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Epidemiology and antibiogram of *Riemerella anatipestifer* isolated from waterfowl slaughterhouses in Taiwan

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Abstract

Introduction: Laryngeal swab samples collected from three waterfowl slaughterhouses in central Taiwan were cultured and suspected isolates of *Riemerella anatipestifer* were identified by API 20NE and 16S rDNA PCR. **Material and Methods:** Serum agglutination was used for serotyping, and antimicrobial susceptibility was tested. **Results:** Seventy-six *R. anatipestifer* isolates were detected, and the prevalences in the ducks and geese were 12.3% (46/375) and 8.0% (30/375), respectively. The positive isolation rates were 65.6% for all arriving waterfowl, 76.0% for birds in the holding area, 1.6% for defeathered carcasses, but zero for degummed carcasses. A PCR examination detected *R. anatipestifer* in the slaughtering area frequently. Serotype B was dominant in both duck (34.8%) and goose (46.7%) isolates, but the wide serotype distribution may very well impede vaccination development. All isolates were resistant to colistin, and 79.7% were resistant to more than three common antibiotics. **Conclusion:** The results proved that most ducks had encountered antibiotic-resistant *R. anatipestifer* in rearing, which suggests that the bacterium circulates in asymptomatic waterfowl. It is worth noting that most waterfowl farms were found to harbour *R. anatipestifer*, and contaminated slaughterhouses are a major risk factor in its spread. Effective prevention and containment measures should be established there to interrupt the transmission chain of *R. anatipestifer*.

Keywords: waterfowl, *Riemerella anatipestifer*, slaughterhouse, contamination, antibiotic resistance, Taiwan.

Introduction

Riemerella anatipestifer causes infections in waterfowl and has an adverse economic effect on waterfowl husbandry. It is a rod-shaped, Gram-negative bacterium infection with which results in 20%–30% morbidity and 5%–20% mortality in waterfowl displaying septicaemia and infectious serositis (12, 16, 17). Respiratory tract and skin wounds are speculated to play important roles in *R. anatipestifer* transmission (29). Poor sanitation, nutrient deficiency, adverse (*e.g.* stressful) environments or climates, and concomitant diseases usually predispose fowl to outbreaks of *R. anatipestifer* infection, especially those under 8 weeks old (3, 26, 34). In a survey of samples of waterfowl in Taiwan between 2006 and 2007, the significant prevalence (84.4%) of *R. anatipestifer* in farms revealed the potential risk of an outbreak (4, 14). *R. anatipestifer* infection in young ducklings results in high morbidity (75%), but older adults usually suffer from chronic subclinical diseases or are even asymptomatic (11).

High genetic diversity and serotype variation was reported among field *R. anatipestifer* isolates, with more than 21 serotypes found (1, 22, 25, 29, 31). Although several kinds of vaccines against *R. anatipestifer* have been developed, such as attenuated, inactivated, and subcellular variants (7, 15, 20, 24, 28), high antigenic diversity and low cross-protection impede their application in the field. Antimicrobial agents and improved biosecurity are currently applied to prevent and control *R. anatipestifer*

infection in waterfowl farming; however, the increasing resistance to common antibiotics in *R. anatipestifer* seriously challenges the treatment (5).

Epidemic studies of zoonotic *Salmonella* and *Campylobacter* in poultry slaughterhouses have been carried out worldwide (23, 32). However, relatively little epidemiological research on *R. anatipestifer* has been undertaken in the field, possibly due to its narrow host range and the scarcity of clinical symptoms in adult birds. In this study, an epidemiological investigation of *R. anatipestifer* in slaughterhouses was conducted, including testing for it in the birds on arrival, the carcasses being processed, the machines, and the environments.

Material and Methods

Sampling methods. First, to evaluate the prevalence of *R. anatipestifer* in waterfowl, laryngeal and conjunctival swab samples (2) were collected from three slaughterhouses in central Taiwan housing and processing ducks and geese that were transported from farms in central and southern Taiwan. Cochran's formula ($n = Z^2PQ/e^2$, where n = sample size, Z = the desired confidence levels, P = expected prevalence, Q = 1−P, and e = the desired level of precision (8)) was used to calculate a representative sample size in this study. Thereafter, brain heart infusion agar was used for the bacterial cultures of 125 ducks (25 birds per flock) at various stages of the slaughtering process, including laryngeal and conjunctival swabs of the birds on arriving and before slaughter, and wash solutions of defeathered or degummed carcasses (sampled by a one-minute shaking with 150 mL of sterile normal saline, followed by bacterial isolation from 15 mL of this solution). The environmental samples survey was performed in two slaughterhouses, where the workspace and machines coming into contact with the birds are disinfected with hot water and sodium hypochlorite after each processing. Before the disinfection, five samples for each of the 16 selected sites were conducted at different sampling spots: (1) the samples of pool water, bottom mud, faeces, drinking water, and fodder in holding areas, (2) surface swabs of the workbench top, defeathering machine, scalding machine, and transport machines, and (3) samples of the waste feathers, water supply, and wastewater from the waterfowl scalding, defeathering, degumming, organ removal, purge, and slaughtering areas.

Isolation of *R. anatipestifer*. Isolation of *R. anatipestifer* from swab samples was performed by the streak method with blood agar plates (Trypticase soy agar with 5% sheep blood). Solution samples were pelleted at 3,000 × g for 10 min, vortexed for 15 s with 10 mL of sterile normal saline, spun again to remove supernatants, and then streaked on blood agar plates. The agar plates were first cultivated at 37ºC supplemented with 5% CO_2 for 24 h, then the plates and MacConkey agar were used to cultivate the suspicious bacterial colonies for morphological identification and selection tests. All isolates were identified by a commercial API 20NE (bioMérieux, France).

Molecular identification of *R. anatipestifer*. To prepare the DNA, a bacterial colony was picked up and suspended in 500 µL of TAE buffer, which was then boiled for 10 min and centrifuged at 16,000 × g for 1 min. The supernatant was directly used as the DNA template for the PCR. A 16S rDNA amplification PCR method previously described (18) used to molecularly identify *R. anatipestifer* isolates. Reactions containing an RA-5' (TCG AGA TTT GCA TCA CTT CGC ATT G) primer paired with an RA-3' (GCT AGT CTT GAG TAT AGT TGA GCT AGC) primer were performed on a MyCycler thermal cycler (Bio-Rad, USA) with the following amplification settings: initial denaturation at 94ºC for 5 min, 35 cycles of amplification (denaturation at 94ºC for 5 s, annealing at 50ºC for 50 s, and extension at 72ºC for 50 s), and a final extension at 72ºC for 10 min. The amplified products (645 bp) were electrophoresed on 2% agarose gels, stained with ethidium bromide, and then recorded and analysed using a GeneFlash system (Syngene, UK).

Serotyping of *R. anatipestifer*. *R. anatipestifer* isolates were serotyped by the Animal Health Research Institute (AHRI), Taiwan, using the slide agglutination method. In short, 20 µL of standard antiserum for *R. anatipestifer* typing was mixed with the same volume of single colony suspension from each isolate. A positive reaction was indicated by clumping of bacteria within 1 to 2 min.

Antibiotic susceptibility assay. A disc diffusion assay was applied to determine the antibiotic susceptibility of *R. anatipestifer* isolates. Twenty-one antibiotic discs (Oxoid, UK) were used, including: 30 µg of amikacin (AK30), 30 µg of amoxicillin/clavulanic acid (AMC30), 10 µg of ampicillin (AM10), 10 µg of bacitracin (B10), 30 µg of cephalothin (KF30), 30 µg of cefuroxime sodium (CXM30), 30 µg of cefotaxime (CTX30), 5 µg of ciprofloxacin (CIP5), 2 µg of clindamycin (CC2), 10 µg of colistin (CL10), 15 µg of erythromycin (E15), 10 µg of gentamicin (GM10), 2 µg of lincomycin (MY2), 30 µg of nalidixic acid (NA30), 30 µg of neomycin (N30), 10 µg of norfloxacin (NOR10), 5 µg of ofloxacin (OFX5), 10U of penicillin G (P10), 10 µg of streptomycin (S10), 23.75/1.25 µg of sulphamethoxazole/trimethoprim (SXT25), and 30 µg of tetracycline (TE30). Among these antibiotics, amoxicillin/clavulanic acid, ampicillin, colistin, erythromycin, gentamicin, lincomycin, nalidixic acid, neomycin, penicillin, streptomycin, sulphamethoxazole, trimethoprim, and tetracycline are approved for use in waterfowl in Taiwan (19). A 0.5 McFarland bacterial suspension in sterile normal saline was inoculated on Mueller Hinton Agar supplemented with 5% sheep blood (Becton Dickinson, USA), and the antibiotic discs were placed on the agar plates, which were then

cultured for 24 h at 37ºC with 5% CO_2. The inhibition zone diameters were interpreted according to the interpretive criteria in the Clinical and Laboratory Standards Institute (USA) guidelines (33).

Results

The results of a pilot survey for studying the prevalence of *R. anatipestifer* in fowls living in slaughterhouses demonstrated that 375 duck samples in 15 flocks and the same number of goose samples and flocks are required for an accepted error of 10% and a confidence level of 95%. In this study, 25 swabs for each of duck and goose flocks were collected in three waterfowl slaughterhouses in central Taiwan between 2009 and 2010. The flock prevalences of *R. anatipestifer* in living ducks and geese in slaughterhouses were 60.0% (9/15) and 53.3% (8/15), while the individual prevalences in ducks and geese were 12.3% (46/375) and 8.0% (30/375), respectively. Almost all (97.4%, 74/76) *R. anatipestifer* isolates were cultured from laryngeal specimens, including 44 from ducks and 30 from geese, whereas two were isolated from conjunctivas of ducks (Table 1). Seasonal distribution of *R. anatipestifer* in fowls in slaughterhouses showed the highest incidence in spring (69.6% in ducks, 36.7% in geese, subtotal 56.6%) and the lowest in autumn (0% in ducks, 16.7% in geese, subtotal 6.6%) (Table 2).

To analyse the presence of *R. anatipestifer* in fowl during the slaughtering process, 125 ducks collected from 5 equal groups were examined by both bacterial isolation and PCR detection. The isolation rates for birds on arrival, birds in holding, and birds in the defeathering and degumming stages were 65.6%, 76.0%, 1.6%, and 0%, respectively; and PCR detection rates were correspondingly 65.6%, 76.0%, 21.6%, and 11.2%. No discrepancies were detected in living fowls; however, a rate discrepancy between bacterial isolation and PCR detection was found in carcass samples (Table 3).

Additionally, an environmental survey of *R. anatipestifer* in waterfowl slaughterhouses was also conducted. Various samples including pool water, bottom mud, faeces, drinking water, and fodder were inspected by both isolation and PCR examination. No *R. anatipestifer* isolates were found in these samples, but nearly all kinds of samples were contaminated with the DNA of *R. anatipestifer*, with especially high detection rates in the samples from pool water (57.0%), bottom mud (83.0%), and faeces (40.0%) (Table 4). Two swabs of the surfaces of workbenches grew *R. anatipestifer* colonies but no swabs from the various processing machines (defeathering machine, scalding machine, and transport machine) grew any. Thereafter, extracted DNA from swabs on the surfaces of the workbench (7.0%), defeathering machines (30.0%), scalding machines (10.0%), but not transport machines, gave positive results for the presence of *R. anatipestifer* (Table 5). Meanwhile, borehole water and various types of wastewater samples were inspected using both bacterial culture and PCR detection. These samples formed no colonies, but the DNA collected from them regarding waste feathers (26.7%), defeathering waste (10.0%), and slaughter waste (10.0%) showed positive rates (Table 6).

Serotype characterisation by agglutination was performed on all *R. anatipestifer* isolates. The distribution of serotypes in the slaughterhouse isolates showed that serotype B was the dominant serotype in both duck (34.8%) and geese (46.7%) samples, followed by serotypes 3 (30.4%) and 21 (28.3%) in ducks, and serotypes 21 (40.0%) and 2 (23.3%) in geese (Table 7). None of the serotypes 7, 12, 13, 16, or 18 were found in the isolates in this study. Twenty-one common antibiotics were used to evaluate the antibiotic susceptibility of the slaughterhouse isolates. Twelve showed 50% or higher efficacies for the duck isolates, including amoxicillin/clavulanic acid (98%), cefotaxime (96%), cefuroxime sodium (93%), ofloxacin (83%), norfloxacin (80%), cephalothin (78%), ciprofloxacin (78%), tetracycline (65%), penicillin G (57%), clindamycin (52%), erythromycin (52%), and ampicillin (50%); while for the geese isolates, the 10 antimicrobials amoxicillin/clavulanic acid (97%), cefuroxime sodium (90%), cefotaxime (87%), cephalothin (80%), ofloxacin (77%), ampicillin (60%), sulphamethoxazole/ trimethoprim (57%), ciprofloxacin (53%), norfloxacin (53%), and penicillin G (50%) had good efficacies. Most importantly, 50% or more of the isolates had developed resistance against several antibiotics, such as colistin (100%), lincomycin (76%), nalidixic acid (72%), neomycin (70%), gentamicin (67%), and sulphamethoxazole/trimethoprim (50%) in duck isolates, or colistin (100%), lincomycin (83%), nalidixic acid (63%), neomycin (63%), streptomycin (57%), and gentamicin (50%) in geese isolates (Table 8). Notably, all the slaughterhouse isolates were resistant to colistin.

Table 1. The prevalence of *Riemerella anatipestifer* in living waterfowl in Taiwan slaughterhouses

Specimen source	Number of positive samples (%)		
	Duck (n = 375)	Goose (n = 375)	Total (n = 750)
Larynx	44 (11.7)*	30 (8.0)	74 (9.9)
Conjunctiva	2 (0.5)*	0 (0)	2 (0.3)
Total	46 (12.3)	30 (8.0)	76 (10.1)

* $p < 0.05$

Table 2. The prevalence of *Riemerella anatipestifer* in waterfowl in different seasons

Waterfowl	Number of positive samples (%)			
	Spring	Summer	Autumn	Winter
Duck (n = 46)	32 (69.6)*	5 (10.9)*	0 (0)	9 (19.6)*
Goose (n = 30)	11 (36.7)	6 (20.0)	5 (16.7)	8 (26.7)
Prevalence	43 (56.6)	11 (14.5)	5 (6.6)	17 (22.4)

* $p < 0.05$

Table 3. The detection rates of *Riemerella anatipestifer* in waterfowl in various stages in Taiwan slaughterhouses

Flock	Number of positive detections (%) (n = 25)			
	On arriving	Holding	Defeathered	Degummed
1	18 (72.0)	11 (44.0)	11 (44.0)	5 (20.0)
2	6 (24.0)	22 (88.0)	5 (20.0)	3 (12.0)
3	25 (100)	21 (84.0)	5 (20.0)	0 (0.0)
4	21 (84.0)	22 (88.0)	4 (16.0)	4 (16.0)
5	12 (48.0)	19 (76.0)	4 (16.0)	2 (8.0)
PCR	82 (65.6)	95 (76.0)	27 (21.6)	14 (11.2)
Isolation	82 (65.6)	95 (76.0)	2 (1.6)	0 (0)

Table 4. The detection rates of *Riemerella anatipestifer* in environmental samples from holding areas in Taiwan slaughterhouses

Sampling scheme	Number of positive detections (%) (n = 10)				
	Pool water	Bottom mud	Faeces	Drinking water	Fodder
1	7 (70.0)	9 (90.0)	5 (50.0)	0 (0)	0 (0)
2	5 (50.0)	8 (80.0)	3 (30.0)	1 (10.0)	0 (0)
3	5 (50.0)	8 (80.0)	4 (40.0)	2 (20.0)	0 (0)
PCR	17 (57.0)	25 (83.0)	12 (40.0)	3 (10.0)	0 (0)
Isolation	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

Table 5. The detection rates of *Riemerella anatipestifer* in samples from workspace in Taiwan slaughterhouses

Sampling scheme	Number of positive detections (%) (n=10)			
	Workbench top	Defeather machine	Scalding machine	Transport machine
1	2 (20.0)	4 (40.0)	2 (20.0)	0 (0)
2	0 (0)	3 (30.0)	0 (0)	0 (0)
3	0 (0)	2 (20.0)	1 (10.0)	0 (0)
PCR	2 (7.0)	9 (30.0)	3 (10.0)	0 (0)
Isolation	2 (7.0)	0 (0)	0 (0)	0 (0)

Table 6. The detection rates of *Riemerella anatipestifer* in water samples in Taiwan slaughterhouses

Sampling scheme	Number of positive detections (%) (n = 10)						
	Water supply	Waste feather	Waste water				
			Scalding	Defeather	Degumming	Organ purge	Slaughter
1	0 (0)	1 (10.0)	0 (0)	1 (10.0)	0 (0)	0 (0)	0 (0)
2	0 (0)	3 (30.0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (20.0)
3	0 (0)	4 (40.0)	0 (0)	2 (20.0)	0 (0)	0 (0)	1 (10.0)
PCR	0 (0)	8 (26.7)	0 (0)	3 (10.0)	0 (0)	0 (0)	3 (10.0)
Isolation	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

Table 7. The serotype of *Riemerella anatipestifer* isolated from waterfowl slaughterhouses

Serotype	Isolates (%)		
	Ducks (n = 46)	Geese (n = 30)	Total
1	1 (2.2)	1 (3.3)	2 (2.6)
2	0 (0)	7 (23.3)	7 (9.2)
3	14 (30.4)	0 (0)	14 (18.4)
4	2 (4.3)	3 (10.0)	5 (6.6)
5	1 (2.2)	0 (0)	1 (1.3)
6	7 (15.2)	1 (3.3)	8 (10.5)
7	0 (0)	0 (0)	0 (0)
8	8 (17.4)	2 (6.7)	10 (13.2)
9	4 (8.7)	5 (16.7)	9 (11.8)
10	1 (2.2)	0 (0)	1 (1.3)
11	9 (19.6)	0 (0)	9 (11.8)
12	0 (0)	0 (0)	0 (0)
13	0 (0)	0 (0)	0 (0)
14	4 (8.7)	3 (10.0)	7 (9.2)
15	4 (8.7)	0 (0)	4 (5.3)
16	0 (0)	0 (0)	0 (0)
17	1 (2.2)	0 (0)	1 (1.3)
18	0 (0)	0 (0)	0 (0)
19	1 (2.2)	0 (0)	1 (1.3)
20	1 (2.2)	5 (16.7)	6 (7.9)
21	13 (28.3)	12 (40.0)	25 (32.9)
B	16 (34.8)	14 (46.7)	30 (39.5)
Unknown	8 (17.4)	3 (10.0)	11 (14.5)

Table 8. The antibiotic susceptibility of *Riemerella anatipestifer* isolated from waterfowl slaughterhouses (46 ducks, 30 geese)

Antibiotic	Number of positive isolates (%)								
	Resistance			Intermediate			Susceptibility		
	Duck	Goose	Subtotal	Duck	Goose	Subtotal	Duck	Goose	Subtotal
Amikacin (AK30)	35 (76.0)	20 (67.0)	55 (72.4)	1 (2.2)	5 (16.7)	6 (7.9)	10 (21.2	5 (16.7)	15 (19.7)
Amoxicillin/Clavulanic acid (AMC30)	1 (2.2)	1 (3.3)	2 (2.6)	0 (0)	0 (0)	0 (0)	45 (97.8)	29 (96.7)	74 (97.4)
Ampicillin (AM10)	19 (41.3)	7 (23.3)	26 (34.2)	4 (8.7)	5 (16.7)	9 (11.8)	23 (50.0)	18 (60.0)	41 (53.9)
Bacitracin (B10)	6 (13.0)	11 (36.7)	17 (22.4)	19 (41.3)	5 (16.7)	24 (31.6)	21 (45.7)	14 (46.7)	35 (46.1)
Cephalothin (KF30)	2 (4.3)	4 (13.3)	6 (7.9)	8 (17.4)	2 (6.7)	10 (13.2)	36 (78.3)	24 (80.0)	60 (78.9)
Cefuroxime Sodium (CXM30)	1(2.2)	2 (6.7)	3 (3.9)	2 (4.3)	1 (3.3)	3 (3.9)	43 (93.5)	27 (90.0)	70 (92.1)
Cefotaxime (CTX30)	1 (2.2)	4 (13.3)	5 (6.6)	1(2.2)	0 (0)	1 (1.3)	44 (95.7)	26 (86.7)	70 (92.1)
Ciprofloxacin (CIP5)	6 (13.0)	7 (23.3)	13 (17.1)	4 (8.7)	7 (23.3)	11 (14.5)	36 (78.3)	16 (53.3)	52 (68.4)
Clindamycin (CC2)	19 (41.3)	13 (43.3)	32 (42.1)	3 (6.5)	3 (10.0)	6 (7.9)	24 (52.2)	14 (46.7)	38 (50)
Colistin (CL10)	46 (100)	30 (100)	76 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Erythromycin (E15)	12 (26.1)	12 (40.0)	24 (31.6)	10 (21.2)	6 (20.0)	16 (21.1)	24 (52.2)	12 (40.0)	36 (47.4)
Gentamicin (GM10)	31 (67.4)	15 (50.0)	46 (60.5)	2 (4.3)	6 (20.0)	8 (10.5)	13 (28.3)	9 (30.0)	22 (28.9)
Lincomycin (MY2)	35 (76.1)	25 (83.3)	60 (78.9)	9 (19.6)	5 (16.7)	14 (18.4)	2 (4.3)	0 (0)	2 (2.6)
Nalidixic acid (NA30)	33 (71.7)	19 (63.3)	52 (68.4)	5 (10.9)	7 (23.3)	12 (15.8)	8 (17.4)	4 (13.3)	12 (15.8)
Neomycin (N30)	32 (69.6)	19 (63.3)	51 (67.1)	7 (15.2)	11 (36.7)	18 (23.7)	7 (15.2)	0 (0)	7 (9.2)
Norfloxacin (NOR10)	5 (10.9)	11 (36.7)	16 (21.1)	4 (8.7)	3 (10.0)	7 (9.2)	37 (80.4)	16 (53.3)	53 (69.7)
Ofloxacin (OFX5)	3 (6.5)	3 (10.0)	6 (7.9)	5 (10.9)	4 (13.3)	9 (11.8)	38 (82.6)	23 (76.7)	61 (80.3)
Penicillin G (P10)	5 (10.9)	9 (30.0)	14 (18.4)	15 (32.6)	6 (20.0)	21 (27.6)	26 (56.5)	15 (50.0)	41 (53.9)
Streptomycin (S10)	29 (63.0)	17 (56.7)	46 (60.5)	5 (10.9)	5 (16.7)	10 (13.2)	12 (26.1)	8 (26.7)	20 (26.3)
Sulphamethoxazole/ Trimethoprim (SXT25)	23 (50.0)	5 (16.7)	28 (36.8)	4 (8.7)	8 (26.7)	12 (15.8)	19 (41.3)	17 (56.7)	36 (47.4)
Tetracycline (TE30)	7 (15.0)	10 (33.3)	17 (22.4)	9 (19.6)	9 (30.0)	18 (23.7)	30 (65.2)	11 (36.7)	41 (53.9)

Discussion

Although *R. anatipestifer* infection is nonlethal for adult waterfowl, there is an economic rationale for preventing infestations of the bacterium. The microbe's biofilm formation and antibiotic resistance nevertheless makes the eradication of its contamination in the field nearly impossible (13, 29). The insufficient biosecurity and inadequate husbandry methods of waterfowl farms and slaughterhouses increase the exposure of waterfowl to *R. anatipestifer* and even favour its silent circulation in fields, thereby leading to possible outbreaks without effective treatment. This study is the first survey of *R. anatipestifer* in waterfowl slaughterhouses in Taiwan. A few of the healthy ducks and geese that arrived at the slaughterhouses proved to be infected with *R. anatipestifer* (Table 1). Ducks appear to be more susceptible to *R. anatipestifer* than geese, although they are closely related, belonging to the same *Anatidae* family. It was reported that outbreaks of *R. anatipestifer* infection usually occur in spring and winter or after bad weather with a sudden temperature drop (9, 30); this was confirmed by the high incidences observed between November and April in this study (Table 2). Interestingly, most of the isolates (97.4%) were cultured from laryngeal samples of healthy waterfowl. The result was in agreement with previous suggestions that *R. anatipestifer* is a normal part of the pharyngeal flora of some ducks (2, 27). It is worth noting that the comparatively easy method of conjunctiva sampling, which is used to isolate pathogens from sick fowl and may appear to offer a simpler alternative to laryngeal sampling, is not suited for general surveys of *R. anatipestifer*. In agreement with a previous study on *R. anatipestifer* infection in wild birds (2), we suggest that laryngeal swabs are most suitable to isolate the bacteria.

The prevalence of *R. anatipestifer* in ducks being held in a slaughterhouse was higher than that of ducks on arrival, which suggests that some ducks were infected in a contaminated environment during the holding period or possibly that the stress of transportation and crowding increased the detection rate of *R. anatipestifer* in the laryngeal bacteria of healthy ducks. The high-temperature treatments of scalding (64ºC for 3 min) and rosin plucking (120–150ºC for 5 s) are detrimental to the survival of *R. anatipestifer*, therefore only 1.6% of defeathered ducks and no degummed duck carcasses were positively isolated; however, a few carcass washing samples yielded bacteria to positive detection through the PCR method due to the remaining nucleic acids (Table 3). In the environmental survey of *R. anatipestifer* in slaughterhouses, only two isolates were cultured from workbench swabs, but none were isolated from any waste or swab samples. However, most of the environmental samples showed a low positive rate through PCR detection, confirming that any *R. anatipestifer* surviving in contaminated environments may be too fragile to be cultivated (10). Therefore, the bacterial culture method may not truly reflect the level of *R. anatipestifer* contamination in waterfowl farms.

The serotypes of the slaughterhouse *R. anatipestifer* isolates revealed that type B was the major serotype in both ducks (34.8%) and geese (46.7%). Serotype B, which does not belong to the classic 21 serotypes (25), was discovered, classified, and reported as one of the dominant serotypes (serotypes 2, 1, and B) in sick waterfowl in a farm survey study conducted in 2010 (6). Sixteen serotypes were identified in duck isolates, while ten serotypes were found in geese isolates in this study. This suggests that ducks are susceptible to more serotypes of *R. anatipestifer* than geese. Interestingly, multiple serotypes were identified in one waterfowl, which clearly proved that broad cross-protection is not established in *R. anatipestifer* infections. As several serotypes were found circulating in the same slaughterhouses, the development of an effective vaccine may be difficult.

According to the results of disc diffusion assays, 21 antibiograms of the isolates showed that all *R. anatipestifer* isolates are resistant to colistin and more than 70% of the isolates are resistant to lincomycin. These antibiotics were approved for use in feed and for treating bacterial infection in poultry, as well as neomycin (70.0% and 63.0% resistance in duck and geese isolates, respectively). Interestingly, the bacterium did not demonstrate elevated resistance rates to penicillin G, also an approved feed drug. More than half of the isolates had developed resistance against amikacin, gentamicin, nalidixic acid, and streptomycin, which are not allowed for use in feed or environmental treatments. It seems that overuse or misuse of approved antibiotics in food animals is a major cause of the antibiotic resistance problem but not the only one. Moreover, multiple drug resistances were found in most isolates. Antibiotic resistance makes the treatment and control of *R. anatipestifer* difficult in the waterfowl industry. Fortunately, a high percentage (97.4%) of the tested isolates were still susceptible to amoxicillin, which is approved for use in waterfowl.

This study demonstrated that *R. anatipestifer* has substantially contaminated waterfowl slaughterhouses and circulates in waterfowl farms and slaughterhouses in central Taiwan. The high prevalence of colistin resistance in the isolates associates with a risk of dissemination of that resistance gene in agricultural soils, probably through a plasmid-mediated route (21). Therefore, antibiotic therapy action might be suppressed in a zoonotic infection with the bacteria which have acquired the multidrug resistance. It is worth noting that the bacterial isolation method may not suit *R. anatipestifer* surveys on farms. There is a salient and urgent need for training in farm biosecurity with a particular focus on *R. anatipestifer* in

the waterfowl industry. Meanwhile, the severe problem of antibiotic resistance in *R. anatipestifer* requires the attention of the appropriate antimicrobial stewards.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

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