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Fowl Cholera Outbreak in Domestic Poultry and Epidemiological Properties of *Pasteurella multocida* Isolate

Yong-Ku Woo[1,*] and Jae-Hak Kim[2]

[1]*Institute of Laboratory Animal Resources, Seoul National University, San 56-1, Silim-dong, Gwanak-gu, Seoul, 151-742, Republic of Korea*
[2]*National Veterinary Research and Quarantine Services, MAF, 480, An-Yang-6-dong, Manan-gu, An-Yang-City, 467-180, Republic of Korea*

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Symptoms of fowl cholera including orofacial edema, swollen and edematous wattles and combs, and severe respiratory disorders were detected in domestic poultry in two broiler breeder farms: one located in Gyeong-gi Province (October, 2000) and the other in Chung-cheong-nam Province (March, 2001). Gram-negative, bipolar staining bacillus was easily found in a direct smear. The biochemical properties of isolates were examined using a standard diagnosis method, proving that they were 99.7% similar to the *Pasteurella multocida* (*P. multocida*: PM), a pathogenic and causative agent of fowl cholera (FC). As a result, an FC outbreak in domestic fowls was confirmed for the first time in Korea since 1942. Because FC was detected in broiler breeder farms for the first time in 59 years at the same time as an FC outbreak was confirmed in wild birds (October, 2000), our concern was focused on whether the PM strains that originated in wild birds were transmitted into poultry farms. The possibility was tracked down by comparing phenotypic and genetic properties between the two types of PM strains. PM strains of chicken origin showed prominent differences from the PM strains of wild bird origin in both phenotypic and genetic properties. An examination of the origin of the wild bird bacteria was conducted, but no evidence has been identified that PM strains from the wild bird were introduced into domestic poultry farms.

Keywords: fowl cholera, domestic fowl, *P. multocida*, epidemiological properties

The first report about the fowl cholera (FC) outbreak was recorded in the 8th research report by the Institute of Livestock Disease Control and Serum Production (Animal Quarantine Station) under the rule of Japanese imperialism (Kim *et al.*, 1966, Jeung *et al.*, 1987). Nine strains of *P. multocida* (PM) were isolated to conduct the experiment in 1942, and it was suggested at that time that there was a possibility that FC had already broken out in Korea before 1933 (Kim *et al.*, 1966). According to the statistical data on livestock diseases published between 1932 and 1942 by the Ministry of Agriculture and Commerce, the Japanese colonial government stated that FC was detected in 2,543 fowl, all of which were dead at that time. It also stated that domestic poultry were vaccinated against FC in 1940, which suggested how serious the condition was at that time. Since 1942, there have been no reports of FC in domestic poultry until this decade (Kim *et al.*, 1966; Jeung *et al.*, 1987).

According to more recent data, acute septicemia occurred regularly throughout Korea until 1942, but today, chronic types of bacterial infections occur more commonly. Notably, it is stated that no outbreaks of chronic type FC have been reported in Korea since 1942 (Jeung *et al.*, 1987).

A significant number of wild birds died in the Cheon-su bay, Gyeong-gi Province, in the middle of October, 2000. We sent the PM isolates to the National Veterinary Service Laboratory (NVSL), USA and requested an analysis of antigenic and genetic properties. As a result of the analysis, *P. multocida*, the causative agent of FC, was verified, and the data were made public through the mass media as a report on the FC outbreak in wild birds for the first time in Korea.

Also in October of 2000, an outbreak of FC was detected in domestic chickens for the first time since

* To whom correspondence should be addressed.
(Tel) 82-2-880-8151; (Fax) 82-2-886-0578
(E-mail) wooyk@snu.ac.kr

1942. The first FC outbreak occurred in a broiler breeder farm (SM: Isabrown) located in Gyeong-gi Province (Peongtaek area). The FC was mainly observed in domestic fowls about 25 weeks of age. Since their symptoms included orofacial edema, wattle and comb swelling, and respiratory symptoms, they received an initial diagnosis of Coryza from a clinical veterinarian, who subsequently prescribed antibiotic treatments. However, because this treatment was having no apparent effect, a definitive diagnosis on the case was requested from our laboratory.

Because there was a good chance that this FC outbreak was related to that just discovered in wild birds (based on both timing and location), an immediate visit to the farm was paid to examine the clinical symptoms. Autopsies were conducted on diseased chickens; the clinical findings showed mainly lesions of Colibacillosis, such as airsacculitis, pericarditis, peritonitis, and severe pneumonia. Swelling of eyes and wattles, and serious respiratory disorders similar to FC symptoms were also found. However, because these were domestic chickens, any decision on FC needed to be dealt with prudently.

Since more precise identification of the causative agent was needed, material for strain isolation was inoculated into culture media. At the same time, the feather culture method was applied to cause vertical streaking with *Staphylococcus aureus* (*S. aureus*) which generates complete hemolysis to enrich *Heamophilus paragallinarum (H. paragallinarum:* HP*)*. This HP strain is a very fastidious and needs V-factor as a causative agent of Coryza, cultivating each in CO_2 and normal incubating method respectively. When checking these culture agars, a great number of transparent, glossy, and quite big colonies were found on the blood agar plates. They gave off a distinctive and sweet smell, which is characteristic of *P. multocida.* On the contrary, a Coryza strain (HP) was found only around *S. aureus* colonies in the form of small sized colonies (dew drop size, transparent and glossy). Taking these facts into account, the strains isolated from the chickens were strongly suspected to be *P. multocida.* For rapid and definitive species and subspecies identification, colonies were analyzed with a Crystal EN/F Kit (BBL, USA), which confirmed 99.7% positive match with *P. multocida.*

Thus, FC clinical symptoms were confirmed in domestic chickens for the first time since 1942, and PM, the causative agent of FC, was definitively identified in affected organs on laboratory levels. The bacterial isolates were submitted to NVSL (USA), and the genetic analysis and antigenic identification were re-confirmed.

A 2nd FC outbreak was also detected in a broiler breeder farm located in Chung-cheong-nam Province around February of 2001. Chickens expressed clinical symptoms similar to the first FC case, and the overall

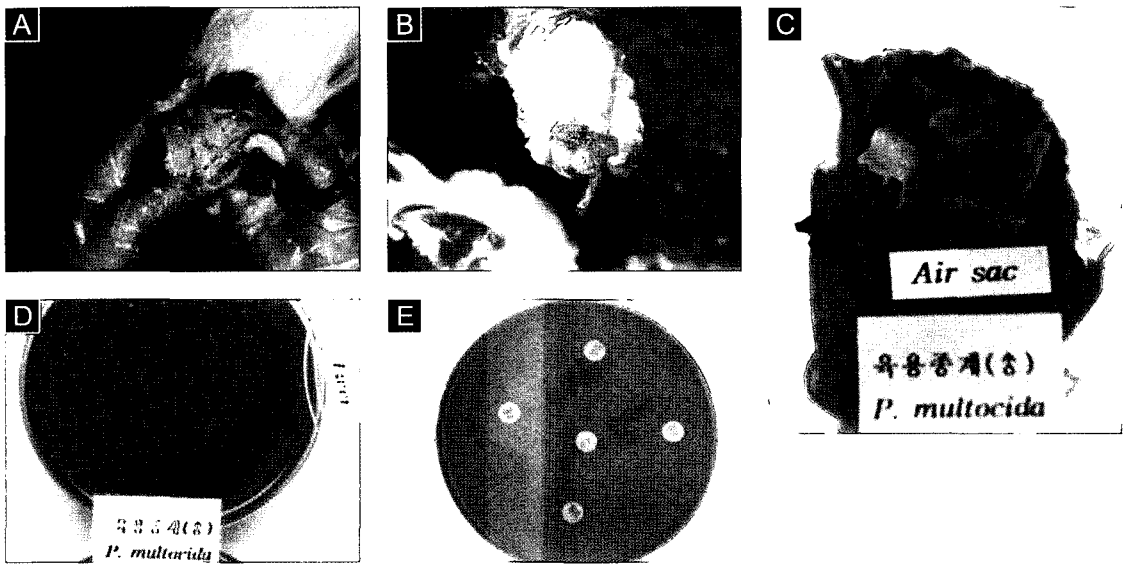

Fig. 1. (A) The cheese like inflammatory exudate both at the infra-orbital sinus and the basal part of wattle in clinically infected chickens.
(B) The inflammatory exudate from the cutting surface of lung, which had the severe pneumonic lesion.
(C) The severe air-sacculitis and pneumonia lesion from the clinically infected chickens.
(D) The pure and clear *P. multocida* colonies cultured from the infected chicken organ (Liver) on sheep blood agar. The pure colonies are non-hemolytic and have a characteristic sweetish odor.
(E) Antimicrobial susceptibility test of *P. multocida* isolates from domestic chickens on blood agar was tested against ampicillin, cefixime, gentamycin, penicillin G, tetracycline.

epidemiological features were almost identical to the first FC case. As might have been expected, it was also diagnosed as Coryza by a clinical veterinarian, who prescribed antibiotic treatments. However, because the treatment was not satisfactory, the clinical veterinarian himself requested that we confirm the causative agent. Immediately, we visited the outbreak farm with the clinical veterinarian to conduct the epidemiological examination of the farm. The examination confirmed that symptoms were mainly observed in male breeder chickens and then spread to the entire chicken population. Clinical symptoms featured combs and one-eye swellings, and mainly respiratory disorders. In the autopsies, viscous inflamed masses were found inside swollen combs, and the inflammatory material infiltrated up to the hypodermic area of the heads (Fig. 1-A). A small number of necrotic and inflammatory foci were also discovered on the liver surface. The cardiac air sac was filled with inflammatory materials, and serious pneumonic lesions were also found at the end of the lung lobe. When pressed, inflammatory materials leaked out from cuts made in the lung lobe. The latter lesions are rarely observed in Coryza, and are regarded as symptoms mainly found in FC (Fig. 1-B, C).

Overall, since the clinical features were similar to those of the first FC outbreak, it was tentatively diagnosed as FC. The chickens' tissues and organs were carried to our lab to be examined. A direct smear was produced, and gram-negative bacillus was cultured and identified on the blood agar using the same methods as in the first outbreak case. Hereby it was confirmed that FC was present, and the causative agent, PM, was isolated and definitively identified (Bergan and Norris, 1978; Shewen *et al.*, 1993; Holt *et al.*, 1994; Blackall and Miflin, 2000).

This decade, two FC outbreaks have been confirmed in domestic chickens for the first time in 59 years. Since FC was first detected in wild birds in 2000, one of the main purposes of this study is to analyze the characteristics each FC outbreak, focusing on possible connections between the causative agents of FC in wild birds and domestic chickens.

Materials and Methods

Sampling and isolation of animals and strains

In the first FC outbreak (Sep. 27, 2000), at the broiler breeder chicken farm, located in Gyeong-gi Province (Peonagtaek area), strains of PM isolated from 25-week-old chickens with FC were announced publicly, with *P. multocidas* directly isolated from various organs and tissues of 50-week-old male breeder chickens in another broiler chicken farm. A second FC outbreak occurred in Chung-cheong-nam Province (Daejeon area) on Feb. 28, 2001. In addition, PM (A:1,12,14) strains isolated from wild birds for the first time were submitted for a comparison test. PM strains of capsular A and D distributed by NVSL (USA) and from domestic pigs were also compared to their characteristics.

Epidemiological features of outbreak farm

General epidemiological features were examined through the visit to the two farms with the FC outbreaks and by interviews with both the owners and farm workers. In regard to items such as farming scale, outbreak period, clinical features, mortality rates, status of drug inoculation and treatment responses, field-work was conducted along with research based on the data collected from the farms. In addition, during field surveys, photographs were taken of chickens that expressed clinical symptoms. Chickens with clinical symptoms were selected for autopsy, and test material was collected and delivered to the lab for bacterial isolation, followed by identification of the pathogenic agent.

Biochemical characterization

Assays for biochemical properties of PM isolates were conducted according to MacFaddin's methods, cultivating various agars for biochemical properties including arabinose, dulcitol, sorbitol, trehalose, and xylose. Analyzed according to Holt classification standards, subspecies were confirmed (Holt *et al.*, 1994; Murray *et al.*, 1994; MacFaddin, 2000). For reliable identification and comparison of results, the BBL crystal E/NF ID system (BBL, USA), a rapid diagnosis kit for commercial use, was simultaneously applied to compare the biochemical responses.

Serotyping

To identify the serotype of PM strains collected from the chickens, capsular typing was conducted using the Rimler's method (Rimler, 1994). First, a susceptibility test was applied to staphylococcal hyaluronidase for the identification of capsular A-type, while an aggregation test was conducted using acriflavine for the identification of capsular D-type. In addition, in accordance with the Heddleston method, the agar-gel diffusion precipitin test (AGDPT) was employed to identify somatic serotype (Heddleston *et al.*, 1972). Because of a lack of relevant experience and technology to perform certain test procedures of standard positive antiserum, the test was entrusted to the NVSL (USA), to secure accuracy. Their results have been cited in this study.

Antibiotic susceptibility test

Susceptibility to all types of antibiotics was tested to

Table 1. Comparison of antimicrobial susceptibility test result of *P. multocida* isolates from wild birds and domestic chickens

Antimicrobial drugs	Code / Disc content in ug	Diameter of zone of inhibition to nearest mm			Wild birds isolates (n=12)	Chicken isolates (n=7)
		Resistant	Intermediate	Susceptible		
Amoxacillin + Clavulanic acid	AMC 30	≤13	14-17	≥18	19.8	21.0
Amikacin	AN 30	≤14	15-16	≥17	18.2	17.9
Ampicillin	AM10	≤13	14-16	≥17	18.4	21.0
Cephalothin	CF 30	≤14	15-17	≥18	23.0	23.4
Cefoperazone	CFP 75	≤15	16-20	≥21	25.8	23.0
Cefixime	CFM 5	≤15	16-18	≥19	19.7	20.0
Ciprofloxacin	CIP 5	≤15	16-20	≥21	21.0	37.3
Colistin	CL 10	≤8	9-10	≥11	13.3	13.6
Doxycycline	D 30	≤12	13-15	≥16	18.8	15.1
Gentamicin	GM 120	≤12	13-14	≥15	13.9	12.3
Kanamycin	K 30	≤13	14-17	≥18	19.3	11.4
Neomycin	N 30	≤12	13-16	≥17	19.5	11.9
Nitrofurantoin	F/M 300	≤14	15-16	≥17	24.4	19.1
Norfloxacin	NOR 10	≤12	13-16	≥17	29.5	19.1
Ofloxacin	OFX 5	≤12	13-15	≥16	31.9	20.0
Oxytetracycline	T 10	≤15	16-18	≥19	22.1	10.0
Penicillin	P 10	-	-	-	22.7	22.1
Polymyxin B	PB 300	≤8	9-11	≥12	14.6	14.1
Trimethoprim + Sulfamethoxazole	SXT	≤10	11-15	≥16	23.5	22.3
Tetracycline	TE 30	≤14	15-16	≥19	22.9	13.1
Trimethoprim	TMP 5	≤10	11-15	≥16	25.8	24.3
Tobramycin	NN 10	≤12	13-14	≥15	17.4	13.3

examine the phenotypes of PM isolates collected from the chickens. For susceptibility tests for antibiotics, a disk diffusion method was applied using Mueller-Hinton agar in accordance with the National Committee for Clinical Laboratory Standard (Bergan and Norris, 1978; NCCLS, 1997). As shown in Table 1, 25 antimicrobial drugs including ampicillin were used; plates were incubated at 37°C for 18 hours. The results were interpreted from measuring the diameter of the inhibitory zone. The same test was conducted on a PM strain of wild bird origin, followed by phenotypic comparison.

Pathogenicity test in vivo on experimental animals

To examine *in vivo* the pathogenicity of PM strains isolated from domestic poultry, SPF-mice (6-week-old, BALB/c and ICR breeds) were used. Isolates from a chicken and a wild bird were injected at 10^7 cfu/ml into the abdominal cavity of SPF-mice, and unlimited foods and water were provided. If the SPF-mice died, the inoculated strains were re-isolated from their organs, such as livers and spleens. For heart blood, blood film specimens were designed to check gram-negative, bipolar staining bacillus through a microscope. Strain isolation was conducted on specimens and confirmed using tryptic soy broth (TSB, Biolife, Italy) and blood culture agar. Multiplied colonies were identified using standard laboratory methods. A variation test was conducted on the basis of Koch's hypothesis: if the PM strains from poultry reveal virulence, and are then re-isolated from the inoculated laboratory animal, the isolated strains should be the same as the inoculated strains. The last test was conducted to verify that PM isolates that

originated in chickens are a pathogenic agent of FC.

Identification of capsular antigen based on multiplex-PCR (M-PCR)

Identification of antigen types through standard antiserum techniques takes significant labor and time, and subjectivity can affect the interpretation of results, so multiplex-PCR (M-PCR) was chosen. This method is convenient, specific, and rapid in identifying capsular antigens A, B, D, E and F. For the first time, capsular antigens of PM strains isolated from chickens were identified. Primers and reaction conditions for M-PCR were conducted based on the Townsend method (Townsend *et al.*, 1998). Sizes of amplified DNA products of M-PCR were examined by electrophoresis, and capsular antigen types were determined by reference strain DNA markers. While strains were being confirmed by the NVSL through diagnosis techniques based on the standard positive antisera, the conditions of M-PCR were optimized. A modified-REA method was applied for this study, and restriction enzymes *Hha*I and *Hpa*II (NEB, U.S.A) were used to digest and analyze the products of M-PCR. PCR-RFLP (restriction fragment length polymorphism), which is used for analyzing a small number of digested DNA fragments, was conducted to examine possible genetic variation between these strains.

DNA fingerprinting based on restriction enzyme digestion (REA)

After PM strains collected from infected chickens were cultured in brain heart infusion broth of 10 ml (BHI, Biolife, Italy) overnight at 37°C, DNA was extracted to be refined for separation with Wilson method (Wilson *et al.*, 1993). REA on purified DNA was implemented based on Christiansen's method (Christiansen *et al.*, 1992). First, purified DNA (2-3 μg) was mixed with reaction buffer (30 μl) and restriction endonucleases (*Hha*I and *Hpa*II) (10U: GibcoBRL, USA) at 1 or 2 μl, respectively. To eliminate revitalization of residue RNA, RNase of 2 μl (10 mg/ml) (Sigma, USA) was added to reaction mixture. After being incubated at 37°C for 3 to 5 hours, a stop mixture of 5 μl (0.25% bromophenol, 0.25% xylene cyanole, 25% Ficoll 400; Sigma, USA) was added to stop the reaction. Electrophoresis in 0.7% agarose was conducted for 15 to 17 hours in TAE buffer at 30 V. The DNA was visualized with ethidium bromide (GibcoBRL, USA), and the gel was photographed under a UV illuminator. The results sent by NVSL confirmed our findings.

Genetic analysis based on pulsed field gel electrophoresis (PFGE)

For a more general level of genetic analysis of PM strains isolated from domestic chicken and wild birds, PFGE was conducted (Woo *et al.*, 2005). *Apa*I (NEB, U.S.A) was selected as a suitable restriction enzyme to analyze large chromosomal DNA of PM strains. After PFGE was completed, a dendrogram was produced based on obtained DNA profiles, and analysis of genetic relations between PM strains was conducted, using GelCompar II software (GenMath, Belgium).

Results and Discussion

Epidemiological surveys of FC outbreak farms

The farm, where the first FC outbreak occurred is located in Gyeong-gi Province (Peongtaek area). With the farming scale totaling 60,000 chickens, it was confirmed that the farm had 40,000 FC cases and 50 chickens per day died. Chickens on this farm had facial swelling and severe respiratory disorders. In regard to age, the diseased chickens averaged approximately 175 days (25 weeks) of age and were from the Isabrown breed. Because they were breeder chickens, they were raised on the ground floor, which upon examination showed that the breeding method severely worsened respiratory disorders. At about 23 weeks of age, peritonitis, air-sac disease, pericarditis and perihepatitis began to be observed. Since swelling of eyes, faces, and wattles and serious pneumonia were found (Fig. 1-A, B, & C), they were diagnosed with colibacillosis and coryza and given antibiotics. However, chickens kept dying. Finally, the investigator attempted to select a more effective antibiotic by using a precise antibiotic susceptibility test.

What is unusual about the findings discovered from epidemiological examination on the outbreak farm is that the first outbreak farm is in an adjacent area to the Cheon-su bay, where the first FC case in wild birds was detected. The outbreak date correlated with the date of the public announcement (after being confirmed by NVSL, USA), that the wild bird isolates were *P. multocida*, a causative agent of FC. This, of course, lead to the assumption that the farm had some relation with the wild birds, and it became urgent to find and trace the source of the chicken farm's isolates, as well as to discover any relation with isolates of wild bird origin.

The second outbreak (Feb. 28, 2001) occurred at a chicken breeding farm located in a town near the Daejeon area. The breeding method and raised breeds were quite similar to those of the first outbreak farm; it was a broiler breeding chicken farm, with chickens breeding on the ground floor. Unusual features found in this farm were that clinical symptoms and mortality rates started to increase in male chickens (Fig. 1-A, B, & C).

It was noted that the outbreak first occurred in henhouses 1 and 2 among a total of 6 henhouses, spreading from these to neighboring ones. Upon clinical observation, these chickens showed similar symptoms to the first outbreak: one-eye swelling, and watery eyes. Male chickens' wattles or combs were severely swollen. Autopsies confirmed features such as pericarditis, perihepatitis, and air-sac disease, which are normally seen in colibacillosis. These lesions were regarded as the result of complicated infection of pathogenic *E. coli*. Chickens with disease symptoms had inflammatory features spread to their head. They also showed features of pneumonia, such as blackened edges of lungs from which, inflamed liquids leaked out. Each organ was collected in aseptic conditions, and delivered to the lab to conduct a strain identification. Common features found were that PM strains were cleanly separated in blood culture agar (Fig. 1-D, E).

Based on the epidemiological findings stated above, the pathogens examined here matched the clinical features, lesion findings, and standard strains shown in reports (Bergan *et al.*, 1978; Namioka, 1978; Shewen *et al.*, 1993; Holt *et al.*, 1994; Murray *et al.*, 1995; Rimer, 1996) conducted by prior researchers. It was concluded that FC was found domestically in the two above-mentioned chicken farms for the first time since 1942.

Biochemical property test of isolates

Tissue smears of organs from the first FC outbreak farm were observed through a microscope. Gram-negative, bipolar staining bacillus was observed, and these strains were separated as pure colonies with blood agar (Fig. 1-D, E). A biochemical test was conducted on independent colonies based on a rapid identification kit and standard laboratory diagnosis method. The result from the Crystal Enteric/ Non-fermenter ID system, a rapid identification kit, was matched with *P. multocida at* 98.6% certainty (Code No. 3441344344). Materials collected from chickens at the second outbreak farm were identified in the same manner. The results also highly matched with *P. multocida* at 99.7% certainty. The PM strains isolated from chickens at the two outbreak farms were confirmed to be the causative agent of FC, which has not been reported since 1942 (Kim *et al.*, 1966; Jeong *et al.*, 1987). Results of the biochemical test on 12 PM strains from domestic chickens were opposite of those from strains which originated in the wild migratory birds in 2000. In regard to biochemical properties, all the strains (100%) originating in chickens had a positive reaction to a sorbitol and trehalose test, contrasting with the findings for the strains originating from the wild bird. Contrary to this, strains originating in wild birds had a negative reaction. FC strains isolated from the chickens were confirmed to have a distinct biochemical makeup from the strains from the wild birds.

Antigen type identification

It was necessary to identify an accurate antigen type, because these PM strains were isolated from domestic poultry for the first time since 1942. However, because of the lack of essential technology for and actual experience with the standard positive antiserum and typing procedures, they were given to the NVSL (USA) to obtain the results. Here the typing results confirmed by the NVSL are presented. Isolates that originated in chickens confirmed that somatic antigen is a common antigen simultaneously having 2 subantigens, antigen 10 and antigen 11. Since the capsular antigen was determined to be type A, the final antigenic type was classified as A:10,11. Although all the PM strains originating in wild birds were also of type A, the classification was finally determined to be A:1,12,14, with a common somatic antigen having 3 subantigens of 1, 12, and 14.

Previous work on, strains cultured in pigs published capsular antigens, while people isolating strains originating in fowl identified only somatic antigens; for many strains, the capsular antigens are not known (Carter and Rundell, 1975; Derieux, 1978; Blackall and Miflin, 2000). Looking into research on PM strain antigens done in different nations, antigens of strains that originate in wild birds in North America are mainly A:1 (Derieux, 1978; Timoney *et al.*, 1988; Christiansen *et al.*, 1992; Murray *et al.*, 1995), while strains isolated from wild birds crossing the Atlantic Ocean were reported to be mainly A:3 or A:3,4 (Rimer, 1996; Rhoades and Rimler, 1999). Based on this limited information, there is no correlation between previous work and the antigens of isolates originating in chickens or wild birds in Korea. Therefore, wild birds were excluded when they came from North America or the Atlantic Ocean or if they flew into Korea by way of North America or the Atlantic Ocean. Current data says that most wild birds flying into Korea come via Siberia or China.

Antimicrobial drugs susceptibility

The results of the antimicrobial drug susceptibility tests are presented in Table 1 and Fig. 1-E. Seven strains were isolated from different organs of chickens from the first and second outbreak farms. These strains somehow acquired a significantly high level of resistance to 7 types of antimicrobial drugs: kanamycin, neomycin, oxytetracycline, tetracycline, tobramycin, doxycycline, and even gentamicin. Contrary to this, none of the 12 strains that originated in wild birds

had acquired significant tolerance to any of 25 types of antimicrobial drugs. However, a weak tolerance to gentamicin (13.9 mm) was only found. This suggests that PM strains from the domestic fowl are strains that have existed in various poultry farms environments for a significantly long time, but we have not detected them.

Since antibiotics can be used without any particular regulations on the domestic poultry farms, we assume that their widespread use killed off those strains without resistance and also allowed resistant strains to flourish. As stated above, symptoms similar to coryza were frequently found at the FC outbreak farms, and antibiotic were commonly prescribed. Therefore, it seems natural to have acquired a high level of resistance to diverse drugs, if strains have been exposed to the environment of domestic chicken farms for a long period. However, considering that the PM strains that caused mass death of wild migratory birds in the Cheon-su bay on October. 22nd, 2000, had not acquired any antibiotic resistance, the wild bird PM strains are thought to have originated in an environment that had no connections with the domestic chicken farms.

Pathogenicity in vivo on SPF-mice

Detailed results for the virulence test are presented in Table 2. 6-week-old BALB/c and ICR breeds were used in the experiments, and PM strains were inoculated into the abdominal cavity, adjusting for the number of strains. After inoculation, unlimited water and food were provided. Strains (PM00637-DC) from domestic chickens showed a 25% mortality rate (one out of four) against ICR mice, while the strain, PM00637-2W-DC, showed a higher mortality rate of 66.6% (20 out of 30). This shows that there is a difference in mortality rate between strains isolated from domestic chickens. The experiment was also conducted using strains from wild migratory birds. Within 17 hours after the inoculation, the entire number (34 out of 34) of inoculated mice died (100%) regardless of breed and the number of inoculated strains, indicating a significantly high virulence.

When direct smear specimens of heart blood from dead mice were observed through the microscope, gram-negative, bipolar staining bacillus were observed in all the specimens, matching with features of inoculated strains. Strains were isolated again from all the specimens. Mice were confirmed to have died from inoculated PM strains. All the results of virulence tests using SPF-mice indicate a remarkable difference in pathogenicity between isolates from domestic chickens and wild birds.

Inoculation experiments on poultry have been postponed, but inoculation tests were conducted on SPF-mice as an alternative method, and the inoculated strains were re-isolated from dead or surviving laboratory mice. The PM strains isolated from experimental mice were the same as the originally inoculated strains.

Identification of capsular antigen with multiplex PCR (M-PCR)

The results of analyzing distribution patterns of capsular antigen of PM strains using M-PCR are shown in Fig. 2. In almost all PM strains isolated from chickens, an amplified DNA band was found at the size of 1,044 bp (belonging to type A). On the contrary, amplified products of DNA identical to a gene encoding capsular antigen were not detected in strains 6 and 8 of Fig. 2. Capsular antigens of strains from wild bird were already confirmed to be type A using standard antisera at NVSL, but this test was also done on them for comparison and confirmation of the specificity of the M-PCR method conducted here. The experiment showed they were amplified products of DNA belonging to type A as shown in Fig. 2. There was a high correspondence between these two tests, and therefore the specificity of the

Table 2. Response of SPF mice to intraperitoneal (ip) inoculation of *P. multocida* isolates from wild birds and domestic chickens

Strains (origin)	Mice breed	Age (weeks)	Dosage/mice (x10^7 cfu/0.5 ml)	Mortality[a] (dead/total)	Response (death time)
PM00627-4 (WB[b])	ICR	6	1.0	4/4 (100%)	Death (16~17th Day)
PM00644-7S (WB)	BALB/c	6	4.4	30/30 (100%)	Death (16~17th Day)
PM00637 (DC[b])	ICR	6	1.0	1/4 (25.0%)	Survived
PM00637-2W (DC)	BALB/c	6	3.0	20/30 (66.6%)	Survived
PM00637-8W (DC)	BALB/c	6	4.4	4/16 (25.0%)	Survived

a: The mortality was measured after a completion of inoculation test.
b: WB and DC means wild birds and domestic chickens, respectively.

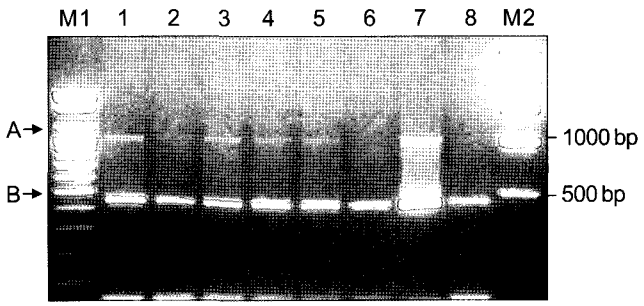

Fig. 2. Multiplex capsular PCR (M-PCR) typing system for *P. multocida*. Chromosomal DNA PCR was performed with wild birds and domestic chicken isolates. Lane M1; 100 bp DNA marker (Bioneer, Korea), M2; 1 kb DNA marker (Bioneer, Korea), lane 1; reference strain of serogroup A (wild bird; NVSL identified with antisera), lane 2; DC-PT-688-2-AS (domestic chicken), lane 3; WB-644-6-L (wild bird), lanes 4~8; domestic chicken isolates from diverse organs (air-sac, liver and lung). The arrow A indicated the amplified DNA products of capsular type A serogroup (1,044 bp) and the arrow B indicated the amplified DNA products (460 bp) of all positive serogroup strains of *P. multocida*.

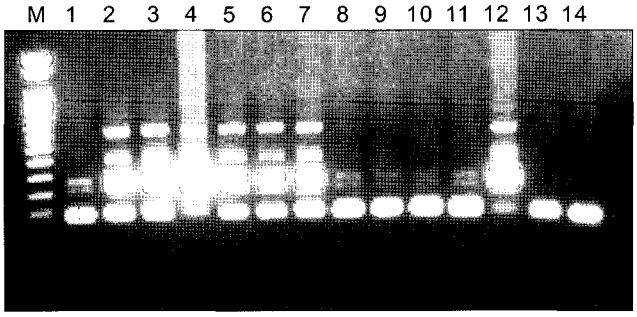

Fig. 3. Patterns of restriction fragment length polymorphism (RFLP) of PCR products using *Hpa*II enzyme of capsular A type avian (wild and domestic birds) and swine *P. multocida* isolates. Lane M; molecular weight marker 100 bp DNA marker (Bioneer, Korea), lane 1; domestic chicken isolates (DJ-3-Lung-3-2-DC: Group I), lanes 2~7; wild birds isolates (Group II), lanes 8~11; domestic chicken isolates (Group I), lane 12; wild birds isolates (Group II), lanes 13~14; *Pasteurella* species isolates from domestic swine (Group III).

M-PCR method was established.

As is well-known, analysis using traditional antiserum methods is necessary to obtain a standard positive antiserum. These methods are available only in the internationally well-recognized research institutes of a few nations (USA and France). It was also difficult to produce an antiserum in Korea itself due to the lack of relevant technology and experience. Considering the above situations and the fact that FC has already been detected in poultry as well as wild birds in Korea, there is no time to waste in preparing the standard antiserum. Instead, we adopted a previously developed method (Townsend *et al.*, 1998) that simultaneously amplifies DNA to rapidly identify genes encoding PM capsular antigens of types A, B, D, and E with a simple PCR reaction. This method was regarded as an acceptable and definitive alternative, given the current laboratory situation.

The acriflavin test, the Carter method (Carter and Subronto, 1973; Carter and Rundell, 1975), or a decision based on the outer appearance of the colony alone were not enough to precisely differentiate the type of capsular antigen. Since there is a high possibility of subjectivity involved, accuracy drops. It is necessary to secure an effective identification method with a high level of specificity and sensitivity.

M-PCR was conducted on strains that were cultured in pigs bred in the Gyeong-buk district for comparison. Analysis determined that strains of type A accounted for 86.5% of the PM population, while strains of type D accounted for just 2.9%. Strains without a capsular antigen accounted for 7%. Therefore, it was confirmed that strains of type A were the most abundant. We suggest that future epidemiological studies on PM strains originating from poultry should involve a study of the relationship with strains from pigs.

Based on the DNA profile (PCR-RFLP) produced through digestion with restriction enzymes *Hha*I and *Hpa*II of the amplified DNA products of M-PCR, differentiation between strains was conducted depending on antigen types. Lanes 2 to 7 and 12 of Fig. 3 show the DNA profile of A-type strains originating in wild bird; the distribution pattern consists of a total of 4 to 5 DNA bands between 200 and 600 bp. However, lanes 1 and 8 to 11 showed the DNA profile of A-type strains isolated from chickens: here, only 2 DNA bands were found clearly between 200 and 250 bp. The patterns (RFLP type I) were relatively easy to analyze. Overall, enzymes like *Hha*I and *Hpa*II made it possible to differentiate DNA patterns, but it was difficult to differentiate effectively between the two strains' relation. *Hpa*II was selected as a more suitable restriction enzyme for PCR-RFLP. Since the PCR-RFLP method produces only a few DNA digestion fragments, it was considered a worthy alternative as opposed to the REA method, which is difficult to analyze with the naked eye.

Analyzing of REA profile

REA was used to identify and quantify the DNA fragments obtained from digesting PM DNA with *Hha*I. As shown in Fig. 4-A, B, the biggest DNA fragment from chickens was 9.4 kb, whereas the largest DNA fragments from wild birds were closer to 5 kb. Differentiation between these two was possible with the naked eye. However, the number of small

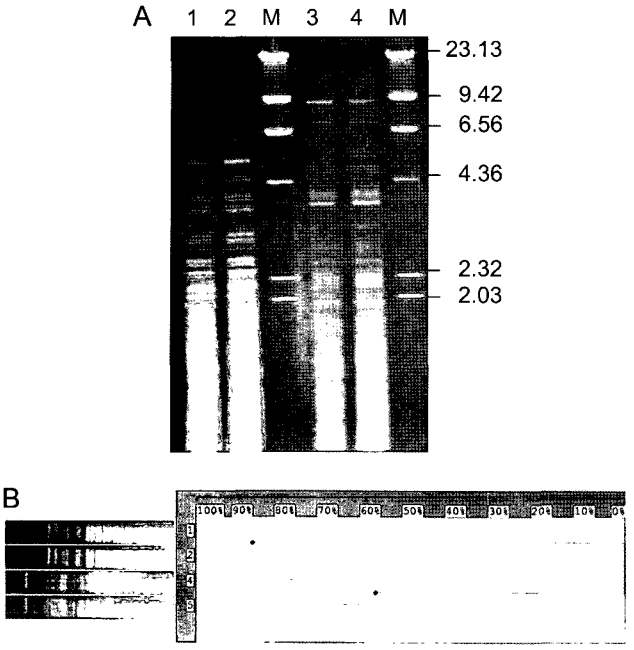

Fig. 4. (A) Agarose gel DNA fingerprint profiles, using *Hha*I enzyme of *P. multocida* isolates from wild birds and domestic chickens. This figure was received by E-mail from the National Veterinary Services Laboratories (NVSL) in USA from Beverly J. Schmitt, D.V.M. Lanes 1 & 2; DNA profiles of wild birds from the Chun-Su bay in Korea (HA-1141 type), lanes 3 & 4; *P. multocida* isolates from domestic chickens (HA-1142 type), M; DNA marker from lambda bacteriophage digested with *Hind*III. Fragment size shown is in kilobase (kb) pairs.
(B) REA profiles, using *Hha*I enzyme (left) and dendrogram (right) generated by the BioID2+ software showing the relationships of *P. multocida* isolates from domestic chickens and wild birds. Lanes 1 & 2; REA profiles (HA-1141 type) of wild birds from the Chun-Su bay, lanes 3 & 4; REA profiles (HA-1142 type) of domestic chickens.

sized DNA fragments was so grate, it was difficult to differentiate between them. Therefore, a specialized analysis program was used. Because it was the first time that these strains were analyzed, a request for help was made of Dr. Wilson at NVSL for more credible analysis. His findings on the DNA profile are presented here in Fig. 4-A. Based on these findings, the genetic relationship between PM strains from chickens and strains from wild birds was analyzed with GelCompar II software. The program predicted that the PM strains isolated from chickens and wild birds are not related genetically (Fig. 4-B).

Strains from the domestic chickens were determined as the genotype HA-1142, whereas strains from wild birds was as the genotype HA-1141 (Fig. 4-A). This new genotype of PM was isolated from these domestic chickens and wild birds that have never before been detected in America. Therefore, this finding suggests that North America should be excluded from the origin source for the PM strains isolated in Korea.

Based on the above results, the influx of PM strains from the wild bird into the domestic poultry farms can be excluded, in spite of their similar isolation period. We also conclude that the source of PM strains isolated from the domestic chickens is of domestic origin and that it has existed undetected in the chicken farm environment for an unknown period.

Analyzing of PFGE profile

PFGE was applied to analyze PM strains originating in the domestic poultry and wild birds at more fundamental gene levels, because overall analysis of the chromosomal DNA of bacteria is made with the method. The two biggest DNA bands were found clearly at a size of 436.5-388 kb in PM strains originating in wild birds, showing PFP (pulsed-field profile) patterns that contrasted prominently with strains from domestic chickens (Fig. 5). In the genetic analysis of the GelCompar II software, a high level of genetic relation (80%) was found between PM strains from wild birds (WB-644-8-S-M, WB-644-8-S-C & WB-644-1-L). This implies that the origin is similar. Because association with PM strains (DC-688-3-E-PT) originating in domestic chickens was 70%, genetic relation between the two was believed to be weak. It was seen that PM strains that originated in the wild bird have a weak association with strains from domestic chickens in viewpoints of genetic level. A much lower level of a relation, 50% was seen between chicken strains. Finally the findings suggest that the origins of the PM strains from the

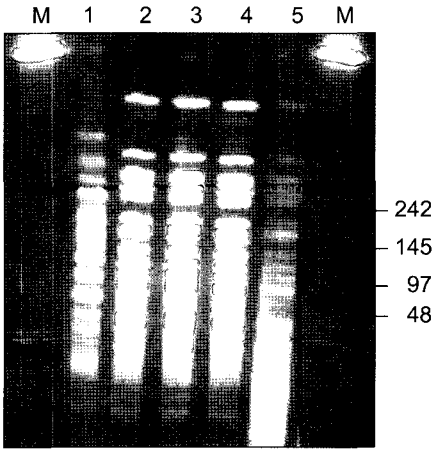

Fig. 5. This photograph of electrophoretic gel for pulsed-field gel electrophoresis (PFGE) of *P. multocida* (PM) isolate, using *Apa*I enzyme (NEB, USA). Lanes M; Bacteriophage lambda ladder PFGE marker (kb; Bio-Rad, USA), lane 1; domestic chicken PM isolate (DC-688-3-E-PT), lanes 2~5; wild birds PM isolates (WB-644-8-S-M, WB-644-8-S-C & WB-644-1-L).

chicken and the wild bird are different.

In conclusion, clinical features and lesions of FC were observed in domestic chickens for the first time since 1942. PM strains, the causative agent, were isolated from the diseased chickens and confirmed by a variety of methods. In phenotypic and genetic analysis, PM strains isolated from domestic chickens were confirmed to be significantly different from PM strains isolated from the wild bird. Finally, the source of PM strains was tracked down and identified. It is believed that among domestic chickens, it has existed for a long time undetected in the chicken farm environment. To effectively control FC in domestic poultry and minimize damage and economic loss of farms, epidemiological studies on the current FC infection in poultry farms should be conducted in a broader and more systematic manner, including pig and dog hosts infected with PM strains. The source of a causative agent should also be clearly established, followed by rapid elimination and the implementation of fundamental prevention measures, along with additional control measures on disease expansion and transmission. In the event of disease transmission throughout the entire nation, manufacture of a vaccine should commence after selecting a representative PM strain. A study to produce a live vaccine utilizing a genetically modified strain should also follow.

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