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Epidemiology of Fowl Cholera in Free Range Broilers

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SUMMARY. Fowl cholera, caused by *Pasteurella multocida*, remains a major problem of poultry worldwide. In the current report, we describe an outbreak in free range organic broilers. In addition to culturing samples from dead broilers, we attempted to isolate *P. multocida* from feral cats trapped on the farm. The isolates were identified by PCR as *P. multocida* and then serotyped using the Heddleston scheme and genotyped using both a multilocus sequence typing (MLST) method and an enterobacterial repetitive intergenic consensus (ERIC)-PCR method. A total of 123 isolates of *P. multocida* were recovered from 12 broilers. All 123 isolates were examined by ERIC-PCR, and only one pattern was identified. A subset of seven broiler isolates were examined by MLST and all were typed as sequence type (ST) 20. A total of 28 isolates of *P. multocida* were recovered from 17 cats, and five ERIC-PCR genotypes were identified, with one genotype (E-1, shared by 19 isolates) being the same as the ERIC-PCR pattern associated with the broilers. One representative cat strain for each ERIC-PCR pattern was subjected to MLST. The cat isolate with the same ERIC-PCR genotype as the broiler isolates was confirmed as having the same MLST result, ST 20. The other five cat ERIC-PCR patterns were allocated to four STs: E-2 and E-5 to ST 265, E-3 to ST 30, E-4 to ST 20, and E-6 to ST 264. Both genotyping methods confirmed that isolates of *P. multocida* were common between the feral cats and the chickens. It was not clear whether the strain was transmitted from the cats to the chicken or whether the cats obtained the strain preying on chicken. The study has shown that cats can harbor *P. multocida* strains with the same genotype found in chickens affected with fowl cholera.

RESUMEN. Epidemiología del cólera aviar en pollos orgánicos—Estudio de un caso.

El cólera aviar, causada por *Pasteurella multocida*, sigue siendo un problema importante en la avicultura a nivel mundial. En el presente reporte, se describe un brote en pollos de engorde de tipo orgánico. Además de cultivar muestras de pollos muertos, se intentó aislar *P. multocida* de gatos salvajes atrapados en la granja. Los aislamientos fueron identificados por PCR como *P. multocida* y luego fueron serotipificados utilizando el esquema de Heddleston y genotipificados utilizando tanto el método de tipificación por secuencia multilocus (MLST) y mediante el método del consenso intergénico repetitivo de enterobacerias (ERIC)-PCR. Se recuperaron un total de 123 aislamientos de *P. multocida* de 12 pollos de engorde. Todos los 123 aislamientos fueron examinados por ERIC-PCR, y se identificó un solo patrón. Un subconjunto de siete aislamientos de pollos de engorde fueron examinados por el método MLST y todos se tipificaron como secuencia tipo (ST) 20. Un total de 28 aislamientos de *P. multocida* se recuperaron a partir de 17 gatos, y se identificaron cinco genotipos por ERIC-PCR, con un genotipo (E-1, compartida por 19 aislamientos) siendo el mismo que el patrón de ERIC-PCR asociado con los pollos de engorde. Una cepa representativa de gato de cada patrón de ERIC-PCR fue sometida a MLST. El aislamiento de gato con el mismo genotipo ERIC-PCR del aislado de pollo de engorde se confirmó que mostraba el mismo resultado por MLST, ST 20. Los otros cinco patrones de ERIC-PCR de aislamientos de gatos fueron asignados a cuatro serotipos: E-2 y E-5 a ST 265, E-3 a ST 30, E-4 a ST 20, y E-6 a ST 264. Ambos métodos de genotipificación confirmaron que los aislamientos de *P. multocida* eran comunes entre los gatos silvestres y los pollos. No estuvo claro si las cepas se transmitían de los gatos al pollo, o si los gatos la obtenían mediante la predación de los pollos. El estudio ha demostrado que los gatos pueden albergar cepas de *P. multocida* con el mismo genotipo encontrado en los pollos afectados con el cólera aviar.

Key words: fowl cholera, organic broilers, feral cats, genotyping, epidemiology

Abbreviations: ERIC = enterobacterial repetitive intergenic consensus; MLST = multilocus sequence typing; RIRDC = Rural Industries Research and Development Corporation; ST = sequence type.

Pasteurella multocida, the causative agent of fowl cholera, is of major economic importance worldwide (5). When present as an acute disease, high morbidity and mortality can occur (6). In this acute form, clinical signs are typically only seen shortly before death. Typical clinical signs seen in the acute form of the disease are fever, ruffled feathers, mucus discharge from the mouth, diarrhea, and increased breathing rate (5). The disease also presents as a chronic form, in which localized infection of joints and sinuses can follow the acute form, or on occasion, be the only form of the disease present in a flock. In the chronic form, the typical signs include swollen wattles, eyes, sinuses, leg or wing joints, or foot pads; twisted necks (torticollis); and respiratory gurgles (rales). In the chronic form, birds that recover become carriers of the disease (5), can remain infected for life, and become a reservoir of infection for further outbreaks (4).

The disease has been recognized as a problem in the emerging free range layers (17,21) and backyard poultry (2) in the developed world and in village chickens in the developing world (13). No reports of fowl cholera outbreaks in free range, organic broiler flocks in the developed world appear to have been reported.

The recurrence of fowl cholera outbreaks on properties has been reported in turkeys (both shedded and free range) and free range layers and ducks (11,17,21). The explanation for these recurring outbreaks could be carrier chickens or environmental persistence of *P. multocida*. An alternative explanation could be a common source reintroducing the strain at intermittent periods.

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Table 1. Time line of sampling and control measures.

Sampling	No. of birds sampled	No. of feral cats sampled	Results of sampling	Control measure
First chicken (C-1)	5	0	*P. multocida*	
				Biosecurity increased
				Vaccine program introduced
First feral animal (F-1)	0	2	*P. multocida*	
Second feral animal (F-2)	0	1[A]	*P. multocida*[B]	
Third feral animal (F-3)	0	5	*P. multocida*	
Fourth feral animal (F-4)	0	2	*P. multocida*	
Fifth feral animal (F-5)	0	4	*P. multocida*	
Second chicken (C-6)/sixth feral animal	4	3	*P. multocida*	
				Avoid cold stress
Third chicken (C-7)	4	0	*P. multocida*	
				Changes in coccidiosis vaccination

[A]A single fox was also sampled.
[B]The *P. multocida* isolate was obtained from the feral cat.

Since all types of birds are susceptible to fowl cholera (5), wild birds are a potential source of reintroduction of strains. It has been shown that sparrows and pigeons can become infected with *P. multocida* from infected chickens and once infected can transmit it to susceptible chicken (16). However, it seems only the isolates of *P. multocida* from pigs and cats that have been shown to be consistently pathogenic for fowl (5). Cats have consistently been identified as a potential source of introduction of *P. multocida* into flocks (3,6). However, definite evidence linking cats with fowl cholera outbreaks does not exist. A genotypic study on isolates from free ranging chicken and their animal contacts, such as cats and dogs, in Tanzania did not indicate that strains of *P. multocida* are widely exchanged between poultry and other animal species even though close contact exists (12).

This case study reports recurring outbreaks of fowl cholera on a free range broiler farm associated with a single strain and investigates the potential of cats as the source of the reintroduction of *P. multocida*.

MATERIALS AND METHODS

Farm. The study farm was a large complex that contained a hatchery, a processing plant, fertilizer composting facilities, and the growing facilities for the broilers. The individual growing sheds were operated on an all-in all-out basis. However, chickens had access to open ranges, the farm was multi-age, and biosecurity with regard to keeping wildlife out was minimal.

Control measures. A number of strategies were employed to try and control the fowl cholera outbreaks. Firstly, considerable effort was made to institute conventional biosecurity procedures within the limits of organic certification and the limits of facilities available to restrict the introduction of infection to flocks. A focus of this activity was an attempt to restrict the entry of feral animals. Control measures included use of electric fencing and traps. In addition, the processing and hatchery waste, as well as the dead chickens, was no longer used in the composting facility. These wastes were buried on site. Subsequent to these biosecurity measures, a fowl cholera vaccination program was instigated. A killed fowl cholera vaccine with aluminum hydroxide gel adjuvant based on an isolate from the initial investigation was used. Field trials evaluated safety (100-bird treatment groups, vaccinated and nonvaccinated, grown to 7 days of age) and efficacy of a single dose vaccine given at 1 day of age (2000 birds were vaccinated mixed into each flock of 20,000 birds), and a full vaccination program was instigated in which all flocks were vaccinated, at 1 day of age, with a single dose of the killed autogenous fowl cholera vaccine. A timeline table that shows the flow of these intervention measures as well as the subsequent samplings of the broilers and feral cats (described in detail in following text) is provided in Table 1.

Broiler samples. Swabs were collected aseptically from the pericardial sac, heart blood, liver, and bone marrow of the femur during necropsies performed on the farm. The swabs were transported on ice to the laboratory and plated on 5% sheep blood agar. The plates were incubated in air at 37 C overnight. Colonies showing typical appearance of *P. multocida* were picked and two sequential single colony subcultures performed.

At the initial investigation five suspect single colonies were taken from the primary isolation plate of the bone marrow of each bird (Table 2; a total of 25 isolates). As well, single colonies from the other three sites were taken (a total of nine isolates).

In follow-up studies, a further two outbreaks of fowl cholera in different broiler flocks over the next 12 mo were examined. In these follow-up investigations, the same set of tissues were examined as in the original outbreak, with four chickens being examined in both cases (Table 2). As in the first investigation, multiple single colonies were taken from the primary plates. In the second outbreak 19 suspect *P. multocida* isolates were obtained, while 70 suspect *P. multocida* isolates were obtained in the third investigation.

Feral animal samples. Feral animals were trapped on the property (Tables 1, 2). The oral cavities of the animals were swabbed, and the swabs transported on ice to the laboratory. The swabs were cultured as for the broiler samples and suspect *Pasteurella* colonies subcultured as for the broiler samples.

Feral animal trapping was performed on six occasions (two sampling periods in 2011 and four sampling periods in 2012), and a total of 17 cats were sampled (Table 2). Samplings 3, 4, 5, and 6 occurred over a few days, and all swabs were submitted to the laboratory at the same time. The sixth sampling of cats occurred at the same time as the second sampling of chickens. A single fox was trapped and sampled (Sampling F-2), all other animals trapped and sampled were feral cats. Multiple colonies of *P. multocida*–like isolates present on the primary plate were picked and purified as described above.

Identification and serotyping. The suspect isolates were examined using one of two *P. multocida*–specific PCR assays (10,19). The isolates from the first chicken sampling were subjected to the assay of Miflin and Blackall (10), while all other isolates were identified by the Townsend *et al.* (19) assay. Each isolate of *P. multocida* was serotyped via the Heddleston scheme (7).

Genotyping. All isolates of *P. multocida* were genotyped using the enterobacterial repetitive intergenic consensus (ERIC)-PCR method as previously described (14). Any difference in the ERIC-PCR banding pattern was regarded as a different pattern, and patterns were numbered in sequence as they were recognized. Repeat ERIC-PCR examinations were performed to confirm that a suspect new pattern was indeed a new pattern. ERIC-PCR patterns were regarded as different when a difference was consistently detected and when a difference was present in an examination that involved the same PCR run, on the same day, and in the same gel.

Table 2. Serovar and genotype profile of *P. multocida* strains from chicken and cats from an organic broiler farm.

Sampling	Host	No. of animals/birds	No. of *P. multocida*	Heddelston serovar	ERIC-PCR pattern	MLST sequence type
C-1	Chicken	5	34	4 (3)[A]	E-1 (34)	ST 20 (5)
C-6[B]	Chicken	4	19	1, 4 (2)	E-1 (19)	ST 20 (1)
C-7	Chicken	4	70	ND[C]	E-1 (70)	ST 20 (1)
F-1	Cat	1	1	1 (1)	E-6 (1)	ST 264 (1)
	Cat	1	0	NA[E]	NA	NA
F-2[D]	Cat	1	1	1 (1)	E-6 (1)	ND
F-3	Cat	5	12	ND	E-1 (12)	ND
		2	3	1 (2)	E-3 (3)	ST 30 (1)
F-4	Cat	2	3	1, 4 (1)	E-1 (3)	ST 20 (1)
		1	2	4 (2)	E-2 (2)	ST 265 (1)
		1	1	4 (1)	E-5 (1)	ST 265 (1)
F-5	Cat	3	4	ND	E-1 (4)	ND
	Cat	1	0	NA	NA	NA
F-6	Cat	1	1	4 (1)	E-4 (1)	ST 20 (1)
	Cat	2	0	NA	NA	NA

[A]The values in parentheses are the number of isolates examined.
[B]This chicken sampling occurred at the same time as sampling F-6. All samplings with a different end number occurred at different times.
[C]ND = not done.
[D]One fox was sampled at this sampling period but no *P. multocida* was isolated.
[E]NA = not applicable.

Selected isolates were examined using the Rural Industries Research and Development Corporation (RIRDC) multilocus sequence typing (MLST) scheme as described by Subaaharan *et al.* (18). The avian *Pasteurella multocida* MLST website (http://pubmlst.org/pmultocida/) developed by Keith Jolley and sited at the University of Oxford (9). The development of this site has been funded by the Wellcome Trust.

RESULTS

Disease outbreaks. During a 12-mo period, flocks on this property repeatedly experienced outbreaks of disease that resembled fowl cholera. Both the incidence and severity of the problem increased to the point that all flocks were involved and the mortality to processing was averaging 55%.

Clinical findings. High mortality outbreaks occurred as early as 30 days of age. Large numbers—averaging over 3% per day—of sudden mortality were commonly seen. Birds in good condition were found depressed, then comatosed and rapidly progressed to death. A few days into the course of the condition, small, depressed, sick birds appeared in large numbers. Cannibalism became a problem; however, it was noted that the disease appeared before the cannibalism emerged. Feral animal (cat and fox) predation was often observed before outbreaks.

Lesions. Principle gross lesions were hydropericardium, multiple white necrotic foci in the liver, and cardiac muscle hemorrhages (Fig. 1).

Presumptive diagnosis. On the basis of the clinical signs and lesions observed, a presumptive diagnosis of peracute or acute fowl cholera was made.

Bacteriology examination—broilers. In the initial sampling five bone marrow samples and three pericardial sac, heart blood, and liver samples (same three birds for all tissues) yielded pure cultures of *P. multocida*, and the colony picks resulted in 34 isolates of *P. multocida*. Later samplings yielded 19 and 70 isolates of *P. multocida* (Table 2).

Bacteriology examination—feral animals. A total of 17 cats were sampled, yielding 28 isolates of *P. multocida* (Table 2). The one fox sampled did not yield *P. multocida*.

Species level identification. All suspect *P. multocida* isolates obtained from broilers (34 in the initial investigation, 19 in the first follow-up investigation, and 70 in the second follow-up investigation) were confirmed as *P. multocida* by PCR (Table 2). The fox sample did not yield a confirmed *P. multocida*. A total of 17 cats were sampled, two at the first sampling period (F-1), one at the second (F-2), and 14 in the last four sampling periods (five at F-3, two at F-4, four at F-5, and three at F-6). These samplings resulted in a total of 28 cat isolates being confirmed as *P. multocida* (Table 2).

Serotyping. A total of three broiler isolates from the initial outbreak were serotyped and identified as serovar 4. Two of the 89 isolates obtained from broilers in the subsequent investigations were

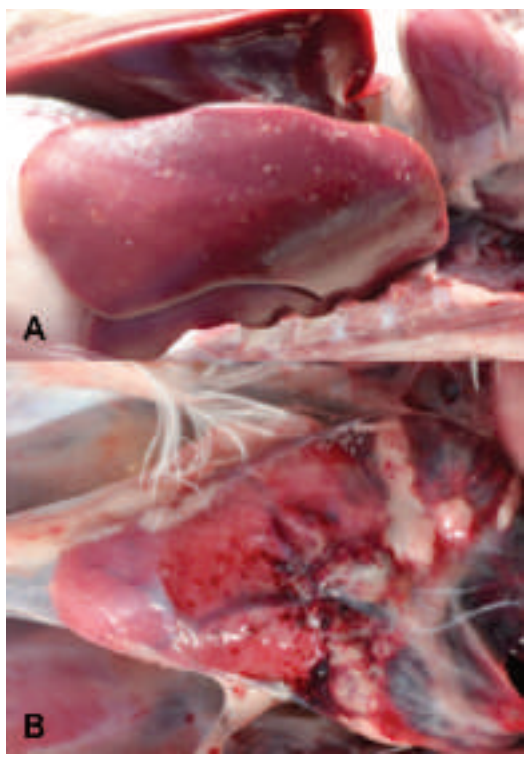

Fig. 1. (A) Liver of affected broiler. Note the multiple white necrotic foci. (B) Heart of affected broiler. Note the multiple hemorrhages.

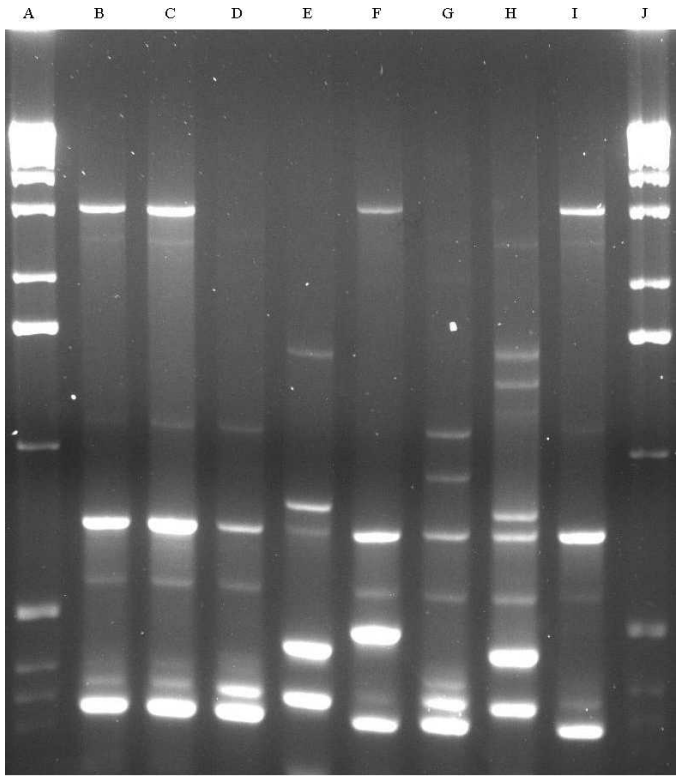

Fig. 2. Representative ERIC-PCR patterns of *Pasteurella multocida* isolates from broilers and cats. Lanes A and J = molecular weight marker; Lane B = PM 1613, ERIC-PCR Pattern 1; Lane C = PM 1614, ERIC-PCR Pattern 1; Lane D = PM 1616, ERIC-PCR Pattern 2; Lane E = PM 1618, ERIC-PCR Pattern 3; Lane F = PM 1620, ERIC-PCR Pattern 4; Lane G = PM 1621, ERIC-PCR Pattern 5; Lane H = PM 1525, ERIC-PCR Pattern 6; Lane I = PM 1422, ERIC-PCR Pattern 1. PM 1422 and PM 1614 are isolates of broiler chicken, while the other isolates are from cats.

serotyped and both were found to cross-react with serovars 1 and 4 (Table 2). The cat isolates selected for serotyping were representatives of the various ERIC-PCR patterns detected. The nine isolates subjected to serotyping were identified as serovars 1 (four isolates), 4 (four isolates), and 1 cross-reacting with 4 (one isolate) (Table 2).

Genotyping. All the broiler *P. multocida* isolates were examined by ERIC-PCR, and all 123 isolates showed the same pattern, termed E-1. All 28 cat *P. multocida* isolates were subjected to ERIC-PCR, and a total of six different patterns were recognized, with 19 of the 28 isolates having the same pattern (E-1) as the broiler isolates. The details of the ERIC-PCR results are presented in Table 2. A representative sample of the ERIC-PCR patterns found in the broiler and cat isolates is shown in Figure 2.

In the initial broiler examination, one isolate per bird (i.e., five isolates in total) was subjected to MLST, and all five isolates were sequence type (ST) 20. One broiler isolate from each of the two follow-up investigations was subjected to MLST, and both were shown to be ST 20 (Table 2). A subset of the cat isolates, selected on the basis of representing different ERIC-PCR patterns (one cat isolate of each ERIC-PCR pattern type) was subjected to MLST analysis. These six isolates were allocated to four different STs (Table 2). The cat isolate that had the same ERIC-PCR pattern as the broiler isolates (isolate from F-4 sampling an E-1 pattern) had the same ST as the broiler isolates (ST 20). Of the other five cat isolates, all with a different ERIC-PCR pattern, the two isolates representing patterns E-2 and E-5 were both allocated to ST 265, the isolate representing pattern E-3 was allocated to ST 30, the isolate for pattern E-4 was allocated to ST 20, while the isolate for pattern E-6 was allocated to ST 264.

Table 3. Available data on the *P. multocida* STs found in this study. Values in parentheses are the number of *P. multocida* isolates.

		Database[A]	
ST	Present data	Host	Country
20	Chicken (7)	Turkey (7)	Australia (7)
	Cat (2)	Chicken (2)	Australia (2)
		Pig (1)	Australia (1)
30	Cat (1)	Chicken (1)	Australia (1)
		Duck (1)	UK (1)
264[A]	Cat (1)	NA[B]	NA
265[A]	Cat (2)	NA	NA

[A]Database (http://pubmlst.org/pmultocida/) accessed on 15 May 2013. ST 264 and ST 265 are unique STs first recognized in the current study with no matches in the database (other than the isolates from this study).

[B]NA = not applicable.

The available data at the RIRDC MLST website (http://pubmlst.org/pmultocida/) on the STs recognized in the current study is provided in Table 3.

Control measures. Despite successful action to reduce the activities of native and feral animals and to generally improve biosecurity, no significant reduction in fowl cholera–linked mortality was gained. The vaccine was deemed safe because no obvious differences were seen at 7 days of age between the vaccinated birds and the control birds. A 65% protection level was achieved in the efficacy trial (10% of a flock vaccinated). Vaccination of the flock with the autogenous vaccine at 1 day old yielded no clinical fowl cholera for a period of over 6 mo.

After 6 mo, a 30% mortality due to fowl cholera was found in one flock. This flock was severely cold stressed at 1 day old. It was rationalized that the cold stress had depressed flock immunity. However, further outbreaks of fowl cholera occurred at a rate of one flock per month with 10%–30% mortality. The clinical findings and lesions were similar to the previous outbreaks. The genotype of *P. multocida* found in the new outbreaks was the same as had been found previously. An observation just prior to the investigated outbreak was the occurrence of poor gut health, with severe enteritis being noted in the days immediately prior to an outbreak. Changes in coccidiosis vaccination resulted in marked improvement in gut health, and no further fowl cholera outbreaks occurred.

DISCUSSION

Fowl cholera is a disease that has long been recognized. According to Glisson *et al.* (6), several outbreaks of a disease that is recognizable as fowl cholera were recorded in Europe in the latter half of the 18th century. In the Australian context, the first clearly identifiable fowl cholera outbreak was identified in 1895 in ducks and fowls (15).

Outbreaks of fowl cholera associated with mortality are typically seen in laying flocks (6). There is strong field evidence of an increasing susceptibility to fowl cholera with age in broilers. This evidence arises from an accidental contamination of an early infectious laryngotracheitis vaccine with *P. multocida*. In 20,000 chickens of less than 16 wk given the contaminated vaccine, there were no mortalities. However, in 70,000 chickens of over 16 wk of age given the vaccine, there was a severe mortality (around 90%) (8).

Hence, the current study in which there were repeated outbreaks of fowl cholera in different flocks of free range organic broilers is somewhat unusual. While vaccination did help control these outbreaks, even autogenous vaccines based on the sole genotype present in the outbreak failed to prevent mortalities at times. While no definitive evidence is available, the field evidence suggests that these vaccine failures were typically associated with stress—with the stress ranging from temperature stress to gut health stress. It is worth noting that an early American report on fowl cholera in turkeys emphasized the role of environmental stressors such as changes in climate, nutrition, injury, and excitement as contributing to the incidence and course of the disease (1).

There have been a number of studies looking at the diversity of *P. multocida* associated with fowl cholera outbreaks—both within an outbreak and over time. It is now clear that the findings of such epidemiological studies can vary—some outbreaks are associated with a single genotype that does not change over many years as reported by Zhang *et al.* (21) for a free range layer farm, while other outbreaks can involve multiple genotypes within an outbreak as recently reported for a free range layer outbreaks and turkey outbreaks (17). The current study involved examination of multiple isolates (a total of 123 isolates) from 12 broilers in three separate outbreaks, and only a single ERIC-PCR pattern was detected. In contrast, there was diversity in the serotyping results seen in the five isolates examined. Since both MLST and ERIC-PCR agreed that a single genotype was involved in this outbreak, it would seem that the diversity in serotyping results may reflect more the difficulty of obtaining consistent, accurate results in the Heddleston scheme rather than true diversity in serovars. There are several reports suggesting problems in the reproducibility or stability of Heddleston serotyping (17,20). Overall, the evidence of the current study is strongly suggestive of a single strain of *P. multocida* causing these repeated outbreaks of fowl cholera.

Because this study used MLST, the RIRDC MLST database can be used to gain insight on what is known of the STs observed (see Table 3). Two STs from the current study have been associated with poultry prior to this study (ST 20 and 30), while in this study only one of the STs was associated with poultry (ST 20). The RIRDC MLST database, while still limited in coverage, now holds data on 638 isolates from over 40 host species and 35 countries. Approximately 18% of the isolates of *P. multocida* in the RIRDC MLST database are from chickens. The strength and robustness of the information contained within the RIRDC MLST database will increase as more isolates are added.

In a prior study looking at village production systems and animal reservoir, the exchange of strains of *P. multocida* was only demonstrated among hosts belonging to the same species (12). In contrast, the current study has shown that cats can harbor the same strain of *P. multocida* that is present in associated chicken. It is known that isolates of *P. multocida* from cats are pathogenic for fowl (5), and cats have consistently been identified as a potential source of introduction of *P. multocida* into flocks (3,6). This is the first study that has found the same strain in both feral cats and chickens. However, it is not clear if the cats infected the chickens or just ate dead chicken and consequently harbored the strain in their oral cavity. Overall, this study has provided proof based on genotype analysis that feral cats can be a potential source of *P. multocida* capable of infecting chickens.

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