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**SEROLOGICAL STUDIES ON MSU TWO ISOLATES OF PASTEURELLA MULTOCIDA**

presented by

**Shengfeng Li**

has been accepted towards fulfillment
of the requirements for

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# SEROLOGICAL STUDIES ON TWO MSU ISOLATES OF *PASTEURELLA MULTOCIDA*

by

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ABSTRACT

SEROLOGICAL STUDIES ON MSU TWO ISOLATES OF
PASTEURELLA MULTOCIDA

By

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Gel-diffusion precipitin test was used for serotyping Pasteurella multocida P1010 and P1020, two isolates from avian microbiology laboratory of Michigan State University. The two isolates were demonstrated as serotype 1 of Pasteurella multocida when the antisera from 15 serotypes of Pasteurella multocida strains were used.

It was shown that antigens from infected liver and plasma were not be able to produce cross reaction pattern when the gel-diffusion precipitin teat was used.

These two serotypes possess the characteristics of biochemical and physiological properties of Pasteurella multocida strains.

The results suggest that the Pasteurella multocida strains, P1010 and P1020, have a same serotype as X-73, which was used as a standard strain for serotype 1. It indicates that the two strains are potential vaccines for prevention of fowl cholera caused by X-73 strain. Either of the two strains or combined, P1010 and P1020, of Pasteurella multocida could produce significant protection to the virulent Pasteurella multocida infection of homologous strain.

# ACKNOWLEDGEMENT

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# INTRODUCTION

Pasteurella multocida is the causative agent of fowl cholera (avian cholera, avian pasteurellosis, avian hemorrhagic septicemia), which is a contagious disease affecting domesticated and wild birds (Heddleston and Rhoades, 1978).

Fowl cholera was recognized as a disease of poultry almost 200 years ago. Pasteur isolated the organism and used it in attempting to prepare one of the first vaccines. Although fowl cholera has been recognized and studied for almost 200 years, it still remains an important and poorly controlled disease of poultry because of the lack of an effective vaccine (Barnes et al, 1979).

The causative agent, Pasteurella multocida, is a Gram negative, nonmotile nonsporing rod often occuring singly, in pairs, and occasionally as chains or filaments (Kenneth, 1984). The antigenic structure of Pasteurella multocida is complex (Blackburn et al 1975). Heat-stable antigens extracted with formalized saline were used in the gel diffusion precipitin test to group Pasteurella multocida associated with fowl cholera into 5 serotypes in 1972 (Heddleston et al 1972b). Afterwards, 15 serotypes have been described on the basis of differences in heat-stable antigen (Rhoades and Rimler, 1984). A new serotype of Pasteurella multocida associated with fowl cholera was reported in 1977 (Brogden et al 1977). To date, 16 serotypes were reported The agar gel-diffusion precipitin test (AGDPT) is a satisfactory method for serotyping Pasteurella multocida (Blackburn et al,

1975, Heddleston et al, 1972b). The heat-stable antigen was demonstrated in the serum of a turkey with acute fowl cholera and in the blood of turkeys that died of acute disease (Blackburn et al 1975). Effective use of the agar diffusion precipitin test to demonstrate specific serological and immunological antigens (endotoxins) from two strains of Pasteurella multocida associated with fowl cholera was shown by Heddleston et al (Heddleston et al, 1966). Some other serological methods which were used for grouping Pasteurella multocida are 1) Serum plate agglutination (Little and Lyon, 1943), 2) Indirect or passive hemagglutination (Carter, 1955) and 3) a combination of the two methods (Namioka and Murata, 1964). A fourth method, passive immunization, is occasionally referred in the literature (Little and Lyon, 1943, Roberts, 1947). Classification of Pasteurella multocida by these methods is often difficult or impossible because of the inability of encapsulated cells to agglutinate (without treatment) or the lack of hemagglutinating antigens on unencapsulated cells (Namioka and Murato, 1964). Cross reaction sometimes occurs among strains, particularly with the agglutination test. Pasteurella multocida strains that differ in fermentation, agglutination, immunogenic and pathogenic characteristics could not be differentiated on the basis of the passive hemagglutination test (Namioka and Murata, 1964).

Two isolates from avian microbiology laboratory of Michigan State University have been shown to be a potential vaccine substitute (Chang 1986, personal communication). Previous work (Heddleston et al, 1972a) showes that emulsified water-in-oil fowl cholera bacterins prepared from 3 strains of formalin-killed

Pasteurella multocida induced a high degree of immunity in turkeys against homologous but not heterologous challenge. Most of the isolates appeared to have only of the antigenic determinants. Currently, many scientists are searching for a more effective vaccine against field outbreak of three serotypes of fowl cholera (types 1, 3 and 4). Two field isolates of Pasteurella multocida in the Avian Microbiology Laboratory of Michigan State University have been tested for antigenic activities. Preliminary test results showed some values in protection of birds against fowl cholera challenge. The objectives of this thesis are: (1) antiserum preparation from chickens against 15 serotypes of Pasteurella multocida. (2) serotyping of these two isolates, P1010 and P1020. (3) identifying the serological properties of these two isolates. (4) determination of biochemical peoperties of these two isolates.

# LITERATURE REVIEW

## The Disease--Fowl Cholera Caused by *Pasteurella multocida*

The fowl cholera usually appears as a septicemic disease associated with high morbidity and mortality, but chronic or benign conditions often occur (Heddleston and Rhoades, 1978). Fowl cholera occurs sporadically or epizootically in most countries. Sometimes, it caused high mortality; at other times, losses are minimal (Heddleston, 1975). Vaught et al (1967) reported that over 1000 wild geese died of fowl cholera in one night. In studying the chronic respiratory form in chickens, Hall et al (1955) observed that mortality was low but infection persisted for at least 4 years.

### The Etiologic Agent

The etiologic agent is *Pasteurella multocida*, a gram-negative, bipolar staining bacillus that grows readily on blood agar but seldom on MacConkey's agar (Barnes et al, 1979). The optimal growth temperature is 37C. The optimal pH range is 7.2-7.8, but it can grow in the range of 6.2-9.2, depending on the composition of the medium (Rhoades amd Rimler, 1984). In liquid media, maximum growth is obtained in 16-24 hours. The broth becomes cloudy and in a few days a sticky sediment collects at the bottom. With some isolates a flocculent precipitate occurs. The bacteria will grow on meat infusion media; growth is enhanced

when the medium is enriched with enzymatic digest of peptone, casein hydrolysate or avian serum (Heddleston and Rhoades, 1978). The organism varies greatly in its antigenic make up, a characteristic that may explain the difficulties in producing effective bacterins and vaccines (Barnes et al, 1979). The virulence depends on the strain, the host species, within-strain variation of Pasteurella multocida, age difference of host, and condition of contact between the host and etiological agent. A strain may be so virulent that the number of organisms is relatively unimportant in inducing fowl cholera; or the host may be so susceptible to infection that an avirulent strain for chickens will induce the disease of fowl cholera in turkeys (Heddleston, 1975). Pasteurella multocida is easily destroyed by many disinfectants and by sunlight, heat and drying. Conversely, the organism persists for months in decaying carcasses and moist soil (Barnes et al 1979).

Transmission, Carrier, vector

The possibility that insects may serve as vectors of fowl cholera has been investigated. Skidmore (1932) experimentally transmitted fowl cholera to turkeys by feeding them flies that had previously been fed on infected blood.

Dissemination of Pasteurella multocida within a flock is primarily by excretions from the mouth, nose, and conjunctiva of diseased birds that contaminated their environment, particular feed and water (Heddleston, 1972a). Iliev et al (1965) demonstrated that Pasteurella multocida labeled with P-32 was

inactivated in the proventriculus and feces contained no viable Pasteurella multocida.

Wild birds, including sparrows and pigeons, are believed to harbor and disseminate the organism. Many mammals, especially pigs, cats, wild rodents, racoons, opossums, dogs and people, are suspected of disseminating Pasteurella multocida (Barnes, 1979).

## Clinical Signs

With acute cholera, the birds may die within 24-48 hours of exposure. The first indication of disease may be an unexpected finding of dead birds. Signs usually observed are the drowsiness, dyspnea, fever, diarrhea, anorexia, cyanosis, and mucous discharge from mouth (Heddleston, 1975). Affected birds often conceal themselves under equipment (Barnes, 1979). Cynosis often occurs immediately prior to death and is most evident in unfeathered areas of the head, such as comb and wattles.

Chronic fowl cholera is usually characterized by localiezd infection. It may appear in a flock after an acute outbreak has diminished or it may result from infection with organisms of low virulence (Heddleston, 1975). Wattle, sinuses, leg or wing joints, foot pads and sternal bursal often bec~)e swollen. Exudative conjunctival and pharyngeal lesion may be observed, and forticollis sometimes occurs (Rhoades and Rimler,1984) In turkeys with chronic cholera, forticollis is a frequent sign.

## Lesions of Infection

Lesions may be absent if the disease is very acute. Usually there are petechial and ecchymotic hemrrhages at a few sites , especially on the heart, under serous membranes, in mucous membranes, in the gizzard or in abdominal fat (Barnes et al, 1979). Livers of acutely affected birds may be swollen and usually contain multiple small focal areas of coagulution necrotic and heterophilic infiltration (Rhoades and Rimler, 1984). Heterophilic infiltration also occurs in lungs and contain other parenchymatous organs.

In chronic cases there may be localized inflammatory lesions. These often involve a joint, tendon sheath, wattle, conjunctival sac, infraorbital sinus, the nasal turbinates, the middle ear or cranial bones at the base of the skull (Barnes et al, 1979). Multinuclear giant cells were often associated with necrotic masses of heterophils in air spaces (Heddleston and Rhoades, 1978).

Turkeys often have cholera as a complication of air sacculitis caused by mycoplasma gallisepticum. In such cases there is usually a marked air sacculitis and a well developed fibrinous pneumonea (Barnes et al, 1979).

# Immunogenic Properties Of *Pasteurella multocida* and The Disease Caused

## Antigenic Structure

The antigenic structure of *Pasteurella multocida* is complex (Blackburn et al, 1975). Soluble fractions of *Pasteurella multocida* strain 1059 were extracted from a single source by four methods, and their immunogenecity was evaluated by challenge exposures in turkeys. The endotoxin content was the highest in LPS-protein, followed by KSCN (potassium thiocynate Na salicylate, and saline extract in the order. The four fractions contained at least one common antigen, which had previously been shown to be a surface-protective antigen (Kodamer et al, 1982). Yaw et al (1957) made a comparison of the protection-inducing factors in chickens and mice of a type 1 strain of Pasteurella multocida. The type-specific capsular polysaccharide of *Pasteurella multocida* has been reported to give protection to mice challenged with virulent microorganisms of homologous type but there was no protection after challenge with bacteria of other serological type. Smooth or fluorescent colonical variants of strains of three different serological types of *Pasteurella multocida*, though equally virulent for mice, vary markedly in their virulent for chicken (Yaw et al, 1956).

## Immunologic Response

Pasteur developed an attenuated *Pasteurella multocida* vaccine

that worked under laboratory conditions, but behaved erratically under field conditions (Harschfield, 1965) It was reported (Hayden, 1914) that the immunity against Pasteurella multocida in rabbits, chicken, and pigeons was produced by injecting an avirulent Pasteurella multocida. Various preparations of Pasteurella have been used as immunizing agents for fowl cholera, including live avirulent vaccine (Bierer et al, 1972; Heddleston et al, 1975; Maheswaran, 1973), killed bacterins (Heddleston et al, 1968; Heddleston, 1972a and Heddleston 1974a), oil-adjuvant bacterins (Gaunt et al, 1977), tissue-propagated bacterins (Heddleston, 1968), potassium thiocyanate extract (Guant et al 1977) and various cell fraction (Brow et al, 1970). In 1978, Chengappa reported a streptomycin-dependent live Pasteurella multocida type-3 vaccine for the prevention of fowl cholera in turkeys (Chengappa et al, 1978).

It was reported by Heddleston (1978) that fowl cholera bacterins prepared with liver and blood from a turkey that died of acute fowl cholera induced immunity in turkeys, though not in mice, against a different immunogenic type of Pasteurella multocida. A bacterin prepared with the bacteria grown on laboratory media did not induce immunity against the immunogenic type, demonstrating that Pasteurella multocida produces a wider spectrum of immunogens in live birds than on laboratory media. Heddleston (1974a) showed that the cross-immunity induced in the turkeys was host specific and the antigen responsible for cross-immunity was produced only when Pasteurella multocida was grown in embryonating turkey eggs. Embryonating chicken eggs and infected chicken and mouse tissue, however, did not produce

bacterins that induced immunity against the different immunogenic type of pasteurella. In 1976, Rebers (1976) reported that modified fowl cholera bacterins prepared by inoculating agar medium with infected liver tissue from birds which died of acute fowl cholera induced 70% cross-protection in turkeys, ie, protection against a different immunogenic type of Pasteurella multocida. Standard bacterins prepared from cultures which had been lyophilized and stored showed variable cross-protection. Repeated subculturing of the standard inoculum on agar reduced cross-protection. The protection with either the modified or standard bacterins was comparable (80-100%) when immunity was challenged with the homologous strains. With lyophilization of Pasteurella multocida and subculturing on agar, it appears that antigens are capable of inducing homologous immunity. The regular Pasteurella multocida strains X-73 and P-1059 bacterins have been found to induce immunity only against the homologous strains (Heddleston, 1962 and Heddleston, 1966). However, good cross-immunity has been induced with formalin-killed in-vivo-propagated Pasteurella multocida (Heddleston, 1972a). These bacterins were produced by treating Pasteurella multocida infected heart blood and liver tissue with formalin. Inoculation of turkeys with tissue bacterins induced the formation of cross-protection IgG antibodies as demonstrated by passive immunity trials with purified serum fractions, whereas cross-protective antibodies were not induced by the regular bacterins. Cross-protective antigens are apparently present with in-vivo-propagated Pasteurella multocida cells that are not present when lyophilized inocula are cultivated with artificial media.

It was also reported (Rimler, 1979a) that crude liver homogenates from turkeys that died of fowl cholera infected by serotype 1 or 3 Pasteurella multocida induced cross- protection. Pasteurella multocida harvested from the blood of infected turkeys by a centrifugal technique were as immunogenic as the liver homogenates. Neither bacterial cell-free blood plasma nor washed Pasteurella from infected turkeys induced significant cross-protection. It was shown (Rimler, 1979b) that the cross-protection factors of Pasteurella multocida were maintained in vitro through at least 9 serial passages. Different growth media and temperature enhanced or repressed the ability of Pasteurella multocida to produce cross-protection factors. It was demonstrated (Rimler, 1979) that certain amino acids were innocuous to expression of cross-protection factor. The plasma of normal turkeys contained a compound or compounds that were responsible for expression and maintenance of cross protection factor.

Bierer and Derieux (1972) stimulated renewed interest in live fowl cholera vaccine administered in drinking water. They demonstrated good immunity in 14-week-old turkeys that were given a live culture of Pasteurella multocida (CU strain) in drinking water 2 weeks before challenge exposure. Best results were obtained by inoculating 8-week-old turkeys with a killed bacterin and then administering the live vaccine 2 weeks later; Maheswaran et al (1973) also induced immunity in turkeys with live vaccines via drinking water. They suggested that the vaccine induced localized but not systemic protection. In other studies, Heddleston et al (1974b) showed that serum from vaccinated via

drinking water induce passive immunity in chicks and turkeys.

## Serological Methods Used in Grouping *Pasteurella Multocida*

Before the agar gel-diffusion precipitin test (AGDPT) was used for serotyping *Pasteurella multocida*, some other serological methods were used. First, serum plate agglutination was performed by Little and Lyon (1943). The method was carried out by use of rapid slide agglutination test with monovalent antiserum. Overnight growth of cultures were suspended in physiological saline at the time of testing. A platinum loop 5 mm in diameter was used to measure and transfer the culture suspension and a loop 1 mm in diameter was used for the serum. Glass slides were flamed to removed surface film, cooled, and rolled into three compartments an inch square. Mixtures of bacterial suspension were made with specific monovalent serum and tested simultaneously. This experiment confirmed the existence of 3 serological distinct types within the nonhemolytic Pasteurella. Second method was indirect or passive hemagglutination described by Carter (1955), who identified *Pasteurella multocida* as three types, A, B, and C on the basis of serological differences. The third one, a combination of the two methods (Namioka and Murata, 1964), was used to analyse the O antigen of Pasteurella. It was shown that there were three serotypes that produced fowl cholera. A fourth method, passive immunization, is occasionally referred to in the literature (Little and Lyon, 1943; Roberts, 1947). Grouping of *Pasteurella multocida* by these methods is often difficult or impossible because of the inability of encapsulated

cells to agglutinate (without treatment) or the lack of hemagglutinating antigens on unencapsulated cells. Cross reactions sometimes occur among strains, particularly with the agglutination test. Pasteurella multocida strains that differ in fermentation, agglutination, immunogenic. and pathogenic characteristics could not be differentiated on the basis of the passive hemagglutination test (Heddleston, 1972a).

The agar-gel-diffusion precipitin test (AGDPT) is asatisfactory method for serotyping Pasteurella multocida (Blackburn et al, 1975 and Heddleston et al, 1972b). In 1972, heat-stable antigens extracted with formalized saline were used in the gel diffusion precipitin test to group asteurella multocida associated with fowl cholera into 5 serotypes by Heddleston et al (1972b). 16 serotypes have been described on the basis of the differences in heat stable antigen (Rhoades amd Rimler, 1984)

16 stock cultures and the serotypes

| Serotype | Stock Number | Host |
|---|---|---|
| 1 | X-73 | Chicken |
| 2 | M-1404 | bison |
| 3 | P-1059 | turkeys |
| 4 | P-1662 | turkeys |
| 5 | P-1702 | turkeys |
| 6 | P-2192 | chicken |
| 7 | P-1997 | herring gull |
| 8 | P-1581 | pine siskin |
| 9 | P-2095 | turkeys |
| 10 | P-2100 | turkeys |
| 11 | P-903 | Swine |
| 12 | P-1573 | human |
| 13 | P-1591 | human |
| 14 | P-2225 | cattle |
| 15 | P-2237 | turkeys |
| 16 | P-2723 | turkeys |

## Methods of Antigen Preparation

The preparation of antigens for serotyping has been described by Heddleston et al (1972b), Brogden et al (1977) and Blackburn et al (1975). The 18-to-24-hour growth from a heavily seeded agar plate was suspended in 1.0 ml of 0.85% NaCl solution containing 0.3% saturated solution of formaldehyde. The suspension of cells was heated in a water bath at 100C for 1 hour. The cells were sedimented by centrifugation, and the supernatant was used for the antigen in the gel diffusion precipitin test.

## Methods of antisera preparation

Preparation of antisera for the immunodiffusion test described by Heddleston et al (1972b), which has been adopted by the National Animal Disease Center (NADC), USDA, to develop a training program in immunotyping of Pasteurella organisms for personnel of state diagnostic laboratories. The method was described as follows:

Antisera were prepared in 12-to-16-week-old New Hampshire roosters. Each strain of Pasteurella multocida was grown 18-24 hours at 37C on DSA (dextrose starch agar), harvested in 0.85% saline containing 0.3% formalin, and adjusted to 10X a McFarland 1 density. This suspension was emulsified with equal amounts of a light mineral oil containing 3.0% Arlacel A (Atlas Powder Company, Wilmington, Del). One milliliter of emulsified bacterin was injected subcutaneously in the neck. Three weeks later, 1 ml of bacterin was injected intramuscularly in the breast, 0.5 ml on

each side of the sternum. The birds were exsanguinated 1 week later. The antisera were separated from the clot within 3 hours by centrifuging 1 hours at 1500xg, and preserved with 0.01% thimerosal and 0.06% phenol.

Method of Agar Gel Diffusion Precipitin Test (AGDPT)

AGDPT is one kind of double immunodiffusion tests, which was first used by Ouchterlony (1948).In the thirties some bacteriologists made attempts to apply the diffusion principles for serological typing of various bacteria species but well-standardized techniques for immunodiffusion (ID) were not established untill the middle of the forties. In 1940 Oudin in France gave his first report on the principles and application of the simple diffusion technique in tubes (Oudin, 1946). Somewhat later, double diffusion methods were introduced in 1947 in Sweden by Ouchterlony (1948). It has been demonstrated (Brogden et al, 1977) that AGDPT is a satisfactory method for serotyping Pasteurella. Heddleston et al (1972b) used this method to group 258 field isolates. A new serotype has been reported using gel diffusion precipitin test (Brogden, 1977). The method used by Heddleston and Brogden is described in detail as follows:

The agar gel consisted of 0.9% Noble agar (Difco) and 8.5% NaCl in distilled water. Five ml of molten agar is to be pipetted onto standard 25x75 mm microscope slides. Two patterns consisting of 7 wells (6 peripherally around a center wells) are to be cut on a single slide. Antisera were placed in the outer wells and antigens in the center wells. The slides are placed in covered

petri dishes, incubated overnight at 37C, and examined under a magnifying glass with background illumination.

# MATERIALS AND METHODS

## Cultures

Pasteurella multocida strains of 15 serotypes used to prepare antisera were received from National Animal Diseases Center, Iowa and two MSU isolates of Pasteurella multocida were from avian microbiology laboratory of Michigan State University.

## Physiological tests

The ability of the two strains to ferment various carbohydrates was tested by inoculating phenol red broth base (Difco) contaning 1 percent of the various carbohydrates. Indol production was tested after 24 hours of growth in a medium that contained 2 percent tryptose (Difco) and 0.85% percent NaCl.

## Preparation of Antigens for serotyping

Lyophilized cultures of 16 Pasteurella multocida strains and MSU isolates P1010 and P1020 were reconstituted in tryptose yeast extract broth (Difco). They were transferred in tryptose yeast extract broth three times, and then streaked on tryptose yeast extract agar. The 24-hours growth from a heavily seeded agar plate was suspended in 1.0 ml of formalied saline solution. The suspension of cells was heated in a water bath at 100C for 1 hour. The cells were sedimented by centrifugation, and the supernatant was used for the antigen in the gel diffusion precipitin test.

## Antisera Used for the Serotyping

Antisera for the immunodiffusion test were prepared as described by Brogden (1977), using Rhode Island Red instead of mature New Hampshire roosters. Each strain of Pasteurella multocida was grown 24 hours at 37C on tryptose yeast extract agar after be transfered three times in tryptose yeast extract broth (Difco) The cells on the agar were harvested in 0.85% saline containing 0.3% formalin. This suspension was emulsified with equal amounts of complete Freund's adjuvant (Difco). One milliliter of emulsified bacterin was injected subcutaneously in the neck of the chickens. Three weeks later, 1 ml of bacterin was injected intramuscularly in the breast, 0.5 ml in each side of the sternum. The birds were exsanguinated 1 week later. Feed was removed from cage the night before the birds were exsanguinated. The antisera were separated from the clot within 3 hours by centrifuging 1 hour at 1500xg.

## Antigens From Plasma Bacteria

Bacteria strains P1010 and P1020 were harvested from infected blood by a differential centrifugation technique similar to that described by Smith et al (1953) and Rimler et al (1979a). Heart blood was collected aseptically from chicken livers 24 hours after inoculation with Pasteurella multocida strains P1010 and P1020 respectively. The blood was collected into 30 ml syringes containing an anticoagulant of 3.0 ml of citrate-saline solution

(5% sodium citrate in 0.85% saline). The blood was immediately dispensed into 30 ml screw-cap centrifuge tubes (15 ml per tube ) and centrifuged at 60xg for 30 min. The supernatant layer was aspirated off, dispensed into fresh centrifuge tubes and recentrifuged two times at the same rate. The final supernatant layer was aspirated off to within 3 mm of the pellet. The pellet was discarded. Formalin was added to half of the supernatant layer to give a final concentration of 0.3%. This portion of supernatant layer is heated in a water bath at 100C for 1 hour. The cells were sedimented by centrifugation and the supernatant was used for the antigen in the gel diffusion precipitin test in experiment two.

Antigen from infected chicken tissue

To prepare a chicken tissue antigen, chickens were inoculated in the breast muscle with 1 ml of P1010 and P1020 solution, respectively, that were grown on tryptose yeast extract agar (Difco) and harvested in 2 ml of 0.85% saline. 24 hours after inoculation, the livers were aseptically removed and homogenized in a blender with 3 volumes of physiological saline. This homogenates were filtered through 6 layers of cheesecloth to remove large particles. Formalin was added to give a final concentration of 0.5%. This solution was heated in a waterbath at 100C for 1 hour. The cells were eliminated by centrifugation and the supernatant was used for the antigen in the gel diffusion precipitin test in experiment three.

## Agar Gel Diffusion Precipitin Test (AGDPT)

The agar gel consisted of 0.9% Noble agar (Difco) and 8.5% NaCl in distilled water. Five ml of molten agar was pipetted onto standard 25x75 mm microscope slides. Antisera were placed in the outer wells or in an opposite way. The slides were placed in covered petri dishes, incubated overnight at 37C, and examined under a magnifying glass with background illumi nation.

## Immunoelectrophoresis

Immunoelectrophresis was conducted at pH 7.6 in a medium consisting of 1% agarose, 0.1% sodium azide, 0.02M Tris on 50x75mm plates. with 50x75 mm. The current was 20 mA. Chicken antiserum was added at completion of run, and incubated at 37c for 2 days. The immunoelectrophresis was used to compare the heat-stable antigens from media preparation (P1010 and P1020) with a infected plasma antigen and tissue preparation.

# RESULTS

## Physiological properties

Each strain of the two MSU isolates, P1010 and P1020, produced indol and fermented glucose, sucrose, xylose, dextrose, dulcitol, fructose and manitol. Neither of the strains fermented lactose, maltose, raffinose, salicin and inositol.

## Serotyping results

Experiment 1. This experiment, performed with antigens from agar media and the antisera for serotyping induced by bacterin, was designed to determine the serotype of two strains of Pasteurella multocida P1010 and P1020 according to the method used by Blackburn et al (1975). Fig.1A shows the reaction between antigen prepared from P1010 and antiserum prepared against the representative of each of the 15 serotypes. The center wells are the heat-stable antigens of P1010(A) and P1020(B) prepared from media. Outer wells contain the 15 serotype sera and the antiserum against P1010(1), P1020(2), normal serum(3), P1059(4), P1662(5), P1072(6), P2192(7), P1997(8), P1581(9), P2095(10), P2100(11), P903(12), X-73(13), P1573(14), P1591(15), P2225(16), P2237(17), P2723(18). The reactions observed are between antigen prepared from P1010 and antiserum prepared against X-73, (well 13), the serotype 1 of Pasteurella multocida. The positive contrast of reaction between antigen prepared from P1010 and the antiserum against P1010 was positive (well 1) and the antigen of P1010 also reacted with P1020(well 2). Fig.1B shows the reaction between

antigen prepared from P1020 instead of P1010 and antiserum prepared against representative of each of the 15 serotypes. The reactions observed are between antigen prepared from P1020 and the antiserum prepared against X-73(well 13), The homologous antiserum P1020 (well 2). In fig 1A and 1B, the cross reaction between the two isolates, P1010 and P1020, was as apparent as the homologous reaction.

Experiment 2. This experiment, performed with antigens from plasma-bacteria and antisera same as in experiment 1, was designed to compare the precipitin antigenic differences of MSU isolates P1010 and P1020 between plasma-bacteria antigen and antigens from media bacteria through immunodiffusion. The results show no difference in the precipitation pattern (date not shown).

Experiment 3. Since no difference in precipitin pattern could be detected between bacterin antigens prepared from blood and the antigen preparation media, a third experiment was designed to understand whether cross reaction could be seen by using antigens of MSU isolates P1010 and P1020 from liver tissues. Both the P1010 and P1020 antigen preparation from liver tissues do not show visible precipitin cross reaction with other serotypes except the cross reaction between P1010 and P1020, which have been demonstrated to be same serotype, serotype 1 of Pasteurella multocida(date not shown).

Immunoelectropgoresis

Lines of identity were observed among the heat stable antigens from media cultures, infected plasma, and infected

liver. The electrophoretic mobility was compared and no differnce was found.

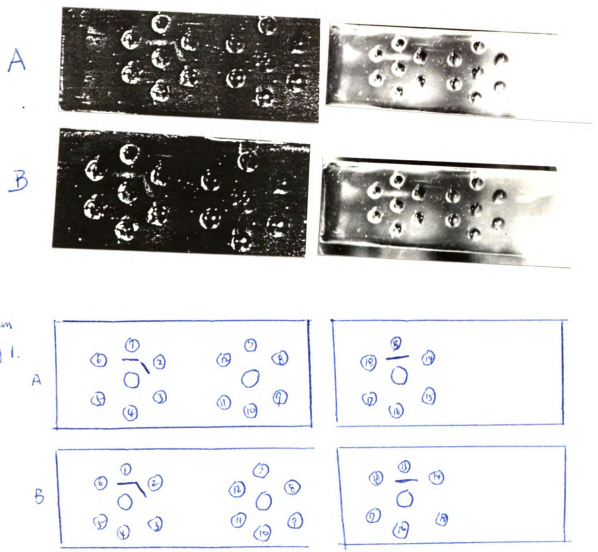


Fig. 1. Precipitin patterns of Pasteurella multocida. heat stable antigens from media, P1010(A) and P1020(B). Outer wells contain the 15 typing antisera and the positive serum against P1010(A) and P1020(B). Details are described in text.

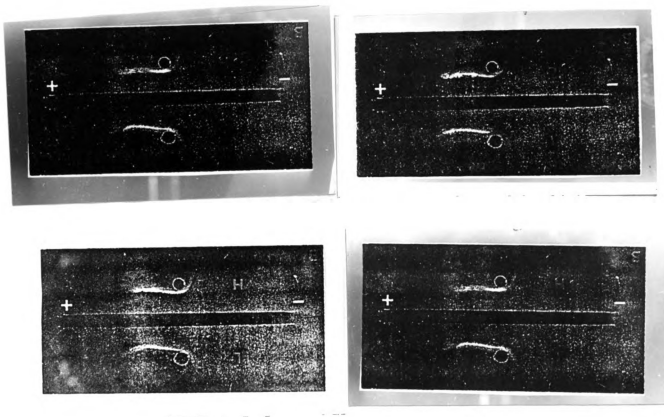


Fig. 2. Immunoelectrophoresis patterns ahowing the electrophoretic mobility of the heat stable antigen P1010 and P1020. A. serotyping antibody in the trough as described above and the antigen in the wells from P1010 media preparation. B. same antiserum in the trough and the antigens in the wells from plasma (H) and infected liver tissues (L). C. serotyping antibody agaisnt P1020 in the trough and the antigen P1020 from media in the outer wells. D. the antiserim against P1020 infected plasma(H) and liver tissues (L). The two strains have lines of idendity.

# DISCUSSION AND CONCLUSION

The results indicate that the MSU isolates P1010 and P1020 of Pasteurella multocida have the same serotype as X-73, the serotype 1 of Pasteurella multocida. Since correlation was good, though not absolute between the serological reaction and the immune response in chicken and turkeys (Heddleston et al, 1972a), the two avirulent isolates could be potential vaccine for prevention of homologous infection. This is under investigation. The gel diffusion test is a good test for serotyping isolants of Pasteurella multocida associated with fowl cholera (Heddleston et al, 1972b). Since the Pasteurella multocida strain X-73 and the P1010 and P1020 could be distributed among the experimental chicken, a test between normal serum and P1010, P1020 and other representative antigen of 15 serotype were performed. However, no visible cross reaction has been seen. It demonstrated the heat-stable antigens were specific and only reacted with homologous antiserum.

As for the serologic techniques employed, it was shown that the AGDPT was superior to the indirect or direct hemagglutination test described by Carter (1955) and serum plate agglutination by Little and Lyon (1943). It is well known from the work of Namiok and Murata (1963) that the O antigen composition of Pasteurella multocida is very complex. In order to obtain "specific factor sera" they had to absorb all of their sera with large quantities of heterologous organisms. However, the AGDPT employed by Brogden et al (1977) and Heddleston et al (1972b) was accurate and convenient. The heat-stable antigen employed in AGDPT in this

experiment was specific.

This experiment also demonstrated that precipitin antigen for serotyping from infected tissues and plasma other than from media did not show any different precipitin pattern in gel diffusion precipitin test. At the beginning of these studies, emphasis was placed on serotyping of the two MSU isolates following the method adopted by Rhoades et al (1977). When the results indicated that both of the two isolates belong to serotype 1, studies were initiated to test the cross reaction with antigen s prepared from infected tissues and plasma-bacteria. However, the cross-reaction was not visible in the gel diffusion precipitin test when performed with antigens from infected liver tissues and plasma-bacteria preparation. It was reported (Rimler et al, 1979b) that crude liver homogenates from turkeys by serotype 1 or 3 Pasteurella multocida induced cross protection against infection of different serotype. The organisms harvested from the blood of infected turkeys were as immunogenic as the liver homogenates. Our results showed that the relationship between the cross protection and cross reaction in the gel diffusion precipitin test could not be correlated. One of the reasons was that the antigenic determinants for cross protection were not heat-stable, and so they were easily destroyed during the antigen preparation. Alternatively, different strains or isolates, although same serotype, could have antigenic structure of minor differences. Some of them could induce cross protection and other could not induce the cross immunity when the vaccine are prepared from infected tissues or plasma-bacteria preparation. The question still

remains for MUS isolates of Pasteurella multocida whether or not the vaccines prepared from tissues and plasma-bacteria can induce cross protection under field conditions.

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