PATHOGENICITY OF *PASTEURELLA MULTOCIDA* ISOLATED FROM MYNA BIRDS AGAINST CHICKENS

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Introduction

Fowl cholera is an infectious disease which affects not only domestic fowl but also wild birds. The disease is world-wide in its distribution and consists of two types, acute or peracute type and chronic one. The acute type of fowl cholera usually induces septicemia with high mortality. The chronic type occurs usually with lower mortality.

In Japan, there has not been any outbreak of fowl cholera for more than thirty years. However, in April, 1976, the disease occurred in myna birds (*Eulabes intermedia*, A. HAY) imported from Thailand shortly after their arrival in Japan.

The authors describe the outbreak of fowl cholera in myna birds and the pathogenicity of the isolated bacteria, *Pasteurella multocida*, against chickens.

An outbreak of fowl cholera in myna birds

On April 22, 1976, 480 myna birds arrived at Tokyo from Thailand by airplane. Within a few days, those birds were distributed to pet shops in various areas. Among 480 birds, a total of 54 birds were confirmed to be dead up to April 26, and 13 out of 54 dead birds were examined bacteriologically and pathologically.

The dead birds showed hyperemia in the trachea and marked edema in the lungs. Clear yellowish exudate was observed in the thoracic cavity. A small number of petechial hemorrhages occurred in the heart and liver, and ecchymoses were observed in the lamina propria of the duodenum in a few cases. Bacterial clumps were seen in exudates in the air capillaries and tertiary bronchi. In the liver, there were small necrotic foci with bacterial clumps. These lesions were accompanied with mild cellular reactions consisting of heterophils and histiocytic cells.

Bacteria were isolated from the heart, spleen, trachea and liver of 7 birds by using sheep blood agar plates. Bacteria forming a small greyish white pure colony were isolated practically from each specimen. The isolates produced bluish iridescent colonies on a trypticase soy agar plate (BBL). Seven strains isolated from the livers of the seven birds had identical biological properties, as follows. They were rod shaped, gram-negative, and stained positive by bipolar staining and capsular staining.

They did not grow on McConkey agar plates. They produced catalase and oxidase, but not urease. They produced almost always indol. They did not hydrolyze esculin or gelatin. They utilized citrate and resisted against KCN. They reduced nitrate and nitrite. They did not produce H_2S in SIM agar, neither did they react to Voges-Proskauer reaction or methyl red test.

They fermented glucose, xylose, arabinose, fructose, galactose, mannose, sucrose, mannitol, and sorbitol, but not maltose, lactose, inositol, dulcitol, salicin or raffinose.

From the results, the isolated bacteria were identified as *Pasteurella multocida*.

Serological typing of the two isolates was made by tube agglutination test using antiserum from rabbit immunized against TS-8 strain of *Pasteurella multocida* (0-5:A) and HC1 treated antigen with

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turbidity equivalent to McFarland No. 2 scale. The isolates were agglutinated with the antiserum. The serotype of the isolates was determined as "0 - 5". Capsular typing was not made but it was presumed to be "A".

Pathogenicity of isolates against chickens by intramuscular inoculation

Two isolated strains derived from the livers of affected myna birds were designated as 68-1-L and 68-2-L, respectively. They were cultured in a trypticase soy broth (BBL) at 37°C for 18 hours. Each of 10^4, 10^6, and 10^8 time dilutions of the cultured broth was inoculated into the pectoral muscle of two 90-day-old specific pathogen free (SPF) White Leghorn chickens maintained at the Poultry Disease Laboratory.

From the results of colony count of each inoculum, 10^8 time diluted inoculum was supposed to contain 4.5 viable cells in 68-1-L strain, and 5.9 in 68-2-L strain.

Most of the inoculated chickens, including those inoculated with only 4 to 6 viable bacteria died with acute fowl cholera within 24 hours after inoculation (Table 1). Two chickens inoculated with a small number of bacteria and killed 7 days after inoculation, however, did not show any clinical sign before slaughter.

Table 1 Pathogenicity of *Pasteurella multocida* isolated from myna birds against chickens*

Inoculum		No. of chickens inoculated	No. of chickens which died**	Time of death after inoculation (hours)
Strain	Approx. No. of *P. multocida*			
68–1–L	$4.5X10^4$	2	2	17, 19
	$4.5X10^2$	2	2	21, 21
	4.5	2	1	23
68–2–L	$5.9X10^4$	2	2	17, 17
	$5.9X10^2$	2	2	21, 21
	5.9	2	1	41

* Ninety-day-old specific pathogen free White Leghorn chickens of the Poultry Disease Laboratory.
** 7 days after inoculation.

Gross lesions:

The liver of affected chickens was darker in color than that of the noninoculated controls and displayed whitish and/or yellowish multiple small necrotic foci. The lungs were edematous with translucent appearance and yellowish watery exudate was also seen on or under the surface of the lungs. Petechiae were often found in the coronary adipose tissue of the heart. Ecchymoses in the duodenum were observed in a few chickens. Yellowish caseous exudates were found between the superficial and profound pectoral muscles accompanied with severe hemorrhage on the surface membrane of both pectoral muscles.

Microscopic lesions:

Liver; The affected liver revealed prominent hyperemia and often multiple necrosis. Most of the necrotic foci were accompanied with moderate heterophil infiltration. In a few cases which had died earlier, however, heterophil infiltration was very slight. Bacterial clumps were found in the sinusoids or necrotic foci and some of the endothelial cells contained a few bacteria in their cytoplasm.

Lungs; The lobules in most dead chickens became thin with pulmonary edema. Marked edema and slight hemorrhage were found around blood vessels in the interlobular connective tissue

of every inoculated bird. Edematous exudate was also found in the lumen of the tertiary bronchi and their atria. Most capillary blood vessels contained some heterophils in the lumen.

Heart; Fresh hemorrhages in the coronary adipose tissue were often seen in most chickens. Edema was frequently observed in the connective tissue within the adipose tissue or cardiac muscles.

Spleen; Hemorrhages in the adenoid sheath and congestion of the red pulp were commonly observed. Sometimes deposition of serous material was found in the sheath or periarteriolar lymphoid tissue. A small number of necrotic foci with heterophils and edema were seen in the white pulp in a few cases.

Duodenum; Severe hemorrhage was often observed in the lamina propria. Serous homologous substance stained lightly with eosin was often seen in the lumen of the duodenum. In a few specimens, bacterial clumps were seen in the lamina propria where hemorrhages occurred.

Pectoral muscles; Pectoral muscles, where the inoculum had been administered, revealed severe necrosis and degeneration with hemorrhage, infiltration of heterophils and macrophages, and numerous bacterial clumps. Some blood vessels in the severely necrotic lesions were destroyed showing numerous bacterial aggregates. In areas far from the necrotic lesions, there was often a moderate to mild lymphoid cell infiltration around the blood vessels. Most blood vessels had swollen and proliferative endothelial and adventitial cells.

Electron-microscopic findings:

Numerous bacteria were frequently found in the blood vessels of the liver, lungs, spleen and pectoral muscles. Some endothelial cells contained a few bacteria in their cytoplasm. Most bacteria showed an electron transparent zone around the bacterial bodies.

From the results described above, the pathogenicity of the isolates from myna birds was confirmed to be so severe as to kill most 90-day-old chickens within 24 hours after intramuscular inoculation with only 4 to 6 viable micro-organisms. The lesions obtained here were similar to those of acute fowl cholera described in the literature.

Difference in pathogenicity by various routes of inoculation

The authors designed this experiment to evaluate the effect of various routes of inoculation on pathogenicity against chickens.

The 68-1-L strain of *P. multocida* was used for the tests. It was cultured in a trypticase soy broth (BBL) at 37°C for 18 hours. Each of 10^2, 10^4, 10^6 time dilutions of the cultured broth was used as inoculum. The number of bacteria in each inoculum was counted by using colony count method.

Two to six SPF White Leghorn chickens 86 to 500 days of age, and raised in the Poultry Disease Laboratory, were inoculated with 0.5 ml of each diluted inoculum through the muscular, nasal, tracheal, oral (*per os*), or dermal route, respectively (Table 2). In the cutaneous administration, the diluted materials were inoculated with a cotton swab onto the surface of intact dorsal skin in some groups ("Intact") or onto the surface of the skin after removing about ten feathers from the dorsal trunk in other groups ("Treated"). Skin inoculation was made so carefully as not to damage the skin.

Inoculated chickens were observed for 8 days after inoculation and those which survived were sacrificed. Both dead and sacrificed chickens were examined histopathologically.

The results of the experiments are shown in Table 2. Even such a small number of bacteria as 10^2 could readily induce death of inoculated chickens through intramuscular and treated (wounded) skin routes. Oral inoculation could hardly induce death without the administration of a large number of bacteria. Even the chickens inoculated through the intact skin route were susceptible to bacterial invasion when a relatively large number of bacteria were administered. On the surface of the dorsal skin, a slight scratch, slight hemorrhage and/or swelling of two or three feather follicles were observed in birds which died following intact skin inoculation.

Intranasal or intratracheal inoculation with a small number of bacteria could not always kill the inoculated chickens.

Table 2 Pathogenicity of *Pasteurella multocida* according to inoculation route

Exp. No.	Age of chickens used (days)	Route of inoculation	Approx. No. of viable bacteria inoculated		
			10^6	10^4	10^2
1	153	Intramuscular			4/4*
		Per os			0/4
2	86	Intramuscular			3/3
		Via skin			
		Treated		3/3	3/3
		Intact		2/3	
		Per os	1/3	0/3	0/3
		Intratracheal			0/3
3	132	Intramuscular			6/6
		Via skin			
		Treated			2/3
		Intact		1/3	0/3
		Per os	2/3		
4	125	Intramuscular			4/4
		Per os			0/4
		Intranasal			1/4
5	500	Intramuscular			2/2
		Intranasal		0/4	0/4

* Number of dead chickens/number of inoculated chickens
Observation was carried out for 7 or 8 days after inoculation.

Death occurred suddenly within 24 to 48 hours without prominent clinical signs; one bird which died of intranasal inoculation, however, displayed mild respiratory signs before death.

Main lesions in all chickens which died of infection were found in the liver, heart, lungs, spleen, and duodenum, and inoculated sites such as pectoral muscles or dorsal skin. The morphological features and incidence of pathological changes obtained in this experiment except for some lesions in the lungs and skin, were similar to those in the previous intramuscular inoculation.

Macroscopically, the inoculated site of the skin was often swollen with reddish or yellowish feather follicles where the feathers had been plucked. All chickens which died of intact skin inoculation showed a slight scratch, hemorrhage and/or swollen feather follicles on the surface of skin where the inoculation had been made. Those scars were also recognized on the subcutaneous side when the skin was removed for histopathological observations. One bird which died of intranasal inoculation showed pneumonia with severe exudate around the lungs.

There were no lesions in the chickens which survived for 7 or 8 days after inoculation and were sacrificed.

Microscopic examination of the lung of chickens which died of intranasal inoculation revealed numerous bacterial clumps in or around the blood vessels in the interlobular tissues and in the tertiary bronchi along with severe serous exudation and heterophil infiltration. The affected skin of the chickens inoculated via treated skin demonstrated severe infiltration of heterophils, and erythrocytes, and bacterial clumps were observed in the follicular cavity. In most chickens, degeneration or desquamation of follicular epidermis was accompanied with the changes described above. In the subcutaneous tissue or follicular connective tissue, there was an infiltration of

heterophils and histiocytes along with hemorrhages and edema. Endothelial and adventitial cells of some blood vessels in the dermis showed proliferation. Such blood vessels were surrounded by infiltrates of histiocytic cells. Only a few feather follicles in chickens which died following intact skin inoculation showed mild to moderate infiltration of heterophils and degeneration of epidermis of follicles where the feathers were not seen in the follicular cavity.

Even in chickens which died following treated skin inoculation, there were some uninfected feather follicles at the site of inoculation and newly formed feathers were seen in the feather follicles.

No histopathological changes were observed in any organs of chickens which survived.

Recovery of *P. multocida* from inoculated chickens

In the experiments No. 1 and No. 4 shown in Table 2, *P. multocida* was recovered from inoculated chickens to determine whether infected chickens could be the source of natural infection. Isolation of the organisms was carried out from the blood, palatine cleft, oral cavity and cloaca in every inoculated chicken at intervals of 3 hours from 3 to 24 hours after inoculation and then every 6 hours after 48 hours, and 72 hours after inoculation in experiment No. 1. Blood sample was collected from the wing vein with a heparinized syringe and was 10 fold diluted by trypticase soy broth (BBL). Each dilution was cultured on a trypticase soy agar (BBL) at 37°C for 18 hours, when the number of colonies was counted. Other samples from palatine cleft, oral cavity, and cloaca were collected with cotton swabs. The swab samples were soaked in trypticase soy broth for 10 minutes and the broths were cultured on trypticase soy agar plates at 37°C for 18 hours.

In the experiment No. 4, the heparinized blood and other swab samples were collected at intervals of 3 to 6 hours until 24 hours after inoculation, and were also collected 2, 3 and 7 days after inoculation.

Diluted blood samples were also cultured on trypticase soy agar plates, and the number of colonies was counted to determine the number of viable cells contained in the blood. The swab materials were directly cultured in trypticase soy broths at 37°C for 18 hours. In this experiment, each cultured broth was inoculated intramuscularly into a commercial chicken at the age of 35 to 40 days, because it was impossible to eliminate other contaminated bacteria from the samples by using any selective medium. The swab sample was estimated to contain some *P. multocida* when the inoculated chicken with its cultured broth died and *P. multocida* alone was isolated from visceral organs such as liver, heart, spleen and lung on a trypticase soy agar plate.

Inoculated chickens with cultured broth containing *P multocida* became slightly depressed with fever about 9 to 12 hours after inoculation and death occurred suddenly within 24 to 48 hours in most chickens.

P. multocida was isolated in a pure form from the blood as early as 12 hours after inoculation in 9 affected birds. The number of bacteria found in the blood 12 hours after inoculation ranged from 10^2 to 10^4 per ml, to about 10^6 to 10^8 per ml at the moribund stage, 3 to 6 hours after the appearance of the organisms in the blood (Tables 3, 4).

P. multocida, if present in the swab materials, could not be distinguished from other kinds of contaminating bacteria on the trypticase soy agar plates in experiment No. 1. Detection of *P. multocida*, however, was successful in samples from the oral cavity, palatine cleft and cloaca in some chickens 12 hours after inoculation when the cultured swab materials were inoculated into the commercial chickens in experiment No. 4 (Table 4). The incidence of the isolation was high in moribund or dead chickens.

Following intranasal inoculation, *P. multocida* was isolated from two birds, but one bird harboured the organisms in the palatine cleft only 18 hours after inoculation. This chicken did not die during the observation period and no lesions could be observed in the nostrils or visceral organs. The other chicken died about 18 hours after inoculation.

P. multocida was not recovered from any site in chickens which survived except in one bird dissected 7 or 8 days after inoculation.

Table 3 Recovery of *Pasteurella multocida* from the blood in inoculated chickens

Chicken No.	Route of inoculation	Hours after inoculation												
		3	6	9	12	15	18	21	24	30	36	42	48	72
13677	Intra-muscular	–	–	–	10^4 *	10^6	+							
13678		–	–	–	–	10^4	10^7+							
13679		–	–	–	–	10^4	10^8+							
13680		–	–	–	–	–	10^4	10^5	10^8+					
1	*Per os*	–	–	–	–	–	–	–	–	–	–	–	–	–
2		–	–	–	–	–	–	–	–	–	–	–	–	–
3		–	–	–	–	–	–	–	–	–	–	–	–	–
4		–	–	–	–	–	–	–	–	–	–	–	–	–

Age of chickens used: 153 days, Inoculation dose: 500 viable cells per bird
* Number of organisms contained in one milliliter of blood
+: Died, –: Negative isolation
Chickens which survived were killed 8 days after inoculation

Table 4 Recovery of *Pasteurella multocida* from four sites in inoculated chickens

Chickens No.	Route of inoculation	Hours after inoculation							
		6	12	15	18	24	48	96	168
15968	Intramuscular	–*1	BNC*2 10^5*3	+*4 (BNOC) 10^6					
15971		–	B 10^2	B 10^4	+(BC) 10^4				
15970		–	BC 10^4	BC + 10^6					
15969		–	BO 10^4	+(BNO) 10^6					
9	Intranasal	–	–		–	–	–	–	–
15972		–	BO 10^3		+(BNOC) 10^5				
11		–	–		–	–	–	–	–
12		–	–		N	–	–	–	–
5	*Per os*	–	–		–	–	–	–	–
6		–	–		–	–	–	–	–
7		–	–		–	–	–	–	–
8		–	–		–	–	–	–	–

Age of chickens: 125 days, Inoculation dose: 100 viable cells per bird
Chickens which survived were killed 7 days after inoculation
*1 Negative isolation of *Pasteurella multocida*
*2 Positive isolation from blood (B), palatine cleft (N), oral cavity (O), and cloaca (C)
*3 Number of organisms contained in one milliliter of blood
*4 Died, ( ) indicates recovery after death

Discussion and summary

Both the 68-1-L and 68-2-L strains of *Pasteurella multocida* isolated from myna birds belonged to the serotype "0-5" and showed so severe pathogenicity as to kill 90-day-old SPF White Leghorn chickens within 24 to 48 hours after intramuscular inoculation with only 4 to 6 viable organisms.

The lesions observed in chickens experimentally inoculated with the isolates were similar to those of acute fowl cholera described by other workers. Multiple focal necrosis with heterophil infiltration and some bacterial clumps in the liver, ecchymoses in the adipose tissue of the heart and in the lamina propria of the duodenum, and edematous or serous exudate in the lungs and spleen were characteristic of the disease in the present study.

The electron transparent zone observed around the bacterial body was presumed to be the capsular substance of the organisms.

Oral inoculation with at least 10^6 viable cells could induce death in 50% of inoculated chickens, although infection by oral route sometimes was unable to induce the disease. It should be emphasized that a small number of bacteria were able to induce death of chicken by intranasal inoculation.

The present experiments showed also that inoculum containing about 10^2 viable bacteria could induce infection in chickens via skin not only when placed on the skin after removing about ten feathers, but also when placed on the intact skin, though half of the chickens were not affected. It was interesting to note that all the chickens which died of infection via intact skin had scratches or swollen feather follicles devoid of feathers on or near the skin where the inoculation had been made. Those small wounds might have been formed immediately after the inoculation and allowed the penetration of *P. multocida* through the skin or feather follicles.

It was also interesting to observe that *P. multocida* was isolated as early as 12 hours after inoculation and that in most of the chickens which died within 3 to 6 hours after the appearance of *P. multocida*, death was induced shortly after its penetration into the body of a host.

It was fortunate that the outbreak of fowl cholera was limited only to one lot of myna birds imported from Thailand and that it did not spread among chicken flocks in Japan.

Discussion

Gupta, B.K. (India): You used anti-serum against the whole cell of *Pasteurella multocida*. Did you use 0 factor 5 specific serum, as there is a possibility of cross-agglutination from other antibodies working in this system.

Answer: Bacteria isolated from myna birds were identified as typical *Pasteurella multocida* by morphological and biochemical tests. Serotype of the strains was detected as 5 (somatic antigen) A (capsular antigen). The standard anti-serum for factor 5 used in this test was prepared by Dr. Namioka and kept in our Institute.

Namioka, S. (Japan): Did you ever make domestic vaccine to prove the efficacy of protective ability?

Answer: No, I didn't.

Ogata, M. (Japan): I would like to know about the distribution of fowl cholera in Thailand.

Answer (Srihakim, S., Thailand): This disease is widespread throughtout the country in the poultry especially in the rural areas where vaccination is not performed. Pigeons, parrots, ducks and geese are affected. The incidence in poultry is particularly high (700,000 ducks have been killed for the past five years). Also, there are many carriers. In this particular instance, it is possible that the infection of the myna birds took place in the quarantine station and that they became carriers of the disease, which might have been induced by the stress consecutive to the transportation.

Gatapia, S.L. (Philippines): Was there any review of the literature made about the occurrence of the disease in myna birds in Thailand?

Answer: No literature is available on pasteurellosis or fowl cholera in myna birds.

Rahman, A. (Malaysia): Was any virological study carried out on the dead myna birds and, if so, was there any significant finding?

Answer: No pathogenic viral agents could be isolated from affected myna birds.