmids were found to be different, while isolates possessing different non-conjugative R-plasmids were indistinguishable by three other molecular typing methods. Wide variations in the carriage of plasmids and lack of correlation between plasmids and virulence suggest that plasmid profiles are of limited use as an epidemiological marker of *P. multocida.*

3.2.2 Chromosomal DNA analysis

Characterization of chromosomal DNA provides significant information about the genetic make up of bacteria (Threllfall et al., 1990) and has the theoretical advantage that all strains are typeable. Restriction endonuclease analysis (REA) and ribotyping have been used widely in epidemiological studies of *P. multocida* during the last 10 years with significant success compared with traditional phenotyping methods (Kim and Nagaraja, 1990; Wilson et al., 1992, 1993; Blackall et al., 1995; Christensen et al., 1998).

3.2.2.1 Restriction endonuclease analysis (REA)

REA has proved to be a valuable component of fowl cholera epidemiologic studies. Initial investigations by Kim and Nagaraja (1990) and Snipes et al. (1990a) showed that REA of whole cell DNA from *P. multocida* could provide information sufficient to differentiate field isolates and vaccine isolates of the same serotype. Through REA it was confirmed that the M-9 vaccine was responsible for outbreaks of fowl cholera in turkey flocks (Christiansen et al., 1992). In a subsequent study, REA-typing revealed a complex web of sources of outbreaks. It was shown in one of three premises with repeated outbreaks of fowl cholera that a strain of identical *Sma*I REA type was responsible for disease. The same REA type was isolated from wildlife which suggested the presence of enzootic wildlife reservoir in the premises. In another premise seven out of 8 outbreaks that occurred were caused by different *Sma*I REA types, in the third premises a non-wild life isolate was isolated. These initial findings

illustrated the potential of REA typing in epidemiological marking of fowl cholera outbreaks. Recently, Christensen et al. (1998) demonstrated transmission of *P. multocida* between wild birds and backyard chickens by REA and ribotyping. Two outbreaks of fowl cholera in wild birds were caused by the same clone of *P. multocida* ssp. *multocida* as evidenced by *Hpa*II REA analysis of isolates. The same clone was subsequently isolated from backyard poultry kept close to outbreak areas. During this project, *Hpa*II REA cuts were used to determine the relatedness of *Pasteurella* species obtained from the same and different hosts. Isolates obtained from the same animals by different methods of isolation were invariably identical (Appendix III). REA results also indicated that a single clone infected each flock and the duck isolates from a single village in Tanzania were clonal and different from those obtained from chickens.

For routine typing purposes, visual comparison of the band patterns is sufficient to characterize REA profiles (Appendix III). However, scanning computer analysis of fingerprint bands may be needed when numerous indistinguishable bands are produced. Despite the problems that may arise from REA results, the technique is highly reproducible and sensitive for discriminating recent changes in the genome of related bacteria (Christensen et al., 1998). Moreover, the relatively low cost and simplicity of the method allow it to be used in routine microbiological laboratories.

3.2.2.2 Ribotyping

Due to a high number of bands produced by REA, DNA fragments are often transferred to a membrane, and hybridized with a well defined 16S rRNA or 23S rRNA *E. coli* derived probe, a technique referred to as ribotyping (Grimont and Grimont, 1986). The ribosomal sequences are highly conserved and a probe from the *Escherichia coli* ribosomal operon can be used over a wide range of species. Ribotyping has been useful for epidemiological typing of *P. multocida* (Snipes et al., 1990a; Blackall et al., 1995; Christensen et al., 1998; Appendix I and III)

Ribotyping of avian isolates from fowl cholera outbreaks in The United States (Carpenter et al., 1991) and Australia (Blackall et al., 1995) demonstrated a

considerable heterogeneity that was enough to discount relatedness of outbreak isolates previously found identical by serotyping and biotyping. In the present investigations, some of the isolates from different animals which were initially demonstrated to be homogenous on biotyping were separated by ribotyping (Appendix II). However, the results also showed ribotyping to be less sensitive than REA in subtyping *P. multocida* strains (Appendix I and III). One chicken strain and duck strains had identical ribotype, just as some epidemiologically unrelated strains from cats had similar ribotype patterns (Appendix III). Some strains of *P. multocida* ssp. *septica* were furthermore shown to have identical ribotypes with *P. multocida* ssp. *multocida*. These findings show that strains with the same ribotype are not necessarily epidemiologically related. However, the limitations of ribotyping have not overcome its advantages of reproducibility, typeability, and delineation of the highly conserved DNA structures. Dissimilarity of strains by ribotype profiles is sufficient evidence to discount relatedness between them (Hunt et al., 2000), whereas strains that appear similar should be further compared by REA, which is more sensitive.

3.2.2.3 Pulse field gel electrophoresis (PFGE)

Although PFGE is considered as 'gold standard' fingerprinting method of molecular epidemiology (Goering et al., 1993), it has not been widely applied for typing *P. multocida*. PFGE has been used to characterize isolates from human beings (Donnio et al., 1994) pigs (Donnio et al., 1999), bovine haemorrhagic septicemia (Townsend et al., 1997) and recently fowl cholera outbreak strains (Gunawardana et al., 2000). Donnio et al. (1994) demonstrated dissimilarity between oropharyngeal *P. multocida* isolates from pig breeders and isolates from their pigs which suffered from pasteurellosis. Subsequent investigations showed that dermonecrotic producing strains of *P. multocida* from human and swine were homogeneous (Donnio et al., 1999). It was suggested that there was exchange of strains between pigs and human beings. PFGE analysis of bovine haemorrhagic septicemia causing strains separated North American and Asian isolates, with the later strains showing a high degree of homogeneity (Townsend et al., 1997). Application of the same methods to avian isolates showed PFGE to be more

discriminatory than repetitive extragenic palindromic PCR (REP-PCR) in distinguishing avian isolates of *P. multocida* (Gunawardana et al., 2000). PFGE appears to be a useful tool for epidemiological studies of fowl cholera, but more studies are needed to validate the use of this method.

3.2.2.4 Polymerase chain reaction (PCR)

PCR-based methods have rarely been used for epidemiological typing of *P. multocida* strains (Hopkins et al., 1998; Gunawardana et al., 2000). By using arbitrary primed PCR (AP-PCR), Hopkins et al. (1998) distinguished the CU vaccine- and fowl cholera outbreak strains. Sensitivity of this AP-PCR was improved by application of radiolabelled primers, however, this has probably limited widespread use of this technique. In another experiment repetitive extragenic palindromic PCR (REP-PCR) permitted recognition of *P. multocida* isolates from fowl cholera outbreak into epidemiologically related and unrelated strains (Gunawardana et al., 2000). Different strains at genomic level were revealed by amplification of repetitive sequences, which showed heterogeneity of the number and distribution of ensuing strain fragments. Although overall REP-PCR did not compare favourably with PFGE in typing of these strains, it was shown in some instances, that REP-PCR was more discriminatory than PFGE. The reasons for discrepancies have not been explained.

The present findings confirmed that only limited information on epidemiology of *P. multocida* strains can be obtained by phenotypic typing (Appendix I and II). Correspondence between phenotypic clusters and ribotypic cluster was demonstrated by quantitative numerical analysis; however, this information has more taxonomic than epidemiological significance. As ribosomal RNA genes are highly conserved, the presence of different bands is sufficient evidence to conclude that the strains are not identical. On the other hand, strains with identical ribotypes should be typed by other genomic methods before concluding that the strains are related. Promising results have been shown by other genomic methods, however, many studies have employed a limited number of strains. More strains need to be characterized by these methods to validate their use for avian strains.

4. Epidemiology of *P. multocida* and fowl cholera disease

4.1 Epidemiology of fowl cholera

4.1.1 Prevalence of *P. multocida* in poultry

Since its first demonstration, *P. multocida* has been associated with fowl cholera in poultry and wild birds as well as septicemia in other domestic animals. In domestic and wild birds *P. multocida* is almost exclusively associated with occurrence of clinical disease in the flocks (Mraz et al., 1980; Curtis et al., 1981). Findings by Mraz et al. (1980) and Curtis et al. (1981) showed carriers of *P. multocida* in the convalescent flocks of chickens and turkeys, respectively. Subsequently, Snipes et al (1988) found a significantly higher prevalence of *P. multocida* in outbreak flocks (11%) compared with 0% in control flocks. It was concluded that healthy poultry are not normal carriers of *P. multocida*. However, in the present study carriers were found in flocks with a history of fowl cholera as well as in flocks without a history of fowl cholera (Appendix I and II). The prevalence of carriers in two commercial flocks with a history of fowl cholera was 73% and 80%, respectively. In the non-outbreak commercial flocks the prevalence of carriers ranged from 3% to 63%. Prevalence of *P. multocida* in commercial flocks in Denmark was higher than that of free-range chickens and ducks in Tanzania (Appendix II).

Close contact between birds has been shown to favour the transmission of *P. multocida* between birds in a flock (Pabs-Garnon and Soltys, 1971b). This may partly explain the difference in prevalence of *P. multocida* between commercial and free ranging poultry. Free-range birds are more likely to be exposed to disease organisms; however, transmission among birds might be lower than in the intensive flocks. *P. multocida* was shown to occur in one of the three climatic zones for reasons yet to be established. Prevalence of *P. multocida* in free ranging ducks was higher compared with chickens, but it was not established if fowl cholera has occurred in that area before or during the study. Being more mobile, free ranging chickens are more likely to be exposed to *P. multocida* from other sources compared with the ducks. Ducks on the other hand tend

to be more aquatic than chickens, which makes it more likely that they may pick up the infection through water (Hazlewood et al., 1978). Experimental investigations simulating village free-range conditions are required to demonstrate the influence of these factors in the epidemiology of *P. multocida* in free- ranging chickens and ducks.

In conclusion, the present investigations have indicated the presence of carriers of *P. multocida* in both disease and non-diseased flocks of poultry. Presence of clonal infections in a flock indicates either introduction of bacteria from a common source and/or transmission of the same strain between the birds. Investigation of commercial flocks in successive outbreaks of fowl cholera showed appearance of a different clone in each outbreak. Epidemiologically this represents a high spreading ability of *P. multocida*, and that infections of *P. multocida* in the flocks are usually clonal and the infection can be eradicated from a farm. More investigations on the carriers and disease flocks are required to understand more about the diversity of *P. multocida* clones and their relationship with fowl cholera disease.

4.1.2 Hosts

All species of domestic birds seem to be susceptible to fowl cholera although turkeys are regarded as more susceptible than fowl; adult birds and late growers are more susceptible than the young stock (Heddleston, 1962; Hungerford, 1968). Ducks and other web-footed birds are also highly susceptible (Rhoades and Rimler, 1989). Fowl cholera has been reported in more than 100 species of wild birds (Botzler, 1991). *P. multocida* ssp. *multocida* has predominantly been isolated from fowl cholera outbreaks in turkeys, chickens, and ducks (Blackall et al., 1995; Christensen et al., 1998; Townsend et al., 2000) and from a variety of wild birds (Hirsh et al., 1990). *P. multocida* ssp. *septica* has been obtained from poultry as well as wild birds with fowl cholera, whereas *P. multocida* ssp. *gallicida* seems to be more prevalent in web-footed birds (Hirsh et al., 1990; Bisgaard, 1993; Appendix I).

4.1.3 Transmission

Infection originates from excretions of carrier birds and clinically diseased poultry

(Rimler and Glisson, 1997). A source of disease is usually healthy carriers that harbour the organism in bacteria in their nasal clefts (Pritchett and Hughes, 1948). Cloacal carriers shown among carrier ducks and chickens (Appendix I) could presumably spread the infection faster through contamination of feed and water. Infection passes from one bird to another by oral, nasal and conjunctival routes and through wounds (Rhoades and Rimler, 1989). Aerosol transmission and sharing of water in the flocks has been shown to help in spreading the disease within the flock (Pabs-Garnon and Soltys, 1971b).

Feed bags and equipment may also serve as a source of infection to the flock just as spread can be through contaminated carcasses (Rimler and Glisson, 1997). Alls (1970) showed that bird contact challenge of *P. multocida* using seeder birds could result into more than 90% transmission rate in susceptible turkey poults. Other animals may also transmit *P. multocida* pathogenic to poultry (Iliev et al., 1964; Christensen et al., 1998). Iliev et al. (1964) experimentally produced fowl cholera in chickens by using isolates obtained from pigs. Epidemiological investigations have recently confirmed that wild birds can transmit virulent strain of *P. multocida* to domestic birds (Christensen et al., 1998). *P. multocida* has been isolated from infected human beings (Hansmann and Tully, 1945; Peel, 1993; Talan et al., 1999), however, the role of diseased or carrier human beings in the transmission of fowl cholera has not been demonstrated.

An exchange of *P. multocida* ssp. *multocida* between wild birds and domestic poultry is reported to be possible, and wild birds are capable of spreading the disease to new areas (Snipes et al., 1990b; Christensen et al., 1998; Christensen et al., 1999). The extent to which the agent is introduced into susceptible flocks is difficult to estimate and will probably be influenced by the form of production. Christensen et al. (1999) demonstrated that more than 80% of the diagnosed cases of *P. multocida* infections in poultry in Denmark during the years 1995 to 1997 included poultry which had been in contact with wild fauna. This included contact with mammals, but the role of these as reservoirs was not confirmed. However, isolates from wild birds have been found to be identical or closely related to those isolated from domestic poultry, these clones have

also been demonstrated to be virulent for poultry (Chirstensen et al., 1998; Christensen et al., 1999; Petersen et al., 2000). Findings by Townsend et.al. (2000) indicated the likely transmission of *P. multocida* ssp. *gallicida* strains between ducks and pigs. Isolates from porcine acute pasteurellosis (Townsend et al., 1998a) and from tonsils of healthy pigs were shown to be identical to fowl cholera outbreak strains from ducks in Vietnam. These strains had phenotypic and genotypic similarities with porcine haemorrhagic septicemia strains (Cameron et al., 1996) obtained from Australia. Migratory birds that are found in both Australia and Vietnam were implicated in the transmission of disease between two countries. The risk of transmission of *Pasteurella* species between industrial cattle and pigs kept with poultry has not been explained, however, these findings indicate the risk of exchange of strains between these animals and domestic poultry.

4.1.4 Free-range poultry management and *P. multocida* epidemiology

Free ranging poultry management is the major form of poultry raising in the developing countries (Sonaiya, 1989). Coupled with poor management practices, lack of housing and feed supplements, these birds are also left freely to fetch for feed (Mwalusanya, 1998). In most families in the villages, chickens and ducks are kept alongside other animals such as dogs, cats, goats and pigs (Mwalusanya, 1998). Since all animals are left free in the area, contact between chickens and ducks from different households, and with other animals cannot be controlled. Occasionally the owners provide feed to the birds, which increase contact with other animals. Similarly, birds are likely to have access to food leftovers from dogs and cats. Transmission of diseases between chickens within and between villages has been reported (Yongolo, 1996). Considering the wide host spectrum of *P. multocida* the exchange of these organisms between different hosts seems likely under these conditions. However, apparently little information is available on epidemiology of fowl cholera in village chickens.

Snipes et al. (1988) showed that wild mammals and birds captured in turkey's premises could be involved in the *P. multocida* infection cycle in turkey flocks. In the village environment where cats and dogs have free access to dead poultry, and in other

instances predate poultry (Mwalusanya, 1998), transmission of *Pasteurella* strains between different flocks by that means is likely to occur. However, the present findings could not confirm that *P. multocida* was widely exchanged between poultry and other animals that are kept in contact in the villages (Appendix III). This could be due to adaptation of some clones of *P. multocida* to chickens, while others have adapted to ducks, or pigs, dogs, and cats. Since infections in birds have been shown to be clonal (Appendix I) bird-to-bird transmission is likely to have a more significant impact in fowl cholera epidemiology than the exchange of strains between different animal species. Control of fowl cholera under improved management should mainly embark on management of chickens and ducks.

5. *P. multocida* pathogenicity and determinants of virulence in birds

5.1 Virulence of *P. multocida* and pathogenesis of fowl cholera

The outcome of *P. multocida* infection depends on a number of bacterial and host factors which may considerably vary the incidence of fowl cholera in a flock. These factors include species of a bird; turkeys and pheasants being more susceptible than chickens (Matsumoto et al., 1991; Rimler and Glisson, 1997; Petersen et al., 2000) - the age of the host (Hungerford, 1968), the genetic line of the host (Sacco et al., 1991; Nestor et al., 1999), the bacterial strain (Rimler and Glisson, 1997; Matsumoto and Strain, 1993; Appendix VI), environmental factors such as crowding (Van Es and Olney, 1940; Hamilton et al., 1996) and concurrent disease (Collins, 1977; Rimler and Rhoades, 1986). Nutritional stress has also been suggested as a possible predisposing factor (Eleveth et al., 1949). In experimentally infected birds, the route of infection and challenge dose have been shown to correlate with the outcome of infection (Pehlivanoglu et al., 1999; Aye et al. 1999; Wilkie et al., 2000).

Correlation between pathogenicity of *P. multocida* and documented virulence factors responsible for fowl cholera development seems to be erratic. Much evidence has accumulated over the years to support the role of capsule as a major virulence factor of *P. multocida*. However, it has also been shown that encapsulated forms from infected birds may be of low virulence (Brogden et al., 1978; Matsumoto and Strain, 1993) just as acapsular forms may be of relatively high virulence (Matsumoto and Strain, 1993). Resistance to serum complement activity has also been regarded as one of indicators of virulence (Snipes and Hirsh, 1986; Hansen and Hirsh, 1989; Lee et al, 1988a and b), but virulent serum sensitive, and avirulent serum resistant strains have also been reported (Hansen and Hirsh, 1989; Wilkie et al., 2000).

The pathogenesis of *P. multocida* infections is poorly understood. However, it is known that the severity of the disease may vary considerably depending on host and bacterial

factors mentioned above. The port of entrance of *P. multocida* in birds is apparently the mucous membranes of the pharynx, nasal passages, conjunctiva, and cutaneous route through the wounds (Rimler and Glisson, 1997). Ascending infections through the cloacal mucosa may also take place (Bisgaard, 1995a). Virulent *P. multocida* initially colonizes the upper respiratory tract (Rhoades and Rimler, 1990b; Rimler and Glisson, 1997), which is followed by invasion and septicaemia. Following intratracheally infection with four different strains, the most virulent strain persisted in the trachea of chickens for longer than the less virulent strains (Appendix IV). The possible explanation could be the presence of adhesion factors to the trachea in the virulent strain (Glorioso et al., 1982). Spread to the lungs and multiplication was shown to precede septicaemia after intratracheal inoculation in turkeys (Matsumoto et al., 1991). Pabs-Garnon and Soltys (1971a) showed that spleen and liver were more favourable sites for bacterial multiplication than blood. In their experiment the mean number of bacteria/gram in the blood rose suddenly to a maximum 28 hours post inoculation, at the same time there was only a gradual increase in the liver and spleen.

Snipes et al. (1987) demonstrated intravascular multiplication to be more important for virulent strains, which was contrary to other workers, who found that the organisms multiply first in the liver and spleen before being released into the blood stream at a terminal phase of infection (Pabs-Garnon and Soltys, 1971b; Tsuji and Matsumoto, 1989). *P. multocida* is assumed to cause death by endotoxic shock (Rhoades, 1964; Heddleston and Rebers, 1964; Collins, 1977), which is concurrent with production of clinical fowl cholera by endotoxin injection (Heddleston and Rebers, 1975; Rhoades and Rimler, 1987a).

5.2 Virulence of *P. multocida* in experimentally infected birds

Challenge infection of susceptible birds is currently the only method of investigating pathogenicity of *P. multocida*. Considerable variation exists among different workers as to the route of infection, dose of challenge, and age of birds used in the experiments. Inoculation of *P. multocida* has been performed by different routes including oral (Alls, 1970), intratracheal (Appendix IV; Petersen et al., 2000), intravenous (Rhoades and

Rimler 1988), intramuscular (Rhoades and Rimler, 1990c) and subcutaneous route (Aye et al., 1999). Highest mortalities in birds have been reported following intravenous administration, and strains avirulent by other methods of infection have been shown to be virulent when given intravenously (Rhoades and Rimler, 1988; Pehlivanoglu et al., 1999; Wilkie et al., 2000). With the exception of a highly virulent strain that caused 100% mortality when inoculated by either of intravenous, intramuscular or intratracheal inoculation (Wilkie et al., 2000), routes of infection seem to have a significant impact on the outcome of experimental infection. It is recommended that virulence of strains determined in various ways should be compared with caution.

A wide range of challenge doses have been used in experimental infection of *P. multocida* and the outcome of infection has not always been consistent with the challenge dose applied. Rhoades and Rimler (1988), showed that a challenge of 10^2 c.f.u intramuscular of a low virulent strain do not cause mortality in turkeys. However, increasing the dose up to 10^7c.f.u, caused mortality to all birds injected. In the same study a highly virulent strain was shown to cause mortality in all doses ranging from 10^1 to 10^6 c.f.u. Considerable variations in the mortality were demonstrated in turkeys injected intravenously with various isolates of *P. multocida* (Snipes et al., 1988; Carpenter et al., 1989). Although some strains were declared avirulent due to failure to cause mortality at high doses, the findings could not precisely explain the differences between low and highly virulent strains. In the present investigations three field strains and a reference strain were used for challenge experiment in chickens (Appendix IV). The chickens were given 10^4 c.f.u bacteria intratracheally and differences in the pathological lesions in chickens infected with different strains were observed 24 hrs and two weeks after infection. The reference virulent strain which was previously shown to cause high mortality in turkeys and pheasants (Petersen et al., 2000) produced chronic fowl cholera in chickens. The natural route of infection of *P. multocida* is presently unknown but it is believed that *P. multocida* enters through respiratory route (Simmensen and Olson, 1980; Gustafson et al., 1998). Consequently intratracheal route was used in the present research (Appendix IV). To avoid endotoxin like reactions

which may mask differences in the strains virulence a relatively low dose 10^4/0.5ml was inoculated in the chickens investigated (appendix IV).

5.3 Determinants of virulence of *P. multocida*

Although possibly all *P. multocida* strains may be regarded as potential pathogens, and under certain circumstances capable of producing disease in birds, some strains appear to be more pathogenic than others (Rhoades and Rimler, 1989; Wilkie et al., 2000). Several potential virulence factors responsible for fowl cholera have been suggested for *P. multocida.* These factors and their significance in fowl cholera are discussed in the following subsections.

5.3.1 The Capsule

P. multocida ssp. *multocida* capsular serotype A is considered to be the main cause of fowl cholera (Rhoades and Rimler, 1987b; Rhoades and Rimler 1989), however, organisms belonging to serotypes B, D, and F have also been isolated from diseased birds although at a lower incidence than serotype A (Rhoades and Rimler, 1987b; Rhoades et al., 1992). The virulence properties of serotype A strains are well documented compared with the other capsular types (Bhasin, 1982; Rhoades and Rimler, 1987b). Different isolates of *P. multocida* capsular serotype A also vary greatly in virulence (Lee et al., 1988a; Snipes et al., 1988; Carpenter et al., 1989) showing that the capsule alone is not adequate for assessing *P. multocida* virulence. Virulence of serotype B has been shown by intramuscular injection in turkeys, however, only low level of infection was produced by instillation on the nasal cleft mucosa (Rhoades and Rimler, 1988). Investigation of the disease potential of five serotype D strains only showed low or moderate level of infection in turkeys by intramuscular infection, but one of the moderately virulent strains was highly virulent, when inoculated into the airsacs (Rhoades and Rimler, 1990a). Variation in the virulence has also been reported among serotype F strains (Rimler and Rhoades, 1987; Pehlivanoglu et al., 1999; Aye et al., 1999).

Lee et al. (1988a) showed that four out of six *P. multocida* strains contained plasmids, which were presumably thought to mediate resistance to turkey complement. Three isolates contained plasmids of 5.2, 3.0 and 2.2 megadaltons, however, their degree of serum resistance was shown to vary. The fourth strain had a 32 megadaltons plasmid in addition to other plasmids, but it could not be related to the level of serum resistance. Further investigations showed only a moderate increase in resistance when a serum sensitive strain of *E. coli* (K12) received a plasmid from complement resistant *P. multocida* strains (Lee and Wooley, 1995). However, the induced serum resistance was not stable, and it could be removed by curing. In the same study, transformation of CU strain with another *P. multocida* plasmid conferred only a moderate increase in the complement resistance and virulence in turkeys. In both strains, plasmid acquisition did not result in detectable change in the major outer-membrane proteins, capsule or carbohydrate fermentation patterns. Furthermore, it has been shown that serum resistant, virulent strains such as strain P-40506 (Christensen et al. 1998), do not harbour plasmids. Considering this evidence the plasmids alone cannot be the determinant of serum resistance in *P. multocida*.

Surface components of Gram-negative bacteria have been related to serum resistance in many bacteria (Munn et al., 1982; Davies, 1991). Major outer-membrane proteins alone or in combination with LPS have been shown to determine complement resistance in *Aeromonas salmonicida* (Munn et al., 1982) and *E. coli* (Taylor and Robinson, 1980). In the present work, the correlation between outer-membrane proteins and serum resistance was investigated (Appendix IV). These findings did not outline specific OMP in *P. multocida* that were specific for serum resistant strains *in-vitro* neither were the expression of novel proteins by serum resistant strains presumably conferring OMPs were demonstrated *in-vivo* (Appendix IV). Investigations in *N. gonorrhoea* have confirmed that OMP porin A is the component responsible for resistance to human complement (Ram et al., 1999). All strains of *P. multocida* investigated in the present study (Appendix IV) were shown to contain outer-membrane proteins of approximately 37 kDa, presumably the *P. multocida* porins (Lugternberg et al., 1984; Chevalier et al., 1993). Further investigations on the structure of *P. multocida* porins are indicated to

clarify its role in serum resistance and virulence.

5.3.4 Toxins

Rhoades (1964) showed that macroscopic lesions found on chickens dying from fowl cholera could be due to endotoxin. Subsequent attempts to determine the structure and effects of endotoxin showed that a complement sensitive vaccine strain and a complement resistant field strain had identical LPS and released similar amounts of endotoxin when complement proteins were present (Lee et al., 1992). However, the release only caused loss of viability of the vaccine strain. These findings suggested that the virulent field strain had septicaemic advantage by being able to grow in the presence of complement proteins, and at the same time release endotoxin, both these elements thereby contributing to the overall pathogenicity of the strain. Inoculations of the strains by an oculo-naso-oral route showed that endotoxaemia could persist longer in birds challenged with the field strain.

Cell-free culture filtrate (CCF) inoculation into the airsacs of turkeys caused diffuse thickening of the air sac wall and accentuation of blood vessels which were similar to those induced by cultures of *P. multocida* (Ficken and Barnes, 1989; Ficken et al., 1991). Microscopically, severe swelling of air sac epithelial and mesothelial cells and thickening of the airsac by proteinaceous fluid and heterophils was observed although the exact toxin in CCF was not identified. However, it seems likely that endotoxin could have been involved. Scarcity of other toxins in *P. multocida* and the type of pathological lesions are suggestive of endotoxins. Generally, there seems to be little doubt that endotoxin may act as a contributory factor to virulence. However, factors associated with invasion and multiplication are clearly necessary before endotoxin is produced in sufficient concentrations to exert its action.

P. multocida toxin (PMT) has been described to be involved in pathogenesis of atrophic rhinitis in pigs and several biological effects in other animals including turkeys (Christensen and Bisgaard, 1997). Nielsen *et al.* (1986) investigated the existence of PMT in *P. multocida* ssp. *multocida*, *P. multocida* ssp. *septica*, *P. multocida* ssp.

gallicida, *P. canis* and *P. avium* using embryonic bovine lung cells. Cytopathic toxin was demonstrated from *P. multocida* ssp. *multocida* strains only. More investigations are, however, needed to reach a definite conclusion.

5.3.5 Molecular determinants of virulence

The genetic basis of fowl cholera and other infections caused by *P. multocida* in avian species is little known, and most of the information available does not deal with the genes responsible for differences between virulent and avirulent isolates. Knowledge about the *P. multocida* toxin (PMT) encoding gene, *tox A* is available (Foged, 1992). However, currently there is no evidence of this gene in fowl cholera causing strains although PMT has been demonstrated (Nielsen et al., 1986). Expression of the gene product of *tox A* was shown to be regulated by promoter and repressor protein encoded by a gene located close to *tox A* (Foged, 1992). Whether mutations in these genes will alter the virulence properties of the strains remains to be shown. Other genes of the *P. multocida* genome have been characterized, including the adenylate cyclase gene, 16S rRNA gene sequences and the *oma87* gene, but at present there is no conclusive evidence that they act as virulence genes (Mock et al., 1991; Ruffolo and Alder, 1996).

It has been shown that avian strains of *P. multocida* may harbour plasmids (Snipes et al., 1990b; Price et al., 1993; Diallo et al., 1995). Although plasmids have been shown to encode antibiotic resistance (Hirsh et al., 1985, 1989), there is no evidence to suggest that the plasmids contribute to virulence. Lee et al. (1988a) found plasmids in serum resistant strains, whereas none of the avirulent strains harbored any plasmid. Another study reported that avian isolates without plasmids could be fully virulent for turkeys, whereas strains carrying plasmids were found to be avirulent (Price et al., 1993). Thus, there is no conclusive evidence that virulence of *P. multocida* for chicken may depend on the possession of plasmids.

6. Prevention and control of fowl cholera

Chemotherapy is widely used in the treatment of fowl cholera, and a variety of chemotherapeutic agents such as sulphonamides (Dorsey and Harshfield, 1959; Alberts, 1948) and antibiotics (Dorsey and Harshfield, 1959; Bierer, 1962) are often effective. However, success in the treatment of fowl cholera is dependent on the form of disease, promptness of treatment and the drugs used (Rhoades and Rimler, 1989; Rimler and Glisson, 1997). Treatment of a peracute case is hardly of value; in less acute disease, treatment is questionable because the drugs are only effective as long as treatment lasts (Rhoades and Rimler, 1989).

Control of fowl cholera throughout the world depends principally on vaccination. Under extensive management systems, like in free-ranging management system (Appendix II) vaccination might be the only option as necessary procedures for maintaining a flock free of exposure might not be feasible. Animal welfare concerns have also increased the use of non-confined production farms in the industrialized world, resulting into significant risk of introducing infections to commercial flocks (Christensen et al., 1999). In efforts to control fowl cholera vaccines both viable (live) and inactivated *P. multocida* preparations have been employed.

Many live and inactivated fowl cholera vaccines have been developed and tested in attempts to control the disease (Rimler and Glisson, 1997). The vaccines normally contain *P. multocida* of serotypes A:1, A:3 and A:4 which has been grown in vitro, emulsified in oil adjuvant or aluminium hydroxide (Homchampa, 1995). Most commercial vaccines are bacterins, as live vaccines are capable of reverting to virulent form and cause disease in immunocompromised birds (Snipes et al., 1990a). Despite the perceived dangers, the live vaccines have the advantage of easy use such as administration of in drinking water, which is convenient for large commercial flocks (Zander et al., 1997). Live vaccines can provide cross immunity, but repeated vaccination might be required to provide long term immunity. Killed vaccines

(bacterins) are expensive to produce and do not fully protect the flocks. However, it limits the incidence and severity of clinical disease (Scott et al., 1999) and often provides long term immunity. In addition the bacterins have to be injected and often result into tissue reactions (Davis, 1987), furthermore they only induce immunity to homologous serotypes (Rebers and Heddleston, 1977). The main live vaccines currently used, primarily in North America are Clemson University strain and its mutant the M-9, both of which are serotype A: 3,4 (Rimler and Glisson, 1997). Attempts have been made to construct temperature sensitive mutants which are not capable of growing at 42°C (Hertmann et al., 1980; Hofacre et al., 1989a), however, their efficacy is still questionable (Hofacre et al., 1989b). Recently, construction of non-reverting auxotrophic mutants of *P. multocida* by mutating *aro*-A gene has been reported (Homchampa et al., 1997). Promising results have been obtained in preliminary vaccination trials with some of these strains (Frost et al., 1999).

7. Conclusions and future perspectives

It is clear from the present findings that *P. multocida* can be obtained from apparently healthy flocks, and in convalescent or disease flocks. This conclusion is reached from isolation of *P. multocida* from commercial flocks with and without history of fowl cholera and in free ranging village chickens and ducks. Contact transmission seems to be important in the transmission of *P. multocida* between birds, as evidenced by the higher prevalence of *P. multocida* in commercial flocks than in free ranging poultry. Strains obtained in four different incidences in one commercial farm showed a different clone in each incidence, indicating that *P. multocida* could be eliminated from a farm. Prevalence of *P. multocida* was higher in ducks than in chickens in the village free ranging management. This could be explained by the aquatic behaviour of ducks, which bring together ducks from different households, facilitating the exchange of strains. Duck strains were shown to be clonal, which indicate that the strains spread among ducks across different households in the village.

Additionally, it has been shown that the prevalence of healthy *P. multocida* carriers in fowl cholera flocks was higher than in flocks without history of fowl cholera and, that some birds remain carriers after disease outbreaks. Among the three subspecies of *P. multocida*, the subspecies *gallicida* was exclusively obtained from web-footed birds, however, *P. multocida* ssp. *multocida* and *P. multocida* ssp. *septica* were also obtained from web-footed birds. Although *P. multocida* was shown to be more prevalent in ducks in the warm zone, similar findings were not observed in other animals investigated. Consequently, climate could not be concluded to be the factor guiding the prevalence of *P. multocida* in the villages.

Isolation of *P. multocida* from poultry seem to be best achieved by mouse inoculation, however, other *Pasteurella* species which were predominantly obtained from dogs and cats were isolated mainly through direct blood agar inoculation. Caution is drawn to the fact that isolation technique should focus on the taxa required, and whenever possible more than one isolation method should be used in isolating *P. multocida* from live

animals. The present investigations also indicated that cloacal mucosa is another habitat of *P. multocida* in live birds. Sampling of cloacal mucosa should always be considered when investigating for carriers of *P. multocida*.

Epidemiological typing of the strains obtained from free ranging chickens and their animal contacts could not provide a definite proof that strains of *P. multocida* are widely exchanged among different animals kept together in the villages. Although the reasons are presently unknown, host adaptation might be important in colonization of *P. multocida* in a host. One strain from chickens in the villages had identical ribotype with strains obtained from ducks, suggesting the presence of bird adapted strains. Cat adapted strain was shown in four cats in one village, this strain was identical by ribotyping and REA-typing.

Caution is drawn to the use of ribotyping as a sole method for studying epidemiology of *P. multocida* strains. Genotypic relatedness by ribotyping was demonstrated among epidemiologically related strains as well as among unrelated strains. REA-typing in conjunction with ribotyping was found to be useful in typing *P. multocida* obtained from different sources. Existence of genetic basis for phenotypes was demonstrated by correspondence between phenotype and ribotype clusters. The present findings also demonstrated close relatedness between trehalose positive *P. multocida* ssp. *multocida* strains and *P. multocida* spp. *septica* as outlined by ribotyping with *Hpa* II. The trehalose positive *P. multocida* ssp. *multocida* strains invariably clustered together with *P. multocida* ssp. *septica*, separate from trehalose negative *P. multocida* ssp. *multocida* strains. These findings draw attention to the relevance of subspeciating *P. multocida* into subspecies, *multocida* and *septica*.

Resistance to complement activity of sera from chickens, turkeys, ducks and pigs was shown among avian and feline *P. multocida* strains as well as canine *P. dagmatis* isolates. Strains that were clonal both phenotypically and genotypically were also shown to have similar serum activity, suggesting genetic basis for serum resistance. This indicates that strain's resistance to avian sera is inherent within strains irrespective

whether the strains are adapted to avian hosts or not. Concurrent with the higher susceptibility of turkeys to fowl cholera, it was shown that turkey serum is less inhibitory to a wide range of *P. multocida* strains than chicken, duck and pig sera. Serum resistance was not shown to correlate with outer-membrane protein expression of the investigated strains. No new proteins were expressed when the strains were grown in the serum *in-vitro* or *in-vivo*, which could be related to serum activity of the strains. The correlation of serum resistance and virulence in chickens was found to be inconsistent. The most virulent of the tested strains was serum resistant, however, lesions were also seen in chickens infected with serum sensitive strains.

Future perspectives

Despite the considerable progress in the development of accurate methods of isolating, identifying and investigation of virulence of *P. multocida*, it is apparent that significant improvements are still required. Isolation and identification are essentially important in the clinical microbiology laboratories where there is a clear and urgent need for cost-effective yet comprehensive identification strategies. It should be noted that knowledge of virulence factors and pathogenesis of *P. multocida* are essential for development of reliable disease control measures. Advances in the taxonomy necessitate reinvestigation of epidemiology, pathogenesis and virulence of *P. multocida* and related species.

Further investigations on the epidemiology of *P. multocida* basing on the current classification are strongly recommended. This will help to establish the reservoir and the true distribution of *P. multocida* in poultry, domestic and wild mammals and wild birds. It also remains to determine the fate of healthy carriers under stressful conditions both in the field and in experimentally infected birds. This might further elaborate the effect of concurrent respiratory and intestinal diseases on *P. multocida* carriage in healthy and development of clinical fowl cholera. Long term studies are also needed to investigate the impact of carrier status on productivity in free ranging systems and commercial systems.

Virulence of *P. multocida* and other species in the genus need to be addressed to understand their impact on the isolation of *Pasteurella* species by mouse inoculation. This should go in conjunction with efforts to develop a specific selective medium for avian hosts to replace the use of mouse passage especially in wide scale epidemiological studies. While advocating for development and adoption of species specific PCR and other molecular techniques for detecting *P. multocida* in live birds, these should not be considered as a total replacement for isolation.

It is essential to ensure that the relationships of the taxa of the genus *Pasteurella* Mutters et al. 1985 are described more accurately. This should be done with reference to phenotypic and genotypic features so as to address their value in classifying and epidemiological typing of these organisms. The subspecies of *P. multocida* need to be further investigated by using strains from different geographical areas and a wide host range. Apart from *Hpa*II, other restriction enzymes should be used in ribotyping and the results compared with extended phenotyping results. It will be of interest to know if trehalose positive strains of *P. multocida* will cluster together when ribotyping is performed after digestion with restriction enzymes other than *Hpa*II. Other molecular epidemiology tools such as PFGE and PCR should be further evaluated to demonstrate their suitability for typing *P. multocida.*

The need to develop a standardized method for investigating serum resistance of *P. multocida* has been indicated in this study. Assays that directly measure the complement deposition on the bacterium cell wall would clearly explain the difference between serum resistant and serum sensitive strains. Investigations on components of major outer-membrane proteins of *P. multocida,* and their role in the activation of complement of the host serum are needed concurrent with standardizing chamber model for investigating *P. multocida in-vivo.* All researchers in this field should uniformly employ standard inoculation dose and route of infecting birds with *P. multocida* so that the results from different workers can be compared in a more logical way.

To fully understand the epidemiology and importance of all *Pasteurella* species widespread adoption of new criteria for identification of *Pasteurella*, application of molecular techniques as well as more sensitive isolation and detection procedures are strongly recommended. Together with challenge experiments and developing methods for investigating virulence factors, more information will be obtained about epidemiology, taxonomy, pathogenesis and virulence of *P. multocida* in poultry and other animals.

8. References

1. Alberts, J.O. & Graham, R. (1948). Sulfamerazine in the treatment of fowl cholera. *North America Veterinarian, 29,* 24-26.

2. Alls, A.A. (1970). A bird-contact method of challenging turkeys with *Pasteurella multocida*. *Avian Diseases, 14,* 172-178.

3. Angen, O., Mutters, R., Caugant, D.A., Olsen, J.E., & Bisgaard, M. (1999). Taxonomic relationships of the [*Pasteurella*] *hemolytica* complex as evaluated by DNA-DNA hybridizations and 16S rRNA sequencing with proposal of *Mannheimia hemolytica* gen. nov., *Mannheimia glucosida* sp.nov., *Mannheimia ruminalis* sp.nov., and *Mannheimia varigena* sp.nov. *International Journal of Systematic Bacteriology, 49,* 67-86.

4. Anon (1996). Det Danske Fjekræræd. *Beretning 1996.*

5. Avril, J.P., Donnio, P.Y., & Pouedras, P. (1990). Selective medium for *Pasteurella multocida* and its use to detect oropharyngeal carriage in pig breeders. *Journal of Clinical Microbiology, 28,* 1438-1440.

6. Aye, P.P., Morishita, T.Y., & Angrick, E.J. (1999). Virulence of raptor-origin *Pasteurella multocida* in domestic chickens. *Avian Diseases, 43,* 279-285.

7. Baldrias, L., Frost, A.J., & O'Boyle, D. (1988). The Isolation of *Pasteurella* -like organisms from the tonsillar region of dogs and cats. *Journal of Small Animal Practise, 29,* 63-68.

8. Batamuzi, E.K. & Kristensen, F. (1996). Urinary tract infection: The role of canine transmissible venereal tumour. *Journal of Small Animal Practise, 37,* 276-279.

9. Beichel, E. (1986). Differenzierung von 130 X-und V-Factor-unabhängingen äviaren bakterienstämmen der familie *Pasteurellaceae* Pohl 1981 unter besonder berücksichtigung neuer taxonomischer erkenntnisse. PhD. Tierärztliche Hochschule Hannover.

10. Bhasin, J.L. (1982). Serological types of *Pasteurella multocida* isolated from turkeys and chickens in Canada. *Canadian Journal of Microbiology, 28,* 1078-1080.

11 Bierer, B.W. (1962). Treatment of avian pasteurellosis with injectable antibiotics. *Journal of American Veterinary Medical Association, 141,* 1344-1346.

12. Bisgaard, M. & Dam, A. (1981). Salpingitis in poultry. II prevalence, bacteriology and possible pathogenesis in egglaying chickens. *Nord Vet - Med, 33,* 81-89.

13. Bisgaard, M. (1982). Isolation and characterization of some previously unreported taxa from poultry with phenotypical characters related to *Actinobacillus* and *Pasteurella* species. *Acta Pathologica, Microbiologica et Immunologica Scandinavica, 90,* 59-67.

14. Bisgaard, M., Mutters, R., & Mannheim, W. (1983). Characterization of some previously unreported taxa isolated from guinea pigs (*Cavia porcellus*) and provisionally classed with the 'HPA-group'. *Les editions INSERM, 114,* 227-244.

15. Bisgaard, M. & Mutters, R. (1986). Characterization of some previously unclassified *Pasteurella spp* obtained from the oral cavity of dogs and cats and description of a new species tentatively classified with the family *Pasteurellaceae* Pohl 1981and provisionally called Taxon 16. *Acta Pathologica, Microbiologica et Immunologica Scandinavica, 94,* 177-184.

16. Bisgaard, M., Abdullahi, M.Z., & Gilmour, N.J.L. (1991a). Further studies on the identification of *Pasteurellaceae* from cattle lungs. *The Veterinary Record, 128,* 428-429.

17. Bisgaard, M., Houghton, S.B., Mutters, R., & Stenzel, A. (1991b). Reclassification of German, British and Dutch isolates so-called *Pasteurella multocida* obtained from pneumonic calf lungs. *Veterinary Microbiology, 26,* 115-124.

18. Bisgaard, M. (1993). Ecology and significance of *Pasteurellaceae* in animals. *Zentralblatt fur Bakteriologie, 279,* 7-26.

19. Bisgaard, M. (1995a). Salpingitis in web-footed birds: prevalence, aetiology and significance. *Avian Pathology, 24*, 443-452.

20. Bisgaard M. (1995b). Taxonomy of the family *Pasteurellaceae* Pohl 1981. *In* W. Donachie and et al. (Eds.), *Hemophilus,Actinobacillus and Pasteurella*. New York, Plenum Press, pp. *1-7*.

21. Bisgaard, M., Hinz, K.H., Petersen, K.D., & Christensen, J.P. (1999). Identification of members of the Pasteurellaceae isolated from birds and characterization of two new taxa isolated from psittacine birds. *Avian Pathology, 28*, 369-377.

22. Blackall, P.J., Pahoff, J.L., Marks, D., Fegan, N., & Morrow, C.J. (1995). Characterization of *Pasteurella multocida* isolated from fowl cholera on turkey farms. *Australian Veterinary Journal, 72*, 135-138.

23. Blackall, P.J., Pahoff, J.L., & Bowles, R. (1997). Phenotypic characterisation of *Pasteurella multocida* isolates from Australian pigs. *Veterinary Microbiology, 57*, 355-360.

24. Blackall, P.J., Fegan, N., Chew, G.T.I., & Hampson, D.J. (1998). Population structure and diversity of avian isolates of *Pasteurella multocida* from Australia. *Microbiology, 144*, 279-289.

25. Blackall, P.J., Fegan, N., Chew, G.T.I., & Hampson, D.J. (1999). A study of the use of multilocus enzyme electrophoresis as a typing tool in fowl cholera outbreaks. *Avian Pathology, 28*, 195-198.

26. Blackall, P.J., Fegan, N., Pahoff, J.L , Storie, G.J., McIntosh, G.B., Cameron, R.D.A., O'Boyle, D., Frost, A.J., Bara', M.R., Marr, G., & Holder, J. (2000). The molecular epidemiology of four outbreaks of porcine pasteurellosis *Veterinary Microbiology, 72*, 111-120.

27 Blackburn, B.O., Heddleston, K.L., & Pfow, C.J (1975). *Pasteurella multocida* serotyping results (1971-1973). *Avian Diseases, 19*, 353-356.

28. Botzler, R.G. (1991). Epizootiology of avian cholera in wildfowl. *Journal of Wildlife Diseases, 27*, 367-395.

29. Bragg, R.R., Greyling, J.M., & Verschoor, J.A. (1997). Isolation and identification of NAD-independent bacteria from chickens with symptoms of infectious coryza. *Avian Pathology, 26*, 595-606.

30. Brogden, K.A., Rhoades, K.R., & Heddleston, K.L. (1978). A new serotype of *Pasteurella multocida* associated with fowl cholera. *Avian Diseases, 22*, 185-190.

31. Brogden, K.A. & Rhoades, K.R. (1983). Prevalence of serologic types of *Pasteurella multocida* from 57 species of birds and mammals in United States. *Journal of Wildlife Diseases, 19*, 315-320.

32. Cameron, R.D.A., O'Boyle, D., Frost, A.J., Gordon, A.N., & Fegan, N. (1996). An outbreak of hemorrhagic septicemia associated with *Pasteurella multocida* subsp *gallicida* in a large pig herd. *Australian Veterinary Journal, 73*, 27-29

33. Carpenter, T.E., Hirsh, D.C., Kasten, R.W., Hird, D.W., Snipes, K.P., & McCapes, R.H. (1989). *Pasteurella multocida* recovered from live turkeys: Prevalence and virulence in turkeys. *Avian Diseases, 33*, 12-17.

34. Carpenter, T.E , Snipes, K.P., Kasten, R.W., Hird, D.W., & Hirsh, D C. (1991). Molecular epidemiology of *Pasteurella multocida* . *American Journal of Veterinary Research, 52*, 1345-1349.

35. Carter, G.R. (1967). Pasteurellosis: *Pasteurella multocida* and *Pasteurella haemolytica*. *Advances in Veterinary Science and Comparative medicine, 11*, 321-379.

36. Carter, G.R. (1955). Studies of *Pasteurella multocida* 1. A hemagglutination test for the identification of serological types. *American Journal of Veterinary Research, 16*, 481-484.

37. Carter G.R., De Alwis M.C.L. (1989). Haemorrhagic septicaemia, *In* C. Adlam and J. M. Rutter. (Eds.), *Pasteurella* and Pasteurellosis. London, Academic Press, pp. *131-160*.

38. Chandrasekaran, S. & Yeap, P.C. (1982). *Pasteurella multocida* in pigs: the serotypes and the assessment of their virulence in mice. *British Veterinary Journal, 138*, 332-336.

39. Chandrasekaran, S., Rhoades, K.R., & Sotoodehnia, A. (1985). *Pasteurella multocida* type B:2 isoalted from poultry in Iran. *Veterinary Record, 117*, 155

40. Chanter N., Rutter J.M. (1989). Pasteurellosis in pigs and the determinants of virulence of toxigenic *Pasteurella multocida*, *In* C. Adlam and J. M. Rutter. (Eds.), *Pasteurella* and Pasteurellosis. London, Academic Press. pp. *161-165.*

41. Chevalier, G., Duclohier, H., Thomas, D., Shechter, E., & Wroblewski, H. (1993). Purification and characterization of protein H, the major porin of *Pasteurella multocida*. *Journal of Bacteriology, 175,* 266-276.

42. Choi-Kim, K., Maheswaran, S.K., Felice, L.J., & Molitor, T.W. (1991). Relationship between the iron regulated outer membrane proteins and the outer membrane proteins of in vivo grown *Pasteurella multocida*. *Veterinary Microbiology, 28,* 75-92.

43. Christensen, J.P. & Bisgaard, M. (1997). Avian Pasteurellosis: Taxonomy of the organisms involved and aspects of pathogenesis. *Avian Pathology, 26,* 461-483.

44. Christensen, J.P., Dietz, H.H., & Bisgaard, M (1998). Phenotypic and genotypic characters of isolates of *Pasteurella multocida* obtained from two outbreaks of avian cholera in avifauna in Denmark *Avian Pathology, 27,* 373-381.

45. Christensen, J.P., Petersen, K.D., Hansen, H.C., & Bisgaard, M. (1999). Occurrence of fowl cholera in Danish wild birds, and poultry production. Possible connections. [in Danish, summary in English]. *Dansk Veterinar Tidskrift, 82,* 342-346.

46. Christiansen, K.H., Carpenter, T.E., Snipes, K.P., Hird, D.W., & Ghazikhanian, G.Y. (1992). Restriction endonuclease analysis of *Pasteurella multocida* isolates from three California turkey premises *Avian Diseases, 36,* 272-281.

47. Collins, F.M. (1977). Mechanisms of acquired resistance to *Pasteurella multocida infections.* A review *Cornell Veterinarian, 67,* 103-138.

48. Curtis, P.E. & Ollerhead, G.E. (1981). Investigation to determine whether healthy chickens and turkeys are oral carriers of *Pasteurella multocida*. *The Veterinary Record, 108,* 206-207.

49. Dabo, S.M., Confer, A.W., & Murphy, G.L. (1997). Outer-membrane proteins of bovine *Pasteurella multocida* serogroup A isolates. *Veterinary Microbiology, 54,* 167-183.

50. Dabo, S.M., Confer, A.W., Montelongo, M., & Lu, Y.S. (1999a). Characterization of rabbit *Pasteurella multocida* isolates by use of whole-cell, outer membrane, and polymerase chain reactiontyping *Laboratory Animal Science, 49,* 551-558.

51. Dabo, S.M., Debey, B.M., Montelongo, M., & Confer, A.W. (1999b). Genomic DNA restriction site heterogeneity in bovine *Pasteurella multocida* serogroup A isolates detected with an rRNA probe *Journal of Medical Microbiology,*

52. Davies, R.L. (1991). Virulence and serum-resistance in different clonal groups and serotypes of Yersinia ruckeri *Veterinary Microbiology, 29,* 289-297.

53. Davis, R.B. (1987). Cholera in broiler breeders. *Poultry Science, 20,* 430-434.

54. De Alwis, M.C.L., Wijewardana, T.G., Gomis, A.I.U., & Vipulasiri, A.A (1990). Persistence of the carrier status in haemorrhagic septicemia (*Pasteurella multocida* serotype 6:B infection) in buffaloes *Tropical Animal Health and Production, 22,* 185-194.

55. De ley, J., Mannheim, W., Mutters, R., Piechulla, K., Tytgat, R., Segers, P., Bisgaard, M., Frederiksen, W., Hinz, K.H., & Vanhoucke, M. (1990). Inter- and intrafamilial similarities of r RNA cistrons of the *Pasteurellaceae*. *International Journal of Systematic Bacteriology, 40,* 126-137.

56. Dewhirst, F.E., Paster, B.J., Olsen, I., & Fraser, G.J. (1992). Phylogeny of 54 representastive strains of of species in the family *Pasteurellaceae* as determined by comparison of 16sRNA sequences. *Journal of Bacteriology, 174,* 2002-2013.

57. Dewhirst, F.E., Paster, B.J., & Fraser, G.J. (1993). Phylogeny of *Pasteurellaceae* as determined by comparison of 16S ribosomal nucleic acid sequences. *Zentralblatt fur Bakteriologie, 279,* 35-44.

58. Diallo, I.S., Bensink, J.C., Frost, J.C., & Spadbrow, P.B. (1995). Molecular studies on avian strains of *Pasteurella multocida* in Australia. *Veterinary Microbiology, 46,* 335-342.

59. Dipeolu, M.A., Enwbetine, D., & Williams, T.J. (1996). Indigenous chicken rearing under village conditions. *International Journal of Animal Science, 11,* 63-67.

60. Domino, P.Y., Goff, C.Le, Avril, J.L., Pouedras, P., & Gras-Rouzet, S. (1994). *Pasteurella multocida*: oropharygeal carriage and antibody responnse in breeders. *Veterinary Research, 25,* 8-15.

61. Domnio, P.Y., Allardet Servent, A., Perrin, M., Escande, F., & Avril, J.L. (1999). Characterisation of dermonecrotic toxin-producing strains of *Pasteurella multocida* subsp. *multocida* isolated from man and swine. *Journal of Medical Microbiology, 48,* 125-131

62. Dorsey, T.A. & Harshfield, G.S. (1959). Studies on control of fowl cholera. *South Dakota University Agriculture Experiments Station Bulletin, 23,* 1-18.

63. Droual, R., Walker, R.L., Shivaprasad, H.L., Jeffrey, J.S., Meteyer, C.U., Chin, R.P., & Shapiro, D.P. (1992a). An atypical strain of *Pasteurella gallinarum*: Pathogenic phenotypic and genotypic characteristics. *Avian Diseases, 36,* 693-699.

64. Droual, R., Shivaprasad, H.L., Meteyer, C.U., Shapiro, D.P., & Walker, R.L. (1992b). Severe mortality in broiler chickens associated with *Mycoplasma synoviae* and *Pasteurella gallinarum*. *Avian Diseases, 36,* 803-807.

65. Dubreuil, J.D., Gilbert, L., & Jacques, M. (1992). Cell surface characteristics and virulence in mice of *Pasteurella multocida*. *Zentralblatt fur Bakteriologie, 276,* 366-373.

66. Eleveth, D.F., Goldsby, A.E., & Nelson, C.L. (1949). Fowl cholera. *Veternary Medicine, 64,* 73-78.

67. Fajfar-Whetstone, C.J., Coleman, L., Biggs, D.R., & Fox, B.C. (1995). *Pasterella multocida* septicemia and subsequent *Pasteurella dagmatis* septicemia in a diabetic patient. *Journal of Clinical Microbiology, 33,* 202-204.

68. Fegan, N., Blackall, P.J., & Pahoff, J.L. (1995). Phenotypic characterization of *Pasteurella multocida* isolates from Australian poultry. *Veternary Microbiology, 47,* 281-286.

69. Ficken, M.D. & Barnes, H.J. (1989). Acute airsacculitis in turkeys inoculated with *Pasteurella multocida*. *Veternary Pathology, 26,* 231-237.

70. Ficken, M.D., Barnes, H.J., & Qureshi, M.A. (1991). Acute air sacculitis in turkeys inoculated with cell free culture filtrate of *Pasteurella multocida*. *Veternary Pathology, 28,* 46-54.

71. Foged, N.T. (1992). *Pasteurella multocida* toxin- the characterization of the toxin and its significance in the diagnosis and prevention of progressive atrophic rhinits in pigs. *Acta Pathologica, Microbiologica et Immunologica Scandinavica (APMIS, 100 Supplentum 25,* 56

72. Food and Agriculture Organization: FAOSTAT Database. www.fao.org, 2000.

73. Frame, D.D., Clark, F.D., & Smart, R.A. (1994). Recurrent outbreaks of a cutaneous form of *Pasteurella multocida* infection in turkeys. *Avian Diseases, 38,* 390-392.

74. Frank G.H. (1989). Pasteurellosis of cattle. *In* C. Adlam and J. M. Rutter. (Eds.), *Pasteurella* and Pasteurellosis. London, Academic Press, pp. *197-222.*

75. Frederiksen, W. (1973). *Pasteurella* taxonomy and nomenclature. *Contrib Microbiology, 2,* 170-176.

76. Frederiksen, W. (1993). Ecology and significance of *Pasteurellaceae* in man-an update. *Zentralblatt fur Bakteriologie, 279,* 27--34

77. Frost, A.J., O'Boyle, D., Wilkie, I.W, and Townsend, K.M. (1999). Live metabolic drift vaccines for pasteurellosis in chcikens and ducks. *In* Proceedings of *Hemophilus,Actinobacillus,* and *Pasteurella* (HAP) Conference13-17 September, Bloemfoentein, p *42.*

78. Ganiere, J.P., Escande, F., Andre, G., & Larrat, M. (1993). Characterization of *Pasteurella* from gingival scrappings of dogs and cats. *Comparative Immunology Microbiology and Infectious Diseases, 16,* 77-85.

79. Gilmour N.J.L., Gilmour J.S. (1989). Pasteurellosis of sheep. *In* C. Adlam and J. M. Rutter. (Eds.), *Pasteurella* and Pasteurellosis. London, Academic Press, pp. *223-262.*

80. Glass, S.E. & Panigrahy, B. (1990). Dermal necrosis caused by *Pasteurella multocida* infection in turkeys. *Avian Diseases, 34,* 491-494.

81. Glisson, J.R. & Cheng, I.H.N. (1991). In vivo antigen expression by *Pasteurella multocida*. *Avian Diseases, 35,* 392-396.

82. Glorioso, J.C., Jones, G.W., Rush, H.G., Pentler, L.J., Darif, C.A., & Coward, J.E. (1982). Adhesion of type A *Pasteurella multocida* and its possible role in rabbit respiratory tract infections. *Infection and Immunity, 35,* 1103-1109.

83. Goering, R.V. (1993). Molecular epidemiology of nosocomial infection: Analysis of chromosomal restriction fragment patterns by pulse filed gel electrophoresis. *Infection Control and Hospital Epidemiology, 14,* 595-600.

84. Grimont, F. & Grimont, P.A.D. (1986). Ribosomal nucleic acid gene restriction patterns as potential taxonomic tools. *Annales de l'institute Pasteur Microbiologie, 137B,* 165-175.

85. Gump, D.W. & Holden, R.A. (1972). Endocarditis caused by a new species of *Pasteurella*. *Ann Intern Med, 76,* 275-278.

86. Gunawardana, G.A., Townsend, K.M., & Frost, A.J. (2000). Molecular Characterization of avian *Pasteurella multocida* isolates from Australia and Vietnam by REP-PCR and PFGE. *Veterinary Microbiology, 72,* 97-109.

87. Gustafson, C.R., Cooper, G.L., Charlton, B.R., & Bickford, A.A. (1998). *Pasteurella multocida* infection involving cranial air spaces white leghorn chickens. *Avian Diseases, 42,* 413-417.

88. Hall, W.J., Heddleston, K.L., Legenhausen, D.H., & Hughes, R.W. (1955). Studies on Pasteurellosis.1. A new species of *Pasteurella* encountered in chronic fowl cholera. *American Journal of Veterinary Research, 16,* 598-604.

89. Hamilton, T.D.C., Roe, J.M., & Webster, A.J.F. (1996). Synergestic role of gaseous ammonia in etiology of *Pasteurella multocida* induced atrophic rhinitis in swine. *Journal of Clinical Microbiology, 34,* 2185-2190.

90. Hansen, L.M. & Hirsh, D.C. (1989). Serum resistance is correlated with encapsulation of avian strains of *Pasteurella multocida*. *Veterinary Microbiology, 21,* 177-184.

91. Hansmann, G.H. & Tully, M.T. (1945). Cat bite and scratch wounds with consequent *Pasteurella* infections of man. *American Journal of Clinical Pathology, 15,* 312-318.

92. Hazlewood, R.M., Oddo, A.F., Pagan, R.D., & Botzler, R.G. (1978). The 1975-76 avian cholera outbreaks in Humboldt County, California. *Journal of Wildlife Diseases, 14,* 229-232.

93. Heddleston, K.L. (1962). Studies on pasteurellosis. V. Two immunogenic types of *Pasteurella multocida* associated with fowl cholera. *Avian Diseases, 6,* 315-321.

94. Heddleston, K.L., Watko, L.P., & Rebers, P.A. (1964). Dissociation of a fowl cholera strain of *Pasteurella multocida*. *Avian Diseases, 8,* 649-657.

95. Heddleston, K.L., Goodson, T., Leibovitz, L., & Angstrom, C.I. (1972). Serological and biochemical characteristics of *Pasteurella multocida* from free flying birds and poultry. *Avian Diseases, 16,* 729-734.

96. Heddleston, K.L. & Rebers, P.A. (1975). Properties of free endotoxin from *Pasteurella multocida*. *American Journal of Veterinary Research, 36,* 573-574.

97. Heddleston, K.L. (1976). Physiologic characterization of 1268 cultures of *Pasteurella multocida*. *American Journal of Veterinary Research, 37,* 745-747.

98. Henderson, A. (1963). *Pasteurella multocida* infection in Man: a review of the literature. *Antonie Van Leeuwenhoek, 29,* 359-367.

99. Hertman, I., Markenson, J., Michael, A., & Geier, E. (1980). Attenuated live fowl cholera vaccine. I. Development of vaccine strain M3G of *Pasteurella multocida*. *Avian Diseases, 24,* 863-869.

100. Hirsh, D.C., Martin, L.D., & Rhoades, K.R. (1985). Resistance plasmids of *Pasteurella multocida* isolated from turkeys. *American Journal of Veterinary Research, 46,* 1490-1493.

101. Hirsh, D.C., Jessup, D.A., Snipes, K.P., Carpenter, T.E., Hird, D.W., & McCapes, R.H. (1990). Characteristics of *Pasteurella multocida* isolated from waterfowl and associated avian species in California. *Journal of Wildlife Diseases, 26,* 204-209.

102. Hofacre, C.L., Glisson, J.R., Kleven, S.H., Brown, J., & Rowland, G.N. (1989a). Evaluation of *Pasteurella multocida* mutants of low virulence.1. Development and pathogenecity. *Avian Diseases, 33,* 270-274.

103. Hofacre, C.L., Glisson, J.R., & Kleven, S.H. (1989b). Evaluation of *Pasteurella multicoda* mutants of low virulence. 2. Immunologic response of turkeys. *Avian Diseases, 33,* 275-278.

104. Homchampa, P. (1995). Construction and vaccine potential of AROA biosynthetic mutants of *Pasteurella*. PhD. Monash University, Melbourne.

105. Homchampa, P., Strugnell, R.A., & Adler, B. (1997). Cross protective immunity conferred by a marker -free aroA mutant of *Pasteurella multocida*. *Vaccine, 15,* 203-208.

106. Hopkins, B.A., Huang, T.H M., & Olson, L.D. (1998). Differentiating turkey postvaccination isolants of *Pasteurella multocida* using arbitrarily primed polymerase chain reaction. *Avian Diseases, 42,* 265-274.

107. Hughes, R.W. & Pritchett, I.W. (1930). The epidemiology of fowl cholera. III Portal of entry of *P. avicida*; Reaction of the host. *Journal of Experimental Medicine, 51,* 239-248.

108. Hungerford, T.G. (1968). A clinical note on avian cholera. *Australian Veterinary Journal, 44,* 31-32.

109. Hunt, M.L., Adler, B., & Townsend, K.M. (2000). The molecular biology of *Pasteurella multocida*. *Veterinary Microbiology, 72,* 3-25.

110. Hussaini, S.N. (1975). Nomenclature and taxonomy of *Pasteurella multocida*. *Veterinary Bulletin, 45,* 403-409.

111. Ikeda, J.S. & Hirsh, D.C. (1988). Antigenically related iron-regulated outer membrane proteins produced by different somatic serotypes of *Pasteurella multocida*. *Infection and Immunity, 56,* 2499-2502.

112. Ikeda, J.S. & Hirsch, D.C. (1990). Possession of identical nonconjugative plasmids by different isolates of *Pasteurella multocida* does not imply clonality. *Veterinary Microbiology, 22,* 79-87.

113. Iliev, T.R., Arsov, G.G., & Iovchev, E. (1964). The carrier state in fowl cholera. *Veterinary Bulletin, 34,* 129-129.

114. Jawetz, E. (1950). A pneumonic pasteurellosis in laboratory animals. I. Bacteriological and serological characteristics. *Journal of Infectious Diseases, 86,* 172-186.

115. Jayaraman, M.S. & Sethumadhavan, V. (1974). The B.L. organism- the causal agent of bovine lymphangitis. *Indian Veterinary Journal, 51,* 347-355.

116. Johnson, R.B., Dawkins, J.S., & Spencer, T.L. (1991). Electrophoretic profiles of *Pasteurella multocida* isolates from animals with hemorrhagic septicemia. *American Journal of Veterinary Research, 52,* 1644-1648.

117. Jones, D.M. (1962). A *Pasteurella*-like organism from the human respiratory tract. *The Journal of Pathology and Bacteriology, 143,* 83-151.

118. Kabatange, M. A. and Katule, A. M. (1990). Rural poultry production systems in Tanzania. *In* Proceedings of an International Workshop on Rural poultry in AfricaNigeria. p *171.*

119. Karmy, S.A., El-Hamed, S.A., Fayed, A.A., & El-Ghani, A.E. (1983). Studies on *Pasteurella multocida* in domesticated animals and birds in Aswan governorate. *Journal of the Egyptian Veterinary Medical Association, 43,* 91-100.

120. Kasten, R.W., Hansen, L.M., Hinojoza, J., Bieber, D., Ruehl, W.W., & Hirsh, D.C. (1995). *Pasteurella multocida* produces a protein with homology to the P6 outer membrane protein of *Hemophillus Influenzae*. *Infection and Immunity, 63,* 989-993.

121. Kasten, R.W., Wakenell, P.S., Ahmad, S., Yilma, T.D., & Hirsh, D.C. (1997). Lack of protection against avian cholera with recombinant P6- like protein from *Pasteurella multocida*. *Avian Diseases, 41,* 972-976.

122. Kelly, P.J., Chitauro, D., Rohde, C., Rukwava, J., Majok, A., Davelaar, F., & Mason, P.R. (1994). Diseases and management of backyard chicken flocks in Chitungwiza, Zimbabwe. *Avian Diseases, 38,* 626-629.

123. Kim, C.J. & Nagaraja, K.V. (1990). DNA fingerprinting for differentiation of field isolates from reference vaccine strains of *Pasteurella multocida* in turkeys. *American Journal of Veterinary Research, 51,* 207-210.

124. Knight, D.P., Paine, J.E., & Speller, D.C.E. (1983). A selective medium for *Pasteurella multocida* and its use with animal and human specimens. *Journal of Clinical Pathology, 36,* 591-594.

125. Knights, J.M., Adlam, C., & Owen, P. (1990). Characterization of envelope proteins from *Pasteurella haemolytica* and *Pasteurella multocida*. *Journal of General Microbiology, 136,* 495-505.

126. Korbel, R., Gerlach, H., Bisgaard, M., & Hafez, H.M. (1992). Further investigations on *Pasteurella multocida* infections in feral birds injured by cats. *Journal of Veterinary Medicine series B, 39,* 10-18.

127. Lee, M.D., Wooley, R.E., Glisson, J.R., & Brown, J. (1988a). Comparison of *Pasteurella multocida* serotype 3,4 isolates from turkeys with fowl cholera. *Avian Diseases, 32,* 501-508.

128. Lee, M.D., Brown, J., Wooley, R.E., & Glisson, J.R. (1988b). The relationship of pathogenicity to the growth of *Pasteurella multocida* serotype 3,4 isolates in normal turkey plasma. *Avian Diseases, 32,* 509-512.

129. Lee, M.D., Wooley, R.E., Brown, J., & Glisson, J.R. (1991). A survey of potential virulence markers from avian strains of Pasteurella multocida. *Veterinary Microbiology, 26,* 213-225.

130. Lee, M.D., Glisson, J.R., & Wooley, R.E. (1992). Factors affecting endotoxin release from the cell surface of avian strains of Pasteurella multocida. *Veterinary Microbiology, 31,* 369-378.

131. Lee, M.D. & Wooley, R.E. (1995). The effect of plasmid acquisition on potential virulence attributes of *Pasteurella multocida*. *Avian Diseases, 39,* 451-457.

132. Lobinoux, J., Lozniewski, A., Lion, C., Garin, D., Weber, M., & Le Faou, A. (1999). Value of enterobacterial repetitive intergenic consensus PCR for study of *Pasteurella multocida* strains from mouths of dogs. *Journal of Clinical Microbiology, 37,* 2488-2492.

133. Lu, Y.S., Lai, W.C., Pakes, S.P., & Stefanu, C. (1991). The outer membrane of *Pasteurella multocida* 3:A protects rabbits against homologous challenge. *Infection and Immunity, 59,* 4517-4523.

134. Lubke, A., Hartmann, L., Schroder, W., & Hellmann, E. (1994). Isolation and partial characterization of major outer membrane proteins in the outer membrane of *Pasteurella multocida* and *Pasteurella hemolytica*. *Zentralblatt fur Bakteriologie, 281,* 45-54.

135. Lugtenberg, B., van Boxtel, R., de Jong, M., Boxtel, R.v., Jong, M.d., Van-Boxtel, R., & De-Jong, M. (1984). Atrophic rhinitis in swine: Correlation of *Pasteurella multocida* pathogenicity with membrane protein and lipopolysaccharides patterns. *Infection and Immunity, 46,* 48-54.

136. Madsen, E.B., Bisgaard, M., Mutters, R., & Pedersen, K.B. (1985). Isolation of *Pasteurella* spp. isolated from lungs of calves with pneumonia. *Canadian Journal of Comparative Medicine, 49,* 63-67.

137. Manning P.J., DiGiacomo R.F., DeLong D. (1989). Pasteurellosis in laboratory animals. *In* C. Adlam and J. M. Rutter, (Eds.), *Pasteurella* and Pasteurellosis. London, Academic Press, pp. *261-302.*

138. Matsumoto, M., Strain, J.G., & Engel, H.N. (1991). The fate of *Pasteurella multocida* after intratracheal inoculation in turkeys. *Poultry Science, 70,* 2259-2266.

139. Matsumoto, M. & Strain, J.G. (1993). Pathogenicity of *Pasteurella multocida:* its variable nature demonstrated by in vivo passages. *Avian Diseases, 37,* 781-785.

140. Mc Allister, H.A. & Carter, G.R. (1974). An aerogenic *Pasteurella*-like organism recovered from swine. *American Journal of Veterinary Research, 35,* 917-922.

141. Minga, U. M, Katule, A. M., Maeda, T. and Musasa, J. (1989). Potential and problems of traditional chicken industry in Tanzania. *In* Proceedings of the 7th Tanzania Veterinary Association Scientific Conference held at Arusha International Conference CentreDecember 3-5, Arusha Tanzania. p *207.*

142. Ministry of Agriculture Tanzania (1994): National Sample Census.

143. Mock, M., Crasnier, M., Duflot, E., Dumay, V., & Danchin, A. (1991). Structural and functional relationships between *Pasteurella multocida* and enterobacterial adenylate cyclases. *Journal of Bacteriology, 173,* 6265-6269.

144. Mohan, K., Sadza, M., Madsen, M., Hill, F.W.G., & Pawandiwa, A. (1994). Phenotypic characterization of Zimbabwean isolates of *Pasteurella multocida*. *Veterinary Microbiology, 38*, 351-357.

145. Mohan, K., Kelly, P.J., Muvavarirwa, P., & Pawandiwa, A. (1997). Phenotype and serotype of *Pasteurella multocida* isolates from diseases of dogs and cats in Zimbabwe. *Comparative Immunology Microbiology and Infectious Diseases, 20*, 29-34.

146. Moore, M.K., Cienjak-Chubbs, L., & Gates, R.J. (1994). A new selective enrichment procedure for isolating *Pasteurella multocida* from avian and environmental samples. *Avian Diseases, 38*, 317-324.

147. Morishita, T.Y., Snipes, K.P., & Carpenter, T.E. (1990). Serum resistance as an indicator of virulence of *Pasteurella multocida* for turkeys. *Avian Diseases, 34*, 888-892.

148. Morris, E.J. (1958). Selective media for some *Pasteurella* species. *Journal of General Microbiology, 19*, 305-311.

149. Mraz, O. (1969). Vergleichende studie der atren *Actinobacillus lignieresii* und *Pasteurella haemolytica*. III *Actinobacillus haemolyticus* (Newsom and Cross, 1932) comb. nov. *Zentralbl bakteriol Parasitekd Infectionskr Hyg Abt 1 orig, 209*, 349-364.

150. Mraz, O., Sisak, F., & Jelen, P (1980). The *Pasteurella* carriers in farm and laboratory animals. *Comparative Immunology Microbiology and Infectious Diseases, 2*, 437-445.

151. Muhairwa, A. P., Mtambo, M. M. A., Christensen, J. P., and Bisgaard, M. (1999). Investigations on the diseases of village chickens and significance of detailed characterization of pathogens obtained. *In* Proceedings of Development workers Course: Poultry as a tool of eradication of Poverty Copenhagen. p 198

152. Munn, C.B., Ishiguro, E.E., Kay, W.W., & Trust, T.J. (1982). Role of surface components in serum resistance of virulent *Aeromonas salmonicida*. *Infection and Immunity, 36*, 1069-1075.

153. Mushin, R., Bock, R., & Abrams, M. (1977). Studies on *Pasteurella gallinarum*. *Avian Pathology, 6*, 415-423.

154. Mutters, R., Ihm, P., Pohl, S., Frederiksen, W., & Mannheim, W. (1985a). Reclassification of the genus *Pasteurella* Trevisan 1887 on the basis of deoxyribonucleic acid homology, with proposal for the new species *Pasteurella dagmatis*, *Pasteurella stomatis*, *Pasteurella anatis* and *Pasteurella langaa*. *International Journal of Systematic Bacteriology, 35*, 309-322.

155. Mutters, R., Piechulla, K., Hinz, K H., & Mannheim, W. (1985b). *Pasteurella avium* (Hinz and Kunjara 1977) comb. nov. *Pasteurella volantium* sp.nov *International Journal of Systematic Bacteriology, 35*, 5-9.

156. Mutters R., Mannheim W., Bisgaard M. (1989). Taxonomy of the group. *In* C. Adlam and J. M. Rutter. (Eds.), *Pasteurella* and Pasteurellosis. London, Academic Press, pp. *3-34*.

157. Mwalusanya, N. A. (1998). Studies on productivity and nutritional status of local chickens under village management condition. Msc. Thesis. Royal Veterinary and Agricultural University Copenhagen Denmark.

158. Namioka S. (1978). *Pasteurella multocida* biochemical characteristics and serotypes. *In* T. Bergan and J. R. Norris. (Eds.), Methods in Microbiology. London, New York, Academic Press, pp. *271-292*.

159. Nestor, K E., Lilburn, M.S., Saif, Y.M. Anderson, J.W., Patterson, R.A., Li, Z., & Nixon, J.E. (1999). Influence of body weight restriction in a body-weight-selected line of turkeys on response to challenge with *Pasteurella multocida*. *Poultry Science, 78*, 1263-1267.

160. Newsom, I.E. & Cross, F. (1931). Some bipolar organisms found in pneumonia in sheep. *Journal of American Veterinary Medical Association, 80*, 711-719.

161 Nielsen, J.P., Bisgaard, M., & Pedersen, K.B. (1986). Production of toxin in strains previously classified as *Pasteurella multocida*. *Acta Pathologica, Microbiologica et Immunologica Scandinavica, 94*, 203-204.

162. Ogunnariwo, J.A., Alcantara, J., & Schryvers, A.B. (1991). Evidence for non-siderophore-mediated acquisition of transferrin- bound iron by Pasteurella multocida. *Microbial Pathogenesis, 11*, 47-56.

163. Ojo, M.O. (1971). Studies on *Pasteurella* species isolated from cases of kerato-conjunctivitis of poultry in the Western State of Nigeria. *Bulletin of Epizootic Diseases of Africa, 19*, 357-363.

164. Olsen, J.E., Brown, D.J., Skov, M.N., & Christensen, J.P. (1993). Bacterial typing methods suitable for epidemiological analysis. Applications in investigations of salmonellosis among livestock. *Veterinary Quarterly, 15,* 125-135.

165. Olson, L.D., McCune, E.L., & Moseley, B.L. (1966). Gross and histopathological description of the cranial form of chronic fowl cholera in turkeys. *Avian Diseases, 10,* 518-529.

166. Olson, L.D. & McCune, E.L. (1968). Experimental production of the cranial form of fowl cholera in turkeys. *American Journal of Veterinary Research, 29,* 1665-1673.

167. Pabs-Garnon, L.F. & Soltys, M.A. (1971a). Multiplication of *Pasteurella multocida* in the spleen, liver and blood of turkeys inoculated intravenously. *Canadian Journal of Microbiology, 35,* 147-149.

168. Pabs-Garnon, L.F. & Soltys, M.A. (1971b). Methods of transmission of fowl cholera in turkeys *Journal of American Veterinary Medical Association, 32,* 1119-1120.

169. Peel, M.M. (1993). Dog-associated bacterial infections in humans: isolates submitted to an Australian reference laboratory, 1981-1992. *Pathology, 25,* 379-384.

170. Pehlivanoglu, F., Morishita, T.Y., Aye, P.P., Porter, R.E.J., Angrick, E.J., Harr, B.S., & Nersessian, B. (1999). The effect of route of inoculation on the virulence of raptorial Pasteurella multocida isolates in Pekin ducks (Anas platyrhynchos). *Avian Diseases, 43,* 116-121.

171. Peters, M. (1989). Phänotypische differenzierung von V-Factor-abhängigen aviären feldstamen der familie *Pasteurellaceae* Pohl 1981 unter Berücksichtigung neuerer taxonomischer erkentnisse.Tierärztliche Hochschule Hannover.

172. Petersen, K.D., Christensen, J.P., & Bisgaard, M. (1998). Phenotypic and genotypic diversity of organisms previously classified as maltose positive *Pasteurella multocida. Zentralblatt fur Bakteriologie, 288,* 1-12.

173. Petersen, K.D., Christensen, J.P., Permin, A.P., & Bisgaard, M. (2000). Investigation on the significance of avifauna in the epidemiology of fowl cholera in domestic animlas species. *Avian Pathology, In press.*

174. Pohl, S., Bertschinger, W., Frederiksen, W., & Mannheim, W. (1983). Transfer of *haemophilus pleuropneumoniae* and the *Pasteurella haemolytica*-like organism causing porcine necrotic pleuropneumonia to the genus *Actinobacillus* (*Actinobacillus pleuropneumoniae* comb. nov.) on the basis of phenotypic and deoxyribonucleic acid relatedness. *International Journal of Systematic Bacteriology, 33,* 510-514.

175. Price, S.B., Freeman, M.D., & Macewen, M.W. (1993). Molecular analysis of a cryptic plasmid isolated from avian strains of *Pasteurella multocida. Veterinary Microbiology, 37,* 31-43.

176. Pritchett, I.W. & Hughes, T.P. (1948). The epidemiology of fowl cholera. IV The spread of epidemic and endemic strains of fowl cholera. *Journal of Experimental Medicine, 51,* 71-78.

177. Ram, S., Mackinnon, F.G., Gulati, S., McQuillen, D.P., Vogel, U., Guttormsen, H.-K., Wetzler, L.M., Oppermann, M., Pangburn, M.K., & Rice, P.A. (1999). The contrasting mechanisms of serum resistance of *Neisseria gonorhhoea* and group B *Neisseria meningitidis. Molecular Immunology, 36,* 915-928.

178. Rebers, P.A. & Heddleston, K.L. (1977). Fowl cholera: induction of cross-protection in turkeys with bacterins prepared from host-passaged *Pasteurella multocida. Avian Diseases, 21,* 50-56.

179. Rhoades, K.R. (1964). Microscopic lesions of acute fowl cholera in mature chickens. *Avian Diseases, 8,* 658-665.

180. Rhoades, K.R. & Rimler, R.B. (1987a). Effects of *Pasteurella multocida* endotoxins on turkey poults. *Avian Diseases, 31,* 523-526

181. Rhoades, K.R. & Rimler, R.B. (1987b). Capsular groups of *Pasteurella multocida* isolated from avian hosts. *Poultry Science, 66,* 165.

182. Rhoades, K.R. & Rimler, R.B. (1988). Virulence of avian capsular serogroup B *Pasteurella multocida* for turkey poults. *Avian Diseases, 32,* 121-123.

183. Rhoades K.R., Rimler R.B. (1989). *Pasteurella multocida, In* C. Adlam and J. M. Rutter. (Eds.), *Pasteurella* and Pasteurellosis. london, Academic Press, pp. *37-73.*

184. Rhoades, K.R. & Rimler, R.B. (1990a). Somatic serotypes of *Pasteurella multocida* strains isolated from avian hosts (1976-1988). *Avian Diseases, 34,* 193-195.

185. Rhoades, K.R. & Rimler, R.B. (1990b). *Pasteurella multocida* colonization and invasion in experimentally exposed turkey poults. *Avian Diseases, 34,* 381-383.

186. Rhoades, K.R. & Rimler, R.B. (1990c). Virulence and toxigenicity of capsular serogroup D *Pasteurella multocida* strains isolated from avian hosts. *Avian Diseases, 34,* 384-388.

187. Rhoades, K.R., Rimler, R.B., & Bagley, R.A. (1992). Fowl cholera epornitic:Antigenic characterization and virulence of selected *Pasteurella multocida* isolates. *Avian Diseases, 36,* 84-87.

188. Ribeiro, G.A., Carter, G.R., Frederiksen, W., & Riet-Coerra, F. (1990). *Pasteurella granulomatis* sp. nov. *In Validation of the Publication of New Names and New Combinations Previously Effectively Published outside the IJSB,* List no 32 *International Journal of Systematic Bacteriology, 40,* 105-106.

189. Rimler, R.B. & Rhoades, K.R. (1986). Fowl cholera. influence of *Bordetella avium* on vaccinal immunity of turkeys of *Pasteurella multocida. Avian Diseases, 30,* 838-839.

190. Rimler, R.B. & Rhoades, K.R. (1987). Serogroup F, a new capsule serogroup of *Pasteurella multocida. Journal of Clinical Microbiology, 25,* 615-618.

191. Rimler R.B., Glisson J.R. (1997). Fowl Cholera. *In* B. W. Calnek, H. J. Barnes, C. W. Beard, L. R. McDougald,and Y. M. Saif. (Eds.). Poultry Diseases. London, Mosby-Wolfe, pp. *143-159.*

192. Rosenbuch, C. & Merchant, I.A. (1939). A study of Hemorrhagic septicemia *Pasteurellae. Journal of Bacteriology, 37,* 69-89.

193. Ruffolo, C.G. & Adler, B. (1996). Cloning, sequencing, expression, and protective capacity of the oma87 gene encoding the *Pasteurella multocida* 87-kilodalton outer membrane antigen. *Infection and Immunity, 64,* 3161-3167.

194. Ryu, E. (1959). Studies on *Pasteurella multocida* I. Inhibitory action of blood on the growth of *Pasteurella multocida. The Japanese Journal of Veterinary Science, 21,* 97-102.

195. Sa'idu, L., Abdu, P A., Umoh, J.U., & Abdullahi, U.S. (1994). Diseases of Nigerian indigenous chickens. *Bulletin of Animal Health and Production in Africa, 42,* 19-23.

196. Sacco, R.E., Saif, Y.M., Nestor, K.E., Anthony, N.B., Emmerson, D.A., & Dearth, R.N. (1991). Genetic variation in resistance of turkeys to experimental challenge with *Pasteurella multocida. Avian Diseases, 35,* 950-954.

197. Saphir, D.A. & Carter, G.R. (1976). Gingival flora of the dog with special reference to bacteria associated with bites. *Journal of Clinical Microbiology, 3,* 344-349.

198. Schlatter, L.K., Brenner, D.J., Steigerwalt, A.G., Moss, C.W., Lambert, M.A., & Packer, R.A. (1989). *Pasteurella caballi* a new species from equine clinical specimens. *Journal of Clinical Microbiology, 27,* 2169-2174.

199. Scott, P. C., Markham, J. F., and Whitjear, K. G. (2000). *Pasteurella multocida aro-A* mutant vaccine in chickens, *In* Proceedings of *Hemophilus, Actinobacillus* and *Pasteurella* (HAP) conferenceSeptember 13-17, Mabula, South Africa. p *43.*

200. Simensen, E. & Olson, L.D. (1980). Aerosol transmission of *Pasteurella multocida* in turkeys. *Avian Diseases, 24,* 1007-1010.

201. Smith, I.M. & Baskerville, A.J. (1983). A selective medium for the isolation of *P. multocida* in nasal specimens from pigs. *British Veterinary Journal, 139,* 476-486.

202. Sneath, P.H.A. & Stevens, M. (1990). *Actinobacillus rossii* sp. nov., *Actinobacillus seminis* sp.nov., nom.rev., *Pasteurella bettii sp.nov., Pasteurella lymphangitis* sp.nov., *Pasteurella mairii* sp. nov., and *Pasteurella trehalosi* sp.nov. *International Journal of Systematic Bacteriology, 40,* 148-153.

203. Snipes, K.P. & Biberstein, E.L. (1982). *Pasteurella testudinis* sp.nov.:a parasite of desert tortoises (*Gopherus agassizi*). *International Journal of Systematic Bacteriology, 32,* 201-210.

204. Snipes, K.P. & Hirsh, D.C. (1986). Association of complement sensitivity with virulence of *Pasteurella multocida* isolated from turkeys. *Avian Diseases, 30,* 500-504.

205. Snipes, K.P., Ghazikhanian, G.Y., & Hirsh, D.C. (1987). Fate of *Pasteurella multocida* in the blood vascular system of turkeys following intravenous inoculation: Comparison of an encapsulated, virulent strain with its avirulent, acapsular variant. *Avian Diseases, 31,* 254-259.

206. Snipes, K.P., Carpenter, T.E., Corn, L.J., Kasten, R.W., Hirsh, D.C., Hird, D.W., & McCapes, R.H. (1988). *Pasteurella multocida* in wild mammals and birds in California: Prevalence and virulence for turkeys. *Avian Diseases, 32,* 9-15.

207. Snipes, K.P., Hirsh, D.C., Kasten, R.W., Carpenter, T.E., Hird, D.W., & McCapes, R.H. (1990a). Differentiation of field isolates of *Pasteurella multocida* serotype 3,4 from live vaccine strain by genotypic characterization. *Avian Diseases, 34,* 419-424.

208. Snipes, K.P., Hirsh, D.C., Kasten, R.W., Carpenter, T.E., Hird, D.W., & McCapes, R.H. (1990b). Homogeneity of characteristics of *Pasteurella multocida* isolated from turkeys and wildlife in California, 1985-88. *Avian Diseases, 34,* 315-320.

209. Sonaiya, E. B. (1989). African network for rural poultry development. *In* 4th International DLG- Symposium on Poultry Production in Hot climates Section 2: Small holder poultry production in developing countries *131.*

210. Talan, D.A., Citron, D.M., Abrahamian, F.M., Moran, G.J., & Goldstein, E.J. (1999). Bacteriologic analysis of infected dog and cat bites. Emergency Medicine Animal Bite Infection Study Group [see comments]. *New England Journal of Medicine,*

211. Taylor, P.W. & Robinson, M.K. (1980). Determinants that increase the serum resistance of *Escherichia coli*. *Infection and Immunity, 11,* 278-280.

212. Threlfall, E.L. & Frost, J.A. (1990). The identification, typing and fingerprinting of *Salmonella* : laboratory aspects and epidemiological applications. *Journal of Applied Bacteriology, 68,* 5-16.

213. Townsend, K.M., Dawkins, H.J.S., & Papadimitrou, J.M. (1997). Analysis of haemorhagic septicaemia-causing isolates of *Pasteurella multocida* by ribotyping and field alternation gel electrophoresis (FAGE). *Veterinary Microbiology, 57,* 383-395.

214. Townsend, K.M., O'Boyle, D., Phan, D.T., Hahn, T.X., Wijewardana, T.G., Wilkie, B.N., Trung, N.T , & Frost, A.J. (1998a). Acute septicemic pasteurellosis in Vietnamese pigs. *Veterinary Microbiology, 63,* 205-215

215. Townsend, K.M., Frost, A.J., Lee, C.W., Papadimitriou, J.M , & Dawkins, H.J.S. (1998b). Development of PCR assays for species- and type-specific identification of *Pasteurella multocida* isolates. *Journal of Clinical Microbiology, 36,* 1096-1100.

216. Townsend, K.M., Hanh, T.X., O'Boyle, D., Wilkie, I., Phan, T.T., Wijewardana, T.G., Trung, N.T., & Frost, A.J. (2000). PCR detection and analysis of *Pasteurella multocida* from the tonsils of slaughtered pigs in Vietnam. *Vet Microbiol, 72,* 68-78.

217. Truscott, W.M. & Hirsh, D.C. (1988). Demonstration of an outer membrane protein with antiphagocytic activity from *Pasteurella multocida* of avian origin. *Infection and Immunity, 56,* 1538-1544.

218. Tsuji, M. & Matsumoto, M. (1989). Pathogenesis of fowl cholera: Influence of encapsulation on the fate of *Pasteurella multocida* after intravenous inoculation into turkeys. *Avian Diseases, 33,* 238-247.

219. Van Es, L. & Olney, J.F. (1940). An inquiry into the influence of environment on incidence of poultry diseases. *University of Nebraska Agricultural Experiment Station Research Bulletin, 118,* 17-21.

220. Van Sambeek, F., McMurray, B.L., & Page, R.K. (1995). Incidence of *Pasteurella multocida* in poultry house cats used for rodent control programmes. *Avian Diseases, 39,* 145-146.

221. Wachsmuth, K. (1986). Molecular epidemiology of bacterial infections: examples of methodology and of investigations of outbreaks. *Reviews of infectious diseases, 8,* 682-692.

222. Weinberg, E.D. (1978). Iron and infection. *Microbiological Reviews, 42,* 45-66.

223. Wilkie, I.W., Grimes, S.E., O'Boyle, D., & Frost, A.J. (2000). The virulence and protective efficacy for chickens of *Pasteurella multocida* administered by different routes. *Veterinary Microbiology, 72,* 57-68.

224. Wilson, M.A., Rimler, R.B., & Hoffman, L.J. (1992). Comparison of DNA fingerprints and somatic serotypes of serogroup B and E *Pasteurella multocida* isolates. *Journal of Clinical Microbiology, 30,* 1518-1524.

225. Wilson, M.A., Morgan, M.J., & Barger, G.E. (1993). Comparison of DNA fingerprinting and serotyping for identification of avian *Pasteurella multocida* isolates. *Journal of Clinical Microbiology, 31,* 255-259.

226. Yadav, M.P., Sharma, V.D., & Sethi, M.S. (1977). An outbreak of fowl cholera due to *Pasteurella gallinarum* in Uttar Pradesh (India). *Avian Diseases, 21,* 323-325.

227. Yongolo, M. G. S. (1996). Epidemiology of Newcastle disease in village chickens in Tanzania. Master of Veterinary Medicine. Sokoine University of Agriculture, Morogoro, Tanzania.

228. Zander D.V., Bermudez A.J., Mallison E.T. (1997). Principles of disease prevention, diagnosis and control. *In* B. W. Calnek, H. J. Barnes, C. W. Beard, L. R. McDougald,and Y. M. Saif. (Eds.), Diseases of Poultry. London, Mosby Wolfe, pp. *3-73.*

229. Zhao, G., Pijoan, C., Choi, K., Maheswaran, S.K., & Trigo, E. (1995). Expression of iron-regulated outer membrane proteins by porcine strains of *Pasteurella multocida. Canadian Journal of Veterinary Research, 59,* 46-50.

APPENDIX I

Investigations on the carrier rate of *Pasteurella multocida* in healthy commercial poultry and flocks affected by fowl cholera

Investigations on the carrier rate of *Pasteurella multocida* in healthy commercial poultry flocks and flocks affected by fowl cholera

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Twenty flocks of web-footed birds (Pekin and Muscovy ducks and geese) and eight flocks of chickens raised under intensive management were examined for the presence of carriers of *Pasteurella multocida*. Five hundred and seventy-eight web-footed birds and 240 chickens from healthy flocks, as well as from flocks affected by fowl cholera, were investigated. A total of 135 isolates (80 from healthy flocks and 55 from flocks affected by fowl cholera) were obtained from the pharyngeal and cloacal mucosae after mouse passage (134 isolates) and culture in selective medium (one isolate). Thirty-five percent (7/20) of the flocks of web-footed birds and 38% (3/8) of chicken flocks had birds carrying *P. multocida* in the pharynx and/or cloaca. Birds from flocks affected by fowl cholera carried *P. multocida* at a significantly higher prevalence in the mucosa of the cloaca ($P < 0.001$) compared with the pharynx, while the opposite was observed in birds from healthy flocks. Extended phenotypic characterization confirmed the presence of *P. multocida* ssp. *multocida*, *P. multocida* ssp. *septica* and *P. multocida* ssp. *gallicida* in the flocks examined. *P. multocida* ssp. *gallicida* was exclusively isolated from Pekin ducks, while *P. multocida* ssp. *multocida* and *P. multocida* ssp. *septica* were obtained from chickens as well as web-footed birds. Each flock was shown to be infected by a single phenotypic clone, but some clones were found in more than one flock. A different clone was found in each of four outbreaks of fowl cholera on one of the farms in the preceding 2 years. Two genotypic and phenotypic clones each of *P. multocida* ssp. *multocida* and *P. multocida* ssp. *septica* were found. This observation indicated that outbreaks are usually clonal and that elimination of *P. multocida* from infected farms is possible. The results suggest that healthy poultry, in addition to convalescent carriers, may also be carriers of *P. multocida*. However, the virulence of *P. multocida* isolates and resistance of carriers to clinical infection needs to be examined. This is the first report of isolation of *P. multocida* from the cloacal mucosa of apparently healthy domestic poultry. Sampling of the cloaca appeared to be more sensitive for detecting carriers of *P. multocida*. Although selective medium was used only to a limited extent, the results suggested that mouse inoculation was a more efficient method of isolating *P. multocida* from poultry than the use of selective media.

Introduction

Despite considerable research on fowl cholera in domestic and wild birds, the source of new infections in the flocks is still uncertain. *Pasteurella multocida* has been consistently found in the upper respiratory tract, spleen, lungs, blood and liver of infected birds (Rhoades, 1964; Hunter & Wobeser, 1980). Studies in chickens and turkeys found *P. multocida* in the mucosae of the pharynx and trachea of carrier chickens and turkeys (Mraz *et al.*, 1980; Curtis & Ollerhead, 1981). Little has been done to investigate *P. multocida* in sites other than the upper respiratory tract in healthy carrier chickens since Iliev *et al.* (1964) failed to detect viable *P. multocida* in the faeces of chickens. However,

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Bisgaard (1995) isolated *P. multocida* ssp. *multocida* from the oviduct of ducks and geese suffering from salpingitis, and also isolated *P. multocida* ssp. *multocida* from the cloacae and penises of geese from a flock with mortalities due to salpingitis and peritonitis.

Comparative studies of flocks of chickens and turkeys either exposed or unexposed to *P. multocida* by Curtis & Ollerhead (1981), Snipes *et al.* (1988) and Carpenter *et al.* (1989) revealed that *P. multocida* carriers were present only in flocks that had previously experienced fowl cholera. In a longitudinal study, Pritchett *et al.* (1930) isolated the bacterium for up to 4 months after a fowl cholera outbreak in a chicken flock. However, it is not known if the same clone was involved because reliable methods for investigating clonality did not exist at that time. Sulong & Maheswaran (1976) detected *P. multocida* by immunofluorescence in tissues of turkeys vaccinated with a virulent strain for up to 3 weeks after vaccination. All these studies suggest that some birds remain carriers of *P. multocida* for some time after exposure.

The recovery of *P. multocida* from different hosts has mainly relied on mouse inoculation and *in vitro* culture in selective medium (Curtis & Ollerhead, 1981; Baldrias *et al.*, 1988). Mouse passage seems to represent a more efficient and widely accepted method of detecting *P. multocida*, although it may only select strains pathogenic for mice (Baldrias *et al.*, 1988). Selective media have been devised for different host species of *P. multocida*, but efficacies of these media have not been compared (Rimler & Rhoades, 1989). Moore *et al.* (1994) demonstrated that selective enrichment coupled with blood agar inoculation have potential for increasing the isolation rate of *P. multocida* from contaminated samples. However, the necessity of employing two media might be a disadvantage in large-scale epidemiological studies. Serological techniques for detection of carriers of *P. multocida* appear to be of little use, as non-carriers may have antibodies to *P. multocida* (Rimler & Rhoades, 1989; Donnio *et al.*, 1994). Furthermore, with the ongoing reclassification of the genus *Pasteurella* (Mutters *et al.*, 1985), application of serological studies to identify the exact species or subspecies of *Pasteurella* might be more difficult.

Serological and biochemical characterization of isolates have been widely applied in epidemiological typing of *P. multocida* (Donahue & Olson, 1972; Heddleston *et al.*, 1972). However, in the past decade, it has been shown that chromosomal DNA studies are more discriminatory than phenotypic features in establishing the clonality of strains of *P. multocida* (Wilson *et al.*, 1995; Christensen *et al.*, 1998). Recent investigations of two outbreaks of fowl cholera in birds in Denmark demonstrated that the two outbreaks which appeared at different localities were caused by the same clone, strongly suggesting spread by migratory birds (Christensen *et al.*, 1998). Subsequent investigations indicated that wild birds can also transmit infection to domestic birds (Christensen *et al.*, 1999). Although cats and dogs are usually asymptomatic carriers (Baldrias *et al.*, 1988; Ganiere *et al.*, 1993), their significance in the epidemiology of fowl cholera remains to be confirmed.

It was the objective of the present work to determine the carrier rate of *P. multocida* in flocks of ducks, geese and chickens that had a confirmed history of fowl cholera and in flocks that had never experienced disease. Isolates were characterized phenotypically and genotypically to look for clonal relationships between strains, within and between the flocks.

Materials and Methods

Study population

Except for a single flock of Muscovy ducks, in which only eight ducks were sampled, 30 birds were sampled from each flock to allow a detection level of 10% with 95% certainty (Cannon & Roe, 1982). A total of 578 web-footed birds from 20 flocks and 240 chickens from eight flocks were sampled. Healthy birds and flocks investigated included three parent flocks of Pekin ducks from different farms, seven flocks of parent Pekin ducks from different houses on the same farm with history of bacteriologically confirmed fowl cholera in the previous flocks, and three independent flocks of Muscovy ducks (one flock of parents and two fattening flocks). Other healthy flocks sampled were two parent flocks of geese representing different farms, five fattening flocks of Pekin ducks originating from five different farms, four broiler parents flocks (one flock vaccinated against fowl cholera with the killed vaccine (Pabac, Fort Dodge Laboratories, Iowa, USA)) and two flocks of layers, all of which originated from different farms. One flock of broiler parents and one flock of brown layers investigated were reported to have mortalities caused by fowl cholera at the time of sampling. With the exception of seven flocks of parent Pekin ducks, healthy flocks were randomly selected and came from different geographical areas.

Isolation and bacteriological investigations

The birds were swabbed separately with sterile cotton-tipped applicators on the pharynx and cloaca. From nine flocks, each swab was streaked on a selective medium (Tryptose blood agar base (Difco) to which 5% citrated bovine blood, 0.02% bacitracin and 1% neomycin were added). The swabs were subsequently placed in 2 ml tryptophan broth (Difco) or veal infusion broth (Difco) and kept on ice during transport. The swabs were subsequently vortexed and 0.25 ml of the material injected intraperitoneally into 21-day-old Balb C white mice. From 19 flocks, attempts to isolate *P. multocida* included only inoculation of mice. In two fattening flocks of Muscovy ducks, the swabs from cloaca and pharynx were mixed before mouse inoculation.

Spleens from the dead mice were aseptically removed and macerated under sterile conditions, and the material cultured on blood agar without antibiotics. After overnight incubation at 37C under aerobic conditions, colonies morphologically resembling those of *P. multocida* and containing non-motile Gram-negative rods, which were facultatively anaerobic, fermented glucose without gas formation, and catalase- and oxidase-positive, were subcultured and stored at −80C. The strains were subsequently characterized by standard phenotypic methods described by Bisgaard *et al.* (1991).

On a farm rearing fattening Muscovy ducks (flock number 5) from which *P. multocida* was isolated in 1998 during this study, fowl cholera had been diagnosed in 1996 and 1997, but not in 1998. Although a multiple age operation, the farm was free of birds once per year for cleaning and disinfection. Two and six strains of *P. multocida* were isolated from Muscovy ducks that died in two distinct outbreaks of

Table 1. *Frequency of isolation and subspecies of* P. multocida *found in flocks infected with* P. multocida

Flock number	Breed of poultry and type	History	Subspecies of *P. multocida*	*P. multocida*					
				Selective medium			Mouse inoculation		
				Pharynx	Cloaca	Pharynx	Cloaca	Pharynx and cloaca	Total (%)
1	Broilers, parents	Fowl cholera	*multocida*	ND	ND	8/30	20/30	4/30	24/30 (80%)
2	Muscovy ducks, parents	Apparently healthy	*multocida*	0/8	0/8	4/8	2/8	2/8	5/8 (63%)
3	Pekin ducks, parents	Apparently healthy	*multocida*	ND	ND	14/30	9/30	4/30	19/30 (63%)
4	Geese, parents	Apparently healthy	*multocida*	ND	ND	1/30	0/30	0/30	1/30 (3%)
5	Muscovy ducks, fattening	Apparently healthy	*septica*	0/30	0/30	(16/30)[a]	(16/30)[a]	(16/30)[a]	16/30 (53%)
6	Brown layers	Fowl cholera	*septica*	0/30	1/30	5/30	21/30	4/30	22/30 (73%)
7	Broilers, parents	Apparently healthy, vaccinated against fowl cholera	*septica*	ND	ND	10/30	4/30	0/30	14/30 (47%)
8	Pekin ducks, parents	Apparently healthy	*gallicida*	ND	ND	1/30	0/30	0/30	1/30 (3%)
9	Pekin ducks, parents	Apparently healthy	*gallicida*	0/30	0/30	1/30	4/30	0/30	5/30 (17%)
10	Pekin ducks, parents	Apparently healthy	*gallicida*	ND	ND	1/30	12/30	0/30	13/30 (43%)
		Total		0/98 (0%)	1/98 (1%)	45/248 (18%)	72/278 (29%)	13/248 (5%)	120/278 (43%)

ND, Not done.

[a](16/30), Swabs from pharynx and cloaca of the same duck were mixed before inoculation, not included in the totals.

fowl cholera in 1996 and 1997, respectively, and were available for comparative investigations.

Restriction endonuclease analysis and ribotyping

Restriction endonuclease analysis and ribotyping were only performed on isolates from the aforementioned farm (Table 1, flock number 5). Chromosomal DNA analysis was performed on all 16 strains recovered from the pharynx/cloaca of a clinically normal flock of fattening Muscovy ducks in 1998, and two and six strains obtained from ducks which died of fowl cholera in 1996 and 1997, respectively. Isolation of DNA and digestion with restriction endonuclease HpaII (Boehringer-Mannheim, Mannheim, Germany) and subsequent electrophoretic separation of DNA fragments by agarose gel electrophoresis was performed as previously described by Christensen *et al.* (1993). The DNA was transferred to nylon membranes (Hybond-N, Amersham Int., Amersham, Bucks, UK) by vacuum blotting. DNA was fixed to the membrane by baking at 80C for 60 min. Ribosomal RNA (16s and 23s) from *Escherichia coli* were bought and labelled with digoxigenin using reverse transcriptase (Boehringer-Mannheim) as reported previously by Christensen *et al.* (1993). The probes were hybridized, and membrane-fixed DNA developed under the conditions described by Christensen *et al.* (1993).

Statistical analysis

Statistical analysis of data to determine significance of results was performed using the Chi-square test.

Results

The prevalence of *P. multocida* in different species of poultry and the distribution of different subspecies of *P. multocida* are summarized in Table 1. *P. multocida* was demonstrated in seven out of 20 apparently healthy flocks of web-footed birds examined (35%) and in three out of eight chicken flocks, two of which suffered from fowl cholera. Forty-five and 72 strains were obtained from pharynx and cloaca, respectively, by mouse inoculation. Sixteen more strains were obtained from a flock of Muscovy ducks from which the swabs from pharynx and cloaca of the same bird were mixed before mouse inoculation. A single strain was obtained from the cloaca by isolation from selective medium (Table 1).

P. multocida ssp. *multocida*, *P. multocida* ssp. *septica* and *P. multocida* ssp. *gallicida* were obtained from the cloaca and/or pharynx of four, three and three flocks, respectively (Table 1). *P. multocida* ssp. *multocida* was demonstrated in parent flocks of broilers, Muscovy ducks, Pekin ducks and geese. *P. multocida* ssp. *septica* was isolated from fattening Muscovy ducks, broiler parents and brown layers, while *P. multocida* ssp. *gallicida* was isolated from birds in three different flocks of parent Pekin ducks on the same farm. The history of the *P. multocida* positive flocks is given in Table 1.

The prevalence of *P. multocida* ssp. *multocida* carriers in the broiler parent flock (80%) affected by fowl cholera was not significantly higher ($P > 0.05$) than that of healthy flocks of Muscovy ducks (63%) and Pekin ducks (63%) (Table 1). However, the prevalence of *P. multocida* ssp. *multocida* in the goose flock was significantly lower ($P < 0.001$) than in other flocks carrying *P. multocida* ssp. *multocida*. Twenty-eight isolates were obtained from 24 broiler parents (flock number 1), where the prevalence of cloacal carriers, 20/24 (83%), was significantly higher ($P < 0.001$) than that of pharyngeal carriers, 8/24 (33%), and that of carriers in both the pharynx and cloaca, 4/24 (17%).

In apparently normal flocks of Pekin ducks, Muscovy ducks and geese (flocks number 2 to 4), 25 carriers of *P. multocida* ssp. *multocida* were found. Nineteen carriers (76%) were shown to harbour *P. multocida* in the pharynx, a significantly higher proportion ($P < 0.01$) than that of cloacal carriers, 11/25 (44%), and of carriers on the mucosa

Table 2. *Phenotypic characteristics separating different clones of* P. multocida

Flock number	1 Broiler parents	2 Muscovy ducks	3 Pekin ducks	4 Geese	5 Muscovy ducks	6 Layers	7 Broiler parents	8 White Pekin ducks	9 White Pekin ducks	10 White Pekin ducks
L(+)-Arabinose	–	–	–	–	(+)	–	–	+	+	+
D (–)-Arabinose	(+)	–	–	–	(+)	(+)	(+)	(+)	(+)	(+)
D(+)-Xylose	+	–	+	+	(+)	+	+	–	–	–
Dulcitol	–	–	–	–	–	–	–	+	+	+
D(–)-Sorbitol	+	+	+	+	–	–	–	+	+	+
L(–)-Fucose	(+)	–	–	–	(+)	(+)	(+)	(+)	(+)	(+)
ONPG[a]	–	+	–	–	–	–	–	–	-	–
Trehalose	+	–	–	–	+	+	+	–	–	–
PNPG[b]	+	–	–	–	+	+	+	–	–	–
Subspecies	*multocida*	*multocida*	*multocida*	*multocida*	*septica*	*septica*	*septica*	*gallicida*	*gallicida*	*gallicida*

+, all strains positive within 1 to 2 days; (+), all strains late positive (3 days); –, all strains negative within 14 days.
[a]ONPG, β-galactosidase.
[b]PNPG, α-galactosidase.

of both pharynx and cloaca, 5/25 (20%). However, the prevalence of carriers of *P. multocida* ssp. *multocida* in the cloaca was significantly higher ($P < 0.005$) than that of carriers in both the cloaca and pharynx.

Three phenotypic variants of *P. multocida* ssp. *multocida* were distinguished: one from geese and Pekin ducks; and two different variants from Muscovy ducks and a flock of broiler parents (Table 2). Differences in fermentation of D(−)-arabinose, D(+)-xylose, L(−)-fucose, trehalose, β-galactosidase (ONPG) and α-galactosidase (PNPG) separated the *P. multocida* ssp. *multocida* variants. Isolates from the same flock had identical phenotypic characteristics.

Of the three flocks with *P. multocida* ssp. *septica*, only the flock of brown layers (flock number 6) was reported to have mortalities caused by fowl cholera (Table 1). The prevalence of *P. multocida* ssp. *septica* in healthy layers in this flock (73%) was significantly higher ($P < 0.001$) than that in flock number 5, the apparently healthy fattening Muscovy ducks (53%), and flock number 7 of vaccinated broiler parents (47%). However, there was no significant difference ($P > 0.05$) between the prevalence of *P. multocida* ssp. *septica* in vaccinated broiler parents compared with fattening Muscovy ducks. Excluding results from the flock of fattening Muscovy ducks, where swabs from the pharynx and cloaca were pooled before mouse inoculation, five chickens from the flock affected with fowl cholera had *P. multocida* ssp. *septica* in the pharynx, 21 in the cloaca, and four chickens had *P. multocida* ssp. *septica* in both the pharynx and cloaca. Thus, a significantly higher number ($P < 0.001$) of chickens from the flock affected with fowl cholera carried *P. multocida* ssp. *septica* in the cloaca compared with the pharynx, while the opposite was observed for the apparently healthy, vaccinated flock of broiler parents.

Two phenotypic variants of *P. multocida* ssp. *septica* separated by late fermentation of L(+)-arabinose were identified in the present study, while strains within each flock appeared to be clonal. Fermentation of L(+)-arabinose was not observed in strains from brown layers and parent broilers, while isolates from fattening Muscovy ducks appeared to be late fermenters. The isolates from fattening Muscovy ducks were shown to be clonal by restriction endonuclease analysis and ribotyping. Comparison of the present (1998) isolates of *P. multocida* ssp. *septica* with outbreak strains from the same farm showed that a different clone of *P. multocida* ssp. *septica* was involved in one of two outbreaks that occurred in 1997. The clones differed in their reactions with L(+)-arabinose, trehalose and PNPG. *P. multocida* ssp. *multocida* was obtained from the same farm in 1996 and in the second 1997 outbreak, but these clones were different in their reactions with lactose, trehalose, ONPG, and PNPG (data not shown).

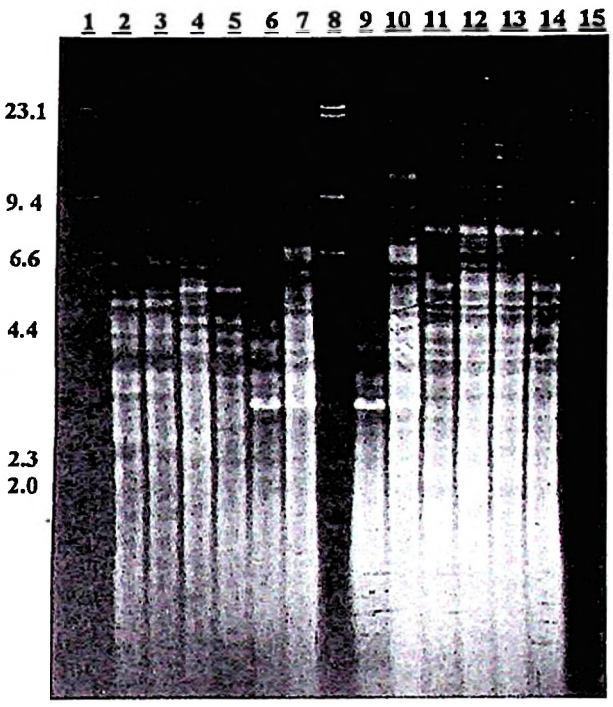

Figure 1. Hpa*II REA profiles of 1996 to 1998 strains from fattening Muscovy ducks. Lane 1, Molecular weight marker phage lambda DNA digested with* Hind*III; lanes 2 and 3,* P. multocida *ssp.* septica *from 1997 outbreak; lanes 4 and 5,* P. multocida *ssp.* multocida *from 1996 outbreak; lane 8, phage lambda DNA digested with* Hind*III; lanes 6 7, 9 and 10,* P. multocida *ssp.* multocida *from 1997 outbreak; lanes 11 to 14,* P. multocida *ssp.* septica *from 1998 carriers; lane 15, phage lambda DNA digested with* Hind*III.*

P. multocida ssp. *gallicida* was found in three flocks of healthy Pekin ducks originating from different houses on the same farm. Previous flocks on this farm had experienced outbreaks of fowl cholera caused by the same subspecies. The prevalence of *P. multocida* ssp. *gallicida* differed significantly ($P < 0.001$) among the three flocks. The highest prevalence was 43% in one flock, and the prevalences were 17 and 3% in the other two flocks (Table 1). Cloacal carriage was shown in 84% (16/19) of *P. multocida* ssp. *gallicida*-positive ducks, significantly higher ($P < 0.001$) than the proportion of pharyngeal carriers, 16% (3/19). No duck was found to carry *P. multocida* ssp. *gallicida* in both the pharynx and cloaca. All isolates of *P. multocida* ssp. *gallicida* obtained in this study belonged to the same phenotypic type.

Selective medium recovered only a single isolate of *P. multocida* ssp. *septica* from the cloaca of a brown layer, while isolates were obtained from 22 of 30 layers by mouse inoculation. Three other flocks (numbers 2, 5 and 9) from which isolates were obtained by mouse inoculation were negative on selective medium. The sensitivity of the selective medium was significantly lower ($P < 0.001$) than mouse passage, but only four *P. multocida*-

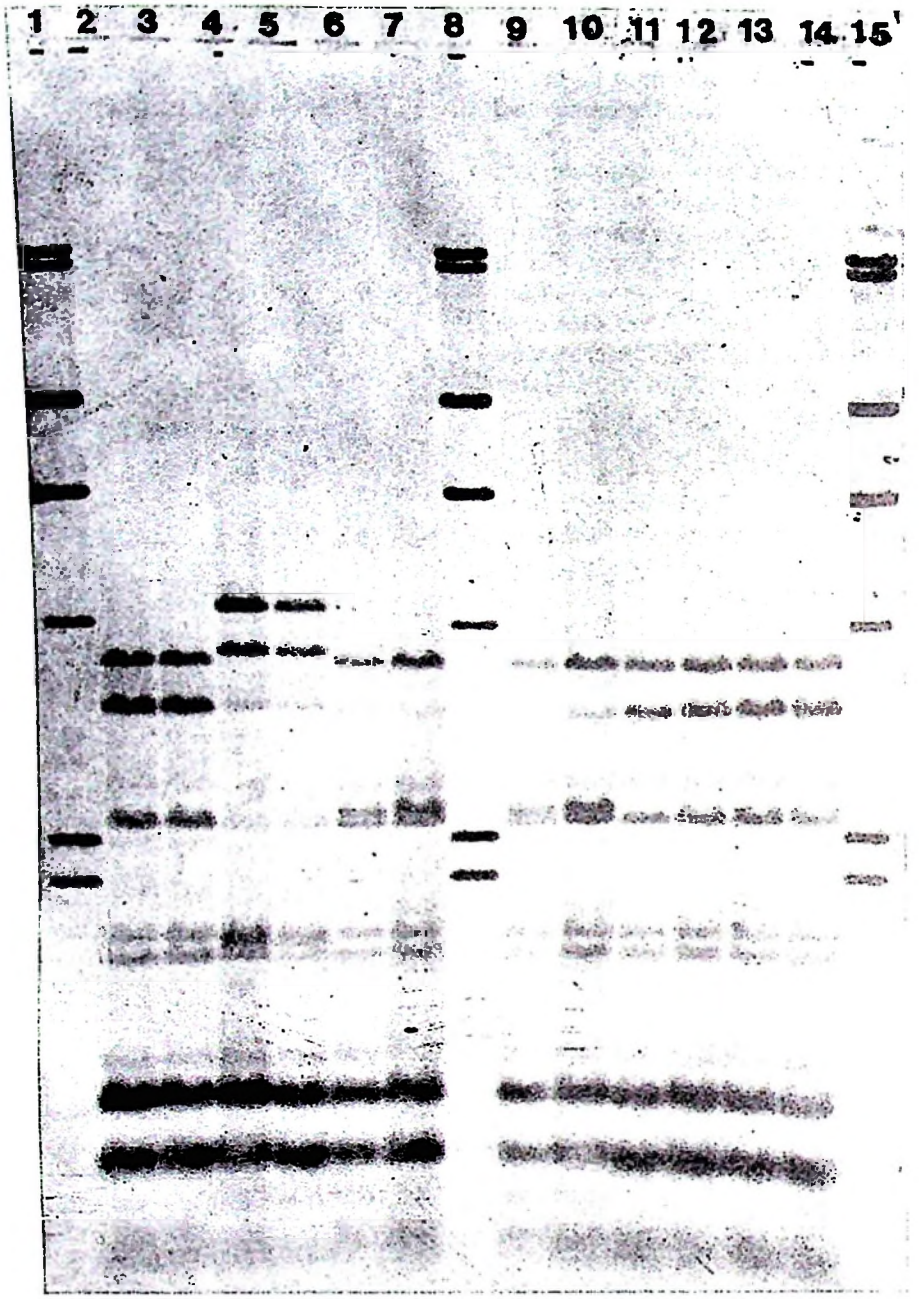

Figure 2. *The ribotypes obtained from digests in Figure 1a.* HpaII *REA profiles of 1996 to 1998 strains from fattening Muscovy ducks. Lane 1, Molecular weight marker phage lambda DNA digested with* HindIII*; lanes 2 and 3,* P. multocida *ssp. septica from 1997 outbreak; lanes 4 and 5,* P. multocida *ssp. multocida from 1996 outbreak; lane 8, phage lambda DNA digested with* HindIII*; lanes 6 7, 9 and 10,* P. multocida *ssp. multocida from 1997 outbreak; lanes 11 to 14,* P. multocida *ssp. septica from 1998 carriers; lane 15, phage lambda DNA digested with* HindIII.

positive flocks were investigated by both mouse inoculation and selective medium (Table 1).

Restriction endonuclease analysis and ribotyping

Restriction endonuclease analysis (REA) and ribotyping of strains from fattening Muscovy ducks showed that the two outbreak strains of *P. multocida* ssp. *multocida* from 1996 were clonal, both by ribotyping and REA typing (Figures 1 and 2). These strains were easily differentiated from four outbreak strains from 1997 on both REA and ribotyping. Differences included several bands in both ribotyping and REA typing. These results agreed with phenotypic findings that the strains were different.

REA profiles of the outbreak clone of *P. multocida* ssp. *septica* from 1997 were also different from the 1998 clone obtained from carrier ducks, but they had the same ribotype pattern. The 1998 clone possessed the REA fragments of

approximately 23 and 16 kb that were not found in the 1997 outbreak clone. More differences were seen in the size and number of smaller fragments (Figures 1 and 2). Each of the two REA types obtained corresponded to a different phenotypic clone.

Discussion

To the knowledge of the authors, systematic investigations on the prevalence of *P. multocida* and its three subspecies have not been performed since the genus *Pasteurella sensu stricto* was reorganized by Mutters *et al.* (1985). Consequently, very little is known about the prevalence of healthy carriers. In addition, very little is known about the number of clones involved in individual outbreaks of fowl cholera. Only recently, the same clone of *P. multocida* ssp. *multocida* was shown to be involved in two outbreaks of fowl cholera in birds in Denmark (Christensen *et al.*, 1998). In the present study, all three subspecies of *P. multocida* (*P. multocida* ssp. *multocida*, *P. multocida* ssp. *septica* and *P. multocida* ssp. *gallicida*) were demonstrated in commercial poultry in Denmark. In each flock, a single phenotypic clone was found. Sixteen strains from a single flock were shown to be clonal by chromosomal DNA analysis.

In flocks affected with fowl cholera, the prevalence of *P. multocida* ssp. *multocida* in broiler parents was 80% and that of *P. multocida* ssp. *septica* in brown layers was 73%. Although these were not significantly different ($P > 0.05$) from some of the clinically normal flocks, most of the other flocks had a significantly lower prevalence ($P < 0.05$). *P. multocida* ssp. *gallicida*, which more frequently seems to be a pathogen of web-footed birds (Mutters *et al.*, 1985), was recovered only from healthy ducks, but previous flocks at the same site had experienced fowl cholera caused by the same clone (Bisgaard, unpublished data). The present results support the findings of Mraz *et al.* (1980), who found a higher prevalence of *P. multocida* in convalescent chicken flocks (25.5%), than in disease-free flocks (6.7%). From a total of 120 different birds found to carry *P. multocida*, almost equal proportions were carrying subspecies *multocida* and *septica* (49 and 52, respectively), and a smaller proportion were carrying subspecies *gallicida* (19 birds). These findings are contrary to those of Fegan *et al.* (1995), who found subspecies *multocida* to be the predominant isolate from poultry while the other two subspecies only occurred sporadically.

In flocks of broiler parents and brown layers suffering from fowl cholera caused by *P. multocida* ssp. *multocida* and *P. multocida* ssp. *septica*, respectively, the proportion of cloacal carriers were significantly greater ($P < 0.05$) than that of pharyngeal carriers. These results confirm previous observations by Bisgaard (1995) but contradict the findings by Iliev *et al.* (1964), who found that *P. multocida* was destroyed in the proventriculus and no viable form was found in the cloaca. However, further studies on colonization and multiplication of *P. multocida* are needed to clarify the pathogenesis. The present findings emphasize the importance of sampling the cloaca of birds when investigating the epidemiology of fowl cholera. Cloacal carriage may also contribute to the shedding of bacteria from the carrier, which may add to the rapid spread of infection in the flock.

The pathogenicity of *P. multocida* has been shown to be increased by bird-to-bird passage over a short period of time (Matsumoto & Strain, 1993). This may indicate that natural passage of *P. multocida* between the birds may enhance the virulence of a normally avirulent strain, leading to clinical disease in a flock of carriers. The presence of distinct clones of *P. multocida* in positive flocks in the current study indicates introduction from a common source, which was possibly followed by bird-to-bird transmission within the flocks. However, the effect of *in vivo* passage on the virulence of subspecies of *P. multocida* for different hosts remains to be investigated. Meanwhile, the presence of *P. multocida* should be viewed as a potential risk factor whenever it is found in a flock of birds.

Significant phenotypic variations have been reported previously for *P. multocida* (Heddleston, 1976; Blackall *et al.*, 1995; Christensen *et al.*, 1998). Although each flock investigated harboured a single phenotypic clone, there were overlaps where one clone affected two or more flocks. Further investigations on *P. multocida* ssp. *septica* from a flock of fattening Muscovy ducks by REA and ribotyping showed that all 1998 isolates were also clonal by DNA analysis. Comparison of phenotypic features with previous fowl cholera outbreak strains from this farm revealed two different clones of *P. multocida* ssp. *multocida* in 1996 and 1997, and a separate *P. multocida* ssp. *septica* in a different outbreak in 1997. All these strains were further shown to differ genotypically (Figure 1a). The clonal nature of *P. multocida* demonstrated in different flocks on this farm (some of which were associated with fowl cholera) suggests that multiple introductions are rare and that clones of low virulence exist. Observations from the same farm over a period of 3 years also indicated that it was possible to eliminate *P. multocida* from infected farms.

Diversity of clones of *P. multocida* involved in different fowl cholera outbreaks has been demonstrated elsewhere (Carpenter *et al.*, 1991; Blackall *et al.*, 1995). Blackall *et al.* (1995) demonstrated various subspecies, biovars and genotypes involved in fowl cholera. In that study, other common factors such as stress were considered to be responsible for precipitating outbreaks of fowl cholera, because no

correlation was found between proximity of the farms and the genotypic clones involved in the outbreaks. The presence of other factors precipitating fowl cholera might partly explain the occurrence of a clone not associated with disease subsequent to different clones associated with outbreaks on the farm of fattening Muscovy ducks (farm number 5). Furthermore, this indicates the risk of outbreaks in flocks with healthy carriers of *P. multocida*.

Induction of immunity by *P. multocida* killed vaccines has been reported to be more specific for homologous strains than for other strains (Heddleston *et al.*, 1970). In the present study, 47% of vaccinated broiler parents were shown to carry *P. multocida* ssp. *septica* on either the pharyngeal and/or cloacal mucosa. Although this prevalence is significantly lower ($P < 0.001$) than that of a flock of brown layers with fowl cholera (73%), it is uncertain whether the strains in the Pabac vaccine, which include only *P. multocida* ssp. *multocida* and *P. multocida* ssp. *gallicida* (serotypes 1,3, and 4 according to Heddleston), might have any effect on immunity to infections caused by *P. multocida* ssp. *septica*. Furthermore, the pathogenicity of the isolated strains needs to be known before any conclusions can be made on the effect of the vaccine on the development of clinical disease.

Selective media have been reported to be efficient in isolating *P. multocida* from dogs and cats (Baldrias *et al.*, 1988; Ganiere *et al.*, 1993). However, in the current study, a higher recovery rate of *P. multocida* was obtained by mouse passage. Moore *et al.* (1994) showed that double selective treatment provided by selective enrichment broth and selective agar lowers the isolation rate of *P. multocida* from both pure and contaminated samples. Inclusion of antibiotics in the medium might select for other bacteria that could inhibit *P. multocida*. In our experiments, we found that the isolates obtained by inoculation of mice, and subsequently inoculated on selective medium, grew well within 24 h of incubation. Additional studies on the interaction between *P. multocida* and the normal microflora of poultry are required to understand its effect on the selection of *P. multocida*.

In summary, this study has established the presence of *P. multocida* in poultry kept under intensive commercial management in Denmark. The birds were found to harbour *P. multocida* in either the cloaca or the pharynx and, in some birds, in both sites. Most of these isolates were obtained by mouse inoculation. Further investigations are needed to develop agar-based selective media for the isolation of *P. multocida* from avian species to avoid using experimental mice. It is recommended that when carriers of *P. multocida* are looked for, both the cloaca and the pharynx should be sampled for isolation.

References

Baldrias, L., Frost, A.J. & O'Boyle, D. (1988). The Isolation of *Pasteurella*-like organisms from the tonsillar region of dogs and cats. *Journal of Small Animal Practice, 29*, 63–68.

Bisgaard, M. (1995). Salpingitis in web-footed birds: prevalence, aetiology and significance. *Avian Pathology, 24*, 443–452.

Bisgaard, M., Abdullahi, M.Z. & Gilmour, N.J.L. (1991). Further studies on the identification of *Pasteurellaceae* from cattle lungs. *Veterinary Record 128*, 428–429.

Blackall, P.J., Pahoff, J.L., Marks, D., Fegan, N. & Morrow, C.J. (1995). Characterization of *Pasteurella multocida* isolated from fowl cholera on turkey farms. *Australian Veterinary Journal, 72*, 135–138.

Cannon, R.M. & Roe, R.T. (1982). *Livestock Disease Surveys: A Field Manual for Veterinarians*. Canberra: Australian Government Publishing Service.

Carpenter, T.E., Hirsh, D.C., Kasten, R.W., Hirth, D.W., Snipes, K.P. & McCapes, R.H. (1989). *Pasteurella multocida* recovered from live turkeys: prevalence and virulence in turkeys. *Avian Diseases, 33*, 12–17.

Carpenter, T.E., Snipes, K.P., Kasten, R.W., Hird, D.W. & Hirsh, D.C. (1991). Molecular epidemiology of *Pasteurella multocida*. *American Journal of Veterinary Research, 52*, 1345–1349.

Christensen, J.P., Olsen, J.E. & Bisgaard, M. (1993). Ribotypes of *Salmonella enterica* serovar *gallinarum* and *pullorum*. *Avian Pathology, 22*, 725–738.

Christensen, J.P., Dietz, H.H. & Bisgaard, M. (1998). Phenotypic and genotypic characters of isolates of *Pasteurella multocida* obtained from two outbreaks of avian cholera in avifauna in Denmark. *Avian Pathology, 27*, 373–381.

Christensen, J.P., Petersen, K.D., Hansen, H.C. & Bisgaard, M. (1999). Occurrence of fowl cholera in Danish wild birds, and poultry production. Possible connections. *Dansk-Veterinaertidsskrift, 82*, 342–346

Curtis, P.E. & Ollerhead, G.E. (1981). Investigation to determine whether healthy chickens and turkeys are oral carriers of *Pasteurella multocida*. *Veterinary Record, 108*, 206–207.

Donahue, J.M. & Olson, L.D. (1972). Characteristics of isolates of *Pasteurella multocida* obtained on consecutive or different years from the same turkey farms. *Avian Diseases, 16*, 529–534.

Donnio, P.Y., Goff, C.L., Avril, J.L., Pouedras, P. & Gras-Rouzet, S. (1994). *Pasteurella multocida*: oropharygeal carriage and antibody response in breeders. *Veterinary Research, 25*, 8–15.

Fegan, N., Blackall, P.J. & Pahoff, J.L. (1995). Phenotypic characterization of *Pasteurella multocida* isolates from Australian poultry. *Veterinary Microbiology, 47*, 281–286.

Ganiere, J.P., Escande, F., Andre, G. & Larrat, M. (1993). Characterization of *Pasteurella* from gingival scrapings of dogs and cats. *Comparative Immunology, Microbiology and Infectious Diseases, 16*, 77–85.

Heddleston, K.L. (1976). Physiologic characteristics of 1268 cultures of *Pasteurella multocida*. *American Journal of Veterinary Research, 37*, 745–747.

Heddleston, K.L., Gallagher, J.E. & Rebers, P.A. (1970). Fowl cholera: immune response in turkeys. *Avian Diseases, 14*, 626–635.

Heddleston, K.L., Goodson, T., Leibovitz, L. & Angstrom, C.I. (1972). Serological and biochemical characteristics of *Pasteurella multocida* from free flying birds and poultry. *Avian Diseases, 16*, 729–734.

Hunter, B. & Wobeser, G. (1980). Pathology of experimental avian cholera in mallard ducks. *Avian Diseases, 24*, 403–414.

Iliev, T.R., Atsov, G.G. & Iovchev, E. (1964). The carrier state in fowl cholera. *Veterinary Bulletin, 34*, 129.

Matsumoto, M. & Strain, J.G. (1993). Pathogenicity of *Pasteurella multocida*; its variable nature demonstrated by in vivo passages. *Avian Diseases, 37*, 781–785.

Moore, M.K., Cienjak-Chubbs, L. & Gates, R.J. (1994). A new selective enrichment procedure for isolating *Pasteurella multocida* from avian and environmental samples. *Avian Diseases, 38*, 317–324.

Mraz, O., Sisak, F. & Jelen, P. (1980). The *Pasteurella* carriers in farm and laboratory animals. *Comparative Immunology, Microbiology and Infectious Diseases, 2*, 437–445.

Mutters, R., Ihm, P., Pohl, S., Frederiksen, W. & Mannheim, W. (1985). Reclassification of the genus *Pasteurella* Trevisan 1887 on the basis of deoxyribonucleic acid homology, with proposal for the new species *Pasteurella dagmatis*, *Pasteurella stomatis*, *Pasteurella anatis* and *Pasteurella langaa*. *International Journal of Systematic Bacteriology, 35*, 309–322.

Pritchett, I.W., Beaudette, F.R. & Hughes, R.W. (1930). Epidemiology of fowl cholera. IV Field observations of the spontaneous disease. *Journal of Experimental Medicine, 52*, 249–258.

Rhoades, K.R. (1964). Microscopic lesions of acute fowl cholera in mature chickens. *Avian Diseases, 8*, 658–665.

Rimler, R.B. & Rhoades, K.R. (1989). *Pasteurella multocida*. In C. Adlam & J.M. Rutter (Eds.), *Pasteurella and Pasteurellosis*, 1st edn (pp. 37–73). London: Academic Press.

Snipes, K.P., Carpenter, T.E., Corn, L.J., Kasten, R.W., Hirsh, D.C., Hird, D.W. & McCapes, R.H. (1988). *Pasteurella multocida* in wild mammals and birds in California: prevalence and virulence for turkeys. *Avian Diseases, 32*, 9–15.

Sulong, A.K. & Maheswaran, S.K. (1976). Studies on *Pasteurella multocida*. IV. Immunofluorescence detection of the organisms in spleen and lungs of turkeys vaccinated with live oral vaccines. *Veterinary Microbiology, 1*, 3–14.

Wilson, M.A., Duncan, R.M., Nordholm, G.E. & Berlowski, B.M. (1995). Serotypes and DNA fingerprinting profiles of *Pasteurella multocida* isolated from raptors. *Avian Diseases, 39*, 94–99.

RÉSUMÉ

Investigations sur les taux de portage de *Pasteurella multocida* dans des troupeaux commerciaux de poulets sains ou affectés par le choléra

Vingt troupeaux de palmipèdes (canards Pékin, canards de barbarie et oies) ainsi que 8 troupeaux de poulets élevés de façon intensive ont été examinés pour rechercher les porteurs de *Pasteurella multocida*. Cinq cent soixante dix huit palmipèdes et 240 poulets appartenant à des troupeaux sains ou affectés par le choléra ont été analysés. Cent trente cinq souches (80 de troupeaux sains et 55 de troupeaux affectés par le choléra) ont été isolées à partir des muqueuses du pharynx et du cloaque après passage sur souris (134 souches) et par culture en milieu sélectif (1 souche). Trente cinq pour cent (7/20) des troupeaux de palmipèdes et 38 % (3/8) des troupeaux de poulets avaient des oiseaux porteurs de *P. multocida* dans le pharynx et/ou le cloaque. Les oiseaux des troupeaux affectés par le choléra étaient porteurs de *P. multocida* au niveau de la muqueuse du cloaque avec une prévalence significativement supérieure ($P < 0,001$) à celle de la muqueuse du pharynx, alors que l'opposé a été observé chez les poulets des troupeaux sains. La caractérisation phénotypique a confirmé la présence de *P. multocida* ssp. *multocida*, *P. multocida* ssp. *septica* et *P. multocida* ssp. *gallicida* dans les troupeaux soumis à analyse. *P. multocida* ssp. *gallicida* a été exclusivement isolée de canards Pékin alors que *P. multocida* ssp. *multocida* et *P. multocida* ssp. *septica* ont été isolées aussi bien chez des poulets que chez des palmipèdes. Il a été observé que chaque troupeau a été infecté par un seul clone phénotypique, mais quelques clones ont été isolés à partir de plus d'un troupeau. Un clone différent a été trouvé à partir de 4 bandes atteintes de choléra dans une des fermes les deux années précédentes. Deux clones génotypiques et phénotypiques appartenant chacun à *P. multocida* ssp. *multocida* et *P. multocida* ssp. *septica* ont été trouvés. Cette observation indique que les cas sont généralement dus à un clone et que l'élimination de *P. multocida* des fermes infectées est possible. Les résultats suggèrent que des volailles saines au contact de porteurs convalescents, puissent également devenir des porteurs de *P. multocida*. Cependant, la virulence des isolats de *P. multocida* et la résistance des porteurs à l'infection clinique nécessitent d'être examinées. Ceci est le premier rapport de l'isolement de *P. multocida* à partir d'une muqueuse cloacale de volaille domestique apparemment saine. Les échantillons de cloaque sont apparus être sensibles pour la détection des porteurs de *P. multocida*. Bien que des milieux sélectifs aient été utilisés à une petite échelle, les résultats suggèrent que l'inoculation à la souris ait été une méthode plus efficace que l'utilisation de milieux sélectifs pour l'isolement de *P. multocida* à partir de volailles.

ZUSAMMENFASSUNG

Untersuchungen über die Rate von Pasteurella multocida-Trägern in gesunden Wirtschaftsgeflügelherden und an Hühnercholera erkrankten Herden

Zwanzig Herden schwimmfüßiger Vögel (Peking- und Moschusenten und Gänse) und 8 Hühnerherden, unter intensiver Betreuung aufgezogen, wurden auf das Vorhandensein von Pasteurella multocida-Trägern untersucht. Fünfhundertachtundsiebzig schwimmfüßige Vögel und 240 Hühner sowohl aus gesunden Herden als auch aus Herden mit Geflügelcholera wurden untersucht. Insgesamt 135 Isolate (80 aus gesunden Herden und 55 aus Herden mit Geflügelcholera) wurden von der Rachen- und Kloakenschleimhaut nach Mäusepassagen (134 Isolate) und nach der Kultivierung auf Selektivnährböden (ein Isolat) gewonnen. In 35% (7/20) der Herden von schwimmfüßigen Vögeln und 38% (3/8) der Hühnerherden gab es Tiere, die P. multocida im Rachen und/oder in der Kloake hatten. Vögel aus Herden mit Geflügelcholera trugen P. multocida mit signifikant höherer Prävalenz in der Mukosa der Kloake ($P < 0,001$) als im Rachen, während das Gegenteil bei den Tieren aus gesunden Herden festgestellt wurde. Ausgedehnte Phänotyp-Charakterisierungen bestätigten das Vorhandensein von P. multocida ssp. multocida, P. multocida ssp. septica und P. multocida ssp. gallicida in den untersuchten Herden. P. multocida ssp. gallicida wurde ausschließlich von Pekingenten isoliert, während P. multocida ssp. multocida und P. multocida ssp. septica sowohl von Hühnern als auch von schwimmfüßigen Vögeln gewonnen wurden. Es wurde nachgewiesen, dass jede Herde mit einem einzigen Phänotyp-Klon infiziert war, aber manche Klone wurden in mehr als einer Herde gefunden. Bei jedem von vier Geflügelcholera-Ausbrüchen in den vorhergehenden Jahren wurde ein anderer Klon festgestellt. Je zwei genotypische und phänotypische Klone von P. multocida ssp. multocida und P. multocida ssp. septica wurden nachgewiesen. Diese Beobachtung zeigte, dass die Ausbrüche gewöhnlich klonal sind, und dass die Eliminierung von P. multocida aus infizierten Farmen möglich ist. Die Ergebnisse lassen darauf schließen, dass neben den Rekonvaleszenzausscheidern auch gesundes Geflügel Keimträger sein kann. Die Virulenz von P. multocida-Isolaten und die Resistenz der Ausscheider gegen die klinische Infektion muss noch untersucht werden. Dies ist der erste Bericht über die Isolierung von P. multocida aus der Kloakenschleimhaut von anscheinend gesundem Hausgeflügel. Kloakenproben schienen für die Ermittlung von P. multocida-Ausscheidern empfindlicher zu sein. Obgleich Selektivnährböden nur in begrenztem Umfang verwendet wurden, lassen die Ergebnisse darauf schließen, dass der Mäuseinfektionsversuch eine leistungsfähigere Methode für die Isolierung von P. multocida aus Geflügel ist als die Verwendung von Selektivnährböden.

RESUMEN

Estudio del indice de portadores de *Pasteurella multocida* en manadas sanas de pollos comerciales y en manadas afectadas por colera aviar

Se examinaron veinte manadas de palmípedas (patos de Pekín, patos almizclados y ocas) y 8 manadas de pollos en régimen de explotación intensiva para determinar la presencia de *Pasteurella multocida*. Se evaluaron quinientas setenta y ocho palmípedas y 240 pollos procedentes de manadas sanas así como de las manadas afectadas por el cólera aviar. Se obtuvo un total de 135 aislados (80 de manadas sanas y 55 de manadas afectadas) a partir de mucosa faríngea y cloacal, después de un pase en ratón (134 aislados) y cultivo en medio selectivo (un aislado). Un treinta y cinco por ciento (7/20) de las manadas de palmípedas y un 38% (3/8) de las manadas de pollos presentaban animales portadores de *Pasteurella multocida* en faringe y/o cloaca. Las aves afectadas por cólera aviar presentaban *P. multocida* con mucho mayor prevalencia en la mucosa de la cloaca ($p < 0.001$) que en la mucosa faríngea, mientras que ocurría lo contrario en las manadas sanas. Una mayor caracterización fenotípica confirmó la presencia de *P. multocida ssp. multocida*, *P. multocida ssp. septica* y *P. multocida ssp. gallicida* en las manadas evaluadas. La *P. multocida ssp. gallicida* se aisló exclusivamente en patos de Pekin, mientras que la *P. multocida ssp. multocida* y la *P. multocida ssp. septica* se aislo en tanto en pollos como en palmípedas. Cada manada estaba infectada por un solo clon

fenotípico, aunque algunos clones se encontraron en más de una manada. En una de las granjas se detectó un clon diferente en cada uno de los cuatro brotes de cólera aviar habidos en los dos años precedentes. Se identificaron dos clones fenotípicos y genotípicos de *P. multocida ssp. multocida* y *P. multocida ssp. septica*. Esta observación indicaba que los brotes son normalmente clonales y que la eliminación de *P. multocida* de las granjas infectadas es posible. Los resultados son sugestivos de que las aves sanas, además de las convalecientes portadoras, pueden ser portadoras de *P. multocida*, aunque la virulencia de los diferentes aislados de *P. multocida* y la resistencia de los portadores a la infección clínica queda por ser estudiada. Este es el primer estudio acerca de el aislamiento de *P. multocida* a partir de mucosa cloacal en pollos aparentemente sanos. Aparentemente la toma de muestras a partir de cloaca presenta una mayor sensibilidad para la detección de portadores de *P. multocida*. Aunque la utilización de un medio selectivo se aplicó sólo en algunos casos, los resultados son sugestivos de que la inoculación en ratón fue mucho más eficiente para *P. multocida* en pollos que el uso de medios selectivos.

APPENDIX II

Occurrence of *Pasteurella multocida* and related species in free ranging village poultry and their animal contacts

Occurrence of Pasteurella multocida and related species in village free ranging chickens and their animal contacts in Tanzania

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Abstract

Investigation was done to determine the presence of Pasteurella multocida and related species in free ranging chickens and ducks, dogs, cats and pigs in three climatic zones (cool, warm and hot) of rural Morogoro, Tanzania. A total of 153 isolates of P. multocida ssp. multocida and related species were obtained by direct culture on blood agar, selective medium and mouse inoculation. P. multocida ssp. multocida was isolated from 0.7% of chickens and 7% of ducks. In dogs and cats, P. multocida ssp. multocida was isolated from 1% and 68%, respectively. One isolate of P. gallinarum was isolated from a duck. Other species obtained were; P. multocida ssp. septica, P. stomatis and taxon 16 from dogs and cats, while P. dagmatis and P. canis were found in dogs only. Prevalence of P. multocida ssp. multocida was significantly higher ($P<0.01$) in ducks of the warm zone (22%) than in ducks of other zones (0%). No significant difference was observed between the prevalence of P. multocida ssp multocida in chickens of the warm zone (2%) and chickens of the cool and hot zones (0%). Extended phenotypic characterization revealed phenotypic similarities between two isolates from chickens and the duck strains. Mouse inoculation appeared to be more sensitive in detecting P. multocida ssp. multocida than blood agar and selective medium. Direct culture on blood agar recovered most of the isolates from dogs. This study has demonstrated for the first time the presence of P. multocida and related species in the village free ranging chickens, ducks, dogs and cats in Tanzania. Other non-classified Pasteurella spp. were also observed in the study, but further characterization is required before the final classification can be made. This paper reports for the first time the isolation of unclassified Pasteurella from dogs and cats in Africa. The results implies that fowl cholera might be occurring in free ranging poultry, and dogs and cats kept in contact might serve as sources of P. multocida to chickens and ducks. Subsequent applications of molecular techniques to analyse the epidemiological relatedness of clones isolated from different host species is indicated.

Key words; Pasteurella multocida, Pasteurellaceae, carriers, chicken-bacteria, ducks, dogs, cats

1. Introduction

Lack of reliable animal diseases statistics in Tanzania as in most developing countries, limits the information of disease prevalence and significance in free ranging village chickens. Also the availability of dead birds from the villages for post-mortem examination is difficult. Most dead birds are either eaten by predators, fed to dogs and cats, or are thrown away so that most cases go unreported (Melewas, 1989). In other incidences sick birds may be slaughtered for human consumption. This means that information on the prevalence and significance of disease agents can only be obtained through indirect studies on the carrier rate of the causal agents in healthy birds or serology

The prevalence of Pasteurella multocida and its disease significance in poultry, cattle, small ruminants, pigs, rabbits and other animals has been studied extensively in the past century (Barnum, 1990). Fowl cholera has been reputed a leading killer of domestic and wild birds in some developed countries (Rimler and Glisson, 1997). The few available reports from the developing world show the presence of fowl cholera in village free ranging chickens in Asia (Thitisaki et al., 1989; Aini, 1990) and Zimbabwe (Kelly et al., 1994).

The potential of domestic cats as natural carriers of Pasteurella in causing Pasteurellosis in feral birds has been indicated by Korbel et al. (1992). Subsequently, Van Sambeek and others (1995) demonstrated pathogenic serotypes of P. multocida in the buccal cavities of cats kept as mousers in poultry farms In wild mammals and birds, Snipes et al. (1988) identified somatic serotypes of P multocida isolates from two out of seven premises examined to be the same as somatic serotypes of infected turkeys in the same location. Recently, Christensen et al. (1998) showed that a single clone of P. multocida ssp. multocida affected several avian species in an outbreak involving different geographic regions. The same clone was subsequently demonstrated in semi -confined commercial poultry (Christensen et al., 1999). Although village free ranging poultry are always in contact with other domestic animals, no documented report is available on the role of this contact in disease occurrence. In addition, no reliable knowledge about the occurrence of fowl cholera in Tanzania or other developing countries is available.

Reclassification of the genus Pasteurella sensu stricto by Mutters et al. (1985) has resulted into 11 recognised species within the genus. The reorganisation has lead into addition of new species and

removal of organisms previously classified under the genus Pasteurella. This has made it difficult to identify with precision organisms reported in previous studies. Thus the epidemiology of these organisms is little known, and reinvestigation of the epidemiology of the organisms is needed (Christensen and Bisgaard, 1997).

It was the objective of the present work to investigate the occurrence of P. multocida and other related species in chickens, ducks, dogs, cats, and pigs kept in the same environment. Since haemorrhagic septicaemia in ruminants seems to be associated with certain geographic areas (Carter and De Alwis, 1989) this study also aimed at observing the effect of climate on the presence of P. multocida in domestic animals in the villages. Moreover, high and low environmental temperatures have been shown to have different effects on the pathogenesis of fowl cholera in turkeys (Simmensen et al., 1980). This paper presents the first report in Tanzania and Africa about the carrier rate of P. multocida and related species after reclassification of the genus Pasteurella by Mutters et al. (1985).

2. Materials and methods

2.1 Study area

The study was conducted in Morogoro Region, Tanzania. The sampling area was divided into three zones; cool, warm and hot zones. In all three zones, heavy rains due to the El-Niño effect dominated throughout the sampling period. In each zone two villages were selected as follows; Langali and Nyandira from the cool highland area (Mgeta), Kiroka and Mkuyuni from the warm zone (Mkuyuni) and Kipera and Kongavikenge from the hot area (Mlali). Differences in the temperatures, altitude and animal population in the three zones are shown in Table 1. Migration of warthogs, hedgehogs, squirrels, buffaloes, monkeys and baboons during the dry season is common in all three zones, and a wide variety of wild birds are found in all three locations.

2.2 Sample size

The study was conducted between November 1997 and June 1998. To raise the number of chickens per unit of population, three households were regarded as a unit of sampling. The required sample size was 110 per location basing on the formula $n=4pq/L^2$. Since the prevalence (P) of the infection was not known, a prevalence of 50% was estimated (Martin et al., 1987), the prevalence could then be established to be within 10% of the true prevalence 90% of the time. Ducks, dogs, cats and pigs, kept by the selected owners and their neighbours were screened as a part of animals kept in contact (Table 2).

2.3 Isolation media

Sheep blood agar (5%) (Columbia; ADSA ^{MICRO}) was used for initial culture and propagation. The modified 8HPG (Smith and Baskerville, 1983) was used as a selective medium, which was made by adding bacitracin (5,000 U/l), polymixin B (200 µg/l), and gentamycin (30 µg/l) to a 5% per cent sheep blood agar. Twenty-one days old Pasteurella-free Swiss strain mice were used for intraperitoneal passage Subsequent isolates from 8HPG and mice were propagated on 5% sheep blood agar.

2.4 Collection of samples, bacterial culture and mouse inoculation

Laryngotracheal and cloacal swabs from a total of 330 chickens and 152 ducks from the three locations were cultured and screened for Pasteurella species. A total of 80 dogs and 37 cats were swabbed around the canine tooth-gingival junction, while nasal swabs were taken from 30 pigs. Each

swab was streaked on two plates of 5% sheep blood agar to obtain single colonies and one plate of selective medium. The swabs were then placed in 3 ml of sterile physiological saline. Blood agar plates were incubated under aerobic conditions for 24 hours at 37°C, whereas selective medium was incubated for 48 hours under the same conditions. Up to four subcultures were made from the blood agar plates and from each selective medium plate.

A physiological saline tube with an infected swab was shaken thoroughly, then 0.5 ml of the contents were injected into Swiss strain mice by intraperitoneal route. The inoculated mice were observed for 48 hours and any mouse that remained alive was euthanised. Spleen samples were aseptically removed and macerated under sterile conditions following dissection of the mice. Using a sterile loop, spleen material was inoculated on blood agar and incubated overnight under aerobic conditions at 37°C. Blood agar and selective medium plates were marked for each animal sampled. However, because of the inefficiency of the method of marking mice not all mice isolates could be matched with respective animals marked on the plates.

2.5 Identification of Pasteurella species

Identification of Pasteurella species was performed according to the methods described by Bisgaard and Mutters (1986) and Bisgaard et al. (1991). Colonies suspected to be P. multocida from blood agar, selective medium and mouse passage were subcultured on blood agar plates. Pure isolates were propagated in tryptophan broth (ADSA^{MICRO}) and were incubated at 37°C for 8 hours. Each isolate in broth culture was inoculated into glucose broth (with Durham tubes to detect gas formation), ornithine decarboxylase, decarboxylase control medium, sucrose, maltose, mannitol, dulcitol, sorbitol, urea broth and urea broth control medium. An overnight tryptophan broth culture was tested for indole production by using dimethylaminobenzaldehyde (Kovac's reagent). Each isolate was again subcultured on blood agar to confirm purity of the culture. All sugar broth cultures were incubated at 37°C for 48 hours. Pure colonies from blood agar were used for motility test, catalase test, oxidase test, Hugh and Leifson's oxidation-fermentation test, and Gram staining. Each strain was tested three times to assess repeatability of the results. All the isolates that were preliminary identified as Pasteurella or Pasteurella-like were subsequently characterized by extended phenotypic methods described by Bisgaard et al. (1991).

2.6 Statistical analysis

A Chi-square test was used to compare the differences on prevalence of Pasteurella species between the zones, host species, and the media of isolation (Martin et al., 1987).

3. Results

3.1 Overall results

A total of 153 isolates of P. multocida and related species were obtained from the 629 apparently healthy animals investigated. Each of the positive chickens and ducks carried a single species, whereas in dogs and cats few carriers of two and three different species were also found, and no isolate came from pigs. Because of inefficiency of mice identification the combination of all three methods in the interpretation of results could not be considered for every animal investigated (Tables 2 and 3). Out of 330 chickens and 152 ducks examined in the three zones, a total of 16 laryngotracheal isolates from the warm zone were confirmed to be P. multocida ssp. multocida and one isolate from ducks was P. gallinarum (Table 2). Pasteurella species were not obtained from the cloaca. Overall prevalence of P multocida ssp. multocida in chickens was 0.7% (2/330), significantly ($P<0.05$) lower than that of ducks (7%). Five isolates were obtained from two chickens. From one chicken, all three methods of isolation demonstrated the P. multocida ssp. multocida, while the second bird yielded P. multocida ssp multocida from the selective medium and from mouse inoculation. From the eleven positive ducks, one bird yielded P. multocida ssp multocida through blood agar inoculation, while the remaining 10 isolations were done through mice (Table 2).

Extended phenotypic characterization confirmed all five isolates from chickens to be P. multocida ssp. multocida. Two isolates from one chicken were identical, but they appeared different from three identical isolates from the second chicken. The first chicken clone (two isolates) was positive for L (+) arabinose and negative for D (+) xylose, trehalose, D (-) arabinose, L (-) fucose and PNPG, the second clone (three isolates) was negative for L (+) arabinose and positive for the other tests. Eleven strains from ducks shared all 80 phenotypic features, and appeared similar to the L (+) arabinose positive clone from chickens. Ducks and chickens carrying P. multocida ssp. multocida were in the same vicinity having boundless contact, despite being kept by different villagers Apart from P multocida ssp. multocida and P. gallinarum, no other species of Pasteurella were recovered from poultry. None of the methods used detected Pasteurella spp. in the 30 pigs investigated.

P. multocida ssp. multocida was demonstrated in 4% (3/80) of dogs and 51% (19/37) of cats investigated (Table 2). Thus, a carrier rate of P. multocida ssp. multocida in cats was statistically significantly higher ($P<0.001$) than those of dogs, chickens, and ducks. P. multocida ssp. septica was

also statistically significantly more prevalent (P<0.001) in cats 30% (11/37) compared with dogs 3% (2/80).

Other species of Pasteurella identified included P. canis, P. dagmatis, P. stomatis, taxon 16, and unclassified Pasteurella (Table 3). The carrier rate of P. canis in dogs was 14% (11/80), which was statistically significantly (P>0.05) higher than in cats where no carrier was detected. P. dagmatis was demonstrated in five out of 80 dogs (6%). The prevalence of P. dagmatis in dogs was statistically significantly (P<0 05) higher in dogs compared with cats where P. dagmatis was not detected. P. stomatis was found in two dogs (3%), a similar prevalence to that of cats 3% (1/37).

Taxon 16 was found in 26% (21/80) of dogs investigated, which was statistically significantly (P<0.01) higher than that of cats 11% (4/37). A variety of unclassified Pasteurella-like strains were found in 14% (11/80) of dogs which is not statistically significant higher (P<0.05) than that of cats 8% (3/37).

The presence of more than one taxon in one animal was shown in 9 out 80 of dogs (11%) and 5 of 37 (13%) cats investigated (data not shown in the tables). The difference in prevalence of multiple carriers between dogs and cats was not statistically significant (P<0 05). In dogs carriers of more than one taxon were found to host the following bacteria; P. multocida ssp. multocida and P. stomatis (1 dog), P. multocida ssp. septica, P. canis and unclassified Pasteurella (1 dog), P. multocida ssp. septica and taxon 16 (1 dog), P. canis and taxon 16 (1dog), P. canis and unclassified Pasteurella (1 dog), P. dagmatis and unclassified Pasteurella (1 dog), taxon 16 and unclassified (3 dogs).

The five cats found to carry more than one species were shown to have; P. multocida ssp. multocida and P. multocida ssp. septica (1 cat), P. multocida ssp. multocida, P. stomatis and taxon 16 (1 cat), P. multocida ssp multocida and unclassified Pasteurella (1 cat), P. multocida ssp. septica and unclassified Pasteurella (1 cat) and P. multocida ssp. septica and taxon 16 (1 cat).

All carriers of more than one species, were detected by either blood agar, selective medium or a combination of both methods. Out of nine multiple carrier dogs, two were detected by blood agar alone and seven by selective medium and blood agar. All cats that carried more than one species were detected by combination of results from blood agar and selective medium.

3.2 Occurrence of Pasteurella spp. in the three zones

The prevalence of P. multocida ssp. multocida in chickens of the warm zone 2% (2/110) was not statistically significantly different (P>0.05) from that of chickens from the other zones (Table 2). However, prevalence of P. multocida ssp. multocida in ducks of the warm zone 22% (11/50) was statistically significantly (P<0.001) higher than that of ducks in the cool and hot zones (0%). Level of differences in prevalence of isolates obtained are indicated in the Tables 2 and 3.

3.3 Efficacy of the methods of isolation

Mouse inoculation was more efficient than the other methods for isolating P. multocida ssp. multocida (Table 4). Thirty-three (52%) isolates were obtained by mouse inoculation which was statistically significantly (P<0.001) higher than blood agar and selective medium which recovered 22% (14) and 25% (16) of the isolates, respectively. Out of 24 isolates of P. multocida ssp. septica 46% (11) were isolated by selective medium which was statistically significantly higher (P<0.05) than that of blood agar (29%) and mouse inoculation (25%). Direct culture on blood agar was generally more efficient that the other methods used in detecting the remaining species of Pasteurella. Efficacy of the three techniques in isolating P. multocida ssp. multocida and other species in different host animals is shown in Table 4.

4. Discussion

Most of the works published before reclassification of the genus Pasteurella sensu stricto (Mutters et al., 1985) do not safely allow the present species and subspecies identification within the genus Pasteurella. This is the first systematic investigation of P. multocida and related species in free ranging chickens and their animal contacts. Contrary to previous investigations in commercial flocks both poultry and contact animals are kept free under village management system which allow optimum interaction between them. According to the recent classification (Mutters et al., 1985) our study demonstrated two subspecies of P. multocida, four species of Pasteurella and at least one taxon of uncertain affiliation (taxon 16) among the animals investigated. We could not accurately compare the organisms described in most previous reports (Saphir and Carter, 1976; Arnbjerg, 1978; Curtis and Ollerhead, 1981; Carpenter et al., 1989) with our findings because of limited number of tests employed in those studies.

Isolation of P. multocida from apparently healthy poultry has been associated with previous outbreaks of fowl cholera in the flock (Curtis and Ollerhead, 1981; Carpenter et al., 1989). In the present investigation, P. multocida ssp. multocida was detected in laryngotracheal swabs in 2% of chickens and 22% of ducks in the warm climatic zone of the studied areas. Extended phenotypic characterization showed that one chicken clone was similar in all 80 phenotypic features with 12 duck strains kept in the same area. This suggests that the strains might be related and there can be exchange of P. multocida ssp. multocida between chickens and ducks, confirming the recent observations of exchange of clones of P. multocida between commercial poultry and the avifauna by Christensen et al. (1999). This exchange of P. multocida ssp. multocida might also have implications on cats and dogs kept in the same area. Three out of seven cats and one of 25 dogs from this zone carried P. multocida ssp. multocida. However, all five cats from the cool zone and 44% of the cats from the hot zone carried P. multocida ssp. multocida while chickens and ducks from these zones sampled negative for P. multocida ssp. multocida. Consequently, exchange of clones of P. multocida ssp. multocida between animal species might not be the same in all zones for reasons which remains to be investigated. Reliable data about the extent to which cats and dogs had access to dead poultry in the different zones were not available. Finally, the relevance of cats and dogs in the epidemiology of P. multocida in birds in the present study remains to be further analysed by molecular characterization of the strains to understand the epidemiological relationship between the clones from different host species under investigation.

The prevalence data of cats in our study support the previous findings that P. multocida ssp. multocida is part of normal flora of oral mucosa in majority of cats. Investigations done in recent years have demonstrated that the prevalence of P. multocida ssp. multocida in cats varies from 21 to 38% (Baldrias et al., 1988; Ganiere et al., 1993; Mohan et al., 1997). High isolation rates in cats of the cool zone may be influenced by the small sample size. P. multocida ssp. multocida has been reported to be less prevalent in dogs compared with cats (Baldrias et al., 1988; Ganiere et al., 1993; Mohan et al., 1997). Agreement of our results with these findings may suggest that dogs seems to represent a less likely source of P. multocida ssp. multocida to poultry compared with cats.

Although P. multocida ssp. septica is scarcely reported in poultry, as also seen in the current study, it has been associated with different outbreaks of fowl cholera in both domestic and wild birds (Hirsh et al., 1990; Blackall et al., 1995). Studies by Snipes et al (1990) and Fegan et al. (1995) found P multocida ssp. septica in less than 1% of all isolates of P. multocida from outbreaks of fowl cholera. Characterization of more isolates from birds in Tanzania and other countries will provide more knowledge about the distribution of P. multocida ssp. septica in birds.

Several reports about the presence of P. multocida ssp. septica in cats and dogs are available (Baldrias et al., 1988; Hirsh et al., 1990; Ganiere et al., 1993). An equal proportion of P. multocida ssp septica to P. multocida ssp. multocida carriers in cats has been documented (Baldrias et al , 1988; Ganiere et al., 1993). A 1:2 ratio of P. multocida ssp. septica to P multocida ssp. multocida was observed in our investigations. However, differences in the sample size and precision of isolation techniques may contribute to the variation in the results between our study and others. Cats in the warm zone were not found to harbour P. multocida ssp. septica, but since P. multocida ssp multocida was isolated in cats, ducks and chickens in this zone, it may be too early to speculate that climate is the factor guiding the prevalence here. Subculture of more isolates per plate might have documented the existence of more species than four subcultures per plate in the current study.

Previous reports of carriers of P. multocida in poultry have been associated with previous occurrence of disease in the flocks (Curtis and Ollerhead, 1980; Carpenter et al.1989). Thus our results may be considered an indication of previous fowl cholera in village free ranging poultry, although the clinical form of the disease was not seen during the period of study. A report on carriers of P. multocida which were not associated with previous occurrence of disease in the flocks has been published recently

(Muhairwa et al., 2000). P. multocida ssp. multocida from cats has been reported to cause disease in feral birds (Korbel et al., 1992). Since free contact between dogs and cats and free ranging poultry might favour exchange of pathogenic strains leading into clinical disease, P. multocida ssp. multocida found in cats and dogs in the present study might have significant impact on village free ranging chickens.

For the first time this study has shown the presence of P. gallinarum in an apparently healthy duck. Although this finding does not rule the possible presence of convalescent carriers or the beginning of infection, it demonstrate that ducks can also be infected by P. gallinarum. Previous reports associated the bacterium with pathological lesions in chickens (Hall et al.,1955; Mraz et al. 1980; Droual et al. 1992). Further studies in healthy and diseased ducks will be necessary to confirm the importance of P. gallinarum in ducks.

P. canis, P. dagmatis, P. stomatis, and taxon 16 have never been reported in chickens and ducks (Mutters et al., 1985; Bisgaard, 1993). P. canis was predominantly isolated from dogs and in equal proportions in the three zones. The present results show that P. canis is more common than P. multocida in dogs. This agrees with the results of Ganiere et al. (1993). Low occurrence of P. dagmatis and P. stomatis in dogs confirms previous observations by Ganiere et al. (1993), who found 11% P. dagmatis, and Mohan et al. (1997) who found less than 1% P. stomatis in dogs. The organism tentatively designated taxon 16 by Bisgaard and Mutters, (1986) was found in twenty-one dogs and in four cats in this study. Ganiere et al. (1993) recovered an organism from dogs with features related to taxon 16 which they named atypical P. stomatis. Seemingly a prevalent taxon in dogs, taxon 16 could represent misclassified P. gallinarum and unidentified Pasteurella reported in dogs (Baldrias et al., 1988).

Unclassified Pasteurella described represent organisms the biochemical patterns of which failed to match with any of species in the new classification of Mutters et al. (1985). Similar organisms have also been reported in dogs and cats in Europe (Bisgaard and Mutters, 1986; Ganiere et al., 1993) and Australia (Baldrias et al. 1988). To the author=s knowledge this is the first report on the occurrence of unclassified Pasteurella in dogs and cats of Africa. It is possible that some of these organisms might later form new taxa or classify in the known species of Pasteurella. Thus, subsequent characterization by extended phenotypic characterization and molecular techniques is required to decide the taxonomic

position of these organisms.

Simultaneous isolation of P. dagmatis and P. multocida from human wounds related to cat bites was reported by Zbinden et al. (1988). Subsequently, Ganiere et al. (1993) demonstrated multiple carriers of Pasteurella species/ subspecies in 19% of dogs and 30% of cats examined. The present results show that, 11% of dogs and 13% of cats are double carriers and therefore confirms the previous findings. However, it is presumed that swabbing of multiple sites in the oral cavity, and subculturing on more plates and several colonies per plate could significantly improve the results.

The use of different isolation methods has been reported to increase the combined isolation rate of Pasteurella species (Baldrias et al., 1988). Thirty-three out of 63 strains of P. multocida ssp. multocida in the present investigation were collected by mouse inoculation. Comparison of our results with those of Baldrias et al. (1988) may suggest that mouse inoculation is superior to other methods we used for isolating P. multocida ssp. multocida. However, the possibility that different clones of P. multocida ssp. multocida could vary in their pathogenicity to mice needs to be addressed. Consequently, the genetic relatedness of clones obtained by different methods of isolation should be investigated to reach a firm conclusion.

The present findings indicate that all three methods of isolation applied can isolate P. multocida ssp. septica from cats. Although the selective medium isolated more strains than direct culture on blood agar and mouse inoculation, the number of strains examined is too low reach a safe conclusion. In addition, the virulence of these organisms for mice needs to be addressed. Generally, the present results show that blood agar is more sensitive for the isolation of Pasteurella other than P. multocida.

Baldrias et al. (1988) isolated a total of 21 strains of P. canis from dogs by using blood agar only. In the same study 20 out of 27 strains of P. canis from cats came from blood agar, only seven strains came from selective medium and mouse inoculation. These findings correlate with our results where we have obtained eight of 14 strains of P. canis from dogs by direct culture on blood agar and three isolates each, from selective medium and mouse inoculation. Lack of pathogenicity to mice and susceptibility to drugs in the selective medium may be the reasons for inefficiency of mouse inoculation and selective medium in isolating P. canis.

In conclusion, isolation of P. multocida ssp. multocida from village free ranging chickens and ducks suggest that fowl cholera might be common in the village poultry of Tanzania. More efforts should be directed in understanding the impact of the P. multocida and other species in village birds. The presence of P. multocida in dogs and cats has never been observed to influence the epidemiology of fowl cholera in free ranging chickens and ducks. However, their potential as a source of disease is still there. Studies using molecular techniques are needed to clarify the significance of dogs and cats in the epidemiology of fowl cholera in free ranging poultry.

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References

Aini, I. , 1990. Indigenous chicken production in Southeast Asia. World's Poultry. Sci. J. 46, 51-57.

Arnbjerg, J., 1978. Pasteurella multocida from canine and feline teeth, with a case report of glossitis calcinosa in a dog caused by P. multocida. Nord. Vet. -Med. 30, 324-332.

Baldrias, L., Frost, A. J., O'Boyle, D., 1988. The Isolation of Pasteurella-like organisms from the tonsillar region of dogs and cats. J. small. Anim. Prac. 29, 63-68.

Barnum, D. A., 1990. Socioeconomic significance of the HAP group. Can. J. Vet. Res 54, S1-S2

Bisgaard, M., Mutters, R., 1986. Characterization of some previously unclassified Pasteurella spp. obtained from the oral cavity of dogs and cats and description of a new species tentatively classified with the family Pasteurellaceae Pohl 1981 and provisionally called Taxon 16. Acta Path. Microbiol. Immunol. Scand. Sect. B 94, 177-184

Bisgaard, M., Houghton, S. B., Mutters, R., Stenzel, A., 1991. Reclassification of German, British and Dutch isolates so-called Pasteurella multocida obtained from pneumonic calf lungs. Vet. Microbiol. 26, 115-124.

Bisgaard, M., 1993. Ecology and significance of Pasteurellaceae in animals. Zbl. Bakt. 279, 7-26.

Blackall, P.J., Pahoff, J.L., Marks, D , Fegan, N., Morrow, C.J., 1995. Characterization of Pasteurella multocida isolated from fowl cholera on turkey farms. Aust.Vet. J. 72, 135-138.

Carpenter, T. E., Hirsh, D. C., Kasten, R. W., Hird, D. W., Snipes, K. P., McCapes, R. H , 1989. Pasteurella multocida recovered from live turkeys: Prevalence and virulence in turkeys. Avian. Dis. 33, 12-17.

Carter, G.R., De Alwis, M.C.L. 1989. Haemorrhagic septicaemia. In: C Adlam and J M. Rutter Eds.) Pasteurella and Pasteurellosis, Academic Press, London, pp. 131-60.

Christensen, J. P., Bisgaard, M., 1997. Avian Pasteurellosis: Taxonomy of the organisms involved and aspects of pathogenesis. Avian Path. 26, 461-483.

Christensen, J. P., Dietz, H., Bisgaard, M., 1998. Phenotypic and genotypic characters of isolates of Pasteurella multocida obtained from two outbreaks of avian cholera in avifauna in Denmark. Avian Path. 27, 373-381.

Christensen, J.P., Petersen, K.D., Hansen, H.C., Bisgaard, M., 1999. Occurrence of fowl cholera in Danish wild birds, and poultry production. Possible connections. Dansk Veterinaertidsskrift 82, 342-346.

Curtis, P. E., Ollerhead, G. E., 1981. Investigation to determine whether healthy chickens and turkeys are oral carriers of Pasteurella multocida. Vet. Rec. 108, 206-207.

Droual, R., Shivaprasad, H.L., Meteyer, C.U., Shapiro, D.P. and Walker R.L. 1992. Severe mortality in broiler chickens associated with Mycoplasma synoviae and Pasteurella gallinarum. Avian.Dis. 36 (3):803-807.

Fegan, N., Blackall, P. J., Pahoff, J. L., 1995. Phenotypic characterization of Pasteurella multocida isolates from Australian poultry. Vet. Microbiol. 47, 281-286.

Ganiere, J. P., Escande, F., Andre, G., Larrat, M., 1993. Characterization of Pasteurella from gingival scrapings of dogs and cats. Comp. Immun. Microbiol. Infect. Dis. 16, 77-85.

Hall, W. J., Heddleston, K. L., Legenhausen, D. H., Hughes, R. W., 1955. Studies on Pasteurellosis.1. A new species of Pasteurella encountered in chronic fowl cholera. Am. J. Vet. Res.16, 598-604.

Hirsh, D. C., Jessup, D. A., Snipes, K. P., Carpenter, T. E., Hird, D. W., McCapes, R. H., 1990. Characteristics of Pasteurella multocida isolated from waterfowl and associated avian species in California. J. Wild. Dis. 26, 204-209.

Kelly, P. J., Chitauro, D., Rohde, C., Rukwava, J., Majok, A., Davelaar, F., Mason, P. R., 1994. Diseases and management

of backyard chicken flocks in Chitungwiza, Zimbabwe. Avian. Dis. 38, 626-629.

Korbel, R., Gerlach, H., Bisgaard, M., Hafez, H.M., 1992. Further investigations on Pasteurella multocida infections in feral birds injured by cats. J. Vet. Med. B. 39, 10-8.

Martin, W. S., Meek, A. H., Willeberg, P., 1987. Veterinary Epidemiology: Principles and Methods, Iowa State University Press, Iowa. pp. 22-47.

Melewas, J. N., 1989. The contribution of poultry industry to the national economy. In: Msolla, P. M. (Ed.) Proceedings of the 7th Tanzania Veterinary Association scientific conference. 7, 13-35.

Mohan, K., Kelly, P. J., Muvavarirwa, P., Pawandiwa, A., 1997. Phenotype and serotype of Pasteurella multocida isolates from diseases of dogs and cats in Zimbabwe. Comp. Immun. Microbiol. Infect. Dis. 20, 29-34.

Muhairwa, A P., Christensen J P., Bisgaard, M 2000 Investigations on the carrier rate of P. multocida in healthy commercial poultry flocks and flocks affected by fowl cholera. Avian Path. **29**, 133-142.

Mraz,O, Sisak, F., Jelen, P. 1980. The Pasteurella carriers in farm and laboratory animals. Comp. Immun. Microbiol. Infect. Dis. 2, 437-445.

Mutters, R., Ihm, P., Pohl, S., Frederiksen, W., Mannheim, W., 1985. Reclassification of the genus Pasteurella Trevisan 1887 on the basis of deoxyribonucleic acid homology, with a proposal for the new species Pasteurella dagmatis, Pasteurella stomatis, Pasteurella anatis and Pasteurella langaa. Int. J. Syst. Bacteriol. 35, 309-322.

Rimler, R. B., Glisson, J. R 1997. Fowl cholera. In: Calnek, B. W., Barnes, H. J., Beard, C. W., McDougald, L. R., and Saif, Y. M. (Eds), Poultry Diseases, Mosby-Wolfe, London. pp. 143-159.

Saphir, D. A., Carter, G. R., 1976. Gingival flora of the dog with special reference to bacteria associated with bites. J. Clin. Microbiol. 3, 344-349

Simmensen, L., Olsen, D., Hahn, G. L., 1980. Effects of high and low environmental temperatures on clinical course of fowl cholera in turkeys Avian Dis. 24, 1007-1010.

Smith, I. M., Baskerville, A. J., 1983. A selective medium for the isolation of P. multocida in nasal specimens from pigs. Br. Vet. J. 139, 476-484.

Snipes, K. P., Carpenter, T. E., Corn, L. J., Kasten, R. W., Hirsh, D. C., Hird, D. W., McCapes, R. H., 1988. Pasteurella multocida in wild mammals and birds in California: Prevalence and virulence for turkeys. Avian. Dis. 32, 9-15.

Snipes, K. P., Hirsh, D. C., Kasten, R. W., Carpenter, T. E., Hird, D. W., McCapes, R. H., 1990. Homogeneity of characteristics of Pasteurella multocida isolated from turkeys and wildlife in California, 1985-88. Avian. Dis. 34, 315-320.

Thitisak, W., Janvirisayopak, O., Morris, R. S., von Krueder, R., Srihakim, S., 1989. Profile study on healthy and productivity of native chickens in Northeast Thailand. In: Riest, U. (Ed.) 89' Proceedings on small holder poultry production in developed countries. 4, 162-170.

Van Sambeek, F., McMurray, B. L., Page, R. K., 1995. Incidence of Pasteurella multocida in poultry house cats used for rodent control programmes. Avian. Dis. 39, 145-146.

Zbinden, R., Sommerhalder, P., Von Wartbug, U., 1988. Co-isolation of Pasteurella dagmatis and Pasteurella multocida from cat bite wounds. Eur. J. Clin. Microbial. infect. Dis. 7, 203-204.

Table 1. Climate and estimated population size in the zones investigated.

Zone	Location	Temperature	Altitude	Chickens	Ducks	Dogs	Cats	Pigs
Hot	Mlali	23-32°C	760m	6674	1800	100	25	-
Warm	Mkuyuni	18-25°C	1200m	9500	1300	88	8	-
Cool	Mgeta	12-18°C	1500m	3440	1200	65	14	200

Table 2. Prevalence of P. multocida ssp. multocida and P. multocida ssp. septica in the chickens, ducks, dogs, and cats investigated.

		P. multocida ssp. multocida				P .multocida ssp. septica			
Host	Zone	BA	SM	MI	n/N	BA	SM	MI	n/N
Chickens	Cool	-	-	-	0/110	-	-	-	0/110
	Warm	1(1%)	2(2%)	2(2%)	2/110 (2%)	-	-	-	0/110
	Hot	-	-		0/110	-	-	-	0/110
	Subtotal	1(0.3%)	2(0.7%)	2(0.7%)	2/330	-	-	-	0/330
Ducks[1]	Cool	-	-	-	0/50	-	-	-	0/50
	Warm	1(2%)	-	10(20%)	11/50 (22%)***	-	-	-	0/50
	Hot	-	-	-	0/52	-	-	-	0/52
	Subtotal	1(0.7%)	-	10(7%)	11/152	-	-	-	0/152
Dogs	Cool	-	-	1(4%)	1/25 (4%)	-	-	-	0/25
	Warm	1(4%)	-	-	1/25 (4%)	-	-	-	0/25
	Hot	-	-	1(3%)	1/30 (3%)	-	2(7%)	-	2/30
	Subtotal	1(1%)	-	2(3%)	3/80	-	2(3%)	-	2/80
Cats	Cool	5(100%)	5 (100%)	5(100%)	5/5 (100%) *	-	-	-	0/25
	Warm	3(43%)	2(29%)	3(43%)	3/7 (43%)[2]	-	1(14%)	1(14%)	1/7 (14%) **
	Hot	3(12%)	7(28%)	11(44%)	11/25 (44%)[3]	7(28%)	8(32%)	5(20%)	10/25 (40%) **
	Subtotal	11(30%)	14(38%)	19(51%)	19/37	7(19%)	9(24%)	6(16%)	11/37

* P<0.05, ** P<0.01 , *** P<0.001 Significantly different compared with two other zones.

n/N=number of positive animals/Number animals sampled. The higher prevalence was used where identification failed to match (see text).

[1] one isolate of P. gallinarum obtained in the warm zone from blood agar is not listed in the Table.

[2] prevalence by findings on blood agar and selective medium plates. [3] prevalence by mice inoculation findings.

BA: blood agar. SM: selective medium. MI: mouse inoculation.

Table 3. Occurrence of Pasteurella species other than P. multocida in dogs and cats in the three zones.

		Dogs				Cats			
	Zone	BA	SM	MI	n/N	BA	SM	MI	n/N
P. canis	Cool	2(8%)	2(8%)	-	3/25 (12%)	-	-	-	0/5
	Warm	1(4%)	1(4%)	3(12%)	3/25 (12%)	-	-	-	0/7
	Hot	4(13%)	-	-	4/30 (13%)	-	-	-	0/25
	Total	7(10%)	3(4%)	3(4%)	10/80	-	-	-	0/37
P. dagmatis	Cool	2(8%)	-	-	2/25 (8%)	-	-	-	0/5
	Warm	-	-	1(4%)	1/25 (4%)	-	-	-	0/7
	Hot	2(3%)	-	-	2/30 (7%)	-	-	-	0/25
	Total	4(4%)	-	1(4%)	5/80	-	-	-	0/37
P. stomatis	Cool	-	-	-	0/25	-	-	-	0/5
	Warm	-	1(4%)	-	1/25 (4%)	-	1(14%	-	1/7 (14%) ***
	Hot	-	-	1(3%)	1/30 (3%)	-	-	-	0/25
	Total	-	1(1%)	1(1%)	2/80	-	1(3%)	-	1/37
Taxon 16	Cool	5(20%)	2(8%)	-	7/25 (32%)	-	-	-	0/5
	Warm	2(8%)	2(8%)	-	4/25 (16%)**	1(14%)	1(14%)	2(28%)	2/7 (28%)**
	Hot	9(30%)	4(13%)	-	10/30 (33%)	2(8%)	-	-	2/25 (8%)
	Total	16(20%)	8(10%)	-	21/80	3(8%)	1(3%)	2(5%)	4/37
Unclassified	Cool	1(4%)	1(4%)	-	2/25 (8%)*	-	-	-	0/5
	Warm	3(12%)	-	-	3/25 (12%)	1(14%)	-	-	1/7 (14%)
	Hot	6(20%)	-	-	6/30 (20%)	2(8%)	-	-	2/25 (8%)
	Total	10(8%)	1(1%)	-	11/80	3(3%)	-	-	3/37

* P<0.05, ** P<0.01 , *** P<0.001 Significantly different compared with two other zones.
n/N= number of positive animals /Number of animals sampled BA: blood agar. SM: selective medium. MI: mouse inoculation.

Table 4. Efficacy of media used (%) for isolation of Pasteurella spp. from chickens and their animal contacts

	Taxon (n)	BA	SM	MI
P. multocida ssp. multocida	Chickens (5)	20	40**	40**
	Ducks (11)	9	-	91***
	Dogs (3)	33	-	66***
	Cats (44)	25	32	43**[1]
P. multocida ssp. septica	Dogs (2)	-	100	-
	Cats (22)	32	41*[2]	27
P. canis	Dogs (13)	54***	23	23
P. dagmatis	Dogs (5)	80***	-	20
P. stomatis	Dogs (2)	-	50**	50***
Taxon 16	Dogs (24)	67***	33	-
	Cats (6)	50***	17	33***[3]
Unclassified Pasteurella	Dogs (11)	91***	9	-
	Cats (3)	100***	-	-

* P<0.05, ** P<0.01 , *** P<0.001 [1]Significantly higher than BA only [2] Significantly higher than MI only [3] Significantly higher than Selective medium only

APPENDIX III

Relationships among *Pasteurellaceae* isolated from free ranging chickens and their animal contacts as determined by quantitative evaluation of phenotypic data, ribotyping and REA-typing.

Relationships among Pasteurellaceae isolated from free ranging chickens and their animal contacts as determined by quantitative phenotyping, ribotyping and REA-typing

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Key words; Pasteurella multocida, characterization, ribotyping, epidemiology, chicken-bacteria, ducks, dogs, cats.

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Abstract

One hundred and forty-three Pasteurella spp. strains and ten unclassified strains obtained from free ranging poultry, dogs and cats were investigated by extended phenotypic characterization. One hundred and forty-nine of these strains were selected for further studies using ribotyping and REA-typing to evaluate the role of dogs and cats in P. multocida transmission. Seven and six type strains were included for comparison in phenotyping and genotyping, respectively. Eleven clusters and six unclustered strains were revealed by phenotyping. Ribotyping outlined twelve clusters and six unclustered strains. A correlation between clusters obtained by phenotyping and ribotyping was demonstrated which indicated that a genetic basis exists for clusters outlined by quantitative evaluation of phenotypic data. Similarities and differences in hosts, phenotype, ribotype, and zone of isolation were demonstrated among Pasteurella strains investigated. Isolates of P. multocida from ducks were shown to be clonal by both phenotyping and ribotyping. These strains were identical to one of the chicken isolates. REA-typing, however, showed that the chicken isolates was different underlining that exchange of clones of P multocida between avian species rarely happens under village conditions. Management practise in the villages suggest the potential for exchange of P multocida between poultry and animals kept in contact. The present findings, however, did not indicate that clones of P. multocida are widely exchanged between poultry and other animal species even though close contact exists. In the present investigation exchange of clones of P. multocida was only demonstrated among animals belonging to the same species. Caution is drawn to the use of ribotyping as the sole method for epidemiological typing and tracing of P. multocida. The present results also underline the importance of proper phenotyping in the identification of P. multocida and related species.

1. Introduction

Pasteurella multocida is the cause of fowl cholera in domestic and wild birds (Rimler and Rhoades, 1989) and respiratory diseases and septicaemia in ruminants and pigs (Carter and De Alwis, 1989; Chanter and Rutter, 1989). The habitat of P. multocida, however, is broad involving mainly the respiratory tracts of mammals, birds and people (Bisgaard et al., 1994). Significant variations in the physiologic properties of P. multocida (Heddleston, 1976) lead to confusion in the delineation and identification of species within the genus Pasteurella. Although the genus was redefined by Mutters et al. (1985), identification on species level still presents considerable problems (Bisgaard, 1993; Petersen et al., 1998). Caution is therefore recommended in interpretation of the literature on epidemiology and ecology of P. multocida.

Outbreaks of fowl cholera associated with exchange of P. multocida strains between wild birds and domestic poultry have been reported (Snipes et al., 1990; Christensen et al., 1998), but the significance of strains from other animals such as dogs, cats and pigs in the epidemiology of fowl cholera is unclear. However, indications exist that these animals might be an important reservoir of avian pasteurellosis (Snipes et al., 1988; Van Sambeek et al., 1995). Carriers of strains of P. multocida that are pathogenic to poultry have been demonstrated in farm cats (Curtis and Ollerhead, 1982) and in wild animals captured in turkey farms (Snipes et al., 1988; Christiansen et al., 1992). Recent investigations have also shown that a high carrier rate may exist in healthy commercial poultry flocks and that P. multocida may be excreted through faeces (Muhairwa et al., 2000). Healthy animals may consequently act as a reservoir of P. multocida for poultry, and pose a risk of direct transmission through bites (Korbel et al., 1992) as well as indirect transmission through faecal contamination. Free ranging village chicken management in Africa allows contact between domestic poultry, dogs, cats, pigs, goats as well as wild animals (Mwalusanya, 1998). However, no data is available on the role of animals kept in contact with poultry in the epidemiology of fowl cholera in free ranging village chickens in Tanzania and other African countries. One report from Egypt showed the presence of different serovars of P. multocida in different domestic mammals and poultry (Karmy et al., 1983).

Inefficiency of serotyping in the identification and determining the epidemiology of P. multocida has

been detailed (Snipes et al., 1988, 1990; Wilson et al., 1993). These studies demonstrated genotypic discrepancies among strains of identical serotypes. Subsequent study by Blackall et al. (1998) further demonstrated the lack of correlation between serovars and ribotypes and multilocus enzyme electrophoresis results among field isolates and the type strains of P. multocida. Consequently, the use of serology in typing of P. multocida should no longer be practised for that purpose. In recent years biotyping (Christensen et al., 1998) combined with genomic typing (Gardner et al., 1994; Diallo et al., 1995; Christensen et al., 1998) has been useful in establishing relationships among isolates of P. multocida. The use of restriction endonuclease analysis (REA) of chromosomal DNA and ribotyping have been employed in comparing and separating isolates of P. multocida from different origins (Snipes et al., 1988; Kim and Nagaraja 1990). Using REA, Kim and Nagaraja, (1990) differentiated field strains from vaccinal strains of P. multocida which belonged to serotype A:3,4. However, difficulty of interpreting complex profiles which may consist of hundreds of bands limits the application of REA for epidemiological typing of bacteria (Maslow et al., 1993; Olsen et al., 1993). A combination of REA and ribotyping was of value in back-tracing outbreaks of fowl cholera in wild and domestic birds (Christensen et al., 1998). Efficiency of these methods has been shown to vary with the restriction enzymes used in cutting genomic DNA (Wilson et al., 1992). HpaII has been shown to produce fragments that are distinct and better separated compared with HhaI (Wilson et al., 1992; Christensen et al., 1998). In both studies HpaII was able to separate isolates that appeared clonal by using HhaI. Consequently HpaII alone was used in the present study.

In a previous study on the prevalence of P. multocida and related species in free ranging village chickens and their animal contacts, strains were identified based upon extended phenotypic characterization (Muhairwa et al., 2000 in press). Since a genetic basis has been indicated for clusters outlined by using quantitative evaluation of phenotypic data (Angen et al, 1997a; Petersen et al., 1998) the aims of the present study were to: (1) determine the relationships among the isolates from free ranging village chickens and ducks, dogs and cats by quantitative evaluation of phenotypic data (Angen et al., 1997a) and DNA fingerprinting in the form of ribotyping to examine the value of characters used in separation of taxa, and (2) to further investigate by REA whether different clones of P. multocida are exchanged between different species of animals.

2. Materials and Methods

2.1 Bacterial strains

A total of 143 strains of Pasteurella spp. and 10 unclassified strains isolated from free ranging village chickens and ducks, dogs and cats from three subclimatic zones (Hot, Warm and Cool) in Morogoro, Tanzania were investigated (Table 1). Seven type strains were included for comparison. Details of the prevalence of P. multocida and other species in animals in the three zones have been published elsewhere (Muhairwa et al.; 2000, in press).

2.2 Bacteriological investigations

All strains had been stored at -80°C since their original isolation. Lyophilized cultures were inoculated on blood agar (Tryptose blood agar base [Difco, Detroit, USA]) containing 5% bovine blood and incubated aerobically at 37°C for 24 h to control that all strains represented pure cultures. Phenotypical characterization had been done as previously described by Bisgaard et al., (1991) and reported previously by Muhairwa et al. (2000 in press).

2.3 Restriction endonuclease analysis (REA) and ribotyping

One hundred and forty-nine field isolates and 6 reference strains were selected for ribotyping. Isolation of DNA, digestion of the DNA with restriction enzyme HpaII (Boehringer- Mannheim, Mannheim, Germany and Amersham, Buckinghamshire, UK) and subsequent electrophoretic separation of the DNA fragments by agarose gel electrophoresis (AGE) were performed as described previously (Christensen et al., 1993) DNA was visualized by staining the gel with 1μg/mL of ethidium bromide (Sigma^{R}). Polaroid photographs were taken during exposure to 256nm ultraviolet light illumination (Maniatis et al., 1982) DNA was transferred to nylon hybridization membranes (Hybond-N, Amersham) by vacuum blotting as recommended by the supplier (Pharmacia, LKB, Uppsala, Sweden). DNA was fixed by baking at 80°C for 1 h. Ribosomal RNA (16S and 23S) from Escherichia coli (Sigma, St Louis, USA) was purchased and labelled with digoxigenin by using reverse transcriptase (Boehringer Mannheim) as reported previously by Christensen et al. (1993). A digoxigenin-labelled lambda phage (Boehringer-Mannheim) cut with HindIII was used as molecular size marker. REA and Ribotyping based upon new DNA extracts were repeated at least twice for

each strain.

2.4 Data analysis

2.4.1 Numerical analyses

The phenotypical results were transformed to a 5-level numerical scale as previously described by Angen et al. (1997a). Briefly, a strongly positive reaction was scored as 5, a delayed positive reaction as 4, a late and weak reaction as 3, very weak and probably negative reactions as 2, and negative reaction as 1. The analyses were performed by using the taxonomy programme NTSYS (Rohlf, 1993). The average Euclidian distance between the strains was calculated from the 5 level scores. Cluster analyses were performed on the distance matrix using Unweighed Average Linkage/UPGMA (Unweighted Paired Group Method of Arithmetic Averages).

2.4.2 Ribotype patterns

The ribotyping filters were scanned and entered in the Gel Compar (version 4.0, Applied Maths:BVBA) statistical programme. Band positions were analysed using fine optimization and a position tolerance of 1%. The similarity between band patterns of individual strains was estimated using the Dice coefficient, and dendrograms were drawn from the obtained similarity matrix (UPGMA).

2.4.3 REA-patterns

Since REA-typing has been shown to be more discriminatory than ribotyping (Christensen et al., 1998; Dabo et al., 1999) the REA patterns of strains having identical ribotypes were subsequently compared visually to investigate if these strains were clonal.

3. Results

3.1 Phenotyping results

Characters common to all strains identified as Pasteurella spp. are shown in Table 2, while characters separating all the different taxa are shown in Table 3. A dendrogram indicating the phenotypic relationships among the taxa is shown in Figure 1. Eleven phenotype clusters (IA-IE, IIA -IIE and III) and 6 unclustered strains can be seen in the Figure.

Cluster IA contained 34 strains of P. multocida ssp. multocida, including the type strain NCTC 10322^T. These strains were exclusively trehalose positive, D(+) xylose positive, PNPG positive, and PGUA negative. With exception of the type strain and four of the field strains, all strains in this cluster were also D(-) arabinose and L(-) fucose positive (Table 3). Four strains, including one of the four aberrant D(-) arabinose negative and L(-) fucose negative strains, could be differentiated from the other strains by positive reaction in maltose. The isolates in this cluster included 29 cat isolates (16 isolates from hot zone; 6 from warm zone and 7 from cool zone) Three chicken isolates were obtained from one chicken in the warm zone and one strain from a dog obtained from cool zone.

Cluster IB consisted of twenty-five strains of P. multocida ssp. septica including the type strain NCTC 11995^T. All strains had negative sorbitol reaction. Two strains of P. multocida ssp. septica could be distinguished from the remaining strains by being positive in maltose (Table 3). Cluster IB included two strains from dogs obtained from the hot zone, 22 cat isolates (20 isolates from hot zone, 2 from warm zone) and the type strain of P. multocida ssp. septica.

Cluster IC contained four strains of P. multocida ssp. multocida. These strains were D(+) xylose and trehalose positive like members of cluster IA. However, they could be distinguished by being PNPG negative and PGUA positive. All four strains in this cluster were obtained from cats in the cool zone.

Cluster ID consisted of eleven strains of P. multocida ssp. multocida that were D(+) xylose-positive and negative in trehalose and PNPG. Eight of these strains could be separated from the others within the cluster by positive fermentation of maltose. Of the remaining strains two could be distinguished by PGUA positive reaction, while the last strain differed from the others by negative reactions in D(-)

arabinose and L(-) fucose. One P. multocida ssp. multocida was obtained from a dog in the cool zone, while the rest were obtained from cats. Two cat isolates were obtained from warm zone and the remaining eight strains from cool zone. A phenotypically aberrant strain of maltose positive P. multocida ssp. multocida (strain T74) was found unclustered between clusters ID and IE. This was the only strain of P. multocida ssp. multocida investigated which was catalase negative (data not shown); it was also positive in trehalose and PNPG but negative in D(+) xylose and PGUA. This strain was obtained from a dog in the warm zone. Cluster IE included fourteen P. multocida ssp. multocida isolates obtained from ducks, chicken and a cat. All strains of P. multocida ssp. multocida under this cluster had negative reactions in D(+) xylose, trehalose, PGUA and PNPG, and with exception of a cat strain (T88), positive fermentation of L(+) arabinose (Table 3). Eleven strains from ducks and two chicken isolates which were phenotypically identical, were obtained from the warm zone. The cat strain was obtained from hot zone.

The type strain of P. multocida ssp. gallicida NCTC 10204^T appeared as a separate branch clustering deeply with cluster IE. This strain differed from other subspecies of P. multocida by fermentation of dulcitol.

Cluster IIA consisted of 19 strains which included 13 field strains and the type strain (NCTC 11621^T) of P. canis, 3 field strains and the type strain (NCTC 11623^T) of P. stomatis, and an atypical isolate of P. dagmatis. Three strains of P. canis including the type strain, could be separated from the other P. canis strains investigated by negative reactions in both trehalose and PNPG. One of the remaining strains differed from other strains by being negative in PNPG. Although the three field strains of P. stomatis and type strain NCTC 11623^T were included in cluster IIA, they could be separated from P. canis by negative reactions in oxidase and ornithine decarboxylase. Two field strains of P. stomatis had weak raffinose fermentation, while the others were negative. The atypical strain of P. dagmatis had positive reactions in ornithine and urea, but negative reactions in maltose and dextrin (Table 3). P. canis field strains which were obtained from dogs only, included four strains from cool zone, 5 from hot zone and 4 strains from hot zone. Two strains of P. stomatis were obtained from a dog and cat, respectively, in the warm zone, whereas the hot zone isolate came from a dog. The atypical P. dagmatis strain was obtained from a dog in the hot zone.

Cluster IIB contained exclusively strains of taxon 16. All thirty strains in this cluster had positive reactions in dextrin, maltose, trehalose and ribose (Table 3). However, some strains had weak positive reactions on raffinose, D(-) arabinose and L(-) fucose. Twenty-four strains in this cluster were obtained from dogs. These included thirteen, four and seven strains from hot, warm, and cool zone, respectively. The cat isolates included two strains from warm zone and four strains from hot zone.

Cluster IIC included four phenotypically identical strains of unclassified Pasteurella which showed negative sucrose reactions. All strains were obtained from dogs. Two strains were obtained from hot zone and a single isolate each from cool and warm zones. Cluster IID contained four strains of P. dagmatis all of which were oxidase negative. One strain (T142) differed from the others by being negative in trehalose and PNPG. These strains were obtained from dogs only; two isolates from the hot zone and one isolate each from the warm and cool zone.

Cluster IIE contained two strains of P. gallinarum, a duck isolate from warm zone and the type strain, ATCC 13361^T. The field strain of P. gallinarum differed from the type strain by positive reactions in D(+) xylose and ONPG, and by being negative in raffinose, L(-) fucose and PNPG (Table 3). The type strain of P. volantium NCTC 3438^T, remained unclustered, but was related with cluster IIE.

Cluster III contained seven unclassified strains which were indole negative and lacked fermentation of carbohydrates with the exception of D(-) ribose and D(+) glucose. One strain (T156) could be separated from the remaining strains by negative reactions in oxidase, and ribose. Five strains, in this cluster including strain T156, showed positive arginine dihydrolase reactions. Cluster III strains included 5 isolates from dogs and 2 from cats. The dog strains included two strains each, from warm and hot zones, and one strain from cool zone. The cat strains were obtained each from hot and warm zone.

Three strains T158, T159 and T160, remained unclustered and were only distantly related with cluster III. These strains made up a heterogenous group, IV, which had negative reactions in fructose and

phosphatase (data not shown). Strain T158, which was obtained from a dog in the hot zone, had negative reactions in porphyrin and alanine aminopeptidase, but hydrolysed arginine. Strain T159 produced gas from glucose, in addition to being positive in trehalose, maltose, ONPG and ONPF. This strain was obtained from a cat in the hot zone. Strain T160 reacted positively in Aesculin Fe, trehalose, NPG, α-galactosidase, α-mannosidase and ONPF. This strain was isolated from a dog in the hot zone.

3.2 Ribotyping results

A total of 100 ribotypes were obtained with 155 strains investigated. Number of bands ranged from 2 to 13 bands, where most of the strains had 8 to 10 bands. Twelve major clusters, 1-12 and five unclustered strains were outlined (Figure 2). The clusters were mainly defined at a similarity level of 70% or higher. The relationships between phenotype and genotype are shown in Figure 2

Ribotype cluster 1 consisted of 13 strains of P. multocida ssp. multocida which corresponded to phenotype cluster IE. All thirteen strains had identical ribotypes. This cluster corresponded to the L(+) arabinose positive strains within phenotype cluster IE. Ribotyping showed that the L(+) arabinose negative strain T88 in phenotype cluster IE was different from the remaining strains within the cluster.

Ribotype cluster 2 consisted of eleven strains of P. multocida ssp. multocida from cats representing 6 different ribotypes. Nine strains within this cluster corresponded to phenotype cluster ID, while one strain corresponded to phenotype cluster 1A. The type strain of P. multocida ssp. multocida NCTC 10322^T made up the last strain. This cluster represented maltose positive strains, except one of the nine strains linked to phenotype cluster 1D and the type strain of P. multocida ssp. multocida.

Ribotype cluster 3 included of 27 ribotypes representing thirty-two isolates of P multocida ssp. multocida and twenty-three strains of P. multocida ssp. septica. P. multocida ssp. multocida strains in this cluster included twenty-eight of thirty-four strains of P. multocida ssp. multocida which made up phenotype cluster IA, and four strains that corresponded to phenotype cluster IC. The strains of P. multocida ssp. septica which include the type strain NCTC 11995^T corresponded to all but two

of the strains in phenotype cluster IB. In general, ribotype cluster 3 corresponded to all P. multocida strains that were defined by similarities in positive fermentation of trehalose, D(-) arabinose, L(-) fucose, and D(+) xylose. Although D(-) sorbitol is used for separation of P. multocida ssp. multocida and P. multocida ssp. septica, this clear separation was not confirmed by ribotyping. In four cases P. multocida ssp. multocida and P. multocida ssp. septica had identical ribotypes but with different REA-profiles.

Ribotype cluster 4 included five ribotypes representing five strains. These strains included four P. multocida ssp multocida strains from phenotype cluster IA. and one P. multocida ssp. septica strain (T55) that was located in cluster 1B. Three of the four P multocida ssp. multocida strains differed from the other strains in phenotype cluster IA in fermentation of D(-) arabinose and L (-) fucose. The remaining strain of P. multocida ssp. multocida and the P. multocida ssp. septica strain could not be distinguished by phenotypic characters from the other strains of phenotype clusters IA and IB, respectively.

Ribotype cluster 5 included 3 ribotypes representing three strains of P. multocida ssp. multocida and the type strain of P. multocida ssp. gallicida (NCTC 10204^T). The three strains of P. multocida ssp. multocida corresponded to maltose negative strains in phenotype cluster ID. The type strain of P. multocida ssp. gallicida NCTC 10204^T represented a different ribotype which seemed to correspond to differences in fermentations of D(+) xylose, L(+) arabinose, dulcitol, and PNPG reaction.

One strain of P. multocida ssp. septica (T53) representing phenotype cluster IB, and one unclassified strain (T156) which was not closely linked to any of the phenotype clusters, appeared as unclustered strains between ribotype clusters 5 and 6. P. multocida ssp. septica strain T53 was maltose positive. The unclassified strain (T156) differed from the other strains of phenotype cluster III in oxidase and ribose fermentation test.

The type strain P. gallinarum ATCC 13361^T and the field strain which appeared as phenotype cluster IIE made up ribotype cluster 6. A total of five bands, however, separated these strains, which could correspond to phenotypical differences observed between the strains (Table 3).

Ribotype cluster 7 consisted of thirteen ribotypes representing 14 strains of P. canis and a strain of P. multocida ssp. multocida (T74). Although P. canis strains were closely related phenotypically, a wide diversity of the ribotype patterns was observed. Strain T74 which remained unclustered phenotypically, branched deeply within ribotype cluster 7. At least two bands separated this strain from those of P. canis.

Ribotype cluster 8 contained 4 ribotypes representing six unclassified strains. These might represent CDC group EF-4 which has been excluded from the family Pasteurellaceae (Mutters et al., 1989). Four of these strains corresponded to phenotype cluster III while the remaining two strains (T158 and T160) did not cluster with any of phenotype groups. With exception of strain T160 all strains in this cluster hydrolysed arginine.

Ribotype clusters 9 and 10 contained strains of taxon 16 which corresponded to phenotype cluster IIB. Ribotype cluster 9 contained 16 ribotypes representing 18 strains, while ribotype cluster 10 had 11 ribotypes representing 12 strains. Both clusters included dog and cat strains. Sixteen dog and two cat strains were in cluster 9, and eight and four strains, respectively, were in cluster 10. Generally, strains in ribotype cluster 9 included two extra bands which were not seen in strains of ribotype cluster 10. However, these differences were not clearly reflected by phenotype markers. A single unclassified strain (T159), which did not link with any phenotype cluster, appeared between ribotype clusters 9 and 10.

Ribotype cluster 11 contained four ribotypes representing four strains of P. dagmatis which corresponded to phenotype subcluster IID. The atypical P. dagmatis strain (T103) which appeared unclustered was linked to ribotype cluster 11 at a similarity level of 45%. This strain corresponded to P. dagmatis in urease reaction, although phenotypically it had many similarities with P. canis (Figure 1). The type strain (NCTC 11623^T) which was the only strain of P. stomatis investigated by ribotyping remained unclustered between strain T103 and ribotype cluster 12.

Ribotype cluster 12 consisted of 4 ribotypes represented by single strains of unclassified Pasteurella that corresponded to phenotype cluster IIC. One unclassified strain (strain T151) also remained

unclustered by ribotyping. Ribotyping, however, separated this strain from the other two strains of phenotype cluster III.

3.3 Correlation between ribotypes, host and geographical origin of strains

Both similarities and differences in the hosts, phenotype, ribotype, and the zone of isolation were demonstrated among isolates of Pasteurellaceae investigated. Strains of P. multocida ssp. multocida included in ribotype cluster I were obtained from ducks and a chicken in one area within the warm zone. These strains were identical by phenotypical markers and ribotypes. REA-typing, however, separated the chicken isolates from the duck isolates. Strains that classified under ribotype cluster 2 represented maltose positive P. multocida ssp. multocida isolates from cats. Isolates from four cats which were obtained from the same village in the cool zone had identical phenotypes and ribotypes. These isolates also shared the same REA-type underlining a common origin.

Strains in ribotype cluster 3 included chicken, dog and cat isolates. With exception of chicken isolates which were obtained from the warm zone only, the other isolates were obtained from dogs and cats in all three zones. The ribotypes in this cluster generally showed about 80% genetic similarity irrespective of the subspecies (Figure 2). The chicken isolates in ribotype cluster 3, which were obtained from one chicken, had phenotypical features and identical ribotype patterns which were different from the remaining strains. Common ribotype patterns were observed among some isolates from different cats obtained from the same zones, and also some cats from different zones. However, only cat isolates from the same zone shared the same REA-type indicating that transmission of *P. multocida* may take place among cats within the same area. Ribotypes of strains of P. multocida obtained from the same cat by different methods of isolation were similar. One isolate of P. multocida ssp. multocida from a dog and one P. multocida ssp. septica isolate from a cat had identical ribotype patterns although they were obtained from different zones. However, REA profiles of these strains are clearly different. One cat isolate and the type strain of P. multocida ssp. septica NCTC 11995^T had identical ribotype, but different REA profiles.

Four P. multocida ssp. multocida strains in ribotype cluster 4 that were obtained from separate cats

in the hot zone could not be related by ribotype pattern. Two strains from different cats in the warm zone in the ribotype cluster 5 had identical ribotypes but different REA-types. These two strains were not related to a dog strain obtained from the same zone. Only three strains of P. canis which were isolated from the same dog by different methods in the warm zone had identical ribotypes whereas each dog carried a unique ribotype of P. canis. None of the isolates of P. dagmatis showed identical ribotypes. One cat and one dog isolate of unclassified strains (ribotype cluster 8) from warm zone had identical ribotypes and REA profiles. These strains were represented by two ribotypic bands, and REA patterns made of three major bands and numerous small bands. Of taxon 16 strains in ribotype cluster 9, only two ribotype profiles represented isolates from more than one animal. The first ribotype pattern represented one cat isolate and one strain from a dog in the warm zone, whereas the second ribotype pattern represented isolates from two dogs from cool zone. Strains with identical ribotypes also had identical REA-types. Two strains of taxon 16 in ribotype cluster 10, which were obtained from different dogs of hot zone had identical ribotypes and REA-profiles. Comparison of ribotypes among strains obtained from the same host by different methods of isolation showed coexistence of more than one ribotype of taxon 16 in three dogs. All three ribotypes represented strains which were phenotypically similar. Combination of different typing methods seems to indicate that transmission of Pasteurella spp. between animal species rarely takes place under village conditions.

4. Discussion

Within past few years, new insights have emerged into the taxonomy of the Pasteurellaceae and hence into identification of isolates (Bisgaard, 1995). Identification of species/taxa belonging to this family, however, still represents considerable problems for the clinical bacteriologist. Since improving of our understanding of the epidemiology and virulence of these organisms depends on correct criteria for identification, characters used in identification were also subjected to evaluation in the present investigations. These investigations confirmed a correlation between taxa identified by extended phenotypical characterization and subsequent quantitative evaluation of phenotypic data and ribotyping, supporting the existence of a genetic basis for the taxa identified based upon the reclassification of the genus Pasteurella (Mutters et al., 1985). Although use of ribotypes for calculation of taxonomic distances has been questioned (Swofford and Olsen, 1990) other researchers have found ribotyping to represent a useful taxonomic tool (Saunders et al., 1988; Gerner-Smidt, 1992; Lester et al., 1993; Angen et al., 1997b). Discrimination power of ribotyping in differentiating bacteria species is generally considered high due to the presence of specific number of ribosomal operons corresponding to species of bacteria (Woese, 1987). The present results confirm this by showing a good correlation between ribotyping and phenotypic methods for different species of Pasteurella characterized.

Ribotyping has been shown to be useful in epidemiological typing of P. multocida (Wilson et al., 1992; Blackall et al. 1998), however, the ability of this method in judging the clonality of bacteria has been questioned. The highlighting of conserved rRNA genes which may remain unchanged over a long period may make evolutionary different strains look similar (Maslow, 1993). Thus, comparison of REA patterns was used to complement ribotyping in tracing the epidemiologic relationship between the strains investigated in the current study. Isolates with identical ribotypes were compared for REA patterns, phenotypical features, host and zone of isolation. Strains of the same species/subspecies obtained from the same host by different methods of isolation (Muhairwa et al., 2000 in press) were invariably identical by REA, confirming similarities observed in ribotyping and phenotyping.

Phenotypic and genotypic investigations have shown that isolates from chickens and ducks from the same area were closely related, as depicted in ribotype cluster 1. These avian strains were the only L(+) arabinose strains of P. multocida ssp. multocida among the collected strains. Previous investigations by Heddleston (1976) showed that arabinose positive strains were predominant among avian isolates of Pasteurella, whereas none of the canine and feline isolates were arabinose positive. However, these findings did not indicate which isomer of arabinose was employed in the investigations. Therefore, the confirmation of Heddleston's results by the present findings will merely be speculative. The presently obtained L(+) arabinose positive strains were all shown to have identical phenotypes and ribotypes with minor differences in the REA patterns, separating chicken from duck strains. Since all ducks were obtained from the same location, the genomic relationship observed supports the possibility of exchange of strains among ducks under free range management (Muhairwa et al. 2000 in press). Christensen et al. (1998) found close genomic relationship among strains obtained from outbreaks of fowl cholera in chickens, ducks and turkeys. However, the clones obtained in the present study had neither spread to other avian hosts nor to non-avian hosts that were examined. Sampling of dogs and cats were limited, however, by the absence of tame dogs and cats in most of households in the villages.

Inefficiency of ribotyping in subtyping of P. multocida was shown in the strains that fell under ribotype cluster 3, which included all trehalose positive P. multocida ssp. multocida and all strains of P. multocida ssp. septica. Although phenotypically these strains clearly differed by sorbitol fermentation, four of the ribotypes in this cluster were shown to include both strains of P. multocida ssp. multocida and P. multocida ssp. septica. The presence of conserved rRNA genes within the species P. multocida could explain the heterogeneity between these subspecies. However, caution should also be drawn to the relevance of subspeciation of P. multocida into multocida and septica, which seem to be premature from the present findings. According to Mutters et al. (1985) the subspecies multocida is separated from subspecies septica by positive fermentation of sorbitol, and whereas all P. multocida ssp. septica are regarded as trehalose positive, this reaction is variable among P. multocida ssp. multocida. From ribotyping results it seems that trehalose positive strains of P. multocida ssp multocida, are closer genomically with P multocida ssp septica than with trehalose negative strains of P. multocida ssp multocida regardless of similarity in sorbitol

fermentation. The complexity of subspecies of P. multocida has been previously demonstrated by Petersen et al. (1999). Reinvestigation of more isolates representing the different clusters of these subspecies by 16s rRNA analysis and DNA/DNA hybridization would conclude the fate of subspecies in the taxonomy of P. multocida.

The strains of P. multocida in the ribotype cluster 3 also demonstrated wider host spectrum than the strains in the remaining clusters. Chicken isolates in this cluster were shown to have identical ribotypes, but unrelated to ribotypes obtained from dogs and cats. The broad host range of this cluster is highly suggestive of a potential of these strains to adapt to different hosts, although the genetic basis of host diversity of these strains has not been identified. Concluding with certainty that exchange of P. multocida among different animals species can result into successive colonization or disease will require infection studies on pathogen free animals by using heterologous strains.

The prevalence of P. multocida in different animal species investigated (Muhairwa et al., 2000 in press) and management practises of free ranging poultry (Mwalusanya, 1998), indicates that poultry and other animals kept in contact may exchange strains of P. multocida. Total free range management as practised in the villages allows predation of poultry by dogs, cats and wild animals (Mwalusanya, 1998), as well as access to dead poultry by these animals. Besides the risk of bites to chickens and ducks, dogs and cats have access to water and supplementary feed which is occasionally provided to poultry by the owners (Mwalusanya, 1998). Such ways may provide a convenient route of P. multocida strains from dogs and cats to poultry. Since strains from other animals including cats have been demonstrated to be pathogenic to poultry (Snipes et al., 1988), dogs and cats should be regarded as a possible agent for transmission of P. multocida to free ranging village poultry. On the other hand if there is a fowl cholera outbreak in a village, dogs and cats may also help spreading the disease between the flocks through moving of carcasses. Furthermore, these animals may also become transient carriers of P. multocida from poultry and transmit the disease to other flocks. Direct evidence of exchange of P. multocida between poultry and contact animals was not shown in this study, neither were clinical cases of fowl cholera. However, management practises and ecology of P. multocida strains obtained does not exclude both the exchange of strains and presence of disease in the villages. Moreover, presence of identical clone of P. multocida among the ducks in the village

shows that transmission of P. multocida within the same animal species across the village is possible.

The only field strain of P. gallinarum which was obtained from a duck represented a D(+) xylose and ONPG positive variant of P. gallinarum (Table 3). Mraz et al. (1977) and subsequently Droual et al. (1992) described atypical strains of P. gallinarum from chickens that had similar reactions in D(+) xylose and ONPG. Ribotyping results supported the relationship between this strain and the type strain of P. gallinarum ATCC 13361^{T}. The present results support that D(+) xylose and ONPG positive strains may represent a variant of P. gallinarum as indicated by REA and protein profile findings of Droual et al. (1992). Although both Mraz et al. (1977) and Droual et al. (1992) associated this variant with mortality and lesions in chickens, no evidence of disease association in ducks was obtained in the present study. More investigations on the prevalence and pathogenicity of P. gallinarum in ducks are required to understand the importance of this variant in ducks.

With exception of P. dagmatis, which has recently been isolated from an avian host (Bisgaard et al. 1999), the remaining taxa P. canis, P. stomatis, taxon 16 and unclassified taxa have not been previously reported in poultry. The present investigation seems to indicate that these taxa do not colonize poultry even under close contact conditions. Taxon 16 whose final taxonomic position remains to be investigated, was separated into two clusters based on differences in ribotypes. The two groups of taxon 16 could not be separated by phenotypical differences. These findings confirm and extend the findings by Bisgaard and Mutters (1986) that taxon 16 represents a separate taxon within the genus Pasteurella. Unclassified strains included two groups which were separated by biochemical reactions and ribotyping. Two unclassified strains obtained from a dog and a cat were found to be identical, however, due to small number of ribotype bands demonstrated it is presumed that use of another or more than one restriction enzyme (Bingen et al., 1994) could discriminate the strains. Phenotype cluster III (ribotype cluster 8) may represent CDC Group EF-4 bacteria (Tatum et al., 1974), the taxonomic position of which has not yet been ascertained. Hydrolysis of arginine places organisms in this cluster in biovar "a" of EF-4 described by Corboz et al. (1993) and Ganiere et al. (1995). Phenotype cluster IIC (ribotype cluster 12) organisms, might represent a new taxon within the family Pasteurellaceae Pohl 1981. These organisms have tentatively been named Bisgaard's taxon 38 and future isolates from other parts of the world should be kept to improve further taxonomic

investigations.

In conclusion, an overall correlation between phenotyping and ribotyping results was demonstrated. The results also showed phenotypical and genotypical heterogeneity of the strains of P. multocida ssp. multocida and P. multocida ssp. septica. The present findings seem to indicate that exchange of clones of P. multocida between animal species might not take place to an extent expected under close contact for reasons that are presently unknown. Demonstration of atypical strains of P. multocida and unclassified strains by phenotypical features which was supported by ribotyping underlines the importance of extended phenotyping in identification of organisms within the family Pasteurellaceae.

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6. References

Angen, Ø., Aalbæk, B., Falsen, E., Olsen, J.E., Bisgaard, M. 1997a. Relationships among strains classified with the ruminant Pasteurella haemolytica-complex using quantitative evaluation of phenotypic data. Zbl. Bakt. 285, 459-479.

Angen, O., Caugant, D.A., Olsen, J.E., Bisgaard, M. 1997b. Genotypic relationships among strains classified under the (Pasteurella) haemolytica-complex as indicated by ribotyping and multilocus enzyme electrophoresis. Zbl Bakt 286, 333-354.

Bingen, E.H., Denamur, E. Elion, J. 1994. Use of ribotyping in epidemiological surveillance of nosocomial outbreaks. Clin. Microbiol. Rev. 7, 311-327.

Bisgaard, M., Mutters, R. 1986. Characterization of some previously unclassified Pasteurella spp. obtained from the oral cavity of dogs and cats and description of a new species tentatively classified with the family Pasteurellaceae Pohl 1981 and provisionally called Taxon 16. Acta Path. Microbiol. Immunol. Scand. Sect. B. 94, 177-184.

Bisgaard, M., Houghton, S.B., Mutters, R., Stenzel, A. 1991. Reclassification of German, British and Dutch isolates so-called Pasteurella multocida obtained from pneumonic calf lungs. Vet. Microbiol. 26, 115-124.

Bisgaard, M. 1993. Ecology and significance of Pasteurellaceae in animals. Zbl. Bakt. 279, 7-26.

Bisgaard, M., Frederiksen, W., Mannheim, W., Mutters, R. 1994. Zoonoses caused by organisms classified with Pasteurellaceae. In: G.W. Beran and J.H. Steele (Editors), Handbook of Zoonoses. CRC Press, London, pp. 203-208.

Bisgaard, M.1996. Taxonomy of the family Pasteurellaceae Pohl 1981. In Donachie, W., Lainson, F.A; Hodgson, J.C (Editors) Hemophilus, Actinobacillus and Pasteurella. pp 1-7. New York, Plenum Press.

Bisgaard, M., Hinz, K.H., Petersen, K.D., Christensen, J.P. 1999. Identification of members of the Pasteurellaceae isolated from birds and characterization of two new taxa isolated from psittacine birds. Av. Path. 28, 369-377.

Blackall, P.J., Fegan, N., Chew, G.T.L., Hampson, D.J. 1998. Population structure and diversity of avian isolates of Pasteurella multocida from Australia. Microbiol. 144, 279-289.

Carter, G.R., De Alwis, M.C.L. 1989. Haemorrhagic septicaemia. In: C. Adlam and J.M. Rutter (Editors), Pasteurella and Pasteurellosis. Academic Press, London, pp. 131-160.

Chanter, N., Rutter, J.M. 1989. Pasteurellosis in pigs and the determinants of virulence of toxigenic Pasteurella multocida. In: C. Adlam and J.M. Rutter (Editors), Pasteurella and Pasteurellosis. Academic Press, London, pp. 161-165.

Christensen, J.P., Olsen, J.E., Bisgaard, M. 1993. Ribotypes of Salmonella enterica serovar Gallinarum and Pullorum. Av. Path. 22, 725-738.

Christensen, J.P., Dietz, H.H., Bisgaard, M. 1998. Phenotypic and genotypic characters of isolates of Pasteurella multocida obtained from two outbreaks of avian cholera in avifauna in Denmark. Av. Path. 27, 373-381.

Christiansen, K.H., Carpenter, T.E., Snipes, K.P., Hird, D.W., Ghazikhanian, G.Y. 1992. Restriction endonuclease analysis of Pasteurella multocida isolates from three California turkey premises. Avian. Dis. 36, 272-281.

Curtis, P.E., Ollerhead, G.E. 1982. Pasteurella multocida of cats on poultry farms. Vet. Rec. 110, 13-14.

Corboz, L., Ossent, P., Gruber, H. 1993. Isolation and characterization of group EF-4 bacteria from various lesions in cat, dog, and badger. Zbl. Bakt. 279, 140-145.

Dabo, S.M., Debey, B.M., Montelongo, M., Confer, A.W. 1999. Genomic DNA restriction site heterogeneity in bovine Pasteurella multocida serogroup A isolates detected with an rRNA probe. J. Med. Microbiol. 48. 279-286.

Diallo, I.S., Bensink, J.C., Frost, J.C., Spadbrow, P.B. 1995. Molecular studies on avian strains of Pasteurella multocida in Australia. Vet. Microbiol. 46, 335-342.

Droual, R., Walker, R.L., Shivaprasad, H.L., Jeffrey, J.S., Meteyer, C.U., Chin, R.P., Shapiro, D.P. 1992. An atypical strain of Pasteurella gallinarum: Pathogenic, phenotypic and genotypic characteristics. Avian. Dis. 36, 693-699.

Ganiere, J.P., Escande, F , Andre, F G., Larrat, M , Filloneau, C. 1995. Characterization of group EF-4 bacteria from the oral cavity of dogs. Vet Microbiol. 44, 1-9.

Gardner, I.A., Kasten, R., Eamens, G.J., Randall, R.J. 1994. Molecular fingerprinting of Pasteurella multocida associated with progressive atrophic rhinitis in swine herds. J. Vet. Diagn. Invest. 6, 442-447.

GelCompar., Applied Maths BVBA. (4.0) 1996. Kortrijk, Belgium.

Gerner-Smidt, P. 1992. Ribotyping of Acinetobacter calcoaceticus-Acinetobacter baumanii complex. J. Clin. Microbiol. 30, 2680-2685.

Heddleston, K.L. 1976. Physiologic characterization of 1268 cultures of Pasteurella multocida. Am. J Vet. Res. 37, 745-747

Kamny, S.A., El-Hamed, S.A., Fayed, A.A., El-Ghani, A.E. 1983. Studies on Pasteurella multocida in domesticated animals and birds in Aswan governorate. J Egyptian Vet. Med. Ass. 43, 91-100.

Kim, C.J , Nagaraja, K.V. 1990. DNA fingerprinting for differentiation of field isolates from reference vaccine strains of Pasteurella multocida in turkeys. Am. J Vet. Res. 51, 207-210.

Korbel, R., Gerlach, H , Bisgaard, M , Hafez, H.M. 1992. Further investigations on Pasteurella multocida infections in feral birds injured by cats. J. Vet. Med. B. 39, 10-18.

Lester, A , Gerner-Smidt, P , Gahrn-Hansen, B , Sogard, P., Schmidt, J., Frederiksen, W. 1993. Phenotypical characters and ribotyping of Pasteurella aerogenes from different sources. Zbl Bakt. 279, 75-82.

Maniatis, T , Fritsch, E.F., Sambrook, J , 1982. Molecular Cloning, a Laboratory Manual.Cold Spring Laboratory. New York.

Maslow, J.N., Mulligan, M E , Arbeit, R.D 1993. Molecular epidemiology: Application of contemporary techniques to the typing of microorganisms. Clin. infec. Dis. 17, 153-164.

Mraz, O , Jelen, P., Bohacek, J. 1977 On species characteristics of Pasteurella gallinarum Hall et al., 1955. Acta Vet. Brno. 46, 135-147

Muhairwa, A.P., Mtambo, M.M.A , Christensen, J.P., Bisgaard, M. Occurrence of Pasteurella multocida and related species in free ranging village chickens and their animal contacts in Tanzania. Vet. Microbiol. In press,

Muhairwa, A P., Christensen, J.P , Bisgaard, M 2000 Investigations on the carrier rate of Pasteurella multocida in healthy commercial poultry flocks and flocks affected by fowl cholera. Av Path. 29, 133-142.

Mutters, R , Ihm, P , Pohl, S , Frederiksen, W., Mannheim, W. 1985. Reclassification of the genus Pasteurella Trevisan 1887 on the basis of deoxyribonucleic acid homology, with proposal for the new species Pasteurella dagmatis, Pasteurella stomatis, Pasteurella anatis and Pasteurella langaa. Int. J. Syst. Bacteriol. 35, 309-322.

Mutters, R., Mannheim, W., Bisgaard, M. 1989. Taxonomy of the group. In: C. Adlam and J.M. Rutter (Editors), Pasteurella and Pasteurellosis. Academic Press, London, pp. 3-34.

Mwalusanya, N. A. 1998. Studies on productivity and nutritional status of local chickens under village management condition. Royal Veterinary and Agricultural University Copenhagen Denmark. Msc Thesis. pp. 22-40

Olsen, J.E., Brown, D.J., Skov, M.N., Christensen, J P. 1993. Bacterial typing methods suitable for epidemiological analysis. Applications in investigations of salmonellosis among livestock. Vet. Q. 15, 125-135.

Petersen, K.D., Christensen, J.P., Bisgaard, M. 1998. Phenotypic and genotypic diversity of organisms previously classified as maltose positive Pasteurella multocida. Zbl. Bakt. 288, 1-12.

Petersen, K.D., Christensen, H., Christensen, J.P., Bisgaard, M. 1999. Investigations on the genetic relationship between subspecies of Pasteurella multocida as demonstrated by ribotyping and 16S rRNA sequencing. In: HAP conference proceedings 13-17 September 1999, Mabula South Africa.University of Orange Free State,Bloemfontein. p6

Rimler, R.B., Rhoades, K.R. 1989. Pasteurella multocida. In: C. Adlam and J.M. Rutter (Editors), Pasteurella and Pasteurellosis. Academic Press, London, pp. 37-73.

Rohlf, F. J. NTSYS-pc. Numerical taxonomy and multivariate analysis system. (1.80). 1993. New York

Saunders, N.A., Harrison, T.G., Kachwalla, N., Taylor, A.G. 1988. Identification of species of the genus Legionella using cloned rRNA gene from Legionella pneumophila. J Gen. Microbiol. 134, 2363-2374.

Snipes, K.P., Carpenter, T.E., Corn, L.J., Kasten, R.W., Hirsh, D.C., Hird, D.W., McCapes, R.H. 1988. Pasteurella multocida in wild mammals and birds in California: Prevalence and virulence for turkeys. Avian. Dis. 32, 9-15.

Snipes, K.P., Hirsh, D.C., Kasten, R.W., Carpenter, T.E., Hird, D.W., McCapes, R.H. 1990. Homogeneity of characteristics of Pasteurella multocida isolated from turkeys and wildlife in California, 1985-88. Avian. Dis. 34, 315-320.

Swofford, D., Olsen, G.J. 1990. Phylogeny reconstruction. In: D. Hills and C. Moritz., (Editors), Molecular sytematics. Sinauer Associates Inc., Sunderland, Mass., pp. 411-501.

Tatum, H.W., Ewing, W.H., Weaver, R.E. 1974. Miscellaneous Gram-negative bacteria. Manual of Clinical Microbiology. American Society for Microbiology, Washington, D.C., pp. 270-294.

Van Sambeek, F., McMurray, B.L., Page, R.K. 1995. Incidence of Pasteurella multocida in poultry house cats used for rodent control programmes. Avian. Dis. 39, 145-146.

Wilson, M.A., Rimler, R.B., Hoffman, L.J. 1992. Comparison of DNA fingerprints and somatic serotypes of serogroup B and E Pasteurella multocida isolates. J. Clin. Microbiol. 30, 1518-1524.

Wilson, M.A., Morgan, M.J., Barger, G.E. 1993. Comparison of DNA fingerprinting and serotyping for identification of Avian Pasteurella multocida isolates. J. Clin. Microbiol. 31, 255-259.

Woese, C.R. 1987. Bacterial evolution. Microbiol. Rev. 51, 221-271.

Table 1. Strains investigated and designations.

Taxon	Host	No. of strains analysed by		Strain designations
		Phenotyping	Ribotyping	
P. multocida ssp. multocida	Chickens	5	5	T17-T19, T86, T87.
	Ducks	11	11	T75-T85
	Dogs	3	3	T29, T63, T74
	Cats	44	44	T1-T16, T20-T28, T30-T33 T59-T62, T64-T73,T88
NCTC 10322^T	Pig	1	1	T90
P. multocida ssp. septica	Dogs	2	2	T48 &T49
	Cats	22	22	T34-T47, T50-T57
NCTC 11995^T	Human	1	1	T58
P. multocida ssp. gallicida				
NCTC10204^T	Bovine	1	1	T89
P. canis	Dogs	13	13	T91-T104
NCTC 11621^T	Dog	1	1	T105
P. dagmatis	Dogs	5**	5**	T144-T147
P. stomatis	Dogs	2	-*	T106 & T108
	Cat	1	-*	T107
NCTC11623^T	Dog	1	1	T109
P. gallinarum	Duck	1	1	T148
ATCC13361^T	Chicken	1	1	T149
P. volantium				
NCTC3438^T	Chicken	1	-	T150
Taxon 16	Dogs	24	24	T112-T123, T126-T130, T133-T139,
	Cats	6	6	T110, T111, T124, T125, T131, T132
Unclassified strains	Dog	11	10*	T140-T143,T152, T153, T155-T157, T158,T160
	Cats	3	3	T151, T154, T159
		160	155	

* :Field isolates of P. stomatis and a single unclassified strain were not digestible by the restriction enzyme (HpaII)

** :Including atypical strain of P. dagmatis.

Table 2. Common phenotypic characters of 143 Pasteurella species investigated in the present study

Gram negative rods	Non motile at 22°C and 37°C
Positive reactions	
Catalase	Production of acid from:
Fermentative (Hugh &Leifson)	D(-) fructose
Nitrate reduction	D(+) galactose
Alanine aminopeptidase	Glucose (without gas formation)
Phosphatase	Mannose
Porphyrine	
Negative reactions	
β-hemolysis	D(+) fucose
Citrate, Simmon's	L(+) rhamnose
Malonate, base	L(-) sorbose
H_2S production	Cellobiose
KCN, growth	Lactose
MR, 37°C	D(+) melibiose
VP, 37°C	D(+) melezitose
Arginine dihydrolase	D(+) glycogen
Lysine decarboxyalse	Inulin
Phenylalanine deaminase	Aesculin
Gelatinase	Aesculin (Fe)
Tween 20	Amygdalin
Tween 80	Salicin
Pigment formation	Arbutin
Production of acid from:	Gentiobiose
Meso-erythritol	D(+) turanose
Adonitol	Methyl-α-D-glucopyranoside
Xylitol	NPG (β-glucosidase)
L(-) xylose	ONPX (β-xylosidase)
Meso(-)inositol	α-galactosidase
	α-mannosidase

Table 3. Phenotypic characters separating the investigated taxa

Test / Taxon	Subspecies of *P. multocida*					*P. canis*	*P. canis*^{T}
	multocida	*multocida*^{T}	*septica*	*septica*^{T}	*gallicida*^{T}		
Oxidase	+	+	+	+	+	+	+
Urease	-	-	-	-	-	-	-
Ornithine decarboxylase	+	+	+	+	+	+	+
Indole	+	+	+	+	+	+	+
Glycerol	d	-	d	+	(+)	-	-
D(+) arabitol	-	-	-	-	-	-	-
D(+) xylose	d	+	+	+	-	-	-
L(+) arabinose	d	-	-	-	+	-	-
D(-) arabinose	d	-	(+)	+	-	-	-
D(-) ribose	+	+	+/(+)	+	+	+	+
Dulcitol	-	-	-	-	+	-	-
D(-) mannitol	+	+	+	+	+	-	-
D(-) sorbitol	+	+	-	-	+	-	-
L(-) fucose	d	-	+/(+)	+	-	-	-
ONPG (β-galactosidase)	-	-	-	-	-	-	-
Sucrose	+	+	+	+	+	+	+
Maltose	d	-	d	-	-	-	-
Trehalose	d	+	+	+	-	d	-
Raffinose	-	-	-	-	-	-	-
Dextrin	-	-	-	-	-	-	-
ONPF (β-fucosidase)	-	-	-	-	-	-	-
PNPG (α-galactosidase)	d	+	+	+	-	d	-
PGUA (β-glucuronidase)	d	-	-	-	-	-	-

\+ :All strains positive within 1-2 days

(+) :All strains positive within 3-14 days

– :All strains negative within 14 days

d :Both positive and negative strains observed

* :One strain different

** :Two strains different

§ :Some of the strains negative in D(-) fructose, D(-)galactose and mannose

Table 3.

Test / Taxon	*P. stomatis*	*P. stomatis*^{T}	*P. gallinarum*	*P. gallinarum*^{T}	*P. dagmatis*	Taxon 16	Unclassified Cluster IIC	Unclassified Cluster III§
Oxidase	d	+	+	+	- *	+	+	+
Urease	-	-	-	-	+*	-	-	-
Ornithine decarboxylase	-	-	-	-	-*	-**	-	-*
Indole	+	+	-	-	+	+**	-	+
Glycerol	d	-	(+)	(+)	d	-*	-	-
D(+) arabitol	-	-	+	+	-	-	-	-
D(+) xylose	-	-	+	-	-	-	-	-
L(+) arabinose	-	-	-	-	-	-	-	-
D(-) arabinose	-	-	(+)	(+)	(+)*	d	-	-
D(-) ribose	(+)	(+)	+	+	+	+/(+)	+	+**
Dulcitol	-	-	-	-	-	-	-	-
D(-) mannitol	-	-	-	-	-	-	-	-
D(-) sorbitol	-	-	-	-	-	-	-	-
L(-) fucose	-	-	-	(+)	+*	d	-	-
ONPG (β-galactosidase)	-	-	+	-	-	-	-	-
Sucrose	+	+	+	+	+	+	-	-
Maltose	-	-	+	+	+*	+/(+)	-	-
Trehalose	+	+	+	+	d	+/(+)	-	-
Raffinose	d	-	-	(+)	(+)	d	-	-
Dextrin	-	-	+	+	+*	+/(+)	-	-
ONPF (β-fucosidase)	-	-	-	-	-	-	-**	-
PNPG (α-galactosidase)	+	+	-	+	d	+	-	-
PGUA (β-glucuronidase)	-	-	-	-	-	-	-	-

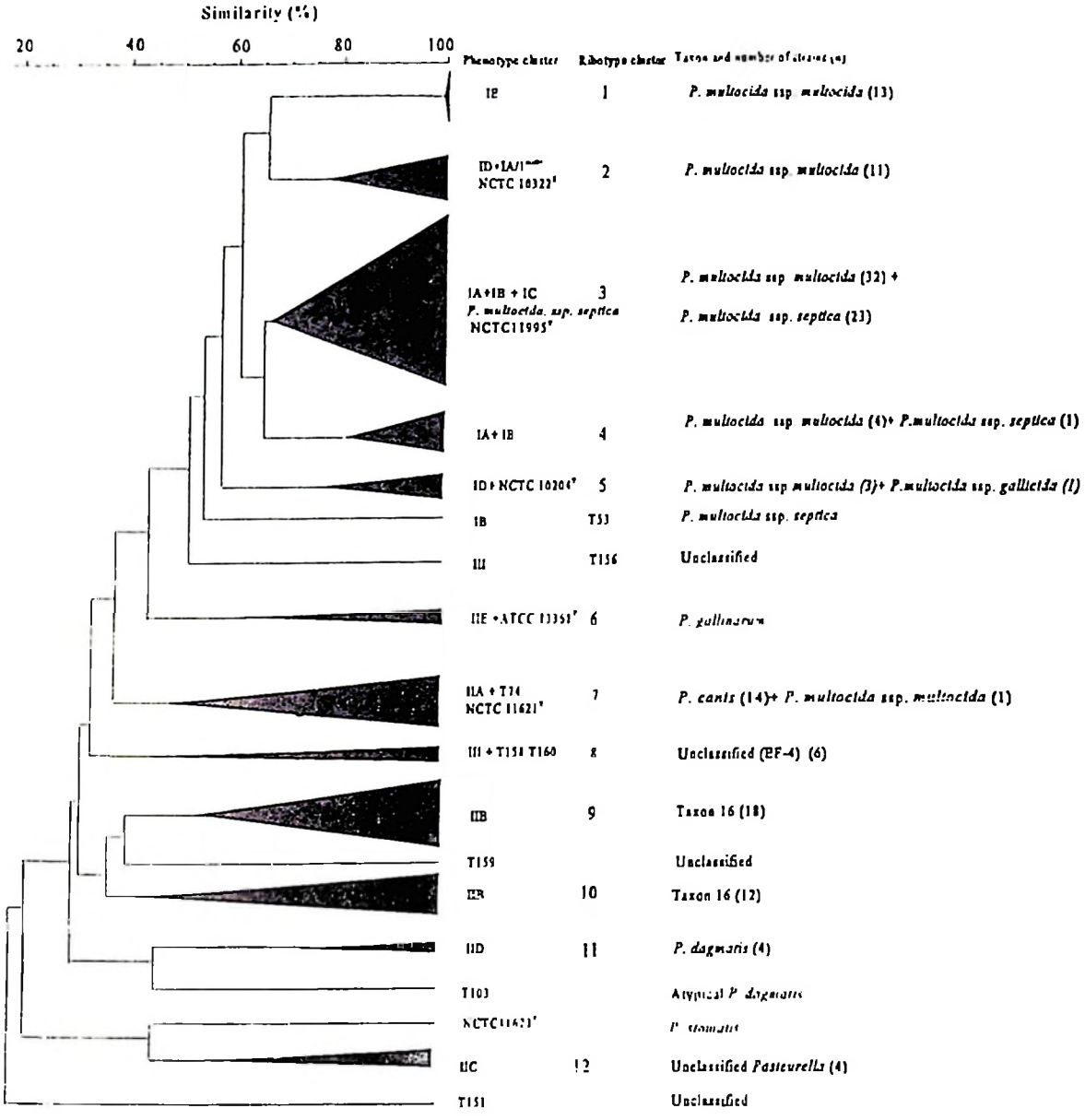
Similarity (%)
20
40
60
80
100
Phenotype cluster
Ribotype cluster
Taxon and number of strains (no.)
IE
1
P. multocida ssp. multocida (13)
ID+IA/1
NCTC 10322T
2
P. multocida ssp. multocida (11)
IA+IB + IC
P. multocida. ssp. septica
NCTC11995T
3
P. multocida ssp. multocida (32) +
P. multocida ssp. septica (23)
IA+IB
4
P. multocida ssp multocida (4)+ P.multocida ssp. septica (1)
ID+NCTC 10204T
5
P. multocida ssp multocida (3)+ P.multocida ssp. gallicida (1)
IB
T53
P. multocida ssp. septica
III
T156
Unclassified
IIE +ATCC 13351T
6
P. gallinarum
IIA + T74
NCTC 11621T
7
P. canis (14)+ P. multocida ssp. multocida (1)
III + T151 T160
8
Unclassified (EF-4) (6)
IIB
9
Taxon 16 (18)
T159
Unclassified
IIB
10
Taxon 16 (12)
IID
11
P. dagmatis (4)
T103
Atypical P. dagmatis
NCTC11621T
P. stomatis
IIC
12
Unclassified Pasteurella (4)
T151
Unclassified

Figure 2

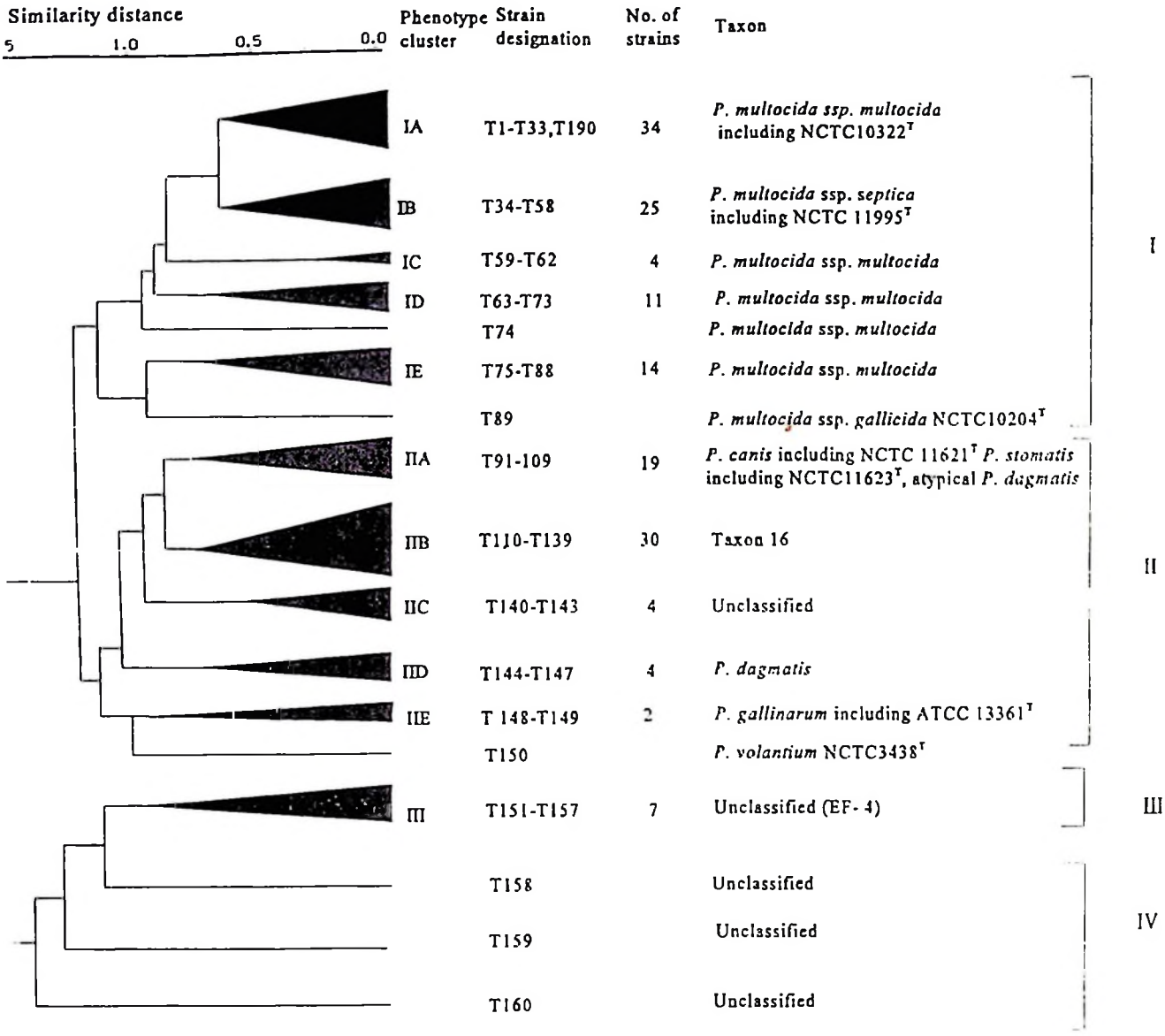
Similarity distance
5
1.0
0.5
0.0
Phenotype cluster
Strain designation
No. of strains
Taxon
IA
T1-T33,T190
34
P. multocida ssp. multocida including NCTC10322T
IB
T34-T58
25
P. multocida ssp. septica including NCTC 11995T
IC
T59-T62
4
P. multocida ssp. multocida
ID
T63-T73
11
P. multocida ssp. multocida
T74
P. multocida ssp. multocida
IE
T75-T88
14
P. multocida ssp. multocida
T89
P. multocida ssp. gallicida NCTC10204T
I
IIA
T91-109
19
P. canis including NCTC 11621T P. stomatis including NCTC11623T, atypical P. dagmatis
IIB
T110-T139
30
Taxon 16
IIC
T140-T143
4
Unclassified
IID
T144-T147
4
P. dagmatis
IIE
T 148-T149
2
P. gallinarum including ATCC 13361T
T150
P. volantium NCTC3438T
II
III
T151-T157
7
Unclassified (EF- 4)
III
T158
Unclassified
T159
Unclassified
T160
Unclassified
IV

Figure 1

Captions

Figure 1. Simplified dendrogram indicating the phenotypic relationships among 160 strains investigated based upon Euclidian distances and UPGMA. Phenotypic clusters, unclustered strains as well as higher order clusters are indicated

Figure 2. Ribotype clusters obtained from analysis of 155 ribotype patterns representing different isolates of P. multocida, other Pasteurella spp. and unclassified strains based on HpaII digestion using fine optimization, 1% position tolerance, Dice coefficient and clustering by UPGMA. Relationships between ribotype clusters and phenotypic groups are indicated.

APPENDIX IV

Investigations on the correlation between serum resistance, outer-membrane proteins and virulence in *Pasteurella multocida*

Investigations on the correlation between serum resistance, outer-membrane proteins and virulence in *Pasteurella multocida*

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Abstract

Serum resistance of *P. multocida* in the sera from chickens, turkeys, ducks and pigs was determined and correlated with *in vitro* and *in-vivo* outer-membrane proteins expression and virulence in chickens. Eighty-seven field strains of *Pasteurella* and nine reference strains representing different clones were grown in sera from chickens, ducks, turkeys and pigs. Serum activity was measured by changes in the optical density of the serum after inoculation and incubation at 41 °C for chicken, turkey and duck serum and 39 °C for pig serum. The strains were classified into High serum resistant (R), moderate serum resistance (M), and serum sensitive (S) by comparing with strains of known serum activity. Strains of identical genotype by Restriction endonuclease analysis were found to have identical growth curves and the same maximum OD values, when cultured in serum from the same host species. Turkey serum was shown to be less inhibitory to a wide range of *P. multocida* strains than chicken, duck and pig sera. Serum resistant strains were demonstrated among avian as well as mammalian strains, with the proportion of serum resistant strains being higher in fowl cholera outbreak strains than in non-outbreak avian strains. A range of minor and major outer-membrane proteins were common among the selected serum resistant and serum sensitive strains, when cultured in BHI, *in-vivo* and in chicken serum. However, no specific OMP expressed *in vitro* or *in vivo* was consistent with serum resistance or sensitivity among the strains investigated. Although most severe lesions in experimentally infected chickens were produced by a serum resistant strain, lesions were also found in chickens infected by serum sensitive strains, indicating the involvement of multiple factors in the virulence of *P. multocida*. Further investigations on serum resistance should also relate other host and bacterial factors responsible development of fowl cholera.

Introduction

Attempts have been made with limited success in understanding the determinants of virulence factors of *P. multocida* obtained from fowl cholera. The role of capsule as a virulence-contributing factor in fowl cholera has been recognized for long time however, other factors such as serum resistance, which also seems to be important in virulence (Lee *et al.*, 1988a; Morishita *et al.*, 1990), have not received comparable attention. Correlation between serum resistance and virulence in animals has been demonstrated for some strains of *P. multocida* (Lee *et al.*, 1988a; Diallo & Frost, 2000) and other Gram-negative bacteria such as *Escherichia coli* (Ellis *et al.*, 1988) and *Yersinia ruckeri* (Davies, 1991). Lee *et al.* (1988a) demonstrated that highly virulent strains of *P. multocida* obtained from turkeys were resistant to turkeys' serum reaching a higher optical density (OD) value than the avirulent strains. Subsequent investigations by Morishita *et al.* (1990) showed the presence of serum resistant virulent *P. multocida* strains among isolates obtained from wild animals in turkey premises. In the same study less virulent strains had lower survival rate in turkeys serum. Serum resistant strains are thus assumed to have a survival advantage in the blood system of the host, which allow them to proliferate and produce disease (Taylor, 1983).

Severity and incidence of *P. multocida* infection is known to vary considerably in different species of birds (Matsumoto *et al.*, 1991; Rimler and Glisson, 1997). Turkeys are considered most susceptible together with ducks while chickens seem to be the least susceptible of the three. Extensive comparison of serum resistance among these birds has not been attempted. Comparing growth of similar strains in sera from different avian hosts may provide more insights about susceptibility of different avian species to *P. multocida* infection. Knowledge of the behaviour in avian sera of strains from hosts supposed to be of importance in the epidemiology of fowl cholera may also be of help in the identification of hosts of importance in the epidemiology of fowl cholera.

Concurrent with the role of serum resistance in virulence, investigations on the determinants of serum resistance in Gram-negative bacteria have also been conducted (Munn *et al.*, 1982; Hansen and Hirsh, 1989). Hansen and Hirsh (1989) showed that the A-type capsule of *P.*

multocida, which mainly consists of hyaluronic acid (Carter and Annau, 1953), was responsible for resistance to bactericidal action of turkey serum to avian strains of *P. multocida.* However, this does not completely explain the cause of serum resistance because non-capsulated strains have also been shown to be serum resistant (Diallo & Frost, 2000). DNA transformation of serum sensitive *Neisseria gonorrhea* strains to serum resistant resulted into structural and antigenic changes of the major outer-membrane protein 39 kDa, of the transformed strain. It was suggested that serum resistance might be related to possession of a 39 kDa subunit of characteristic molecular weight and antigenicity. The role of outer-membrane proteins in *P. multocida* serum resistance is not known. However, variations of outer-membrane proteins expression among strains of *P. multocida* have been demonstrated to vary with environment in which the bacteria are growing (Choi-Kim *et al.*, 1991).

The aims of the present study were to examine if serum resistance of *P. multocida* in the sera from chickens, ducks and pigs correlate with virulence. Expression of outer-membrane proteins in chicken serum, and intraperitoneal chamber in chickens were also investigated to find out if serum resistance and virulence of *P. multocida* might be explained as result of special outer-membrane proteins.

Materials and methods

Serum resistance assays

Bacteria

A total of 87 field strains of *Pasteurella* and nine reference strains (Table 1) were investigated for growth in different sera obtained from chickens, ducks, turkeys and pigs. Field strains included isolates from free ranging chickens and ducks in Tanzania, and dogs and cats kept in contact (Muhairwa *et al.*, 2000b in press), and strains obtained from commercial poultry flocks in Denmark (Muhairwa *et al.*, 2000a). The selected field strains represented clones defined by REA-patterns and ribotypes as described previously (Muhairwa *et al.*, 2000b and c, in press). Reference strains included serum sensitive strains of *Escherichia coli* (K12) (Diallo & Frost, 2000) *P. multocida* (CU vaccine strain), and a serum resistant strain of *P. multocida* strain (P-1059) (Hansen and Hirsh, 1989). Other reference strains included an outbreak clone (P-40605) from wild birds in Denmark, the type strains of *P. multocida* ssp. *multocida* (NCTC 10322^{T}), *P. multocida* ssp. *septica* (NCTC 11995^{T}), *P. multocida* ssp. *gallicida* (HIM 830-7^{T}), *P. gallinarum* (ATCC 13361^{T}), *P. canis* (NCTC 11621^{T}) and *P. stomatis* (HIM 657^{T}).

Collection of serum

Healthy broilers (5 weeks-old), ducks (7 weeks-old) and turkeys (20 weeks-old) from farms with no previous history of *P. multocida* infection were used for blood collection. The collected blood was allowed to clot at room temperature for 1 hour, then cooled to 4 °C for 1 hour followed by centrifugation and filter sterilization (0.2 µl). Pooled pig serum was obtained from *P. multocida* free pigs kept at State Veterinary Laboratory (SVS) Copenhagen, Denmark. All sera were stored at -20 °C and each time before use they were thawed and filter sterilized.

Measurement of serum activity of *Pasteurella* strains

Preliminary assays were conducted using serum sensitive and serum resistant reference strains to determine the concentration of bacteria required for inoculation into the sera. The growth of these strains in serum from individual birds was also examined. Clonal strains were also tested to determine if serum activity might vary within strains of the same genotype.

Lytic action of complement in chicken, duck, turkey and pig sera was monitored by inoculation

of 10 µl of test organisms containing approximately 10^6 c.f.u/ml into 200 µl of normal serum, heat inactivated serum (56 °C for 30 minutes) and Brain-heart infusion (BHI) broth in a microtiter plate. Duplicate suspensions of each strain were subsequently placed in Bioscreen microplate turbidometer (Labsystems, Finland), and incubation was done overnight at 41°C for chicken, duck and turkey serum and 37 °C for porcine serum. Bacterial lysis and growth were monitored by changes in turbidity every 10 minutes, which were automatically recorded in BioLink (Labsystems Computer software). The data were subsequently transferred to Excel program (Microsoft Corporation, 1999) and growth curves were analysed. The experiment was repeated twice in separate days to test reproducibility of the results

The strains were classified into serum resistant and serum sensitive by comparison with the reference serum resistance strain *P. multocida* (P-1059) and serum sensitive CU vaccine strain (Hansen and Hirsh, 1989).

Effect of capsule on serum resistance

Five serum sensitive strains and five serum resistant strains were treated with hyaluronidase to study the effect of capsule on serum resistance. Two previously serotyped strains P1059 (A:3) and P 40506 (A:3) were among the serum resistant strains as reference for capsulated strains. The capsular material was removed from encapsulated strains by growth in BHI broth containing 100 units ml^{-1} hyaluronidase (Sigma Chemical Co.) (Poermadjaja & Frost, 2000). The presence of capsule in the normal and digested bacteria was demonstrated by staining with 1% crystal violet (Sigma) as described by Jasmin (1945). After hyaluronidase treatment, the serum activity of the strains were determined as described above.

Experimental infection of chickens

Chickens

Fifty 18-weeks old layers from a *P. multocida*-free flock were used for experimental infection. Prior to the experiment all chickens were swabbed on the trachea and cloaca, and the presence of *P. multocida* was examined by mouse passage as described by Muhairwa *et al.* (2000a). Briefly, the swabs were transferred into BHI and subsequently vortexed before 0.25 ml was injected intraperitoneally into Balb Cj mice. Chickens were divided into 5 groups of 10 chickens, four

groups were inoculated with different strains of *P. multocida* (Table 3), while the control group was inoculated with sterile BHI broth.

Selection of strains

Four *P. multocida* ssp. *multocida* strains were selected for experimental infection of chickens (Table 3). These included two serum resistant strains, one obtained from a cat (MC 6BA) while the other represented a clone (P-40605) obtained from fowl cholera in wild birds (Christensen *et al.*, 1998), and two serum sensitive strains obtained from a duck (Mamo 2) and a cat (KC 14Hpg). All stock strains had been kept at -80°C since the original isolation. The strains were checked for purity by culture on 5% citrated calf blood agar.

Preparation of cultures for inoculation

Before each experiment deep frozen (-80 °C) strains for inoculation were spread on 5% calf blood agar (Tryptose blood agar Base; Difco) and incubated aerobically at 37 °C overnight to check for purity. Three to five colonies were subsequently inoculated into 10 ml BHI broth and incubated overnight at 37 °C with shaking at 115 rpm. The overnight broth was diluted 1:50 in BHI broth preheated to 41°C and incubated with shaking at 105 rpm to mid-exponential phase. When OD_{410} corresponding to 2 $x10^8$ was reached, one ml was diluted serially in 9 ml broth to a final concentration of 2 $x10^4$ c.f.u/ml. Estimate of bacterial count in the inoculum was obtained from two dilution arrays on blood agar. The chickens in each group were separately inoculated intratracheally with 0.5 ml containing approximately 10^4c.f.u of the respective strain. Control birds were inoculated with 0.5 ml of sterile BHI.

Parameters measured

Twenty-four hours after inoculation 5 chickens in each group were killed by decapitation and post-mortem examination performed to assess the development of the lesions. The remaining birds were screened for tracheal, and cloacal carriers, at 24 hrs, 7 days and 14 days after infection (Table 3). Detection of *P. multocida* carriers in the trachea and cloaca was done by mouse inoculation as described above. At the end of the experiment all the remaining birds were also killed by decapitation and subjected to post-mortem examination

Outer-membrane proteins preparation

Chickens and *P. multocida* strains

The four *P. multocida* ssp. *multocida* strains used for experimental infection (Table 3) were also used for *in vivo* and *in vitro* outer-membrane proteins expressions.

Preparation of bacteria for chambers inoculation

Bacteria for chamber inoculation were grown in 10 ml brain heart infusion (BHI) broth (Difco). Incubations were carried out at 37° C overnight with shaking. One ml of the overnight broth was then taken and subcultured in fresh BHI solution for 2 hrs to mid-exponential phase, which corresponded to 10^7 c.f.u/ml. The organisms were placed in 1.5 ml screw capped Eppendorf tubes, centrifuged and washed twice in normal saline by centrifugation at 4000 rpm for 10 minutes and removing the supernatants. The organisms were resuspended in 1.5 ml normal saline for chamber implantations.

Chamber implants

Chamber implants were prepared and inserted as previously described by Chart *et al.* (1993). Briefly, the 1.5 ml normal saline suspension of bacteria in screw cap Eppendorf tubes were sealed and immersed in 10 ml of 70% aqueous ethanol for 10 minutes. Eppendorf tubes were subsequently washed in sterile saline and placed into sterile dialysis tubing (20 cm long x 1.5 cm diameter), both ends of the tubes were then sealed with a double knot, Eppendorf tubes were uncapped to release the bacteria into the dialysis tube.

Implantation of the chambers

Six weeks-old broilers from *P. multocida*-free sources were used for chamber experiments. Before the experiment the chickens were screened for the presence of *P. multocida* by mouse inoculation and culture on blood agar. The chickens were anesthetized by using isofluorane before surgical preparation of the incision site. A four cm incision was made on the caudal aspect of the ventral abdomen to allow insertion of the implants into the abdominal cavity and the incision was sutured. In each bird 4 chamber implants containing the same strain of bacteria were inserted. To confirm whether growth takes place in the chambers in the abdominal cavity, pilot

experiments hours were conducted. Surface plate counts were performed for samples taken before insertion of chambers and after two hours *in vivo* incubation.

For the major experiment chambers were kept in place for 24 hrs during which time the chickens were monitored for any discomfort of morbidity. At the end the chickens were killed by cervical dislocation and the implants were removed. All chamber implantation experiments were repeated twice to confirm the results.

Samples from the chambers inoculated with *P. multocida* were collected in Eppendorf tubes and were centrifuged at 11000 *g* and the supernatant discarded. The pellets were washed twice by suspension in physiological saline and centrifugation at 4000 g for 10 minutes. Pellets were collected for outer-membrane protein analysis.

***In vitro* grown strains**

For *in vitro* outer-membrane proteins expression, each of the four strains was inoculated in 10 ml BHI and chicken serum. The cultures were incubated overnight with shaking at 37 °C and the cells collected as above.

Preparation of outer-membrane proteins

Detergent insoluble outer-membrane proteins were prepared by a rapid outer-membrane proteins extraction procedure as described by Blackall *et al.* (1990). All centrifugation steps were performed in a microcentrifuge at 14000 g (Carlone *et al.*, 1986). Briefly, 1.5 mg of lysozyme and 85 µl 0.1 M EDTA were added to the washed cells and incubated at room temperature for 20 minutes. The cells were then centrifugated for 5 minutes in microcentrifuge. The supernatant was discarded before resuspension of cells in 1ml cold (4°C) 10 mM HEPES (*N*-2-hydroxylethylpiperzine-*N'*-ethanosulfonic acid; pH 7.4). While held on ice the cells were sonicated (three bursts of 20 seconds each at 50% power on 60% pulsed cycle) with an ultrasonic cell disruptor (Model W- 375 Heat systems Inc., New York). Unbroken cells and debris were removed by centrifugation (2 minutes at 4°C) and the supernatant was centrifuged again for 30 minutes at 4°C. The gel like pellet was resuspended in 200 µl of 10 mM HEPES (pH 7.4) and a

further 200 µl of 10mM HEPES containing 2% (vol: vol) triton X-100 was added. The suspension was left to stand at room temperature for 30 minutes with occasional shaking, centrifugated (30 minute at 4 C) and the obtained pellet was washed without resuspension in 500 µl of 10mM HEPES. The pellet was finally solubilized in 30 µl of double strength electrophoresis sample buffer (Laemli Buffer: Biorad) by heating at 100°C for 5 minutes.

Electrophoresis and Staining

Wells were loaded with approximately 6 µl of the protein sample. Electrophoresis was carried out for about 35 minutes in Mini-PROTEAN ^{R} II Electrophoresis Cell (BIO-RAD, US) at a constant current of 200 volts. SDS-PAGE molecular weight standards, low range (BIORAD), Phosphorylase B (97.4 kDa), Serum albumin (66.2), Ovalbumin (45.0), Carbonic anhydrase (31.0) Trypsin inhibitor (21.5) and Lysozyme (14.4) were used as molecular weight markers.

After electrophoresis, the gels were stained for 3 hours in a staining solution containing 0.1% w/v Coomassie blue G (BDH) in 25% methanol, 10% acetic acid in water (v/v/v). Gels were destained in the same solution but omitting the stain, until the background was clear.

Statistical analysis

Chi square test was used to compare the proportions of serum resistant and serum sensitive *P. multocida* strains in chicken, duck, turkey and pig sera.

Results

Serum resistance study

Preliminary serum activity studies

No variation in the growth pattern and maximum OD values was observed when the same strain was inoculated in sera from different birds of the same species, consequently sera of each species were pooled together for use in the major study. Strains of identical Rea-types were found to have identical growth curves and the same maximum OD values, when cultured in serum from the same host species. Different concentrations of inocula attempted before the major experiment showed that final concentration of about 10^4 c.f.u/ml in the sera and BHI broth resulted in a smooth growth curve. Very low doses resulted into a prolonged lag phase.

Growth of the *Pasteurella* strains in sera from different animals

In BHI broth and heat-treated sera from chickens, ducks, turkeys, and pigs all strains were able to grow as indicated by changes in the turbidity of samples. No *Pasteurella* strain was completely killed in normal chicken and pig serum. However, one *P. multocida* ssp. *multocida* strain and one *P. canis* strain were killed in duck serum and two *P. canis* strains were killed in turkey serum. Maximum OD values of investigated strains ranged from 0.5 to 0.7 in BHI broth; 0.3 to 2 in chicken serum; 0.2-2.0 in duck serum, 0.1-2.0 in turkey serum and 0.2 to 1.2 in pigs' serum (Table 2). Heat inactivated sera resulted into shorter lag phase and slightly higher maximum OD values than those of normal unheated sera. The serum sensitive reference strain *E. coli* K12 demonstrated a short phase of growth followed by inhibition in chickens' serum and was completely inhibited in sera from other animals. However, this strain grew fully in all heat inactivated sera and BHI indicating the presence of complement bactericidal effect in the non-activated sera. The serum sensitive reference strain of *P. multocida*, the CU vaccine strain, grew in all test sera, but the maximum OD values obtained were lower than those of the reference serum resistant strain P-1059 (Table 2).

According to the OD values obtained with the serum resistant and serum sensitive reference strains of *P. multocida*, the remaining strains were classified into high serum resistance (R), moderate serum resistance (M), and serum sensitive (S). Highly serum resistant strains included

strains that had OD values equal or above that of strain P-1059, moderate resistant strains had OD values below that of P-1059 but above that of CU strain. The strains with OD's equal to or below that of CU strain were considered to be serum sensitive (Table 2). Serum activity classification of investigated strains in chicken, duck, turkey and pig sera is shown in Table 1.

Significance of the capsule

Digestion of the capsule by treatment with hyaluronidase was demonstrated from strains P1059 and P 40605. Typical capsules were not demonstrated in any of the remaining eight strains neither before nor after hyaluronidase treatment, however, there was no change in the maximum OD values of all 10 strains tested.

Statistical analysis

The number of *P. multocida* strains that were serum resistant (high and moderate) in turkey serum was statistically significant higher ($P< 0.001$) than the number of strains that were serum sensitive. The number of strains that were serum resistant in turkeys was statistically significant higher ($P<0.001$) than that of serum resistant strains in the chicken, duck and pigs serum.

Experimental infection results

Pathological findings

In the group inoculated with the serum resistant strain (MC 6BA) one chicken was found dead after 24 hours, while no mortality was observed in the remaining groups (Table 3). Variations in the severity of pathological lesions were evident among the chickens infected with different strains both after 24hrs and after two weeks.

Lesions observed in chickens inoculated with serum resistant strains

Twenty-four hours after infection the chickens infected with serum resistant strains had congestion in the trachea, and three out of five chickens infected with strain P-40506 had haemorrhagic tracheitis. The Cat isolate, MC 6BA, caused unilateral fibrinous pleuritis and airsacculitis in all chickens. Two chickens had hepatomegaly with diffuse multifocal greyish spots on the liver surface. Enlarged spleens with multifocal greyish spots were also observed. *P. multocida* was reisolated from spleen of two chickens although not from the chicken that was

found dead. The strain P-40605 caused more severe lung lesions characterized by unilateral fibrinous pleuropneumonia. Livers in four chickens were pale, enlarged and friable and *P. multocida* was reisolated from spleens of all five chickens. Multifocal greyish spots were observed on spleen of one chicken only.

In chickens killed after two weeks, those inoculated with strain MC 6BA strain had unilateral mild pleuritis, which was localized on the caudal margin of the lung lobes. The chickens infected with strain P-40605 were emaciated with one bird on sternal recumbency. All these chickens had unilateral lesions in the lungs, which included oedema and fibrinous pleuritis. The recumbent chicken had unilateral diffuse lung necrosis, and *P. multocida* was reisolated from the spleen.

Lesions in chickens infected with serum sensitive strains

In chickens killed after 24 hours, strain KC 14Hpg caused fibrinopurulent pleuritis, and airsacculitis, which were unilateral in two chickens, and bilateral in the remaining three chickens. Comparatively, the lesions were more severe than those of strain MC 6BA but less than those of strain P-40605. Liver and spleen enlargement were conspicuous in one chicken, which also had multifocal greyish spots on the spleen. *P. multocida* was reisolated from the spleen in the two out of five chickens infected with strain KC 14Hpg. Strain Mamo 2 caused least severe lesions which included marginal pleuritis in the caudal lobes of the lungs in all infected birds. *P. multocida* was isolated from the spleen of one chicken only.

Chickens killed after two weeks in groups infected with strain KC 14Hpg had Fibrinous pleuritis in all birds and unilateral diffuse necrosis of the lungs in two chickens. Slight oedema was observed in the lungs of chickens infected with strain Mamo 2. In both groups *P. multocida* was not reisolated from the spleen.

Carriers of different strains following infection

With the exception of the group infected with strain MC 6BA, *P. multocida* was reisolated from at least one chicken in the other groups (Table 3). However, only strain P-40605 persisted in the trachea of chickens until the end of the experiment. Only one bird that was on sternal recumbency two weeks after infection had *P. multocida* in both the trachea and cloaca, while all

others sampled negative for *P. multocida* from the cloaca.

Outer-membrane proteins

Figure 1 shows the outer-membrane protein profiles of the 4 isolates of *P. multocida* cultured in BHI and intraperitoneal chambers in chicken. All isolates shared a range of minor and major outer-membrane proteins. The most obvious proteins were of the size 35 kDa and 45 kDa, which were shared by all strains when cultured in BHI, *in-vivo* and in chicken serum. An outer-membrane protein of approximately 100 kDa was observed in strains MC 6BA (SR) and KC 14Hpg (SS), in both BHI and in-vivo grown samples (Lanes 2 to 5).

Discussion

Studies of complement activity to *P. multocida* have been done by inoculation of a suspension of viable organisms into serum and determination of surviving organisms by either counting viable cells (Morishita *et al.*, 1990; Diallo and Frost, 2000) or measuring changes in the optical density (Lee *et al.*, 1988a and b). Classification of strains into serum sensitive and serum resistant seems to be arbitrary, because of variations in detection techniques, inoculation dose and incubation time used by different workers (Morishita *et al.*, 1990; Diallo & Frost, 2000; Lee *et al.* 1988b). Lack of a standardised technique for estimating serum activity of *P. multocida* makes meaningful comparison between studies difficult. However, the obtained findings from both viable cell counts and OD changes have been successfully correlated with virulence in poultry (Lee et al., 1988a and b; Morishita et al., 1990). Both methods do not indicate the actual amount of complement deposited on the bacterium. How this affects the classification of strains into serum sensitive and serum resistant is not known, but failure to correlate serum activity with virulence has been reported (Morishita et al., 1990; Diallo and Frost, 2000). Direct detection of deposited complement components C6 and C9 on the bacterial surface by immunofluorecence (Kraiczy *et al.* 2000) was more elaborative in determining the activity of human serum on *Borrelia burgdorferi*. Application of a similar complement detection technique may help to improve studies of *P. multocida* serum resistance in different animal species.

This study has for the first time demonstrated that turkey serum was less inhibitory to a wide range of *P. multocida* strains than chicken, duck and pig sera (Table 1). Sixty-seven out of 80 strains of *P. multocida* (including all three subspecies) investigated had higher OD values than the avirulent serum sensitive CU vaccine strain (Hansen and Hirsh, 1989) in the turkey serum. In chicken, duck and pig sera, only ten, fifteen and thirteen *P. multocida* strains, respectively, had OD values higher than CU vaccine strain. Earlier studies showed that chicken serum was not inhibitory to *P. multocida* while cattle, horse, swine and rabbit sera had various degrees of inhibition to *P. multocida* (Ryu, 1959). Findings based on a single strain by Diallo and Frost, (2000) showed that a *P. multocida* strain sensitive to chicken serum was resistant in turkey, sheep, bovine and rabbit serum. Different assay techniques and limited number of strains employed in those studies does not allow safe comparison with the present results. However, the

results indicate that sera from different animals varies in their reactions to *P. multocida*, and it can be concluded that higher susceptibility of turkeys to fowl cholera correlates with serum activity of *P. multocida* strains.

The present study, has shown that three out of five (60%) *P. multocida* strains obtained from fowl cholera outbreaks were serum resistant in all birds sera, while all nine carrier strains were serum sensitive. Separate studies with outbreak strains found that 60% of the strains were resistant to turkeys' serum (Lee et al., 1988) and 88% resistant in chicken serum (Diallo & Frost, 2000). It can be postulated that serum resistant strains are more prevalent among fowl cholera outbreak strains, for reasons yet to be determined. Serial passage of strains in serum and live birds and comparison of a wider collection of avirulent and virulent host adapted strains is required to reach a sound conclusion on the effect of host on serum resistance to *P. multocida*. Increase in serum resistance subsequent to serial passage in human serum has been demonstrated in *N. gonorrhoea* strains (Ram et al., 1999). A similar change has not been observed for *P. multocida*. However, a change in the virulence of *P. multocida* through a serial intravenous passage in turkeys has been reported (Matsumoto & Strain, 1993). It is possible that through repeated transmissions among birds a strain may adapt to serum components and grow better than newly introduced strains. However, this cannot fully explain the serum resistance of *P. multocida* to avian sera as the present results have shown that resistant strain are present among non-avian isolates, and *P. dagmatis* strains. This suggests the involvement of other determinants of resistance to *P. multocida* to serum bactericidal activity.

In the present study outer-membrane proteins were not demonstrated to correlate with serum resistance of *P. multocida*. No novel outer-membrane proteins were expressed in chicken serum or *in-vivo* chamber that was found to correspond to serum activity. These results imply that serum resistance in *P. multocida* is not determined by existing outer-membrane proteins, or by newly expressed outer-membrane proteins. However, a major outer-membrane protein of approximately 35 kDa, presumably the porin of *P. multocida* (Chevalier *et al.*, 1993; Luo-Yugang *et al.*, 1997) was demonstrated in all strains under different growth conditions. A porin protein in *N. gonorrhoea*, which appears in two main isoforms, Por1A and Por1B, has been linked with serum resistance in human beings (Ram *et al.*, 1999). Por1A has been shown to bind

to factor H a critical regulator of the alternative pathway of the complement system (Ram *et al.*, 1998), thereby inhibiting formation of membrane attack complex. The extent to which *P. multocida* porin or its components are involved in serum activity of *P. multocida* cannot be concluded from the present findings. Characterization of porin proteins from both serum sensitive and serum resistant strains and determining their complement acitivity is required to investigate to what extent they are involved in serum resistance of *P. multocida.*

Early findings by Griffiths *et al.* (1974) suggested RNA machinery to be the target for complement activity to *P. multocida*, but the role of RNA in non antibody-mediated complement killing of serum sensitive and serum resistant strains has not been determined. Subsequently, attempts were made to transform *P. multocida* serum sensitive strains to serum resistant by cloning with plasmids from serum resistant strains (Lee and Wooley, 1995). However, transformants obtained were of intermediate serum resistant compared with serum resistant field isolate. This indicated involvement of other factors in serum resistance of *P. multocida.*

Although the outbreak clone P-40605, which is serum resistant, caused more severe lesions in experimentally infected chickens, serum resistance cannot be concluded to be a determinant of virulence of *P. multocida.* Lesions caused by the serum sensitive strain KC 14Hpg were more severe than serum resistant strain MC 6BA, as well as serum sensitive strain Mamo 2. Strain MC 6BA was not recovered from trachea of the chickens twenty-four hours after infection, whereas the remaining strains were reisolated. However, it was only the outbreak clone that remained in the trachea for up to 7 days, which indicated that the clone was able to colonize in the trachea mucosa for long time. These findings indicate that serum resistance coupled with the ability to colonize the trachea may influence virulence of *P. multocida* strains to chickens. Glorioso et al. (1992) demonstrated the presence of adhesion factors on the cell surface were responsible for colonization to the pharyngeal mucosa by *P. multocida* strains that cause respiratory tract infections in rabbits. Further investigations on the combined influence of adhesion factors and serum resistance might increase the understanding of virulence of *P. multocida.*

In conclusion this study has shown that resistance of *P. multocida* to serum activity occur among strains from different hosts. Although the serum resistant strains are not clonal, strains with the

same genotype have the same serum activity, suggesting that the trait is heterogenous within the species *P. multocida.* Serum resistance in *P. multocida* is not directly correlated with outer-membrane proteins, however, a 35 kDa outer-membrane protein, which was shown in all strains, needs more attention as to its role in serum resistance. Further investigations of serum resistance should also relate other host and bacterial determinants of factors responsible for fowl cholera.

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References

Blackall, P.J., Rogers, D.G., & Yamamoto, R. (1990). Outer-membrane proteins of *Haemophilus paragallinarum*. *Avian Diseases, 34,* 871-877.

Carlone, G.M., Thomas, M.L., Rumschlag, H.S., & Sottnek, F.O. (1986). Rapid microprocedure for isolating detergent-insoluble outer membrane proteins from *Haemophilus* species. *Journal of Clinical Microbiology, 24,* 330-332.

Carter, G.R. & Annau, E. (1953). Isolation of capsular polysaccharides from colonial variants of *Pasteurella multocida*. *American Journal of Veterinary Research, 14,* 475-478.

Chart, H., Conway.D., & Rowe, B. (1993). Outer-membrane characteristics of *Salmonella enteritidis* phage type 4 growing in chickens. *Epidemiology and infection, 111,* 449-454.

Chevalier, G., Duclohier, H., Thomas, D., Shechter, E., & Wroblewski, H. (1993). Purification and characterization of protein H, the major porin of *Pasteurella multocida*. *Journal of Bacteriology, 175,* 266-276.

Choi-Kim, K., Maheswaran, S.K., Felice, L.J., & Molitor, T.W. (1991). Relationship between the iron regulated outer membrane proteins and the outer membrane proteins of in vivo grown *Pasteurella multocida*. *Veterinary Microbiology, 28,* 75-92.

Christensen, J.P., Dietz, H.H., & Bisgaard, M. (1998). Phenotypic and genotypic characters of isolates of *Pasteurella multocida* obtained from two outbreaks of avian cholera in avifauna in Denmark. *Avian Pathology, 27,* 373-381.

Davies, R.L. (1991). Virulence and serum-resistance in different clonal groups and serotypes of *Yersinia ruckeri*. *Veterinary Microbiology, 29,* 289-297.

Diallo, I.S. & Frost, A.J. (2000). Survival of avian strains of *Pasteurella multocida* in chicken serum. *Veterinary Microbiology, 72,* 153-161.

Ellis, M.G., Arp, L.H., & Lamont, S.J. (1988). Serum resistance and virulence of *Escherichia coli* isolated from turkeys. *American Journal of Veterinary Research, 49,* 2034-2037.

Glorioso, J.C., Jones, G.W., Rush, H.G., Pentler, L.J., Darif, C.A., & Coward, J.E. (1982). Adhesion of type A *Pasteurella multocida* and its possible role in rabbit respiratory tract infections. *Infection and Immunity, 35,* 1103-1109.

Griffiths, F. (1974). Metabolically controlled killing of *Pasteurella septica* by antibody and complement. *Biochimica et Biophysica Acta, 362,* 598-602.

Hansen, L.M. & Hirsh, D.C. (1989). Serum resistance is correlated with encapsulation of avian strains of *Pasteurella multocida*. *Veterinary Microbiology, 21,* 177-184.

Heddleston, K.L., Watko, L.P., & Rebers, P.A. (1964). Dissociation of a fowl cholera strain of *Pasteurella multocida*. *Avian Diseases, 8,* 649-657.

Jasmin, A.M. (1945). An improved staiining method for demonstrating bacterial capsules, with particular reference to *Pasteurella*. *Journal of Bacteriology, 50,* 361-363.

Kraiczy, J., Hunfeld, K., Breitner-Ruddock, S., Wurzner, R., Acker, G., & Brade, V. (2000). Comparison of two laboratory mehtods for the determination of serum resistance in *Borrelia burgdorferi*. *Immunobiology, 201,* 406-419.

Lee, M.D., Wooley, R.E., Glisson, J.R., & Brown, J. (1988a). Comparison of *Pasteurella multocida* serotype 3,4 isolates from turkeys with fowl cholera. *Avian Diseases, 32,* 501-508.

Lee, M.D., Brown, J., Wooley, R.E., & Glisson, J.R. (1988b). The relationship of pathogenicity to the growth of *Pasteurella multocida* serotype 3,4 isolates in normal turkey plasma. *Avian Diseases, 32,* 509-512.

Lee, M.D. & Wooley, R.E. (1995). The effect of plasmid acquisition on potential virulence attributes of *Pasteurella multocida*. *Avian Diseases, 39,* 451-457.

Luo-YuGang, Glisson, J.R., Jackwood, M.W., Hancock, R.E.W., Bains, M., Cheng, I.H.N., Wang-ChinLin, Luo, Y.G., & Wang, C.L. (1997). Cloning and characterization of the major outer membrane protein gene (ompH) of *Pasteurella multocida* X-73. *Journal of Bacteriology, 179,* 7856-7864.

Matsumoto, M., Strain, J.G., & Engel, H.N. (1991). The fate of *Pasteurella multocida* after intratracheal inoculation in turkeys. *Poultry Science, 70,* 2259-2266.

Matsumoto, M. & Strain, J.G. (1993). Pathogenicity of *Pasteurella multocida:* its variable nature demonstrated by in vivo passages. *Avian Diseases, 37,* 781-785.

Morishita, T.Y., Snipes, K.P., & Carpenter, T.E. (1990). Serum resistance as an indicator of virulence of *Pasteurella multocida* for turkeys. *Avian Diseases, 34,* 888-892.

Muhairwa, A.P., Christensen, J.P., & Bisgaard, M. (2000a). Investigations on the carrier rate of *Pasteurella multocida* in healthy commercial poultry flocks and flocks affected by fowl cholera. *Avian Pathology, 29,* 133-142.

Muhairwa, A.P., Mtambo, M.M.A., Christensen, J.P., & Bisgaard, M. (2000b). Occurrence of *Pasteurella multocida* and related species in free ranging poultry and their animal contacts in Tanzania. *Veterinary Microbiology, in press.*

Muhairwa, A.P., Christensen, J.P., & Bisgaard, M. (2000c). Relationships among *Pasteurellaceae* isolated from free ranging poultry and their animal contacts as determined by quantitative phenotyping and by ribotyping. *Veterinary Microbiology, in press.*

Munn, C.B., Ishiguro, E.E., Kay, W.W., & Trust, T.J. (1982). Role of surface components in serum resistance of virulent *Aeromonas salmonicida*. *Infection and Immunity, 36,* 1069-1075.

Poermadjaja, B. & Frost, A. (2000). Phagocytic uptake and killing of virulent and avirulent strains of *Pasteurella multocida* of capsular serotype A by chicken macrophages. *Veterinary Microbiology, 72,* 163-171.

Ram, S., McQuillen, D.P., Gulati, S., Elkins, C., Pangburn, M.K., & Rice, P.A. (1998). Binding of complement factor H to loop 5 of porin protein 1A: a molecular mechanism of serum resistance of non-sialylated *Neisseria gonorrhoeae*. *Molecular Immunology, 35,* 398

Ram, S., Mackinnon, F.G., Gulati, S., McQuillen, D.P., Vogel, U., Guttormsen, H.-K., Wetzler, L.M., Oppermann, M., Pangburn, M.K., & Rice, P.A. (1999). The contrasting mechanisms of serum resistance of *Neisseria gonorhhoea* and group B *Neisseria meningitidis*. *Molecular Immunology, 36,* 915-928.

Rimler R.B., Glisson J.R. (1997). Fowl Cholera. *In* B. W. Calnek, H. J. Barnes, C. W. Beard, L. R. McDougald,and Y. M. Saif. (Eds.), Poultry Diseases. London. Mosby-Wolfe. pp. *143-159.*

Ryu. E. (1959). Studies on *Pasteurella multocida* I. Inhibitory action of blood on the growth of *Pasteurella multocida*. *The Japanese Journal of Veterinary Science, 21,* 97-102.

Taylor, P. (1983). Bactericidal and bacteriolytic activity of serum against Gram-negative bacteria. *Microbiological Reviews, 47,* 46-83.

Table 1. Serum resistance observed for different species in the serum from chickens, ducks, turkeys and pigs. Serum resistant (R), moderate serum resistant (M) and serum sensitive (S) strains. Strains P-1059, CU vaccine, and *E. coli* K12 are not shown in the Table.

Species	Host, source and number of strains	Chicken serum			Duck serum			Turkey serum			Pig serum		
		R	M	S	R	M	S	R	M	S	R	M	S
P. m .ssp *multocida*	Chickens (3)	-	-	3	-	-	3	-	1	2	-	-	3
	Ducks -Tanzania (3)	-	-	3	-	-	3	-	-	3	-	-	3
	Ducks -Denmark (3)	2	-	1	2	-	1	2	1	-	2	-	1
	Outbreak P-40605	1	-	-	1	-	-	1	-	-	-	-	1
	Cats (37)	3	-	34	3	2	32	3	31	3	2	3	32
	Dogs (2)	-	-	2	-	-	2	-	1	1	-	-	2
	NCTC 10322^T	-	-	1	-	-	1	-	1	-	-	-	1
P. m. ssp. *septica* (29)	Duck (3)	-	-	3	-	2	1	-	2	1	-	1	2
	Dogs (2)	-	-	2	-	-	2	-	2	-	-	-	2
	Cats (23)	4	-	19	3	2	18	4	16	3	2	2	19
	NCTC 11995^T	-	-	1	-	-	1	-	1	-	-	1	-
P. m. ssp. *gallicida* (1)	HIM 830-7^T	-	-	1	-	-	1	-	1	-	-	-	1
	Subtotal *P. multocida*	10	-	70	9	6	65	10	57	13	6	7	67
P. gallinarum (2)	ATCC 13361^T	-		1	-	1	-	-	1	-	-	1	-
	Duck -Tanzania (1)		1	-	-	-	1	-	-	1	-	-	1
P. canis (5)	Dogs (4)	-	2	2	-	-	4	-	1	3	-	-	4
	NCTC 11621^T	-	1	-	-	-	1	-	-	1	-	-	1
P. stomatis (2)	Dog -Tanzania (1)	-	1	-	-	1	-	-	1	-	-	-	1
	HIM 657^T	-		1	1	-	-	-	1	-	-	-	1
P. dagmatis (4)	Dogs (4)	3	-	1	1	3		4	-	-	3	-	1
	Total	13	5	75	11	11	71	14	61	18	9	8	76

Table 2. Maximum OD values of representative *Pasteurella* strains showing serum resistant (R), moderate (M), and sensitive (S) strains in chicken, duck, turkey and porcine sera. Comparative OD values of the strains in BHI are also shown.

No	Strain	Taxon	Host	Reference	BHI	Chicken		Ducks		Turkey		Pigs	
					OD	OD	SA	OD	SA	OD		OD	SA
1	P 1059	*P. m. multocida*	Turkey	Hansen &Hirsh (1989)	0.7	1.3	R	1.3	R	1.6	R	0.65	R
2	KC 22 Hpg	*P. m. multocida*	Cat	c	0.7	1.8	R	1.2	R	2	R	0.6	M
3	74782-1	*P. m. multocida*	Duck	a	0.67	1.8	R	2.1	R	1.75	R	0.65	R
4	MMC 2	*P. m. multocida*	Cat	c	0.5	1.7	R	1.4	R	2	R	0.7	R
5	71840-1	*P. m. multocida*	Duck	a	0.7	1.7	R	2.1	R	1.75	R	0.65	R
6	P 40605-1	*P. m. multocida*	Eider duck	Christensen *et al.* (1998)	0.65	1.5	R	1.3	R	1.9	R	0.4	S
7	MC 6BA	*P. m. multocida*	Cat	c	0.6	1.8	R	2	R	1.9	R	0.7	R
8	KC 23aBA	*P.m.septica*	Cat	c	0.5	1.6	R	2	R	1.9	R	0.75	R
9	KC 19 Hpg	*P.m.septica*	Cat	c	0.6	1.8	R	1.2	M	1.9	R	0.4	S
10	MMC 3	*P.m.septica*	Cat	c	0.4	1.8	R	2.1	R	1.9	R	0.85	R
11	MC 2 Hpg	*P.m.septica*	Cat	c	0.6	1.7	R	1.2	R	2	R	0.6	S
12	MMD 21	*P. dagmatis*	Dog	c	0.7	2	R	1.2	M	1.9	R	0.85	R
13	KD 21cBA	*P. dagmatis*	Dog	c	1	1.9	R	1.3	R	1.9	R	1.2	R
14	ND 11bBA	*P. dagmatis*	Dog	c	0.5	1.8	R	1.2	M	1.9	R	0.2	M
15	Mao 26	*P. gallinarum*	Duck	c	0.6	0.9	M	0.4	S	0.1	S	0.3	M
16	MD 20c BA	*P. canis*	Dog	c	0.7	1	M	0.4	S	-	S	0.3	S
17	KMD 25	*P. stomatis*	Dog	c	0.4	0.9	M	1.2	M	1	M	0.4	S
18	CU strain	*P. multocida*	Turkey	Hansen &Hirsh, (1989)	0.5	0.75	S	0.55	S	0.65	S	0.45	S
19	MBO 3	*P. m. multocida*	Chicken	c	0.6	0.7	S	0.3	S	1.1	M	0.35	S
20	71660 3a	*P. m. multocida*	Duck DK	a	0.5	0.55	S	0.4	S	1.1	M	0.4	M
21	MD 11c BA	*P. m. multocida*	Dog	c	0.6	0.5	S	-	S	0.4	S	0.3	S
22	NC 4BA	*P. m. multocida*	Cat	c	0.5	0.45	S	0.3	S	0.3	S	0.3	S
23	Mbmo 42	*P. m. multocida*	Chicken	c	0.7	0.35	S	0.4	S	0.6	S	0.4	S
24	Mamo 2	*P. m. multocida*	Duck	c	0.7	0.35	S	0.4	S	1.1	M	0.4	S
25	MBO 39 Hpg	*P. m. multocida*	Chicken	c	0.7	0.3	S	0.2	S	0.6	S	0.4	S
26	77263	*P.m.septica*	Duck DK	a	0.7	0.6	S	0.4	S	0.5	S	0.5	M
27	BM 3	*P.m.septica*	Duck DK	a	0.6	0.55	S	1.1	M	1.1	M	0.4	S
28	ND 15 b	*P. canis*	Dog	c	0.6	0.7	S	-	S	1.1	M	0.35	S
29	ND 13bBA	*P. dagmatis*	Dog	c	0.5	0.2	S	2.4	R	2	R	0.9	R
30	K12	*E coli*			0.8	0.5*	S	-	S	-	S	-	S

a Muhairwa *et al.* (2000a): c :Muhairwa *et al.* (2000c) OD optical density, SA serum activity.

* Complete killing followed a short phase of growth.

- Bacteria were completely killed.

Table 3. Lesions and carrier status of experimentally infected chickens with different strains of *P. multocida* ssp. *multocida*.

Strain	Mortality	Serum acitivity	Isolation of *P. multocida* from the spleen		Trachea carriers**		
			24hr	14days	24hr	7days	14days
MC 6BA	1/5	SR	2/5	0/5	0/5	0/5	0/5
P 40605-1	0/5	SR	5/5	1/5	4/5	5/5	1/5*
KC 14Hpg	0/5	SS	2/5	0/5	3/5	0/5	0/5
Mamo2	0/5	SS	1/5	0/5	1/5	0/5	0/5
Control BHI	0/5	-	0/5	0/5	0/5	0/5	0/5

* *P. multocida* isolated also from trachea and cloaca.

** With one exception (See*) all chickens sampled negative from the cloaca.

Figure1. Comparison of SDS-PAGE profiles of Outer-membrane protein extracts from serum resistant and serum sensitive *P. multocida* strains. Lanes 1 and 10 Molecular weight standards; 2. Serum resistant strain MC 6BA in BHI; 3. MC 6BA grown in intraperitoneal chamber. 4 Serum sensitive strain KC 14Hpg in BHI 5. KC 14 Hpg grown in intraperitoneal chamber. 6 Serum sensitive strain Mamo 2 in BHI; 7. Mamo 2 grown in intraperitoneal chamber 8. Serum resistant strain P- 40605 in BHI; 9. P-40605 grown in intraperitoneal chamber