ISOLATION, IDENTIFICATION AND ANTIBIOGRAM STUDY OF *PASTEURELLA MULTOCIDA* FROM CHICKENS AT DINAJPUR DISTRICT OF BANGLADESH

A THESIS
BY

SULTANA RIFAT FERDOUS
REGISTRATION NO.: 0905072

SESSION: 2009-2010

SEMESTER: MARCH-AUGUST, 2010

MASTER OF SCIENCE (M.S.)
IN
MICROBIOLOGY

DEPARTMENT OF MICROBIOLOGY

FACULTY OF VETERINARY AND ANIMAL SCIENCE

HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY
UNIVERSITY, DINAJPUR-5200

AUGUST 2010

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AUGUST 2010

DEDICATED

TO
MY

BELOVED PARENTS

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The author
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ABSTRACT

The present research work was performed for the isolation and characterization of *Pasteurella multocida* from recent outbreak of chickens and demonstration of antibiotic sensitivity of the selected isolates. The entire research work was conducted primarily in the Bacteriology laboratory of the Department of Microbiology, Hajee Mohammad Danesh Science and Technology University (HSTU), Dinajpur. The samples were aseptically carried out to the laboratory and were subjected to different cultural, morphological and biochemical examinations.

A number total of 106 samples inluding liver, spleen and heart were collected from suspected sick chickens (60 samples) and suspected dead chickens (46 samples) from different selected areas of Dinajpur district. From these samples, 25 (42 %) from suspected sick chickens and 11 (24 %) from suspected dead chickens were found to be positive for *Pasteurella multocida.*

The isolated organism from chickens produced whitish, opaque, round, flat, translucent colonies of sticky mucoid or dry consistency about 1-3 mm in diameter on Nutrient Agar and whitish, opaque, round, translucent colonies without hemolysis on Blood Agar and were able to ferment dextrose, mannitol and sucrose but unable to ferment lactose and maltose on biochemical examinations.

Form the antibiogram study, it was revealed that among the isolated *Pateurella multocida* from chicken, 100% were highly sensitive to Ciprofloxacin and Levofloxacin. 83% were to Neomycin, 78% were to Clindamycin and 3% were to Gentamycin. 75% were moderately sensitive to Doxycicline, 61% were to Gentamycin, 22% were to Clindamycin, 17% were to Neomycin and 14% were to Erythromycin. On the other hand 61% were less sensitive to Erythromycin; 17% were to Tetracycline; 13% were to Gentamicin, 25% were to Doxycicline and 8% were to Bacitracin. 100% isolates were resistant to Amoxycillin, 83% were to Tetracycline; 92% were to Bacitracin, and 25% were to Erythromycin.

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ACRONYMS USED

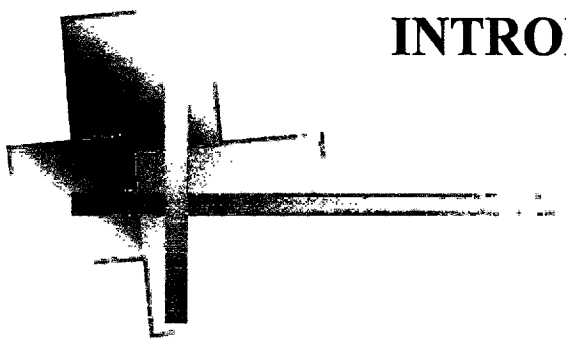
-	=	Resistance
-	=	Negative
%	=	Percentage
+	=	Positive
+	=	Less sensitive
++	=	Moderately sensitive
+++	=	Highly sensitive
µg	=	Microgram
µl	=	Micro litter
AML	=	Amoxycillin
B	=	Bacitracin
BA	=	Boold Agar
BD	=	Bangladesh
C	=	Celsius
CIP	=	Ciprofloxacin
CN	=	Gentamicin
et al.	=	Associates
Fig.	=	Figure
DNA	=	Deoxy ribonucleic acid
DO	=	Doxycycline
DA	=	Clindamycin
E	=	Erythromycin
eg.	=	Example
ELISA	=	Enzyme Linked Immunosorbent Assay
gm	=	Gram
G/L	=	Gram/Litter
HSTU	=	Hajee Mohammad Danesh Science and Technology University
LEV	=	Levofloxacin
MC	=	MacConkey
mg	=	Milligram

ACRONYMS (cont.)

MR	=	Methyl red
min.	=	Minute
NA	=	Nutrient Agar
N	=	Neomycin
NB	=	Nutrient Broth
No.	=	Number
°C	=	Degree centigrade
OIE	=	Office International des Epizooties
PBS	=	Phosphate buffered saline
P. multocida	=	*Pasteurella multocida*
pp	=	Page number
SL	=	Serial
TE	=	Tetracycline
TSI	=	Triple Sugar Iron
V-P	=	Voges-Proskaure
MIU	=	Motility Indol Urea

CHAPTER 1

INTRODUCTION



CHAPTER 1

INTRODUCTION

Poultry industry in Bangladesh is growing day by day. It is an excellent agribusiness, started practically during 1980s in Bangladesh (Haque, 2001). There has been a tremendous development of this sector since last decades (1996-2006) in the country (Rahman, 2003). The sector has already proved itself as a potential income generator and poverty alleviator. At present, there are more then 135 hatcheries producing millions of day old-chicks per week continuously and about one million commercial broiler and layer farms supplying million kgs of meat and about 12 million table eggs per week. But it is true to say that there are factors which are hindering expansion of poultry industries in Bangladesh. Of these, infectious diseases are considered as the most leading causes of economic loss and discouraging poultry rearing in this country (Das *et al.*, 2005). Among the infectious diseases, fowl cholera is a major threat to the poultry industry.

Fowl cholera is a bacterial disease of domesticated and wild avian species caused by *Pasteurella multocida*. The disease is also known as avian cholera, avian pasteurellosis, and avian hemorrhagic septicemia (Rimler and Glisson,1997). It usually appears as a septicemic disease associated with high morbidity, but chronic or benign conditions often occur.

The acute case due to this bacterial agent is characterized by sudden death and the clinical symptoms including fever, diarrhea, anorexia, ruffled feathers, drowsiness, dyspnea, cyanosis and mucous discharge from mouth. Fecal materials initially remain watery and of whitish color which may become greenish later and contain mucous.

The chronic cases may either follow the acute case or due to infections with organisms of lower virulence (Gordon and Jordan, 1982) and is characterized by local infections. In such cases wattle, sinuses, conjunctiva, limbs, joints, foot-pads and sternal bursa often become swollen. Torticollis may occur sometimes.

The gross lesions include hyperemia in veins of abdominal viscera. Petechial and echymotic hemorrhages are widely distributed in the internal organs. There are hemorrhages in lungs, abdominal fat and intestinal mucosa. Peritoneal and pericardial

fluids are also present in increased in quantity. Liver become swollen and contains multiple necrotic foci, coagulative necrosis and sometimes infiltrations are observed in lungs. Ovaries are flaccid and contain thick blood vessels and are hyperemic.

In chronic cases lesions are observed in long bones, hock joints, sternal bursa, foot pad and peritoneal cavities.

The causal agent *Pasteurella multocida* is a Gram negative, non-motile, non-spore forming rod shaped organism occurs in single or in pairs or occasionally as chain form. A capsule can be demonstrated using indirect method of staining of freshly cultured bacteria by Hiss's stain (Calnek et al., 1997). The organisms show bipolar character in Leishman's stain.

A serological classification has been developed for *Pasteurella multocida* in which serotypes were identified on the basis of differences in capsular and somatic antigen. The capsular antigen designated A, B, C, D, E, and F, while the somatic or lipopolysaccharide antigens are designated by number 1 to 16 (Hedlleston *et al*,. 1972). The serotypes B: 2 and E: 2 cause Haemorrhagic Septicemia and A strains are associated with pneumonic disease in cattle. Capsular D strain causes atrophic rhinitis in swine and serotypes A:1, A:3, A:4, A:5, causes fowl cholera in birds. This organisms causes fowl cholera in chicken, turkey, duck and geese. (Chowdury *et al*,.1988, Heddleston & watko, 1965).

Pasteurella multocida is characterized by cultural, biochemical, pathological, serological and molecular study. Culturally this organism is characterized by its whitish, opaque, circular and translucent colonial appearance without haemolysis on Blood agar media. Biochemical characterization of *Pasteurella multocida* is generally performed using sugar fermentation reaction, Methyle Red (MR), Voges Proskauer (VP) and Indol test etc. Serologically, it is characterized by identification of capsular antigens by passive haemagglutination test (Carter, 1955) and somatic antigens by gel diffusion precipitation test (Hedileston *et al*. 1972).

Pathogenicity or virulence of *Pasteurella multocida* is complex and variable, depending on the strain, host species (Rimler and Glisson, 1997). The ability of *Pasteurella multocida* to invade and reproduce in the host is enhanced by the presence of capsule that

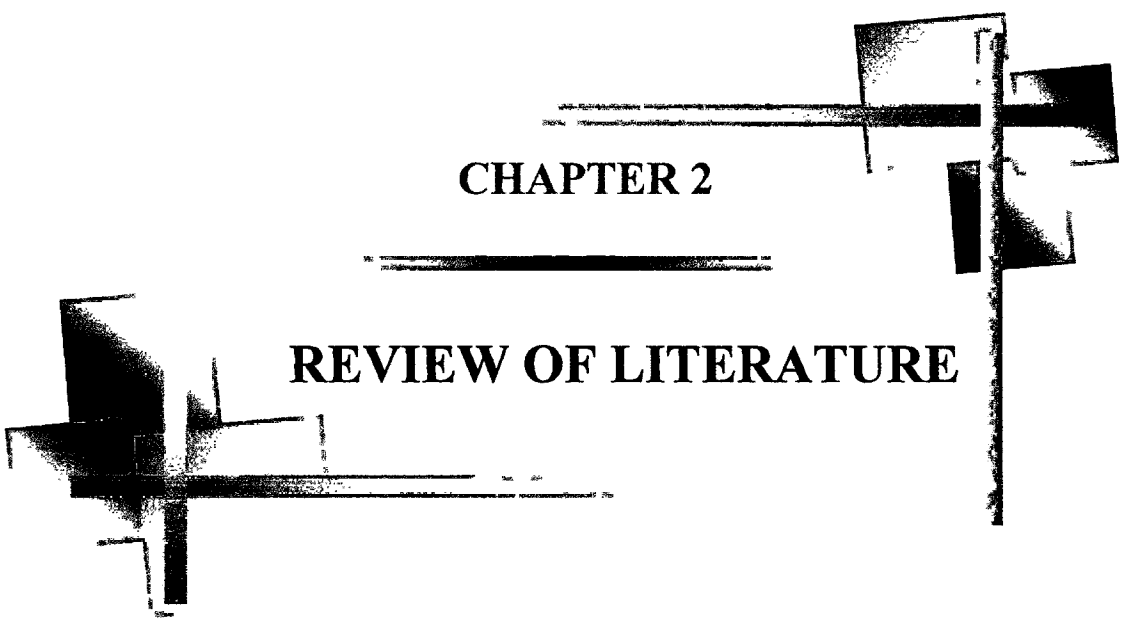
surrounds the organism (Manninger, 1919). Loss of ability of a virulent strain to produce capsule results in loss of virulence (Heddleston *et al.*, 1964).

Result of treatment of Pasteurellosis by antibiotic is not satisfactory in this region because of random use of antibiotics. Therefore it is necessary to study the sensitivity of organisms against some selected antibiotics.

As the Fowl cholera is considered as an economically important disease of chickens of different ages and breeds in Bangladesh, so proper isolation and characterization of the diseases are prerequisite for adapting successful control measure of the disease.

The present study is undertaken with the following objectives:

1) To isolate and identify *Pasteurella multocida* from the suspected sick and dead chickens of different areas of Dinajpur District.
2) To study the prevalence of *Pasteurella multocida* at Dinajpur District of Bangladesh.
3) To study the antibiotic sensitivity of the selected isolates.



CHAPTER 2

REVIEW OF LITERATURE

CHAPTER 2

REVIEW OF LITERATURE

The purpose of this Chapter is to provide a selective review of the past work conducted so far in relation to the present research title "Isolation, Identification and Antibiogram study of *Pasteurella multocida*". The literatures related to the present study are briefly presented under the following headings:

2.1 Historical background of *Pasteurella:*

Pasteur (1880) for the first time isolated the *Pasteurella multocida* by growing pure cultures in chicken broth.

Trevisan (1887) suggested a generic name "Pasteurella" after the name of Louis Pasteur for his remarkable contribution in the field of this organism.

Heddleston (1962) conducted a study to determine biochemical, serological, immunological and pathogenic differences between strains of *Pasteurella* organisms. The bio-chemical and serological studied suggested that the fowl cholera organisms could be divided into at least 2 serological groups and roughly identified by the reaction in dulcitol and xylose medium.

Dorsey (1963) studied the correlation between biochemical classification, serological and immunologic nature of avian *Pasteurella multocida* strains, The strains selected for study were classified bio-chemically (according to their fermentative action on xylose, arabinose and dulcitol) as group I,II or III. A correlation was found between biochemic group and the agglutinative or immunizing action of the strains studied. He, however, remarked that the correlation was not complete and mentioned that some strains, homogenous as to group, were of different serologic or immunogenic type. Based on this, the author concluded that the biochemic grouping would be useful only as a means for roughly selecting strains for more specific serologic or immunologic identification.

Heddleston and Watko (1964) found that besides the avian species, this organism may kill rabbits and mice on experimental infections, mice and rabbits died of acute septicemia after an intranasal infection with *Pasteurella multocida* isolated from acute case of fowl

cholera. They also mentioned that similar infections did not show any clinical response in rats, ferrets and guinea pigs.

Higgins (1969) in a study of fowl cholera in Hongkong, evaluated a blue tetrajolium stained *Pasteurella multocida* whole blood agglutination test to detect infection. He also observed that the isolates of field cases were of a number of bio-chemical types and there were changes in types of organisms within flocks.

2.2 Source and host of *Pasteurella* infection:

Esquinas and Iregui (2007) observed that *Pasteurella multocida* was a multispecies pathogen, that cause serious diseases in wild and laboratory animals as well as in human. This bacterium is etiological agent of diseases such as fowl cholera in chicken and turkey, hemorrhagic septicemia in cattle, atropic rhinitis in swine and infections in human by cat and dog bites. This agent is responsible for the annual losses of hundreds of millions of dollars in animal production, only in United States. In the rabbit, *Pasteurella multocida* can be a virulent fatal pathogen inducing septicemia, pneumonia, chronic rhinitis, otitis as well as abscesses. However, the role of each of these bacteria or some of their virulence factors, like lipopolysaccharides (LPS), in the induction of the disease has not been determined.

Muhaiwam *et al* (2001) determined 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.

Mizan *et al.* (2000) mentioned that most isolates of *Pasteurella multocida* produced sialidase activity, which might contribute to colonization of the respiratory tract or the production of lesions in an active infection.

Hofstad et al. (1976) observed that *Pasteurella multocida* cause infections in a variety of domestic and wild birds like chickens, turkeys, ducks and geese causing a disease known as fowl cholera. He also mentioned that small feral birds like sparrows, crows, starlings and robins etc as well as water fowl and other birds kept in zoological gardens are susceptible to this organism.

Haddleston and Watko (1965) observed that turkeys were more susceptible to *Pasteurella multocida* than chickens and mature chickens were more susceptible than young chickens.

2.3 Transmission:

Blanchong *et al.* (2006) reported that wetlands are unlikely to serve as a long term reservoir for *Pasteurella multocida* because the bacterium dose not persist in wetlands for long time periods following avian cholera outbreak.

Leotta *et al.* (2006) reported the possible cause of outbreak of avian cholera occurred in Hope Bay area, located on the tip of the Antarctic Peninsula, and was that nonbreeder kelp gulls carried *Pasteurella multocida gallicida* to Hope Bay, and avian cholera was transmitted through water to skuas and penguins.

Woo and Kim (2006) they conducted an examination of the origin of the wild bird bacteria but no evidence has been identified that *Pasteurella multocida* strain from the wild bird were introduced into domestic poultry farms.

Morishita *et al.* (1990) found that the organisms with higher survival value were more virulent than that with lower survival value. The authors also reported that birds to bird's passage of bacteria increased the survival value leading to more virulence.

Ficken and Barnes (1989) concluded from their experiment that the air sac might be portal of entry of *Pasteurella multocida* in birds from which the organisms enter into the systemic circulation.

2.4 Clinical signs and gross lesions:

Mizan *et al.* (2000) mentioned that most isolates of *Pasteurella multocida* produced sialidase activity, which might contribute to colonization of the respiratory tract or the production of lesions in an active infection.

Aiello (1998) observed no premonitory sings except death of birds in acute cases of fowl cholera. Mucus discharge from mouth, ruffled feather, anorexia, depression, diarrhea and increased respiratory rate were usually common in acute cases where the sings were appeared.

Poernorno and Sarosa (1996) isolated *Pasteurella multocida* from 28-31 days old broiler chicken which died with clinical signs of lameness and difficult in breathing.

Gordon and Jordon (1985) observed that no premonitory signs in chicken died of fowl of cholera but found dead in good bodily condition. In the acute form depression, anorexia, mucus discharge from the orifices, diarrhea and cyanosis were shown by the affected birds and the authors concluded that those birds survived because of low virulent organisms showed depression, lameness, torticollis and dyspnea, white diarrhea which later become green.

Walker *et al.* (1979) found that *Pasteurella multocida* organisms may also be associated with arthrosynovitis and tendosynovitis in broiler chicken. Thus, they found that infection due to this bacteria in a batch of 6000 chicken of 5 week age were exhibited by a weakness symptom, lameness leading to inability, decumbency, depression and finally death. They also observed sero-purulent and greenish lesions in post-mortem examination and that histopathological examination confirmed acute suppurative arthrosynovitis, lesions including bacteria located in tendon, heart, liver, and spleen. *Pasteurella multocida* isolated from 9/10 joints, 2/9 liver showed Gram-negative cocobacillary form with bi-polarity. They however, could not isolate any organism from heart blood.

Curtis (1979), While presenting an observation on Pasteurellosis of chicken, turkeys, ducks and geese and other birds in Britain, Curtis described that acute cases in chickens (adult birds and broilers) showed hemorrhagic lesions of the type associated with classical fowl cholera, He also mentioned that the chromic cases in chicken manifested classical head lesions affecting comb and wattles, endocardities, egg peritonitis, sinusitis, torticollis and hock lesions.

Djakov (1964) observed during study of experimental and natural infections of acute fowl cholera that there was considerable circulatory disturbance and degenerative lesions in spleen, kidney and liver. The degenerative lesions were prominent as the disease progressed. He further observed that peri-vascular lymphoid cell proliferation were present in liver but less common in heart and kidney.

2.5 Isolation of *Pasteurella multocida:*

Poernomo and Sarosa (2007) identified and estimated of prevalence of *Pasteurella multocida* organisms in different animals and avian species in India. Out of 418 samples collected from different outbreaks suspected to cause by *Pasteurella multocida*, a total of 206 bacterial cultures are identified as *Pasteurella multocida* on the basis of cultural, morphological and biochemical characteristics. All the 206 cultures were isolated from different domestic animal species (cattle, buffalo, sheep, goat, pig and rabbit), avian species (chicken, duck, quail, turkey, goose).

Radad and Moustafa (2006) reported that *Pasteurella multocida* and several bacterial agents associated with some problems in ducks were investigated in different governmental and private farms in Assiut Governorate in Egypt [date not given]. Samples were taken from different tissues showing pathological evidence of infection such as liver, heart, trachea, lungs and spleen, as well as from blood. All samples were subjected to bacterial, mycoplasma and viral isolation. It was found that the total percentages of isolated bacteria (51.7%) were *Pasteurella multocida* (25%). *Escherichia coli* (16.87%), *Campylobacter jejuni* (3.3%) and mixed infection of *Pasteurella multocida* and E coli (6.7%). The present study showed the role of *Pasteurella multocida* as a causative agent in some problems of duck farms at the Assiut Governorate and their sensitivity to some antibiotics.

Muhairwa *et al.* (2001) reported that an investigation was done between November 1997 and June 1998 to determine the presence of *Pasteurella multocida* and related species in free ranging chickens (n=330) and ducks (152), dogs (80), cats (37) and pigs (30) in three climatic zones (cool, warm and hot) of rural Morogoro, Tanzania. A total of 153 isolates of *Pasteurella multocida* ssp. and related species were obtained by direct culture on blood agar, selective medium and mouse inoculation. *Pasteurella multocida* ssp was isolated from 0.7% of chickens and 7% of ducks. In dogs and cats, *Pasteurella multocida* ssp was isolated from 1 and 68^{0}%, respectively. One isolate of *Pasteurella gallinarum* was isolated from a duck.

Asis (2005) observed that Khaki Campbell (KC) and Vigova Super-M (VSM) ducks from an organized farm in Tripura, India, that suffered a total of 8 outbreaks of duck cholera during the period of January 1999 to January 2001 and were presented to the Disease

Investigation Laboratory, Abhoynagar, Agartala, formed the material for this study. Heart blood, spleen, liver and pericardial fluids were collected for histopathological examination, bacterial isolation in blood agar and brain heart infusion agar, pathogenicity test and antibiotic sensitivity test. A total of 18 isolates of *Pasteurella multocida* were obtained. A high percentage (77.77%) of *Pasteurella multocida* isolates were recovered from ducklings as early as 6 weeks of age. Out of the 8 outbreaks, 7 outbreaks occurred in flocks of KC ducks and only one outbreak was recorded in adult VSM ducks. The clinical symptoms observed were loss of appetite, ruffled feathers, whitish followed by greenish colored diarrhea. Mortality ranged from 1120% in different flocks.

Popovici and Mihailescu (1965) observed during an investigation conducted for the isolation of *Pasteurella multocida* from fowl with chronic local infections (sinusitis wattle edema) coming from flocks without a history of fowl cholera it was indicated that the local infections did not precede or follow a fowl cholera out-break. They also observed that out of the 9 strains studied; only those isolated from cases of edema of the wattle increased their virulence under experimental conditions.

2.6 Identification and characterization of *Pasteurella multocida* :

2.6.1 Identification of *Pasteurella* by Gram's staining:

Esquinas and Iregui (2007) observed that *Pasteurella multocida* is Gram negative coccobacilli with several identified virulence factors, like capsule, sialidasa, filamentous haemagglutinin, flagellum, neuraminidaseporins, dermonecrotic toxin, thermolabile toxin (PMT), outer membrane proteins (ONW) and LPS. The lack of effective prevention and control of this type of diseases makes a research of the pathogenesis of the bacteria and its interactions with the host necessary.

Boyce *et al.* (2002) reported that *Pasteurella multocida* is a Gram-negative bacteria pathogen, which causes diseases of economic importance in a wide range of animal species. The response of *Pasteurella multocida* to the host environment has been analyzed at the transcription level, using DNA microarrays, and at the protein-expression level.

Calnek *et al.* (1997) demonstrated that fowl cholera is a Gram-negative, non- motile, non-spore forming rod occurring in single, pairs and occasionally as chains or filaments. A capsule can be demonstrated in recently isolated cultures in indirect method of staining.

Choudhury *et al* (1985) isolated and identified the causal agent of fowl cholera from natural outbreaks in Mymensingh and Dhaka districts. The organisms were identified on the basis of morphology, staining, cultural, biochemical and serological characteristics.

Walker *et al.* (1979) found *Pasteurella multocida* isolated from 9/10 joints, 2/9 liver showed Gram-negative cocobacillary form with bi-polarity. They however, could not isolate any organism from heart blood.

2.6.2 Cultural characterization of *Pasteurella* :

Carter (1976) reported that *Pasteurella multocida* is a heterogenous species occurring widely in animals and associated with a variety of infection. He also suggested that five different varieties or bio-types can readily be identified by the presence or absence of following characters: hyaluronidase, decapsulation, acreflavin floculation, colonial iridescence, fermentation of several carbohydrates, mouse pathogenicity and serum protection test. He also proposed that 5 bio-types of these organisms could be referred as:

1) Mucoid type,
2) H.S. bio-type,
3) Porcine bio-type,
4) Canine bio-type,
5) Feline bio-type.

Bond *et al.* (1970) studied colonial features of *Pasteurella multocida* and their use in diagnosing fowl cholera in Turkey. They mentioned that viewed through stero-microscope with an oblique source of light, colonies of *Pasteurella* isolated from turkey were classified into 3 types, iridescent, intermediate and blue and that no grey colony were seen. They also recorded that the method followed for observing bacterial colonies were highly accurate for presumptive identification of *Pasteurella multocida* in approximately 3000 examination of suspected colonies on dextrose starch agar.

2.5.3 Biochemical, molecular, serological and immunological characterization of *Pasteurella* :

Tang X *et al.* (2009) obtained a total of 233 isolates of *Pasteurella multocida* from 2,912 cases of clinical respiratory disease in pigs in China, giving an isolation rate of 8.0%. Serogroup A *P. multocida* isolates were isolated from 92 cases (39.5%), and serogroup D

isolates were isolated from 128 cases (54.9%); 12 isolates (5.2%) were untypeable. *P. multocida* was the fourth most frequent pathogenic bacterium recovered from the respiratory tract, after *Streptococcus suis, Haemophilus parasuis*, and *Escherichia coli*. All isolates were characterized for their susceptibilities to 20 antibiotics and the presence of 19 genes for virulence factors (VFs). Use of PCR showed that colonization factors (ptfA, fimA, and hsf-2), iron acquisition factors, sialidases (nanH), and outer membrane proteins occurred in most porcine strains. The VFs pfhA, tadD, toxA, and pmHAS were each present in <50% of strains. The various VFs exhibited distinctive associations with serogroups: concentrated in serogroup A, concentrated in serogroup D, or occurring jointly in serogroups A and D. These findings provide novel insights into the epidemiological characteristics of porcine *Pasteurella multocida* isolates and suggest that the potential threat of such multi resistant bacteria in food-producing animals should not be neglected.

Kehrenberg *et al* (2008) investigated three florfenicol-resistant *Pasteurella multocida* isolates from Germany, two from swine and one from a calf, for the genetics and transferability of florfenicol resistance. The isolates were investigated for susceptibility to antimicrobial agents and plasmid content. Florfenicol resistance plasmids carrying the gene floR were identified by transformation and PCR. Plasmids were mapped, and a novel plasmid type was sequenced completely. PFGE served to determine the clonality of the isolates. In one porcine and the bovine *Pasteurella multocida* isolate, florfenicol resistance was associated with the plasmid pCCK381 previously described in a bovine *P. multocida* isolate from the UK. The remaining porcine isolate harboured a new type of floR-carrying plasmid, the 10 226 bp plasmid pCCK1900. Complete sequence analysis identified an RSF1010-like plasmid backbone with the mobilization genes mobA, mobB and mobC, the plasmid replication genes repA, repB and repC, the sulphonamide resistance gene sul2 and the streptomycin resistance genes strA and strB. The floR gene area was integrated into a region downstream of strB, which exhibited homology to the floR flanking regions found in various bacteria. PFGE revealed that the floR-carrying *Pasteurella multocida* strains from Germany were unrelated and also different from the UK strain.

Dziva *et al.* (2008) examined potential diagnostic and selected typing systems for investigating diseases caused by *Pasteurella multocida*. Detection of *Pasteurella multocida* from clinical specimen by; (i) isolation and identification, (ii) polymerase chain reaction (PCR), iii) specific hybridization probes, (iv) serological tests and (v) other alternative methods is critically evaluated. These detection systems provide a wide

spectrum of options for rapid diagnosis and for detecting and understanding of latent infections in herd/flock health control programs, though PCR methods for detecting *Pasteurella multocida* in clinical specimen appear increasingly preferred. For establishing the clonality of outbreak strains, we select to discuss macromolecular profiling, serotyping, biotyping, restriction enzyme analysis, ribotyping and multiplex PCR typing. Although *P. multocida* infections can be rapidly diagnosed with molecular and serological tests, isolation and accurate species identification are central to epidemiological tracing of outbreak strains.

Dabo *et al.* (2007) they classified *Pasteurella multocida* is a pathogenic Gram-negative bacterium into three subspecies, five capsular serogroups and 16 serotypes. *Pasteurella multocida* serogroup A isolates are bovine nasopharyngeal commensals, bovine pathogens and common isolates from bovine respiratory disease (BRD), both enzootic calf pneumonia of young dairy calves and shipping fever of weaned, stressed beef cattle. *Pasteurella multocida* A:3 is the most common serotype isolated from BRD, and these isolates have limited heterogeneity based on outer membrane protein (OMP) profiles and ribotyping.

Townsend *et al.* (2006) reported that the strains of *Pasteurella multocida* serogroup D cause progressive atrophic rhinitis, and produce a potent, intracellular, mitogenic toxin known as *Pasteurella multocida* toxin (PMT), which is encoded by the tox A gene. Authors concluded that the toxigenicity of *P. multocida* is mainly derived from PMT.

Christensen H *et al.* (2005) observed the identification of *Pasteurella multocida*-like bacteria negative for acid formation from sucrose, including isolates from bite wounds caused by large cats, 17 strains were phenotypically and genotypically characterized. Phylogenetic analysis of partially sequenced rpoB and infB genes showed the monophyly of the strains characterized and the reference strains of *Pasteurella multocida*. The sucrose-negative strains formed two groups, one related to reference strains of *P. multocida* and the other related to a separate species-like group (taxon 45 of Bisgaard). DNA-DNA hybridization further documented the species-like nature of this group. Ribotyping showed the heterogeneity of all strains except four strains that shared the same ribotype and that were isolated from bovine lungs. Phylogenetic analysis by 16S rRNA sequence comparison showed the monophyly of the strains characterized and the reference strains of *Pasteurella multocida*. Two strains isolated from leopard bite wounds were

related to the type strain of *Pasteurella dagmatis*; however, they represented a new taxon (taxon 46 of Bisgaard), in accordance with their distinct phenotypic and genotypic identifications. The present study documents that sucrose-negative strains of *P. multocida*-like bacteria belong to two genotypically distinct groups. The study further confirms the phenotypic heterogeneity of *Pasteurella multocida* strains and documents two new species-like taxa of *Pasteurella* related to *Pasteurella multocida*. Until diagnostic tools have been further elaborated, special care should be taken in the identification of *Pasteurella*-like bacteria isolated from bite wounds caused by large cats. The evidence of phenotypic and genotypic divergence calls for the further development of PCR tests and DNA sequencing to document doubtful isolates.

Kumar *et al.* (2004) reported identification and estimation of the prevalence of *Pasteurella multocida* organisms in different animal and avian species in India during November 2000 to July 2003 was carried out. Out of 418 samples collected from different outbreaks suspected to be caused by *Pasteurella multocida*, a total of 206 bacterial cultures were identified as *Pasteurella multocida* on the basis of cultural, morphological and biochemical characteristics. All the 206 cultures were isolated from different domestic animal species (cattle, buffalo, sheep, goat, pig and rabbit), avian species (chicken, duck, quail, turkey, goose) and wild animals such as leopard and deer. Serotyping of *P. multocida* cultures revealed the presence of various serotypes (A:1, A:3, A:1,3, A:4, B:2, D:1and -:1) among the livestock population. *Pasteurella multocida* polymerase chain reaction (PCR) assay applied on different forms of bacterial cultures (bacterial culture lysate, direct bacterial colony and mixed bacterial culture lysate) yielded an amplified product of 460 bp specific for *Pasteurella multocida*. The results of PCR assay correlated well with conventional methods of identification. The present investigation revealed the presence of varied serotypes among livestock and PCR assay was found to be useful for rapid, sensitive and specific diagnosis of pasteurellosis in animals and avian species.

Christensen H. *et al.* (2004), observed that strains deviating in key phenotypic characters, mainly isolated from cases of bovine pneumonia in five European countries, were genotyped in order to examine their genotypic relationship with *Pasteurella multocida*. Ribotyping was used to examine the relationship of the strains, and 13 types, each containing between one and 20 isolates were observed. Identical ribotypes were observed in some cases for *Pasteurella avium* biovar 2 and either *Pasteurella canis* biovar 2 or *Pasteurella multocida* subsp. *septica*. ITS (16S-23S rRNA internal transcribed spacer)

fragment-length profiling showed identity of the majority of strains (47 of 52), representing all four taxa, with only five divergent strains. A 16S rRNA sequence comparison of 11 strains representing the main ribotype clusters showed 99.9 % similarity to the type strain of *Pasteurella multocida subsp. multocida,* but only 97.4 % similarity was obtained to *Pasteurella canis* (biovar 1) and 93.7 % to *Pasteurella avium* (biovar 1). A species-specific PCR test for *P. multocida* gave a positive result with biovar 2 variants of *Pasteurella avium* and *Pasteurella canis*. DNA-DNA hybridizations between strains of *Pasteurella multocida*, biovar 2 variants of *Pasteurella avium* and *Pasteurella canis*, and *Pasteurella multocida* subsp. septica confirmed similarity at the species level. It is proposed, on the basis of genotypic similarity, that *Pasteurella multocida* be reclassified to include the biovar 2 variants of *Pasteurella avium* and *Pasteurella canis* and that the existence of the biovar 2 variants of *Pasteurella avium* and *Pasteurella canis* is highly questionable.

Songserm *et al.* (2003) reported that *Pasteurella multocida* group B, serotype 3, was isolated from sinusitis-affected khaki Campbell ducks. To study the role of *P. multocida* in sinusitis, commercial khaki Campbell ducks were experimentally infected with *Pasteurella multocida* alone or combined with *Escherichia coli.* In Expt. 1, experimental ducks were infected with *Pasteurella multocida* intranasally or ocularly. A comparison was done by intranasal inoculation with pooled nasal discharge from the affected ducks or phosphate-buffered saline. The ducks intranasally inoculated with the nasal discharge or *Pasteurella multocida* showed sinusitis. In Expt. 2, *E. coli* alone or a combination of *Pasteurella multocida* and *E. coli* was intranasally inoculated into experimental ducks. The ducks intranasally inoculated with the combination of *Pasteurella multocida* and *E. coli* had sinusitis.

Weiser *et al.* (2003), found *Pasteurella multocida* is a highly diverse group of bacteria recognized as important pathogens. Although *Pasteurella multocida* is not ordinarily associated with disease in Rocky Mountain bighorn sheep (Ovis canadensis canadensis), numerous isolates were cultured in high numbers from free-ranging bighorn sheep in the Hells Canyon area of Idaho, Washington, and Oregon (USA) during the winter of 1995-96. Animals captured in Hells Canyon and held in captivity, and their offspring, also harbored *Pasteurella multocida.* Biochemical utilization tests on 90 isolates identified three subspecies: *Pasteurella multocida multocida* a (n=54); *Pasteurella multocida multocida* b (n=13); and *Pasteurella multocida gallicida* (n=15); and a non-speciated

biotype, U6 (n=8). Genomic DNA digestion with restriction endonuclease Hha I separated the isolates into 62 unique restriction fragment length polymorphism profiles. Capsular type A was predominant (72% of isolates). Only one isolate type, which may have been transmitted from a feral goat, was capsular type D, possessed the structural gene, toxA, for dermonecrotoxin detected by polymerase chain reaction, and produced toxin as determined by monoclonal antibody immunoblot. In conclusion, bighorn sheep in this study carried diverse types of generally non-toxigenic *Pasteurella multocida* associated with epizootic pneumonia.

Petersen D *et al.* (2001) investigated the genetic diversity of *Pasteurella multocida*, the etiological agent of fowl cholera. The strain collection comprised 69 clinical isolates representing a wide spectrum of hosts and geographic origin. The three type strains for the subspecies of *Pasteurella multocida* were also included. Avian isolates of *Pasteurella multocida* subsp. *multocida* and *Pasteurella multocida* subsp. *septica* did not represent separate lines by HpaII ribotyping and the two type strains of mammalian origin (porcine and cat bite) seemed to be representative of avian strains of *Pasteurella multocida* sub spp. *multocida* and *septica*. By ribotyping, all *Pasteurella multocida* sub spp. *gallicida* strains, except one chicken isolate and the type strain, clustered together. This indicated that the bovine type strain was not representative of this subspecies and that most strains of *Pasteurella multocida* subsp. *gallicida* are genetically related and may be distantly related to other *Pasteurella multocida* isolates, including those of avian origin. By 16S rRNA and atpD sequence comparisons of selected strains, including both *Pasteurella multocida* isolated from birds and mammals and selected distantly related *Pasteurella* species associated with birds and mammals, it was found that *Pasteurella multocida* is monophyletic. Extended DNA-DNA hybridizations are highly indicated since strains may exist which would connect the existing subspecies at species level. The considerable genetic diversity of *P. multocida* fowl cholera isolates is probably related to the colonial nature of this organism, resulting in many divergent lines.

Rimler *et al.* (1989) used passive haemagglutination (PHA) test for specific somatic antigen typing and examined *Pasteurella multocida* inoculated chicken serum as source of antibody.

Choudhury *et al.* (1987) used passive haemagglutination (PHA) test to determine the antibody level in serum following administration of a formalin inactivated fowl cholera vaccine.

Sawada (1985) performed indirect haemagglutination test (IHA) by using sheep RBC for the detection of antibody titers of birds after immunization.

Curtis (1979) recorded that two strains grew on MacConkey agar prepared from a fresh batch of dehydrated medium whereas many more could be grown on MacConkey agar prepared for a batch which had been in store for a considerable time. He also observed that all the isolates were indol, catalase and oxidase positive and all fermented sucrose, dextrose and mannitol without gas production. In the same study Curtis (1979) found that out of the 102 isolates 13 fermented arabinose, seven dulcitol four lactose and three maltose. He could not establish relation between fermentation reactions and species of origin or serotype. He also remarked that the absence of H_2S production did not mean that *Pasteurella multocida* would not produce H_2S if more sensitive methods were employed.

Gupta *et al.* (1978) used 20 isolates from post-mortem cases, field out-breaks and local stock strains in order to sero-type Indian isolates of *Pasteurella multocida* from poultry. Capsular typing revealed that 14 strains belonged to type A and one to type D and 4 to type B. One could not be typed, The somatic typing of Indian strains of avian origin undertaken for the first time revealed that 13 strains fell in '0' group 5, 4 in '0' group 6, and one each in '0' groups 2 and *3,* one could not be typed. They found that the overall serotypes of the strains were, 5: A for 13 strains, 2: D and 3 : A for one strain each and 6 : B for four strains. The isolation of 6: B strain from poultry was considered to be significant as its reservoir status as this serotype was responsible for causing hemorrhagic septicemia in cattle in India. They also considered the possibility of existence of strains of complex antigenic structure with wider host range as were indicated by that investigation.

Curtis (1976) produced specific antisera in fowls to 14 Heddleston type strain (VB 43, abstr. 596), in serotyping British isolates of *Pasteurella multocida* from avian species. and used these in gel diffusion test on antigen prepared from 50 *Pasteurella multocida* isolates from 31 turkeys, 6 ducks, 6 geese, 2 fowls and 5 other birds involved in separate out-breaks. Of these, 14 strains were serotyped 2 and 15 were serotyped 3. He also

recommended serotyping for determining the extent that the new strains were being introduced over a period of time into farms a recurrent cholera problem.

Heddleston (1976) observed the physiologic characteristic of 1,268 culture of *Pasteurella multocida,* 97-1001 of the cultures fermented galactose, glucose, mannitol, mannose, fructose and sucrose, produced hydrogen sulphide and indol and reduced nitrate, He also observed that 6-91% of the cultures fermented arabinose, glycerol, sorbitol, trehatose and xyloseo Fermentation of dextrin, dulcitol, inositol, inolin, lactose, maltose, raffinose, rhamnose and salicin, growth on MacConkey agar, changes on litmus milk, production of unease and haemolysin, liquefaction of gelatin and motility were negative for 97-100% of cultures. Of 200 cases, tested for catalase and oxidase, all were positive. The result indicated that none of these tests would determine the host of origin.

Carter and Runde (1975) identified *Pasteurella multocida* capsular antigen serotype A by hyaluronidase.

Carte and Subronto (1973) determined capsular antigen types for all 17 field strains of *Pasteurella multocida.* They identified capsular antigen serotype D by acriflavin test.

Heddleston (1972) used heat stable antigens extracted with formalinized saline in the gel diffusion precipitation test to group *Pasteurella multocida* associated with fowl cholera into 5 serotypes. Of 258 field isolates tested, 33 were -type 1, 157 were type 3, 28 were type 4, 4 were type 5, and 4 were type 6, He also observed that 27 isolates reacted with type 3 antisera and to a lesser extent with 4 antisera, and 5 isolates did not react with any antisera. Bacterins prepared with *Pasteurella multocida* serotype 1, 3 and 4 induced good immunity in chicken and turkeys against organisms of the same serotypes. A bacterin prepared from serotype 4 culture induced a good immunity against a serotype 5 challenge exposure culture as was induced with homologus bacterin. He also confirmed the serological behavior of heat stable antigens by gel diffusion and immunoelectrophoresis with partially purified lipopolysaccharides protein complex (endotoxin) that had induced specific immunity in turkeys Lines of identity were observed by him in the gel diffusion test but a slight; difference was observed with immuno-etectrophoresis.

Donahue *et al.* (1971) conducted a study to differentiate and compare the bio-chemic, serologic and immunogenic properties of 10 isolates of *Pasteurella multocida* of turkey origin in Missouri. On the basis of their fermentative characters, the isolates were divided

into 3 biochemic groups and with a plate agglutination test they were categorized into 4 serologic groups. In cross protection test in turkeys, 2 of the 10 isolates were immunologically similar. These isolates also belonged to the same serologic grouping. There was little relationship between result of biochemic tests and immunologic tests. With 8 of the 10 isolates, the immunity elicited by the autogenous bacterins was superior or equal to the immunity elicited by the haterologous bacterins or the monovalent bacterins produced from isolates of *Pasteurella multocida* used in commercial bacterins. In view of the differences in mortality, these workers suggested that the cross protection induced by the haterologous bacterins was significant in many instances as compared with that ill the unvaccinated controls. They also observed that two of the isolates induced little autogenous immunity.

Langpap and Matisheck (1970) while reporting, their observations on the prevalence of avian *Pasteurella multocida* serotypes confirmed the findings of Heddleston (1962,68) and mentioned that there were three immunologically distinct, types of the organism. These types were exemplified by Heddleston (1962,68) as strain X-73, P-1059 and P-1662.

2.7 Virulence factors, mortality, morbidity and prevalence:

Tabatabai (2008) reported that four proteins include protective bacterial surface antigen, OMA87 heme-hemopexin receptor,HemR lactate premease, LetP and heptosyl transferase F, RfaF. Both the OMA87 and HemR proteins would be of interest for subunit and modified live vaccine studies, respectively, because of their purported roles as virulence factors for *Pasteurella multocida.*

Shivachandra *et al.* (2006) studied the prevalence of capsular and somatic serotypes among 123 *Pasteurella multocida* strain isolated from chickens (n=94), ducks (22), quails (4), turkeys (2) and geese (1) from different geographical regions of India. All strains exhibited similar cultural and morphological characteristics.

Harper *et al.* (2006) identified the key virulence factors to date which included capsule and lipopolysaccharide. The capsule is clearly involved in bacterial avoidance of phagocytosis and resistance to complement, while complete lipopolysaccharide is critical for bacterial survival in the host. A number of other virulence factors have been identified by both directed and random mutagenesis including *Pasteurella multocida* toxin (PMT), putative surface adhesions and iron inquisition proteins.

Biswas *et al.* (2005) reported that Newcastle disease had the highest proportional mortality rate (15.81%). The proportional mortality caused by fowl pox, fowl cholera, salmonellosis, colibasilosis, aspergillosis, infectious bursal disease, mixed infections and undiagnosed cases were 8.96%, 6.76%, 7.09%, 6.93%, 0.33%, 2.04%, 10.51% and 41.56% respectively.

Borrathybay *et al.* (2003) studied the capsule thickness of avian *Pasteurella multocida* type A strains was determined by transmission electron microscopy after labeling with polycationic ferritin and compared with their pathogenicity for chickens.

Chung *et al.* (2001) observed that the capsule is essential virulence determinant in the pathogenesis of fowl cholera.

Wijewardana *et al.* (1999) recorded that the capsulated form of *Pasteurella multocida* were more virulent than that of non-capsulated forms because the capsulated form of bacteria might easily overcome the humeral defense of the body.

Khan (1997) observed that the virulence of bacteria was enhanced by bird to bird passage and reported that the first passage of bacteria at dose rate of 4x 10^6 CFU inoculated intramuscularly killed 50% of birds while a dose of 5x 10^6 CFU of third passage bacteria killed 100% of chickens.

Rhoades and Rimier (1990) observed that following exposure of chicken to *Pasteurella multocida* via the upper respiratory tract, the organisms were recovered only from the dorsal pharynx at 6 hours after exposure and from the internal organs (lung, heart and liver) at 12 and 24 hours alley exposure.

Tsuji and Matsumoto (1989) reported that the capsule of *Pasteurella multocida* had preventive capacity against rapid phagocytosis and extra cellular killing by humoral immunity. The authors concluded that the capsulated *Pasteurella multocida* produced disease rapidly than the non capsulated one.

Kamal *et al.* (1988) observed 6.43% mortality in chickens due to fowl cholera in Bangladesh Agricultural University (BAU) Poultry Farm, Mymensingh.

Choudhury *et al.* (1985) observed 25% to 35% mortality in chickens of Bangladesh due to fowl cholera.

Wickramasinghe and Peiris (1985) reported that the mortality due to fowl cholera was higher (27.4%) in 62 weeks old birds than the 29 weeks old birds (8.7%). It was found moderately pathogenic in 2 months old chickens when infected intramuscularly.

Curtis *et al.* (1980) studied on the virulence and morphology of *Pasteurella* of avian origin and assessed the virulence of 100, 54 and nine avian isolates for mice, chicken and turkey points respectively by rating on a scale from 0 to 5. They also examined the cultural and colonial morphology of bacteria after mouse passage and observed that there was evidence of a considerable range in the virulence of isolates for the test species, but no correlation between cellular or colonial morphology and virulence was detected.

Coates *et al.* (1977) observed 6.7% mortality in turkeys when a live a virulent stain of *Pasteurella multocida* was inoculated orally at a dose of 1.2×10^{7} bacteria.

Toshkov *et al.* (1975), observed changes in virulence of *Pasteurella multocida* and its influence on the course of epizootics of fowl cholera was observed. They found that strains of *Pasteurella multocida* isolated during recent out-breaks of fowl cholera in Bulgaria, varied in virulence and that the virulence of isolated strains was related to the intensities of disease out-breaks. They further observed that strains of low virulence increased rapidly in virulence upon repeated passage.

2.8 Antibiogram study of *Pasteurella*:

Tang *et al.* (2009) observed the frequency of antimicrobial resistance among *Pasteurella multocida* isolates from swine in China was higher than that reported among *Pasteurella multocida* isolates from swine in from other countries, and 93.1% of the isolates showed multiple-drug resistance. There was a progressive increase in the rate of multi resistance to more than seven antibiotics, from 16.2% in 2003 to 62.8% in 2007. The resistance profiles suggested that cephalosporins, florfenicol, and fluoroquinolones were the drugs most likely to be active against *Pasteurella multocida*. Use of PCR showed that colonization factors (ptfA, fimA, and hsf-2), iron acquisition factors, sialidases (nanH), and outer membrane proteins occurred in most porcine strains. The VFs pfhA, tadD, toxA, and pmHAS were each present in <50% of strains. The various VFs exhibited distinctive

associations with serogroups: concentrated in serogroup A, concentrated in serogroup D, or occurring jointly in serogroups A and D. These findings provide novel insights into the epidemiological characteristics of porcine *Pasteurella multocida* isolates and suggest that the potential threat of such multi resistant bacteria in food-producing animals should not be neglected.

Aarestrup *et al.* (2008) used large amounts of antimicrobial agents are still being used in modern swine production in many countries around the world. This facilitates the emergence and development of antimicrobial resistance. Bacteria causing infections in swine have in several cases acquired resistance to a number of the agents most commonly used for treatment, making it difficult to predict the efficacy of different antimicrobial agents without prior susceptibility testing. This review gives an overview of recent susceptibility data from different parts of the world and discusses the importance of the development of resistance not only in the treatment of infections in swine but also taking into account the human health implications of antimicrobial resistance.

Yokose N and Dan K (2007) observed *Pasteurella multocida* exists in a variety of animals and causes diverse infections in humans due to animal bites and scratches, usually by cats or dogs, and oral and respiratory infection. We report a case of *P multocida* sepsis due to a scratch from a pet cat, complicated with disseminated intravascular coagulation in a post-chemotherapy neutropenic patient with non-Hodgkin lymphoma. The patient was a febrile 79-year-old woman with disturbed consciousness and subcutaneous abscess in her right hand due to a scratch from a pet cat. She was successfully treated with empirical antibiotic therapy with cefepime and administrations of granulocyte colony-stimulating factor and danaparoid. The minimum inhibitory concentration of cefepime against the isolate from this case was <2mg/L. Although a few days are required before a diagnosis of *Pasteurella multocida* infection can be made from a bacteriological study, the infection can be successfully treated against febrile neutropenia with empirical cefepime. In a literature review, 7 cases, including ours, with hematological malignancies complicated with *P. multocida* infection were identified and we summarized the clinical characteristics of these cases. These cases demonstrate the importance of the prevention of close contact between pet animals and immunocompromised hosts such as post-chemotherapy neutropenic patients.

Catry B *et al.* (2006) studied to determine which *Pasteurella* and *Mannheimia* species are present in the upper respiratory tract of healthy calves with no history of antimicrobial treatment prior to sampling. The presence of subpopulations of tetracycline-resistant Pasteurellaceae was also investigated. Nasal swabs from 61 loose group-housed, clinically healthy calves, 1 to 4 months old, from 16 dairy herds were inoculated aerobically on a selective medium (Columbia agar with 5% ovine blood and 16 mg/L bacitracin) with or without 4 mg/L oxytetracycline (OTC). A total of 43 strains belonging to the family Pasteurellaceae were isolated from 38 calves (62.3%) out of 13 herds (81.3%). The predominant organisms were *Pasteurella multocida* subsp. multocida (57.4%), *Mannheimia varigena* (4.9%) and *M. haemolytica* (3.2%). Growth of Pasteurellaceae on the OTC-containing medium was seen only with samples from two herds (6 animals; 9.8%), and on only one farm this proved to be an OTC-resistant subpopulation. Minimum inhibitory concentration (MIC) determinations by means of agar dilution confirmed a low prevalence of OTC-resistant Pasteurellaceae, with overall MIC(50) and MIC(90) values of 0.25 and 32 mg/L, respectively. These data do not support the hypothesis that the relative high frequency of tetracycline-resistant *Pasteurella multocida* isolates from fatal cases of bovine respiratory disease is related to the presence of minor tetracycline-resistance subpopulations within this species.

Asis (2005) observed that Khaki Campbell (KC) and Vigova Super-M (VSM) ducks from an organized farm in Tripura, India, that suffered a total of 8 outbreaks of duck cholera during the period of January 1999 to January 2001 and were presented to the Disease Investigation Laboratory, Abhoynagar, Agartala, formed the material for this study. Antibiotic sensitivity test of all the *P. multocida* isolates revealed that the organisms were highly sensitive to chloramphenicol, enrofloxacin and gentamicin. and resistance to cotrimoxazole, ampicillin and cephalexin.

Catry B *et al.* (2005) studied to measure the level of antimicrobial resistance in potential bovine respiratory pathogens at different production types, nasal swabs were collected from 57 calves of 13 dairy herds, 150 calves of 9 beef cattle herds, and 289 calves of 5 high-density veal calf herds and investigated for the presence of Pasteurellaceae. All calves were less than 6 months old. Susceptibilities of the *Pasteurella* and *Mannheimia* isolates to eight antimicrobials were determined using an agar dilution method. *P. multocida* (37.3%) and hemolytic *Mannheimia* organisms (M. haemolytica sensu lato) (6.3%) were the most frequently detected organisms. The overall prevalence of isolates

resistant to at least one antimicrobial from the dairy, beef, and veal calves were 17.6% (6/34), 21.9% (14/64), and 71.9% (64/89), respectively. In isolates obtained on the veal calf herds, acquired resistance to ampicillin, oxytetracycline, potentiated sulfonamides, gentamicin, tilmicosin, and enrofloxacin was frequently present, and 32.6% of these isolates were resistant to more than two of the tested antimicrobials. Resistance to ceftiofur and florfenicol was not detected. A substantial within-herd variability of species diversity and resistance profiles among isolates belonging to the genera *Pasteurella* and *Mannheimia* was found among the isolates of the veal calf farms.

Welsh *et al.* (2004) srudied between 1994 and 2002, a total of 390 (46.3%) *Mannheimia haemolytica*, 292 (34.7%) *Pasteurella multocida*, and 160 (19.0%) *Histophilus somni* were isolated at the Oklahoma Animal Disease Diagnostic Laboratory from lungs from 6-18-month-old beef cattle with pneumonia. The ratio of *M. haemolytica* isolations to *P. multocida* isolations decreased from 3.1 in 1994 to 0.8 in 2000 while increasing to 1.5 in 2002. *Mannheimia haemolytica* isolations significantly ($P < 0.05$) decreased from 62.5% in 1994 to between 30.6% and 50.4% in 1998-2002. *Pasteurella multocida* isolations significantly ($P < 0.05$) increased from 20.0% in 1994 to between 28.6% and 47.4% in 1998--2002. *Histophilus somni* isolations were <19% except in 1998 (40.8%) and 1999 (23%). For *P. multocida*, antimicrobial susceptibilities significantly declined for erythromycin ($P = 0.0001$), florfenicol ($P = 0.004$), spectinomycin ($P = 0.03$), sulfachloropyridizine ($P = 0.028$), tetracycline ($P = 0.017$), tilmicosin ($P = 0.0001$), and trimethoprim/sulfamethoxazole ($P = 0.0003$).

Kehrenberg *et al.* (2001) investigated Tetracycline-resistant isolates of *Pasteurella multocida* and *Mannheimia* spp. from respiratory diseases in cattle and swine were investigated for the classes of tet gene and their chromosomal or plasmid location. The 34 isolates comprised eight *Pasteurella multocida*, 23 *Mannheimia haemolytica*, two *Mannheimia varigena* and a single *Mannheimia glucosida* isolate. Identification of the tet genes was achieved by PCR analysis and hybridization with specific probes. Transformation and hybridization experiments served to confirm the plasmid location of tet genes. Selected tet genes and their adjacent regions were sequenced. The tet genes tet(B), tet(G) and tet(H) were detected. The gene tet(H) was present in 26 isolates. The 4.4 kb tet(H)-carrying plasmid pMHT1 was detected in six isolates representing all four species. In the remaining 28 isolates, copies of tet (B), tet(G) and tet(H) were identified as chromosomal. No correlation between the tet gene type and the MIC of tetracycline, or

between the number of tet gene copies and the MIC of tetracycline was observed. Tetracycline resistance in *Pasteurella multocida* and *Mannheimia spp.* is mediated by at least three different tet genes. A new type of tet (H)- carrying plasmid, pMHT1, was identified. The detection of pMHT1 in M. glucosida and M. varigena is the first report of resistance plasmids in isolates of these two species. For the first time, tet(G) genes were detected in members of the family Pasteurellaceae.

Kehrenberg *et al.* (2001) observed isolates of the genera *Pasteurella* and *Mannheimia* cause a wide variety of diseases of great economic importance in poultry, pigs, cattle and rabbits. Antimicrobial agents represent the most powerful tools to control such infections. However, increasing rates of antimicrobial resistance may dramatically reduce the efficacy of the antimicrobial agents used to control *Pasteurella* and *Mannheimia* infections. This review presents a short summary of the infections caused by *Pasteurella* and *Mannheimia* isolates in food-producing animals and the possibilities of preventing and controlling primary and secondary pasteurellosis. Particular reference is given to antimicrobial chemotherapy and the resistance properties of *Pasterurella* and *Mannheimia* isolates. The genetic basis of the most predominant resistance properties such as resistance to beta-lactam antibiotics, tetracyclines, aminoglycosides, sulfonamides, and chloramphenicol is discussed. This is depicted with reference to the role of plasmids and transposons in the spread of the resistance genes among Pasteurellaceae and members of other bacterial families and genera.

Willson PJ. (1990) observed clinical isolates of members of the family Pasteurellaceae show resistance to drugs used for therapy of common infectious diseases of animals. Veterinarians want to use an antimicrobial that is effective against the pathogen, continues to provide therapy for several days, and is not too expensive. Resistant bacteria have complicated the problem of selecting the best antibiotics for treatment of livestock. Resistance to some antibiotics, such as those which inhibit ribosome function, may be encoded on the chromosome; however most antibiotic resistance that involves enzymatic pathways is mediated by genetic elements encoded on plasmids and/or transposons. Members of the genera *Haemophilus*, *Actinobacillus* and *Pasteurella* contain transferable plasmids and transposons that confer antibiotic resistance. This means that clones of pathogenic Pasteurellaceae have antibiotic resistance that fluctuates due to transferable plasmids as well as more permanent resistance mediated by chromosomal changes. Effective therapy requires treatment with a combination of long-acting agents.

CHAPTER 3

MATERIALS AND METHODS

CHAPTER 3

MATERIALS AND METHODS

The entire research work was conducted primarily in the Bacteriology laboratory of the Department of Microbiology, Hajee Mohammad Danesh Science and Technology University (HSTU), Dinajpur, Bangladesh during the period of December 2009 to July, 2010. The detailed outline of the Materials and Methods are given below:

3.1 Materials

3.1.1 Study areas:

This study was conducted at the different upazilla of Dinajpur district. The sample were collected from the chickens of selected areas and brought to the laboratory for conducting the experiment.

3.1.2 Field samples:

A total number of 106 field samples comprising liver, heart and spleen (Table 1) were aseptically collected for the isolation and identification of *Pasteurella maltocida*.

Table 1. Number of samples collected from apparently healthy, sick and dead chickens of four upazilla of Dinajpur district of Bangladesh:

Name of the Upazilla	Number of sample collected	Number of suspected sick chickens (heart blood, liver, heart, spleen)	Number of suspected dead chickens (liver, heart. spleen)
Dinajpur sadar	30	18	12
Birgonj	25	15	10
Parbotipur	21	12	9
Chirirbandar	30	15	15
Total	106	60	46

3.1.3 Selection of tissues and organs:

The tissue specimens were collected aseptically and individually on sterile Petridishe and sterile syringes and needle. Impression smear from parietal surface of liver was taken on sterile slides.

3.1.4 Media for culture:

The following bacteriological media were used throughout the isolation and identification procedure.

3.1.4.1 Nutrient broth:

Nutrient broth (NB) was used to grow the *Pasteurella* organisms from the samples collected from the study areas of Dinajpur district .

3.1.4.2 Nutrient agar

Nutrient agar (NA) medium was used to grow the *Pasteurella* organisms from the collected samples.

3.1.4.3 MacConkey agar

MacConkey agar (MC) medium was used for showing no growth of *Pasteurella* organism.

3.1.4.4 Blood Agar:

The Blood agar medium was used to grow the *Pasteurella* organisms from the collected samples.

3.1.4.5 Dorset Egg Medium:

The medium was used to grow the *Pasteurella* organisms and maintenance of stock culture.

3.1.5 Media used for biochemical tests:

In order to identify *Pasteurella multocida* the following media were used for biochemical tests: Sugar media (dextrose, lactose, sucrose, maltose and mannitol) and Triple sugar iron (TSI) agar.

3.1.6 Chemicals, reagents and solutions

These include- Gram's stain, Leishman's stain, Methyle blue stain, Methyl red-Voges Proskaure (MR-VP) solution, Kovac's reagent, phosphate buffered saline (PBS) solution and glycerin.

3.1.7 Hexisol hand rub

Hexisol hand rub (100 ml bottle) was used for disinfection prior to swab sample collection from the selected species.

3.1.8 Glass wares and other necessary instruments

In this research work following glass ware and instruments were used: Test tubes (with and without Durham's fermentation tube), Petri-culture dishes, conical flasks, pipette, glass slides, measuring cylinder, microscope, sterilized cotton, immersion oil, bacteriological incubator, jar, ice boxes, balance, hand gloves, spirit lamps, match lighter and bacteriological loop, glass spreader and forceps.

3.1.9 Antibacterial disc

Ten different antibacterial discs, manufactured by Span Diagnostics Ltd. (Surat) India, were selected for the antibacterial sensitivity study against isolated *Pasteurella*. The antibacterial agents corresponding to eight different discs are those commonly used in the field condition for the treatment of Pasteurella infection, namely- Ciprofloxacin, Clindamycin, Gentamycin, Doxycicline, Erythromycin, Amoxicillin, Tetracycline, Levofloxacin, Bacitracin and Neomycin.

3.2 Methods

3.2.1 Experimental design:

The experimental design is schematically presented in figure 1. The entire study is divided into 4 steps. The first step includes the isolation of *Pasteurella multocida* bacteria from suspected sick and dead chickens of different selected areas of Dinajpur district using cultural characteristics by culturing in Nutrient Broth, Nutrient Agar, Blood Agar, Eosin Mythylene Blue (EMB) Agar, MacConkey Agar.

The 2nd step was engaged for morphological characterization of isolated organism using various staining techniques like Gram's staining, Leishman's staining, Methylene blue staining. Motility test was carried out with hanging drop preparation to confirm the organism was non motile.

In the 3rd step, biochemical characterization of the isolated bacteria was performed using sugar fermentation reaction with basic sugars (Dextrose, Lactose and sucrose, maltose and mannitol), Methyl Red (MR) test, Voges Proskauer (VP) test, Motility Indole Urea (MIU) test and Indole test.

Finally the antibiogram study of the isolated *Pasteurella multocida* done on Nutrient Agar.

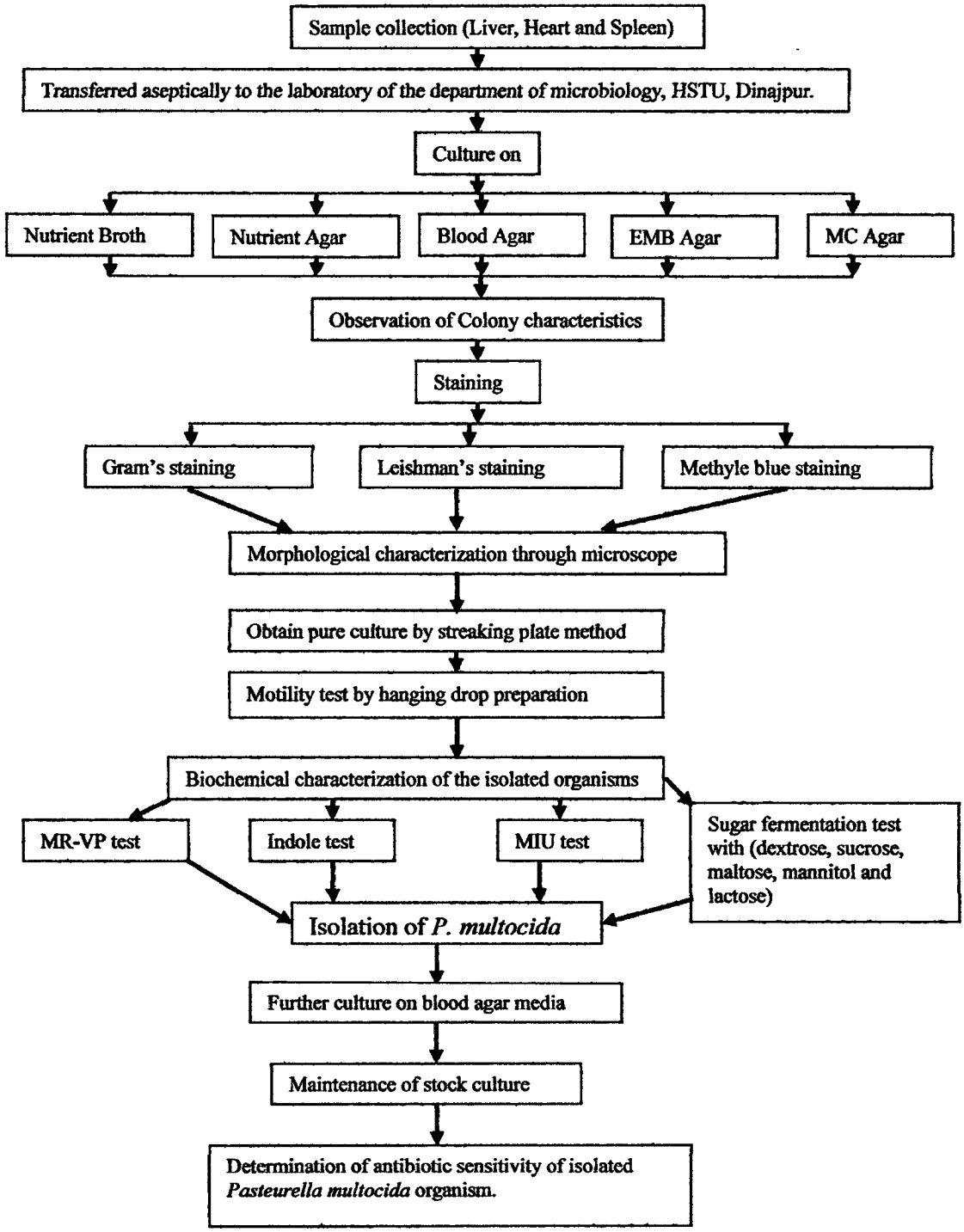


Fig. 1: Schematic illustration of experimental design

3.2.2 Isolation of *Pasteurella multocida* :

3.2.2.1 Collection and transportation of samples:

The liver, heart, heart blood and spleen were collected from the study areas for isolation of the organism. Sterile cotton swab and sterile Nutrient Broth in sterile glass tube was carried to the sampling area. Liver, heart and spleen was taken from the chickens of different ages in aseptic way. Test tubes containing swab samples were then immediately brought to the laboratory for cultural examination.

3.2.2.2 Preparation of culture media and reagents and inoculation of organism:

3.2.2.2.1 Nutrient Broth:

This was prepared by dissolving 13 gms of dehydrated nutrient broth (Hi-media, India) into 1000 ml of distilled water and sterilized by autoclaving at 121°C under 15 pounds pressure per square inch for 15 minutes (Buxton and Fraser, 1977). Then the broth was dispensed into tubes (10 ml/tube). The tubes were then stored at 4°C in the refrigerator until used. Then the Nutrient Broth was inoculated with the organism for revealed the growth of bacteria after 24 hours of incubation at 37°C aerobically.

3.2.2.2.2 Nutrient Agar:

2.8 grams of Bacto-nutrient agar (DIFC)) was suspended in 100 ml distilled water and heated to boiling to dissolve the medium completely. The medium was then distributed into test tubes stoppered with cotton plugs and sterilized in the autoclave for 15 minutes at 15 pounds pressure (121°C). On taking out from the autoclave, the tubes were kept in slant and allowed to solidify. Then the Nutrient Agar plates streaked separately with the organisms for the growth of bacteria after 24 hours of incubation at 37°C aerobically.

3.2.2.2.3 MacConkey agar medium:

51.50 grams powder of MC agar base (HiMedia Laboratories pvt. Ltd.) was added to 1000 ml of distilled water in a flask and heated until boiling to dissolve the medium completely. The medium was then sterilized by autoclaving at 121°C maintaining a pressure of 15-pounds /sq. inch for 15 minutes. After autoclaving, the medium was put into water bath of 45°C to decrease its temperature. After solidification of the medium in the Petri dishes, the Petri dishes were allowed for incubation at 37°C for overnight to check their sterility and then stored in a refrigerator for future use. Then the agar plates streaked separately with the organisms for the growth of bacteria after 24 hours of incubation at 37°C aerobically.

3.2.2.2.4 Blood Agar:

4 grams of Bacto Agar base (DIFCO) were Suspended in 100 ml of distilled water in a flask and boiled to dissolve completely. It was then sterilized by autoclaving at 121°C for 15 minutes at 15 pounds pressure, cooled to 50^0C and added 5% defibrinated bovine blood. The media was then poured in-to sterile Petri dishes and allowed to form thick layer: The sterility of the media was checked by incubating over-night at 37°C. Then the Blood Agar plates streaked separately with the organisms for the growth of bacteria after 24 hours of incubation at 37°C aerobically.

3.2.2.2.5 Eosine Methylene Blue agar:

36gms of EMB agar base (HiMedia Laboratories Pvt. Ltd) was added to 1000 ml of distilled water in a conical flask and heated until boiling to dissolve the medium completely. On sterilization by autoclaving, the medium was poured in10 ml quantities in sterile glass Petri dishes (medium size) and in 15 ml quantities in sterile glass Petri dishes (larger size) to form a thick layer therein. To accomplish the surface be quite dry, the medium was allowed to solidify for about 2 hours with the covers of Petri dishes partially removed. The sterility of the medium was judged and used to store at 4°C in refrigerator for further use. Then the EMB Agar plates streaked separately with the organisms for the growth of bacteria after 24 hours of incubation at 37°C aerobically.

3.2.2.2.6 Dorset Egg Medium:

This was prepared by dissolving 46.7 gms of Dorfet Egg Media base (Hi-media, India) into 1000 ml of distilled water containing 10 ml of glycerol, gently heat to boil to dissolve the media completly and sterilized by autoclaving at 121°C under 15 pounds pressure per square inch (1.2 Kg/Cm^2) for 15 minutes (Buxton and Fraser, 1977). Then the broth was dispensed into tubes (10 ml/tube). The tubes were then stored at 4°C in the refrigerator until used.

3.2.2.2.7 Phosphate buffered saline solution:

For preparation of PBS, 8 grams of NaCl, 2.89 grams of disodium hydrogen phosphate ($Na_2HPO_4.12H_20$), 0.2 grams of KCI and 0.2 grams of potassium hydrogen phosphate (KH_2PO_4) were suspended in 1000 ml of distilled water. The solution was heated to dissolve completely. The solution was then sterilized by autoclaving at 121°C maintaining a pressure of 15-pounnds/sq. inch for 15 minutes and stored at 4-8°C until use. The pH of the solution was measured by a pH meter and maintained at 7.0-7.2 (Cheesbrough, 1985).

3.2.3 Identification of the *Pasteurella multocida* :

3.2.3.1 Staining of organisms:

The smears prepared with heart blood, impressions smears of liver and isolated colonies of organisms were stained by either or all of the following staining methods.

3.2.3.1.1 Gram's staining technique:

This was conducted following (Merchant and Packer, 1968).Smears were fixed by gentle heat: Crystal violet solution was then applied on the smear to stain for two minutes and then washed with water, Lugol's iodine was then added to act as mordant for one minute and then washed with water. Acetone alcohol was afterwards added to act as decolouriser for few seconds, After washing with water, safranine was added as counter stain and allowed to stain for 2 minutes. The preparation was then washed with water, blotted and dried, in air and then examined under microscope.

3.2.3.1.2 Leishman's staining technique:

The technique for this stain comprised of fixing the smear by gentle heating followed by application of Leishman stain so that the smear was flooded. After this an equal amount of distilled water was added. The mixture of stain and water was kept blowing for 5 minutes with the help of a pipettes The preparation was then washed with water, blotted, dried in air and examined under microscope.

3.2.3.1.3 Methylene blue staining technique:

Alkaline methyline blue stain solution was applied on the smear previously fixed by passing over gentle flame. The stain was allowed to act for 5 minutes and then washed with water. The excess water was then blotted, and the smear was dried in air and examined under microscope.

3.2.4 Determination of motility:

This was conducted by -the technique of Hanging drop slide preparation following Merchant and Packer(1968). A loopful of culture of isolated organisms grown overnight in nutrient broth was placed on the centre of a cover slip. Small amount of Vaseline was placed on four corners of cover slip which' was then inverted and placed on the groove of hanging drop slide so that the drop of culture remain hanging in the groove. The preparation was then examined under the microscope to determine the motility.

3.2.5 Bio-chemical tests:

3.2.5.1 Preparation of sugar media:

The medium consists of peptone water to which fermentable sugar was added to the proportion of 1 %. Peptone water was prepared by adding one gram of Bacto peptone (Difco, USA) and 0.5 grams of sodium chloride in 100 ml distilled water. The medium as boiled for 5 minutes, adjusted to pH 7.0, cooled and then filtered through filter paper. Phenol red, indicator at the strength of 0.2 % solution was added to peptone water and then dispensed in 5 ml amount into cotton plugged test tubes containing a Durham's fermentation tubes, placed avertedly. These were then sterilized through autoclaving at 121°C at 15-pound pressure per

sq inch 15 minutes. The sugars used for fermentation were prepared separately as 10 % solutions in distilled water (10 grams sugar was dissolved in 100 ml of distilled water). A little heat was necessary to dissolve the sugar (Buxton and Fraser, 1977).

The sugar solutions were sterilized in Arnold stream sterilizer at 100°C for 30 minutes for three consecutive days. An amount of 0.5 ml of sterile sugar solution was added aseptically in each culture tubes containing sterile peptone water, indicator and Durham's fermentation tube. Before use, the sterility of the sugar media was examined by incubating it for 2 hours at 37°C.

3.2.5.2 Fermentation reaction with five sugars:

The carbohydrate fermentation test was performed by inoculating a loop full of NB culture of the organisms into the tubes containing different sugar media (dextrose, lactose, sucrose) and incubated for 24 hours at 37°C. Acid production was indicated by the colour change reddish to yellow in the medium.

3.2.5.3 Preparation of Methyl Red Voges Proskauer(MR-VP) broth:

A quantity of 1.7 grams of Bacto MR-VP medium was dissolved in 100 ml of distilled water dispensed in 2ml amount in each test tube and then the tubes were autoclaved at 121oC maintaining a pressure of 15-pounds/sq. inch (1.2 kg/cm^2) for 15 minutes. After autoclaving, the tubes containing medium were incubated at 37° C for overnight to check their sterility and then stored in a refrigerator for future use (Buxton and Fraser, 1977).

3.2.5.4 Preparation of Methyl Red solution:

The indicator methyl red solution was prepared by dissolving 0.1 gram of Bacto methyl-red in 300 ml of 95 percent alcohol and diluting to 500 ml with the addition of 200 ml of distilled water (Cheesbrough, 1985).

3.2.5.5 Procedure of Methyl Red (MR) test:

The test was conducted by inoculating a colony of the test organism in 0.5 ml sterile glucose phosphate broth. After overnight incubation at 37°C, a drop of MR solution was added. A red

coloration is positive and indicates an acid pH of 4.5 or less resulting from the fermentation of glucose. A yellow coloration is negative (Cheesbrough, 1985).

3.2.5.6 Procedure of Voges-Proskauer (VP) test:

Two milliliter of sterile glucose phosphate peptone water were inoculated with the 5 ml of test culture. It was incubated at 37°C for 48 hours. A very small amount (knifepoint) of creative was added and mixed. Three milliliter of sodium hydroxide were added and shake well. The bottle cap was removed and left for an hour at room temperature. It was observed closely for the slow development of a pink colour for positive cases. In negative cases there was no development of pink colour (Cheesbrough. 1985).

3.2.5.7 Procedure of Indole test:

Two milliliters of peptone water was inoculated with 5 ml of bacterial culture and incubated for 48 hours. Kovac's reagent (0.5 ml) was added, shaked well and examined after 1 minute. A red color in the reagent layer indicated Indole. In negative case there is no development of red color (Cheesbrough, 1985).

3.2.5.8 Motility Indole Urea test:

18 gms of powder of MIU agar base (HiMedia Laboratories Pvt. Ltd) was added to 950 ml. of distilled water in a flask and heated boiling to dissolve the medium completely. The medium was then sterilized by autoclaving at 121°C maintaining a pressure of 15 pound per sq. inch for 15 minutes. After autoclaving the medium was put in to water bath of 45°C to decrease the temperature. After this medium in the test tube were allowed for incubating at 37°C for overnight to check their sterility and then stored in refrigerator for future use.

3.2.5.9 Preparation of Triple Sugar Iron agar slant:

A quantity of 6.5 grams of Bacto TSI agar (HiMedia Laboratories Pvt. Ltd) was suspended in 1000 ml of distilled water and heated up to boiling to dissolve the medium completely. The solution was then distributed in tubes plugged with cotton plugs and sterilized in an autoclave at 1210C at a pressure of 15-pounds/sq. inch (1.2 kg/cm2) for 15 minutes. After sterilization, the tubes were kept in slanting position in such a manner so as to allow a generous butt after

solidification. The tubes were then incubated at 37°C for overnight to check their sterility and then stored in a refrigerator for future use (Cheesbrough. 1985).

3.2.5.10 Reaction of the organisms in TSI agar slant:

The TSI agar slant was used to detect the lactose fermenters and the saccharose and dextrose fermenter. The medium also helped to determine the ability of the organisms to produce H_2S. The organisms under study were heavily seeded with a platinum needle over the surface of the slants and stabbed into the butt of the tubes containing TSI agar. After an aerobic incubation of 24 hours at 37°C the tubes were examined for changes in the slant or in the butt or in both places. Pinkish slant and yellow butt or black slant and yellow butt were recorded as the positive reaction for *Pateurella multocida* (Cheesbrough, 1985).

3.2.6 Antibiogram study of the isolated *Pasteurella spp*

Susceptibility of isolates of *Pasteurella multocida* to different antimicrobial agents was performed to determine the drug sensitivity pattern and to interpret their disease potential. This method allows for the rapid determination of the efficacy of a drug by measuring the diameter of the zone of inhibition that results from different diffusion of the agent into the medium surrounding the disc. *Pasteurella multocida* isolates were grown overnight on Nutrient Broth. The overnight cultured isolates were inoculated and poured on Nutrient agar and spread uniformly with the help of sterile glass spreader. Antibacterial disc were applied aseptically to the surface of the plate at an appropriate arrangement with the help of sterile forceps and incubated at 37°C for 24 hours, aerobically (Carter, 1979). The following are the antibiotics that were tested against isolated *Pasteurella* spp.

Table 2. Antimicrobial agents and their disc concentration:

Antimicrobial agent	Disc concentration
Doxycicline	10 µg
Amoxicillin	10µg
Gentamicin	10 µg
Ciprofloxacin	5 µg
Clindamycin	2 µg
Erythromycin	15 pg
Tetracycline	30 µg
Levofloxacin	5 µg
Bacitracin	10 µg
Neomycin	30 µg

3.2.7 Maintenance of stock culture:

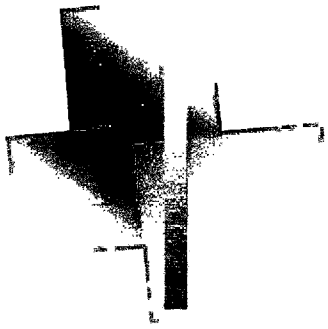
3.2.7.1 On Dorset Egg Medium:

During the experiment it was necessary to preserve the isolated organisms for longer periods. For this purpose the organisms from the pure culture were inoculated into the tubes of Dorset Egg Medium incubated at 37°C for 24 hours. After the growth of organisms the tubes were sealed and kept in the refrigerator at 4°C.

3.2.7.2 Sterile buffered glycerin method:

Sterile buffered glycerin (20%) was performed by mixing 20 parts of pure glycerin and 80 parts of PBS. Then a loopful of thick bacterial culture was mixed with 20% sterile buffered glycerin in small vials and were preserved at -20°C. This method is more appropriate for preserving bacteria with no deviation of their original characters for several years (Buxton and Fraser, 1977).

CHAPTER 4



RESULTS

CHAPTER 4

RESULTS

4.1 Results of Postmortem examination of chickens:

The Postmortem lesions observed in natural outbreaks in chickens yielding *Pasteurella multocida* infection were as follows:

The lesions were characterized by septicemic conditions, blood vascular congestion and hemorrhagic enteritis. Pin pointed hemorrhages were also found in breast muscle and abdominal fat. Pericardial and peritoneal fluids were present in increased amount. Liver was swollen and sometimes congested; multiple necrotic foci were observed on the parietal surface (Plate 1, Page 46). Heart was enlarged and edematous. Trachea and fangs were hemorrhagic and contained increased amount of secretory fluids; viscid mucus were also found in pharynx, crop and intestine. There were also swelling and hemorrhagic enteritis in the deudonal end. Ovaries were flaccid with thick blood vessels.

4.2 Results of cultural examination:

4.2.1 In Nutrient Broth:

In Nutrient broth the growth of bacteria was indicated by the presence of diffused turbidity (Table 3, Page 39, Plate 2, Page 47).

4.2.2 On Nutrient Agar:

On Nutrient agar plates the growth of whitish, opaque, round, flat, translucent colonies of sticky mucoid or dry consistency about 2-3 mm in diameter were observed. Other strain produce small, circular, smooth, iridescent or non iridescent colonies about 1mm in diameter (Table 3, Page 39, Plate 3, Page 47).

4.2.3 On Blood Agar:

The culture of the organism in blood agar yielded small colonies. The other characteristics of these colonies included whitish, opaque circular and translucent appearance. No hemolysis was noticed in blood agar. (Table 3, Page 39, Plate 4, Page 48).

4.2.3 On MacConkey (MC) agar:

Culture on MacConkey agar plate yielded no colonies (Table 3, Page 39, Plate 6, Page 49).

4.2.4 On Eosin Methylene Blue (EMB) Agar:

Culture of *Pasteurella multocida* on EMB agar yielded small, circular, convex, translucent, glistening colonies which had tendency to coagulase. On EMB agar metallic sheen was absent. (Table 3, Page 39, Plate 5, Page 48).

4.3 Result of staining characteristics of bacterial isolates:

4.3.1 Results of Gram's staining technique:

In Gram's staining the organism revealed Gram-negative, pink color, coccobacillary organism when observed under microscope. Similar characters were also observed with organisms grown in various artificial media (Table 3, Page 39, Plate 7, Page 49).

4.3.2 Results of Leishman's staining technique:

Presence of bi-polar characters of *Pasteurella multocida* organisms were manifested after Lishman's staining of the smears prepared from heart blood and liver during necropsy. In case of organisms grown on artificial media, the presence of metachromatic granules providing bipolar characters were not a regular phenomenon.

4.3.3 Results of Methylene blue staining technique:

The organisms following methylene blue staining technique appeared blue with coccobacillary shape with bipolar character (Plate 8, Page 50).

4.4 Results of motility test:

All the isolates were found to be non motile when examined under the hanging drop preparation.

Table 3: Morphology, cultural and staining characteristics of isolated *Pasteurella*:

Sources of Isolates	Colony characteristics				Morphology staining
	Nutrient Agar	EMB Agar	MC Agar	Blood Agar	
Suspected sick chickens	Whitish, opaque, round, flat, translucent colonies of sticky mucoid or dry consistency.	Small, circular, convex, translucent, glistening colonies. No metallic sheen	No colony appears	Whitish, opaque circular and translucent appearance and no hemolysis.	Gram-negative, pink color, coccobacillary bipolar organism.
Suspected dead chickens	Whitish, opaque, round, flat, translucent colonies of sticky mucoid or dry consistency.	Small, circular, convex, translucent, glistening colonies. No metallic sheen	No colony appears	Whitish, opaque circular and translucent appearance and no hemolysis.	Gram-negative, pink color, coccobacillary bipolar organism.

Legends: EMB = Eosin methylene blue.

MC = Mac Conkey.

4.5 Results of biochemical test:

4.5.1 Fermentation reaction with basic sugars:

All the isolates fermented dextrose, sucrose and mannitol, produced only acid. No fermentation was seen in lactose and maltose. Acid production was indicated by the color change from reddish to yellow (Table 4, Page 40, Plate 12, Page 51).

4.5.2 Result of TSI slant reaction:

In the stab culture of TSI agar, the isolated *Pasteurella* organisms produced an alkaline reaction (red) in slant and acid reaction (yellow) in the butt and slightly black color due to H_2S production were also recorded (Table 4, Page 40, Plate 9, Page 50).

4.5.3 Result of Indole test:

In Indole test, the development of red colored ring indicate the positive test. All the organisms revealed positive in Indole test (Table 4, Page 40, Plate 11, Page 51).

4.5.4 Result of Methyl Red (MR) test:

In MR test, the development of red color indicated positive test while appearance of yellow color indicated negative test. All the organisms revealed negative in MR test (Table 4, Page 40, Plate 11, Page 51).

4.5.5 Result of Voges Proskauer (VP) test:

In VP test, the development of pink color indicate the positive test. All the organisms revealed negative in VP test (Table 4, Page 40, Plate 11, Page 51).

4.5.6 Result of Motility Indole Urea (MIU) Test:

All the isolates in the MIU medium clear (non mtile), red colour in the neck (indole positive) and no ureas production. (Table 4, Page 40, Plate 10, Page 50).

Table 4. Biochemical characters of the *Pasteurellae spp.*:

Biochemical Test		**Result**
CHO fermentation	Dextrose / Glucose	+
	Sucrose	+
	Lactose	-
	Maltose	-
	Mannitol	+
MIU	Motility	-
	Indole	+
	Urease	-
TSI	H_2S	+
	CHO fermentation	Only Glucose
Indole		+
MR		-
VP		-

Legends

+ = Positive; - = Negative; MR= Methyl Red; VP= Voges-Proskauer

Table 5: Demonstration of prevalence percentage of *Pasteurella multocida* organism from suspected sick and dead chickens.

Name of samples	No. of samples	No. of positive cases found	Percentage (%)
Heart	40	8	20%
Liver	40	20	50%
Spleen	40	4	10%
Heart blood	16	4	25%
Total	106	36	34%

4.6 Result of antibacterial sensitivity study of the isolated *Pasteurella* organism:

Antibiotic sensitivity pattern of isolated Salmonella organisms were performed against 10 commonly used antibiotics belongings to different groups. After incubation the plates were examined and diameters of the zones of inhibition for individual antibacterial agents were designated as highly sensitive (+++), moderately sensitive (++), less sensitive (+) and resistant (-) (Table 6, Page 41).

Table 6 : Result of antibiotic sensitivity test using various isolates of *Pasteurella multocida*:

Total No. of isolates	Antibiotics disc used									
	CIP	LEV	DA	N	CN	DO	E	AML	TE	B
1	+++	+++	+++	+++	++	++	-	-	-	-
2	+++	+++	+++	+++	++	++	++	-	-	-
3	+++	+++	+++	+++	++	++	++	-	+	+
4	+++	+++	+++	+++	+	++	+	-	+	-
5	+++	+++	+++	+++	++	++	+	-	-	-
6	+++	+++	+++	++	++	+	+	-	-	-
7	+++	+++	++	+++	++	++	+	-	-	-
8	+++	+++	++	+++	+	++	+	-	-	-
9	+++	+++	+++	+++	+	+	-	-	-	-
10	+++	+++	+++	+++	+++	++	-	-	+	-
11	+++	+++	+++	+++	+	++	-	-	-	-
12	+++	+++	+++	++	++	++	++	-	-	-
13	+++	+++	++	+++	++	++	++	-	-	+
14	+++	+++	+++	+++	++	++	-	-	-	-

Total No. of isolates	Antibiotics disc used									
	CIP	LEV	DA	N	CN	DO	E	AML	TE	B
15	+++	+++	+++	+++	++	+	+	-	-	-
16	+++	+++	++	+++	+	+	+	-	+	-
17	+++	+++	+++	+++	+	++	+	-	-	-
18	+++	+++	+++	+++	++	++	+	-	-	-
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33	+++	+++	+++	+++	++	++	-	-	-	-
34	+++	+++	++	+++	++	++	+	-	-	-
35	+++	+++	+++	+++	+	++	+	-	-	-
36	+++	+++	+++	+++	+	++	+	-	-	-

Legends

DO= Doxycycline

AML= Amoxycillin

CIP= Ciprofloxacin

E= Erythromycin

TE = Tetracycline

LEV= Levofloxacin

N= Neomycin

B= Bacitracin

CN= Gentamicin

DA=Clindamycin

+++ = Highly sensitive

++ = Moderately sensitive

+ = Less sensitive

- = resistance

Table 7: Determination of antibiotic sensitivity pattern in percent:

Sensitivity pattern	Percentage of isolated organisms sensitive to various antibiotics									
	CIP	LEV	DA	N	CN	DO	E	AML	TE	B
Resistant	0	0	0	0	0	0	25	100	83	92
Less sensitive	0	0	0	0	36	25	61	0	17	8
Moderately sensitive	0	0	22	17	61	75	14	0	0	0
Highly sensitive	100	100	78	83	3	0	0	0	0	0

Form the antibiogram study, it was revealed that among the isolated *Pateurella* organism from chicken, 100% were highly sensitive to Ciprofloxacin and Levofloxacin, 83% were to Neomycin, 78% were to Clindamycin and 3% were to Gentamycin. 75% were moderately sensitive to Doxycicline, 61% were to Gentamycin, 22% were to Clindamycin,17% were to Neomycin and 14% were to Erythromycin. On the other hand 61% were less sensitive to Erythromycin; 17% were to Tetracycline; 13% were to Gentamycin, 25% were to Doxycicline and 8% were to Bacitracin. 100% isolates were resistant to Amoxycillin, 83% were to Tetracycline; 92% were to Bacitracin, and 25% were to Erythromycin. (Table 7, Page 43; Plate 13-14, Page 52-53).

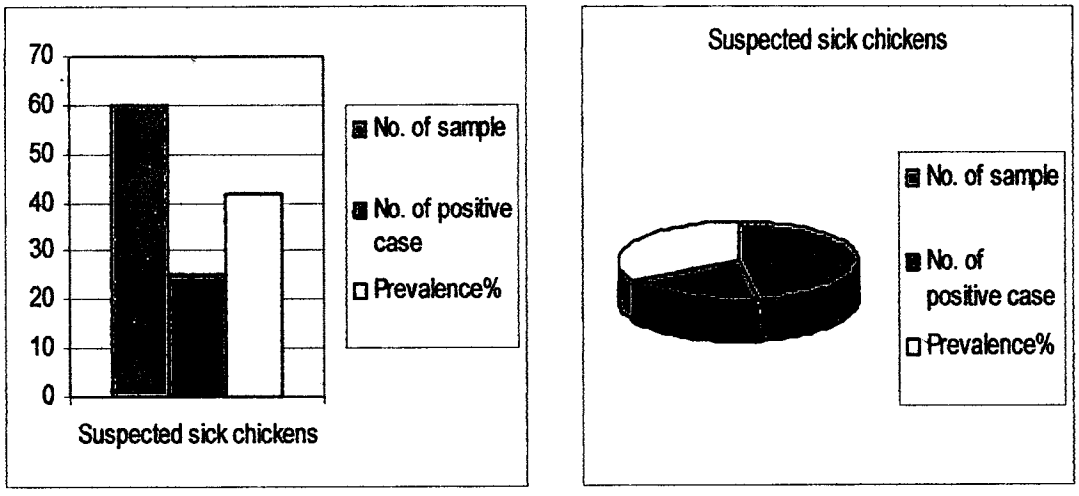


Fig. 2: Histogram showing prevalence percentage of *P. multocida* from suspected sick chickens.

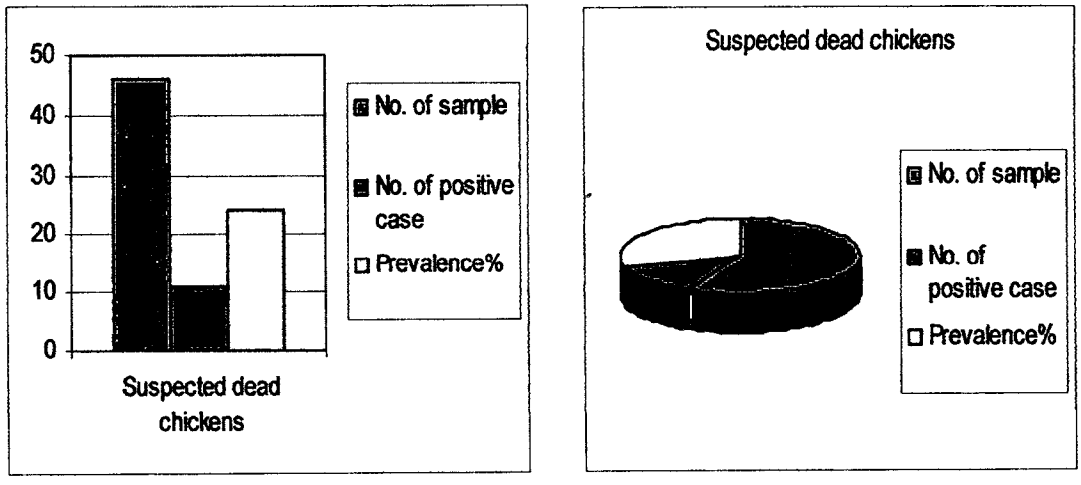


Fig. 3: Histogram showing prevalence percentage of *P. multocida* from suspected dead chickens.

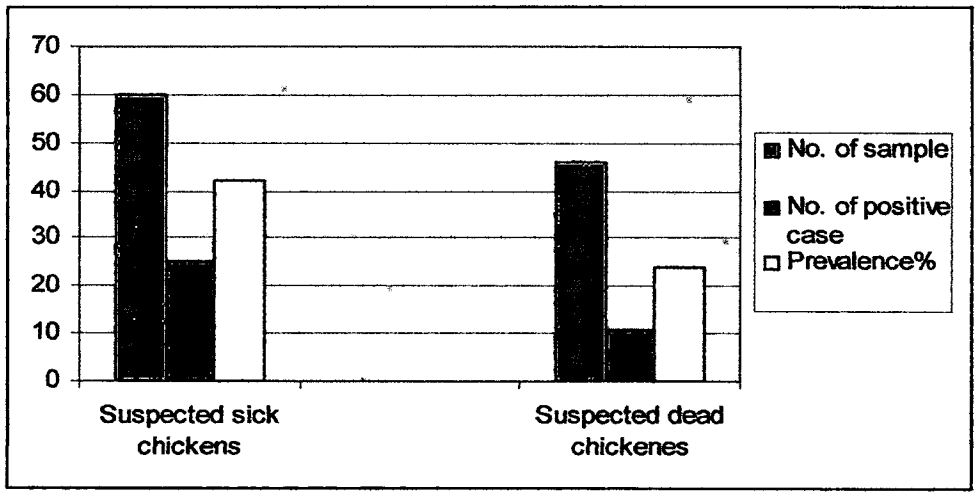


Fig. 4: Histogram showing overall prevalence percentage of *P. multocida* from suspected sick and dead chickens.

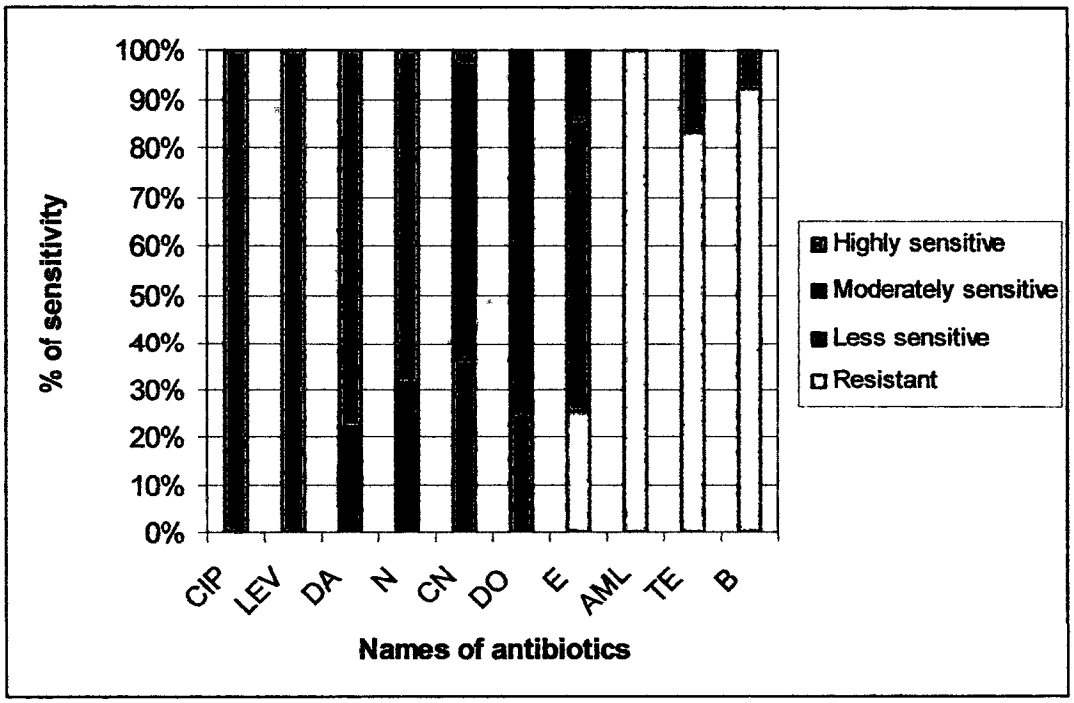
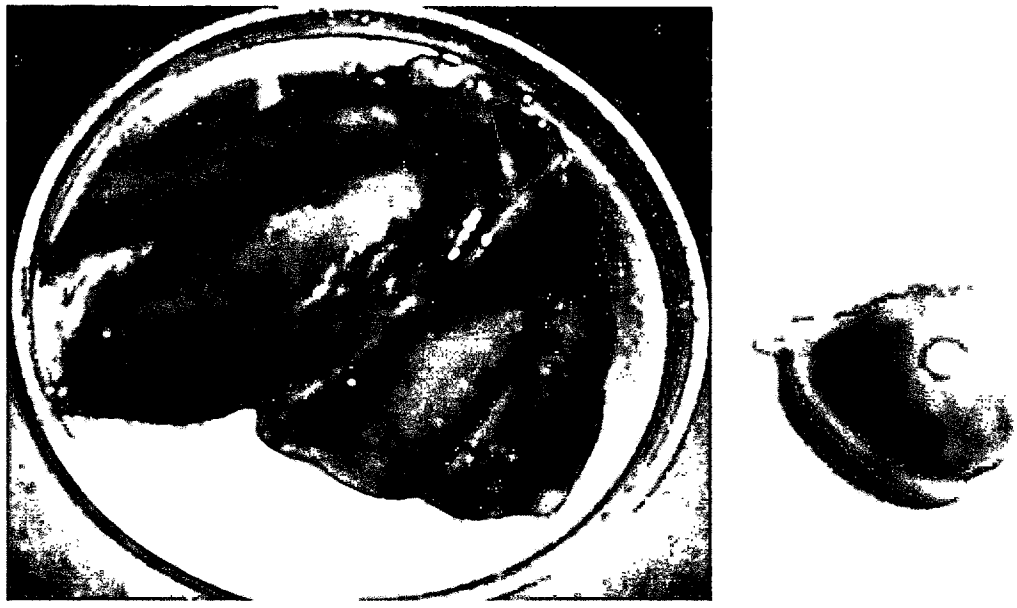


Fig. 5: Bar Diagrammatic pattern of antibiotic sensitivity & resistant of *P. multocida*

Liver



Liver **Spleen**

Plate 1: Samples collected from chickens from suspected cases.

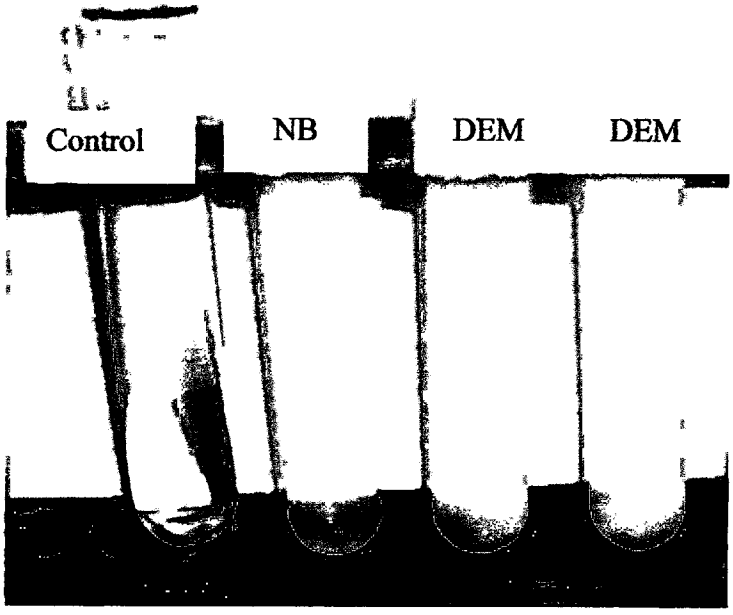


Plate 2: Growth of *Pasteutella multocida* organisms in Nutrient Broth and Dorset Egg Medium indicated by the presence of diffused turbidity.

Plate 3: Growth of *Pasteurella multocida* on Nutrient agar plates showing whitish, opaque, round, flat, translucent colonies.

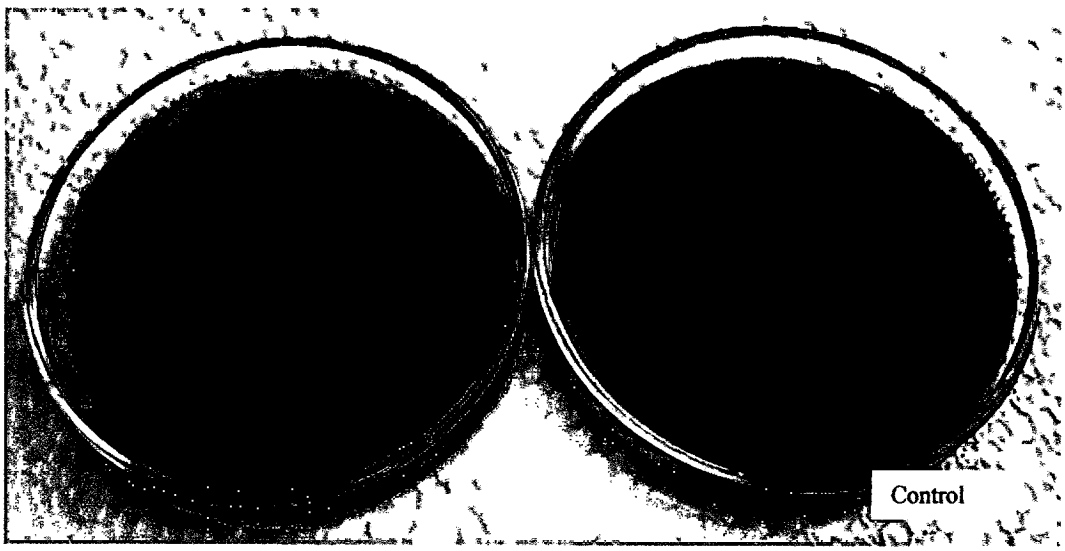


Plate 4: Growth of *Pasteurella multocida* on Blood agar showing whitish, opaque, circular and translucent colony with no hemolysis.

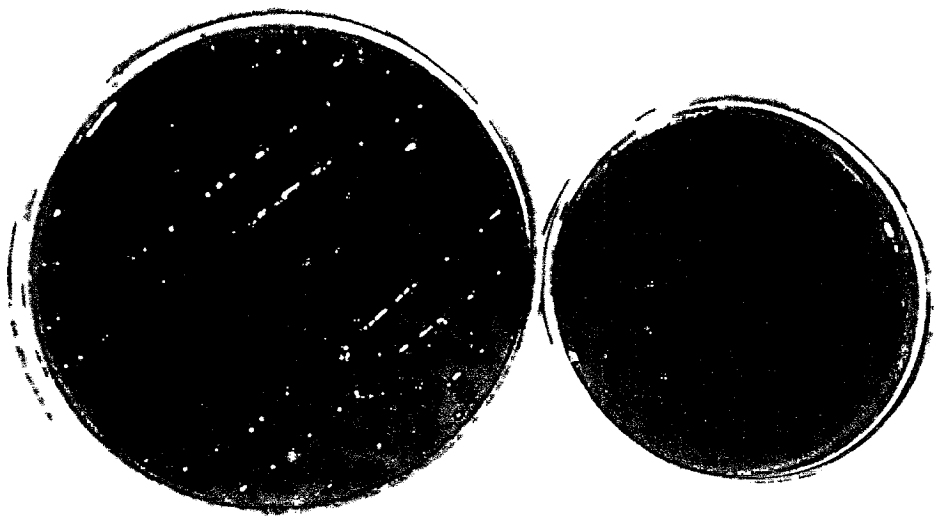


Plate 5: Growth of *Pasteurella multocida* on EMB agar media showing small, circular, convex, translucent, glistening colonies.

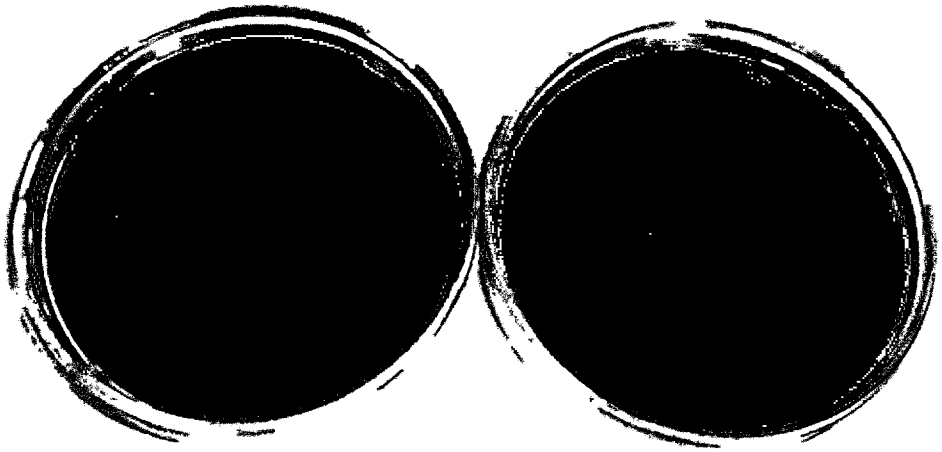


Plate 6: No growth of *Pasteurella multocida* on MacConkey Agar.

Plate 7: Microscopic photograph of *Pasteurella multocida* in Gram's staining.

Plate 8: Microscopic photograph of *Pasteurella multocida* in Methylene blue staining.

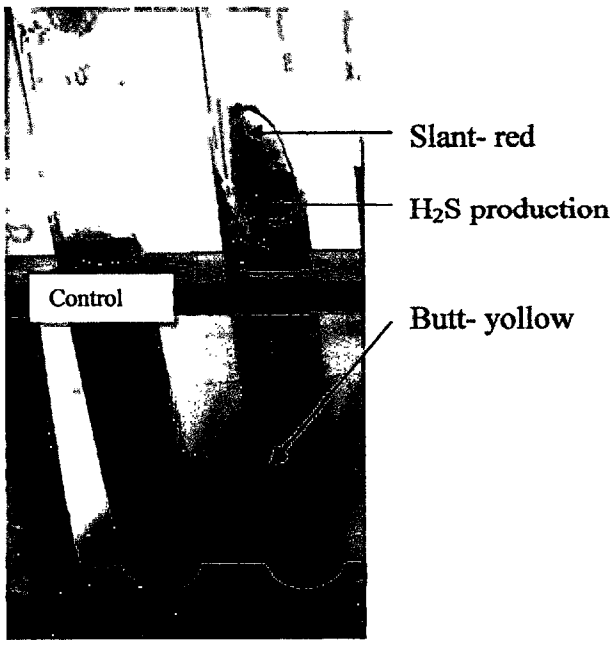


Plate 9: TSI test

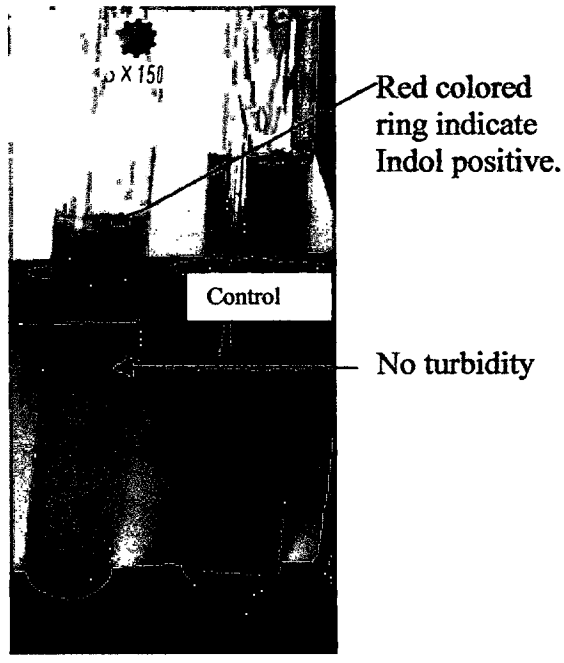


Plate 10: MIU test

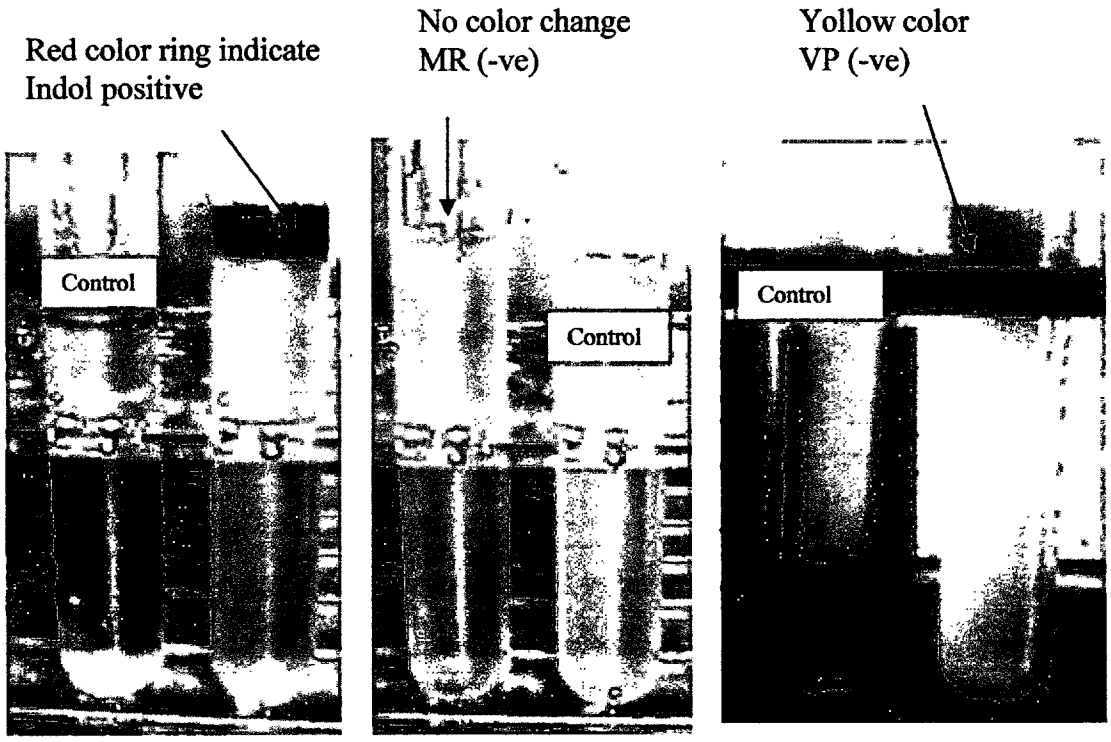


Plate 11 : Biochemical tests; Indol, MIU, TSI and MR-VP test of isolated *Pasteurella multocida.*

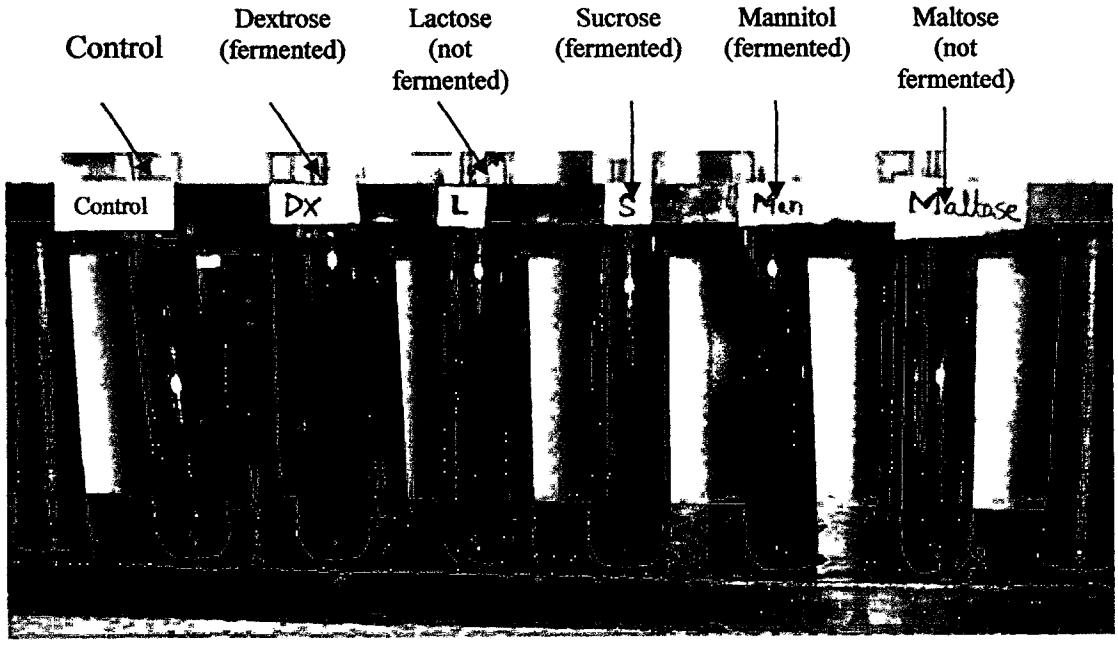


Plate 12: Biochemical tests; sugar fermentation test of isolated *Pasteurella multocida.*

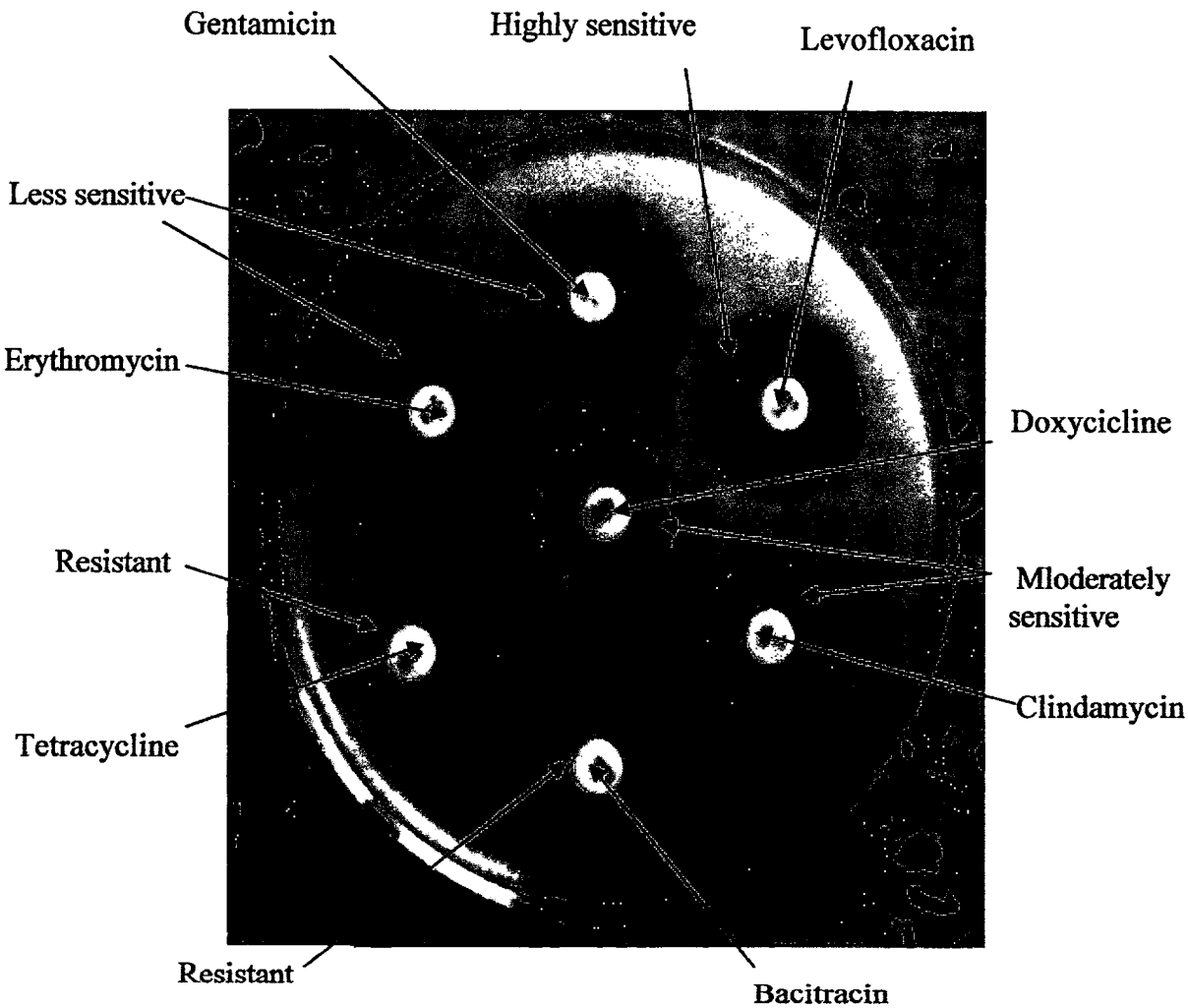


Plate 13: Result of representative samples of antibiogram study of *Pasteurella multocida*

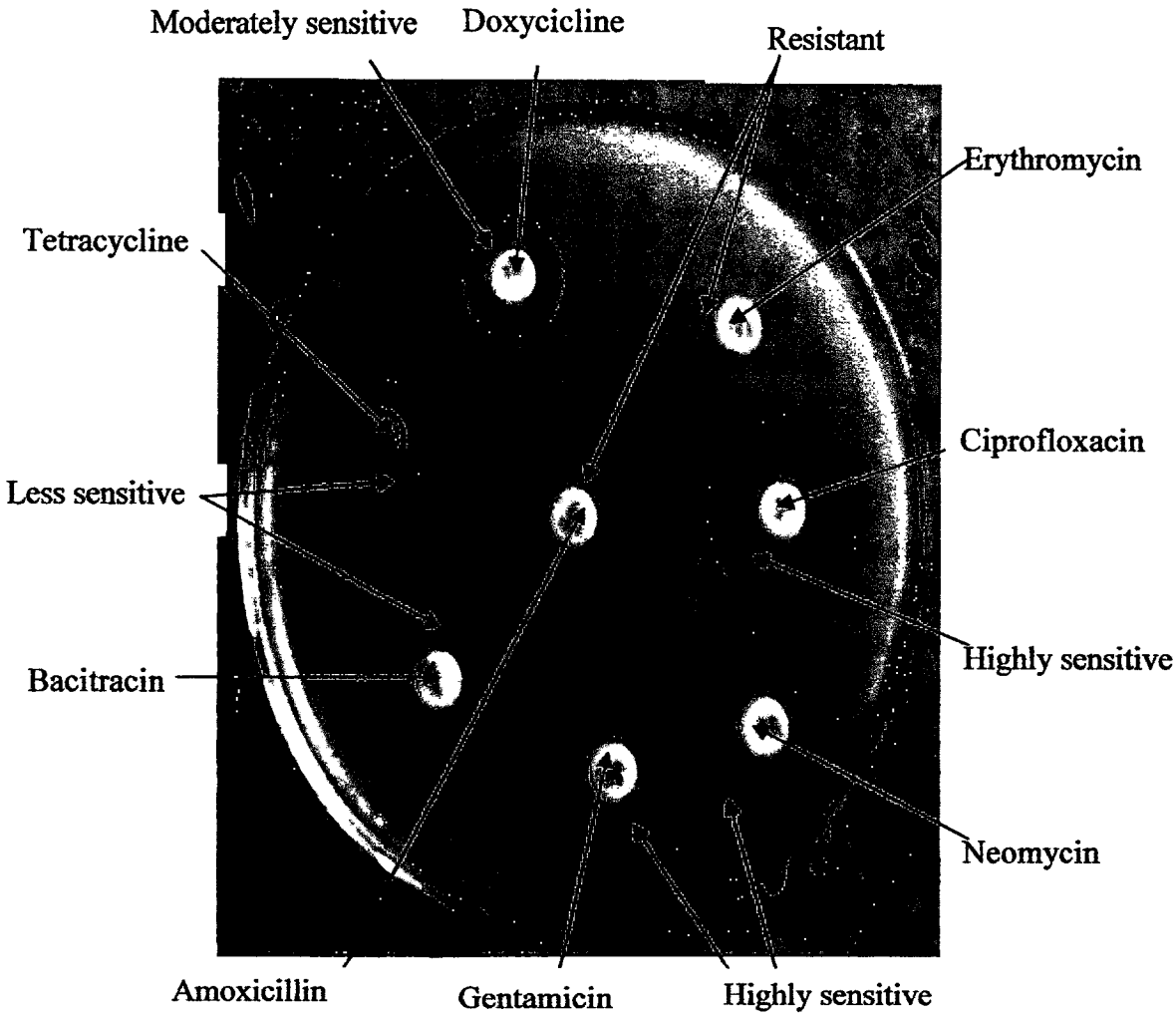


Plate 14: Result of representative samples of antibiogram study of *Pasteurella multocida*

CHAPTER 5

DISCUSSION

CHAPTER 5

DISCUSSION

The aim of the study was to isolate, identify and characterize the *Pasteurella multocida* from chickens and demonstration of antibiotic sensitivity of the selected isolates. The samples were aseptically carried out to the laboratory and were subjected to different cultural, morphological and biochemical examinations.

A total numbers of 106 samples (heart blood, liver, spleen, heart) were collected from suspected sick chickens (60 samples) and suspected dead chickens (46 samples) from different selected areas of Dinajpur district. From these samples, 25 (42 %) from suspected sick chickens and 11 (24 %) from suspected dead chickens were found to be positive for *Pasteurella multocida.* The isolation, identification and characterization procedure of *Pasteurella multocida* from chickens including cultural studies, morphological and staining properties and biochemical characterization were performed as stated by Buxton and Fraser (1977). Specific media and biochemical tests were used for the isolation, identification and characterization of *Pasteurella multocida* from chickens which were previously suggested by a number of works (Buxton and Fraser, 1977; Cowan, 1985).

Identification of *Pasteurella multocida* by colonial feature could better be attained by using sterio-microscope as suggested by Bond. *et al.* 1970.Demonstration of capsule in the freshly isolated culture of organism has been suggested by Hofstad *et al.* 1978. During this study no such investigation was made to demonstrate capsule.

In Nutrient Broth the growth is indicated by the presence of turbidity after 24 hours of post inoculation at 37°C. Cultural characteristics in different agar media, morphology and staining characteristics of the organisms were similar to that of described by Choudhury *et al.* 1985; Buxton and Fraser 1977. The isolated organism from chickens produced whitish, opaque, round, flat, translucent colonies of sticky mucoid or dry consistency about 1-3 mm in diameter on Nutrient Agar. In the present investigation, the selected isolates were found to grow well in bovine BA media producing characteristics colonies of *Pasteurella multocida.* Carter, 1972; Heddleston and Rhoades, 1978 also used bovine blood agar for culturing *Pasteurella multocida* and found similar characteristic colony. On Blood Agar media this organism produced whitish, opaque, circular and translucent colonies without

haemolysis. On Eosin Methylene Blue Agar, *Pasteurella multoida* produced the small, circular, smooth, convex, translucent, glistening colonies which showed the tendency to coagulase. Similar colony characteristics were also recorded by Carter, 1955. MacConkey agar failed to produce any colonies of organisms. Similar observations were also made by Heddleston and Rhoades, 1978.That some strains of *Pasteurella multocida* did not grow in media not containing blood or blood serum were also reported by Merchant and Packer, 1968. However, Curtis, 1979 succeded to grow only two strains of *Pasteurella multocida* on MacConkey agar and observed that the organisms could be grown on such dehydrated medium which were stored for a considerable time.

In Gram's staining the organism revealed Gram-negative, pink color, coccobacillary organism when observed under microscope (Plate 7, Page 49). Presence of bi-polar characters of *Pasteurella multocida* organisms were manifested after Lishman's staining and Methyle blue staining of the smears prepared from heart blood and liver during necropsy(Plate 8, Page 50). The isolates were found to be non motile when examined under the hanging drop preparation and MIU media. (Table 4, Page 40).

The bio-chemical tests conducted in this study were limited to determination of fermentative characters on sugar, production of H_2S and Indol and reaction with MR VP tests (Table 4, Page 40). These were performed for preliminary grouping of *Pasteurella multocida.*

The sugars what were fermented with the production of acid only included glucose, sucrose and mannitol. There was no fermentation of lactose and maltose (Table 4, Page 40). These results were in agreement with Heddleston ,1975 and Merchant and Packer, 1968. However, Heddleston, 1976 while studying the physiological properties of 948 *Pasteurella multocida* cultures of avian origin recorded that 7.4% cultures were positive with arabinose, 2.6% with dulcitol, 0.6% with maltose, 2 .7% with raffinose and 4.1% with trehalose. Further, Curtis (1979) observed that out of 102 isolates of avian origin, 13 fermented arabinose, 7 dulcitol, 4 lactose and 3 maltose.

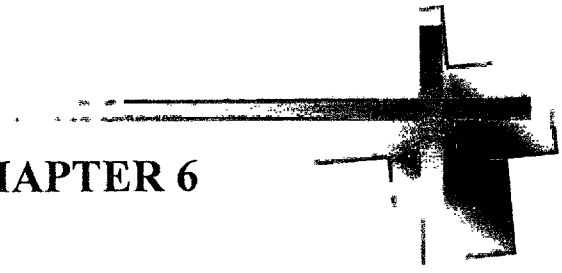
On the other hand, Heddleston and Rhoadas, 1978 mentioned that while fermentation of glucose, sucrose and mannitol without gas is characteristic of *Pasteurella multocida,* lactose is usually not fermented but some avian isolates will ferment lactose. Perhaps, the

compromising view was that of Merchant and Packer, 1968 who observed that the fermentation of lactose, arabinose, dulcitol, sorbitol and fructose might be variable.

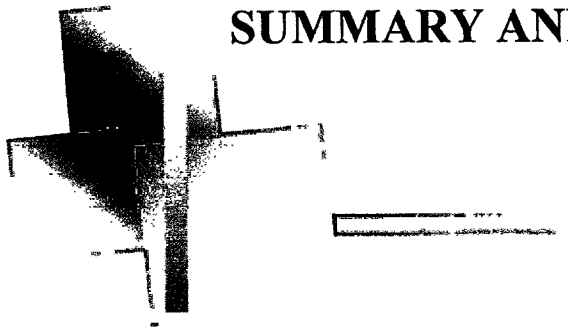
The fermentation of sugars observed in this study were also similar to those of Israil and Quader, 1967 and Ali, 1974 who worked with isolates of *Pasteurella multocida* from chicken and duck respectively.

The presence of H_2S production in triple sugar iron agar (TSIA) observed in this study were encountered by Buxton and Fraser, 1977. In this regard, Curtis, 1979 mentioned that the negative result with triple sugar iron agar did not mean that the *Pasteurella multocida* would not produce H_2S if more sensitive methods were applied. The use of lead acetated paper suspended over the media for detection of H_2S would have provided a more sensitive reaction than mere inoculation of TSIA media (Merchant and Packer, 1968). The production of Indol, absence of motility and negative reaction in MR-VP tests recorded in this study were characteristic of *Pasteurella multocida* as were observed by all the investigators.

Form the antibiogram study, it was revealed that among the isolated *Pateurella* organism from chicken, 100% were highly sensitive to Ciprofloxacin and Levofloxacillin. 83% were highly sensitive to Neomycin, 78% were to Clindamycin and 3% were to Gentamycin. 75% were moderately sensitive to Doxycicline, 61% were to Gentamycin, 22% were to Clindamycin, 17% were to Neomycin and 14% were to Erythromycin. On the other hand 61% were less sensitive to Erythromycin; 17% were less sensitive to Tetracycline; 13% were less sensitive to Gentamycin, 25% were to Doxycicline and 8% were to Bacitracin. 100% isolates were resistant to Amoxycillin, where 83% were resistant to Tetracycline; 92% were resistant to Bacitracin, and 25% were resistant to Erythromycin (Table 7, Page 43).



CHAPTER 6



SUMMARY AND CONCLUSION

CHAPTER 6

SUMMARY AND CONCLUSION

The research work was performed for the isolation, identification and characterization of *Pasteurella multocida* from chickens and demonstration of antibiotic sensitivity of the selected isolates. The entire research work was conducted primarily in the Bacteriology laboratory of the Department of Microbiology, Hajee Mohammad Danesh Science and Technology University (HSTU), Dinajpur, Bangladesh.

A total number of 106 samples (heart blood, liver, spleen, heart) were collected from suspected sick chickens (60 samples) and suspected dead chickens (46 samples) from different selected areas of Dinajpur district.

The isolation, identification and characterization procedure of *Pasteurella multocida* from chickens including cultural studies, morphological and staining properties and biochemical characterization were performed as stated by Buxton and Fraser (1977). Specific media and biochemical tests were used for the isolation, identification and characterization of *Pasteurella multocida* from chickens which were previously suggested by a number of works (Buxton and Fraser, 1977; Cowan, 1985).

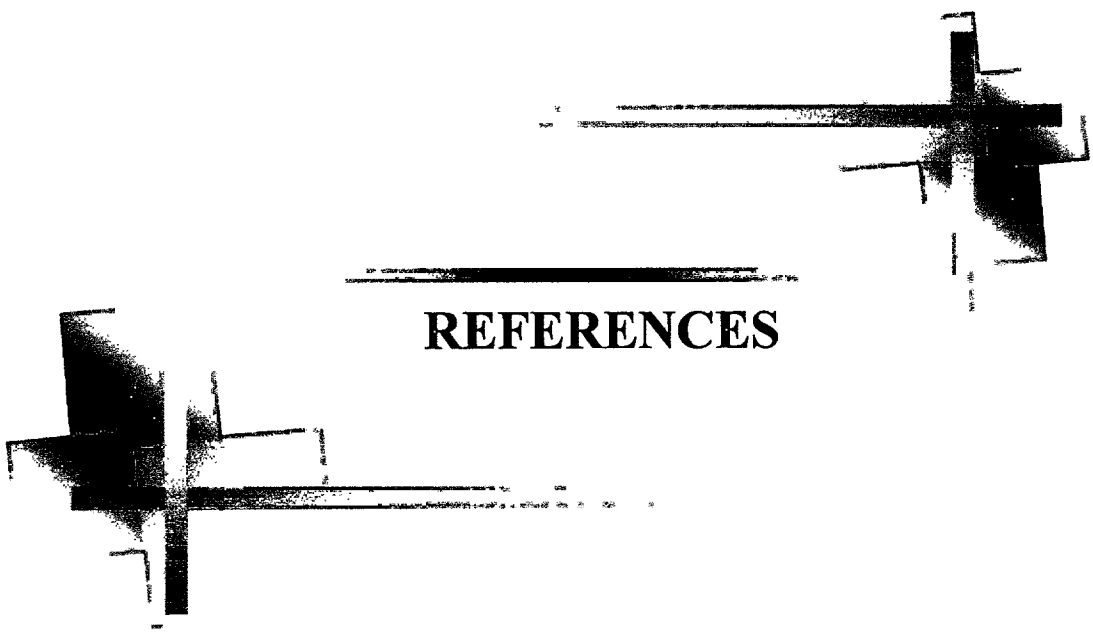
The isolated organism from chickens produced whitish, opaque, round, flat, translucent colonies of sticky mucoid or dry consistency about 1-3 mm in diameter on Nutrient Agar (Plate 3, Page 47) and whitish, opaque, round, translucent colonies without hemolysis on Blood Agar (Plate 4, Page 48) and were able to ferment dextrose, mannitol and sucrose but unable to ferment lactose and maltose on biochemical examinations (Plate 10, Page 51). In NB the growth is indicated by the presence of turbidity after 24 hours of post inoculation at 37°C (Plate 2, Page 47). On EMB agar, *P. multoida* produced the small, circular, smooth, convex, translucent, glistening colonies which showed the tendency to coagulase (Plate 5, Page 48). MacConkey agar failed to produce any colonies of organisms (Plate 6, Page 49).

In Gram's staining the organism revealed Gram-negative, pink color, coccobacillary organism when observed under microscope (Plate 7, Page 49). Presence of bi-polar characters of the *Pasteurella multocida* organisms were manifested after Lishman's

staining and Methyle blue staining of the smears prepared from heart blood and liver during necropsy (Plate 8, Page 50). The isolates were found to be non motile when examined under the hanging drop preparation and MIU media.

The sugars what were fermented with the production of acid only included glucose, fructose. There was no fermentation of lactose. The presence of H_2S production in triple sugar iron agar (TSIA) observed in this study. The production of Indol, absence of motility and negative reaction in MR-VP tests recorded in this study which were characteristic of *Pasteurella multocida* as were observed.

The isolated *Pasteurella multocida* showed resistance to some antibiotics which are used for treatment of poultry.



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APPENDICES

APPENDIX I

Composition of media

1. Blood Agar

Ingredients	g/L
Bacto tryptose	10.0
Beef extract	1.5
Sodium chloride	2.5
Glucose	0.15
Dextrin	0.25
Para aminobenzoic acid	10.0
Agar	7.5
Distilled water	50 ml
Defibrinated blood	50 ml

2. Nutrient borth

Ingredients	g/L
Peptone	5.0
Sodium chloride	5.0
Beef extract	1.5
Yeast extract	1.5
Final pH (at 25^0 C)	7.4±0.2

3. Nutrient agar

Ingredients	g/L
Beef extract	3.0
Peptone	5.0
Sodium chloride	5.0
Agar	20.0
Final pH	7.4±0.2

4. Dorset Egg Medium

Gelatin	20.00
Agar	15
Potassium sulfate	10
Magnesium chloride	1.4
Cetrimid	0.3
Final pH (at 25^0 C)	7.0±0.2 gm

5. MacConkey Agar

Ingredients	**g/L**
Peptone	17.0
Protease peptone	3.0
Lactose	10
Bile salt	1.5
Sodium chloride	5.0
Agar	13.5
Neutral Red	0.03
Crystal violet	0.001
Final pH	7.1±0.2

6. Eosine methylene blue agar

Ingredients	**g/L**
Peptone	100
Lactose	10.0
K_2HPO_4	2.0
Eosin	0.4
Methykene blu	0.065
Agar	20.0
Final pH	6.8±0.2

7. MR-VP medium (Himedia, India)

Composition

Buffered peptone	7.0
Dextrose	5.0
Dipotassium phosphate	5.0
Final pH (at 25^0 C)	6.9±0.2 gm

8. Sugar media

A. peptone water

Bacto-peptone	10.0 mg
Sodium chloride	5.0 gm
0.5% Phenol red	0.1 ml
Distilled water	1000 ml

B. Sugar solutions

Individual sugar	5 gm
Distilled water	100 ml

C. Sugar media preparation

Peptone water	4.5 gm
Sugar solution	0.5 ml

Distilled water 1000 gm

2. Kovac`s reagent for indole preparation

P-dimetheyl aminobenzal	5 gm
Amy alcohol	75 gm
Conc. HCL	25 ml

3. V-P reagent-I

5% alpha-napthanol in absolute ethyl alcohol

4. V-P reagent-II

40% potassium hydroxide 0.3% creatine. The ingredient was dissolved by heating gently Over a steam bath. When in solution, added 0.052 gm of cotton blue dye.

5. Methy red solution

Methy red	0.05 gm
Ethanol (absolute)	28 ml
Distilled water	22 ml

6. Preperation of solutions

a. Stock crystal violet

Crystal violet	10 gm
Ethyl alcohol	1000 ml

b. Stock oxalate

Ammonium oxalate	1 gm
Distilled water	100 ml

Crystal violet working solution: 20 ml of solution no. I mixed with 80 ml of solution no. 2. Additional was made when desired.

c. Lugol`s Iodine solution

Iodine crystal	1 gm
Potassium iodine	2 gm

Dissolved completely in 10 ml distilled water, then added to distilled water to make 300 ml.
Stored in amber bottle.

d. Ethyl alcohol 250 ml

e. Acetone 250 ml

f. Counter stain

Safranine	2.5 ml
Ethyl alcohol (95%)	100 ml

Safranine working solution :

The stock safranine is usually as 1:4 with distilled water.