Development and application of a multiplex PCR assay for rapid detection of 4 major bacterial pathogens in ducks

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ABSTRACT Infections with *Pasteurella multocida*, *Salmonella enterica*, *Riemerella anatipestifer*, and *Escherichia coli* result in high morbidity and mortality, which cause significant economic loss in the poultry industry. It can be difficult to distinguish these pathogens based on clinical signs because these pathogens can cause similar clinical signs and coinfections can occur. Thus, rapid and sensitive detection of these 4 major bacterial pathogens are important in ducks. The aim of this study was to develop a multiplex PCR (mPCR) assay for simultaneously detecting and identifying these 4 pathogenic bacteria in a single tube reaction. The target genes used were KMT1 of *P. multocida*, the invasion protein gene of *S. enterica*, 16S rDNA of *R. anatipestifer*, and the alkaline phosphatase gene of *E. coli*. The detection limit of the assay for all bacterial DNA was 10 pg. The mPCR did not produce any nonspecific amplification products when tested against other related pathogens, including *Staphylococcus aureus*, *Streptococcus pyogenes*, *Clostridium perfringens*, *Mycoplasma gallinarum*, *Mycoplasma synoviae*, and *Mycoplasma gallisepticum*, which can also infect ducks. We applied mPCR to field samples, and the results were the same as the single PCR results. These results suggest that mPCR for the 4 bacteria is a useful and rapid technique to apply to field samples.

Key words: multiplex PCR, bacteria, duck, pathogen

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INTRODUCTION

Bacterial pathogens play an important role in economic losses in the duck industry. Diseases caused by bacteria are a more common cause of mortality in ducks than are those caused by viruses. The frequency of duck mortality events and the variety of infectious bacterial diseases causing that mortality have increased greatly.

A variety of infectious bacterial pathogens, including *Pasteurella multocida*, *Salmonella enterica*, *Riemerella anatipestifer*, and *Escherichia coli*, have been reported to threaten duck health throughout the world. Fowl cholera caused by *P. multocida* is a contagious and septic disease in ducks (Glisson et al., 2008) and is one of the most important diseases in the duck industry because prevalence of *P. multocida* carriers in healthy duck flocks is as high as 63%, and mortality may reach 50% (Tsai and Hsiang, 2005). Salmonellosis, which caused by multiple serotypes of *Salmonella*, is a common duck disease. Ducklings often have an acute onset at 3 wk of age and slightly older; rates of chronically infected ducks were variable from 0 to 66.7% in different flocks depending on the age at *Salmonella* infection (Tsai and Hsiang, 2005). *Riemerella anatipestifer*-induced duck infectious serositis is the most serious bacterial infectious disease caused by acute or chronic exposure infection; ducklings are infected at 2 to 7 wk of age, and mortality can reach 91% (Sarver et al., 2005). *Escherichia coli* duck disease can cause a variety of problems in ducks of all ages, but the most serious incidence occurs at 2 to 6 wk of age and mortality rates up to 43% (Saif et al., 2008). These diseases have many similar clinical symptoms, pathological changes, and epidemiology (Saif et al., 2008). Furthermore, ducks can be infected concurrently with 2 or more of the above bacterial pathogens (Tiong, 1990).

The traditional method of diagnosing bacterial disease is by culturing bacteria on agar plates followed by biochemical and serological testing of the isolates (Saif et al., 2008). This requires growing the patho-

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gen, and culture-based methods are time-consuming, tedious, and show less sensitivity. In particular, some bacteria such as *P. multocida* and *R. anatipestifer* do not have an effective selective medium (Mbuthia et al., 2008; Saif et al., 2008). Molecular techniques such as the PCR are a highly sensitive way to detect specific pathogens in field samples (Townsend, et al., 2000). Individual PCR assays have been developed for detecting and identifying duck bacterial pathogens (Townsend et al., 2000). However, a large number of individual PCR assays are necessary if single primer sets are used on a large number of clinical samples (Kardos et al., 2007). Thus, simultaneously detecting several pathogens with a multiplex PCR (**mPCR**) approach would be relatively rapid and cost-effective and has been widely applied to detect multiple viruses and bacteria in clinical specimens (Malik et al., 2004; Kim et al., 2006; Hu et al., 2011).

However, no study has reported on the simultaneous detection of the 4 aforementioned duck bacterial pathogens in a single tube. Therefore, the aim of this study was to develop a reliable, rapid, and effective mPCR assay for simultaneously detecting *P. multocida*, *S. enterica*, *R. anatipestifer*, and *E. coli* in a single tube. We also evaluated applicability of the method to screen ducks in the field for the presence of the 4 bacterial pathogens before subjecting them to bacterial isolation.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

The bacterial pathogens used in this study are listed in Table 1. The strains of *P. multocida* and *R. anatipestifer* were cultured at 37°C in tryptic soy broth (Merck, Darmstadt, Germany). The *S. enterica* and *E. coli* strains were cultured at 37°C in Luria broth (Difco, Detroit, MI). Other strains from the Korean Veterinary Culture Collection (Anyang, South Korea), American Type Culture Collection (Manassas, VA), Korean Culture Center of Microorganisms (Seoul, South Korea), and Korean Collection for Type Cultures (Daejeon, South Korea) were cultured in appropriate media under recommended culture conditions.

DNA Isolation

Freshly cultured cells were collected from an agar plate with a sterile loop and suspended in 2 mL of PBS, and 200 μL was used for DNA isolation. The DNA extraction procedure was the freezing and boiling method. In brief, cells were resuspended in 200 μL of H_2O and then moved to −80°C to freeze for 10 min and then boiled at 100°C for 5 min. This procedure was repeated 3 times. After cooling on ice for 5 min, the

Table 1. Confirmation of each specific primer pair

Species (description)	Source or reference[1]	PCR results[2]			
		P	S	R	E
Pasteurella multocida (serogroup A)	BA0001832 (KVCC)	+	−	−	−
P. multocida (serogroup B)	12946 (ATCC)	+	−	−	−
P. multocida (serogroup D)	BA0000513 (KVCC)	+	−	−	−
Salmonella enterica (Typhimurium)	14028 (ATCC)	−	+	−	−
S. enterica (Gallinarum)	9184 (ATCC)	−	+	−	−
S. enterica (Pullorum)	19945 (ATCC)		+	−	−
S. enterica (Enteritidis)	12021 (KCCM)	−	+	−	−
S. enterica (Heidelberg)	BA0000506 (KVCC)	−	+	−	−
S. enterica (Senftenberg)	BA0000590 (KVCC)	−	+	−	−
S. enterica (Virchow)	BA0000595 (KVCC)	−	+	−	−
S. enterica (Dublin)	BA0000584 (KVCC)	−	+	−	−
S. enterica (Paratyphi)	BA0000588 (KVCC)	−	+	−	−
S. enterica (Agona)	BA0000470 (KVCC)	−	+	−	−
S. enterica (Infantis)	BA0000469 (KVCC)	−	+	−	−
Riemerella anatipestifer (RA21)	BA0001842 (KVCC)	−	−	+	−
R. anatipestifer (RA25)	BA0001846 (KVCC)	−	−	+	−
Escherichia coli	25922 (ATCC)	−	−	−	+
E. coli (avian pathogenic *E. coli*)	Kwon et al., 2008	−	−	−	+
E. coli (avian pathogenic *E. coli*)	Kwon et al., 2008	−	−	−	+
Staphylococcus aureus	25923 (ATCC)	−	−	−	−
Clostridium perfringens (Type C)	3628 (ATCC)	−	−	−	−
Streptococcus pyogenes	3096 (KCTC)	−	−	−	−
Mycoplasma gallinarum	19708 (ATCC)	−	−	−	−
Mycoplasma synoviae	25204 (ATCC)	−	−	−	−
Mycoplasma gallisepticum	A12-LSF-46 (this study)	−	−	−	−

[1]KVCC = Korean Veterinary Culture Collection (Anyang, South Korea); ATCC = American Type Culture Collection (Manassas, VA); KCCM = Korean Culture Center of Microorganisms (Seoul, South Korea); KCTC = Korean Collection for Type Cultures (Daejeon, South Korea).

[2]P, *P. multocida*; S, *S. enterica*; R, *R. anatipestifer*; E, *E. coli*; +, positive PCR result; −, negative PCR result.

Table 2. Primers used for multiplex PCR

Pathogen	Target gene	Primer designation[1]	Primer sequence (5′–3′)	Product size (bp)	Reference
Pasteurella multocida	KMT1	KMT1-F	GTGTGTTGAGCCAATCTGCT	283	This study
		KMT1-R	GCTGTAAACGAACTCGCCAC		
Salmonella enterica	*invA*	InvA-F	CGGCAGAGTTCCCATTGAAA	527	This study
		InvA-R	TTGTCACCGTGGTCCAGTTT		
Riemerella anatipestifer	16S rDNA	190F	GTATTGAAAGCTCTGGCGG	654	Qu et al., 2006
		843R	TCGCTTAGTCTCTGAACCC		
Escherichia coli	*phoA*	Pho-F	GTGACAAAAGCCCGGACACCAGAAATGCCT	903	Kong et al., 1999
		Pho-R	TACACTGTCATTACGTTGCGGATTTGGCGT		

[1]F = forward; R = reverse; KMT1 = housekeeping gene of *P. multocida*.

samples were centrifuged at 19,000 × *g* for 5 min, and the supernatant was stored at −40°C until assay.

Oligonucleotide Primers

The 190F and 843R primer pair is specific to *R. anatipestifer* and targets the 654 bp 16S rDNA (Qu et al., 2006). The Pho-F and Pho-R primer pair is specific to the 903 bp *E. coli phoA* gene (Kong et al., 1999). The other DNA sequences of KMT1-F/R and InvA-F/R from the GenBank target bacteria sequence were specifically designed using DNAMAN software (Lynnon Corp., St. Louis, MO). All oligonucleotide primers were synthesized at Bioneer (Daejeon, South Korea). The sequences of the 4 PCR primer pairs used for mPCR, their corresponding gene targets, and the sizes of the expected amplification products are shown in Table 2.

Optimization of mPCR Conditions

The mPCR protocol was optimized by varying single parameters while other parameters were maintained. The parameters that were examined were annealing temperature, final primer concentration for each target pathogen, extension time, cycle quantity, and Mg^{2+} and dNTP concentrations. After PCR, the products were transferred to a 1.0% agarose gel, electrophoresed, and DNA was visualized by ethidium bromide staining.

Specificity and Sensitivity of the mPCR Assay

The mPCR specificity was determined by examining the ability of the test to detect and distinguish several bacteria among these 4 bacteria (including 3 *P. multocida* strains, 11 *S. enterica* strains, 2 *R. anatipestifer* strains, and 3 *E. coli* strains). Other strains are shown in Table 1. The mPCR sensitivity was determined by performing serial dilutions of a mixture containing 1,000 ng of the DNA templates from each of the 4 pathogens.

DNA Sequencing

The PCR products were directly sequenced with an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) to verify that the specific primer-pair amplified *P. multocida*, *S. enterica*, *R. anatipestifer*, and *E. coli* DNA. The DNA sequences were analyzed using BLAST and GenBank.

Detecting Pathogens in Field Samples

A total of 123 duck flocks from commercial farms, which included ducks 1 to 21 d of age, were sampled from July 2011 to June 2012. Six to 12 ducks were collected from each flock. Liver samples for bacteriological examination were obtained aseptically and plated on 5% sheep's blood agar (Komed, Masan, South Korea).

All colonies were selected and transferred to a 2-mL microcentrifuge tube with PBS after a 48 h incubation at 37°C. All bacteria from each flock were pooled before DNA extraction. After the freezing and boiling procedure, DNA concentration was adjusted to 200 ng/μL by ASP-2680 (ACTGene, Piscataway, NJ). All DNA from field samples was processed simultaneously by single PCR and mPCR to determine the comparative efficiency of the 2 methods.

RESULTS

Primer Specificity

Primer pair specificity for each bacterium was analyzed initially by single PCR. All species-specific primer pairs produced a single PCR product with an expected product size, indicating species specificity of the designed primers (data not shown). Then, each primer pair was also tested using both single DNA and a mixture of the 4 DNA templates. When different combinations of the 4 bacteria (using 1 to 3 randomly selected bacteria) were used in the mPCR, the respective bacterial amplicons were produced and were separated on agarose gel electrophoresis (Figure 1).

Development of the mPCR Protocol

The mPCR products were 283 bp for *P. multocida*, 527 bp for *S. enterica*, 654 bp for *R. anatipestifer*, and 903 bp for *E. coli*. The mPCR assay required careful optimization of several parameters, including annealing temperature, extension time, Mg^{2+} concentration, Taq DNA polymerase concentration, and primer concentrations. Optimization allowed for simultaneous amplification of the 4 targets. The optimum Mg^{2+} concentration for mPCR was determined by adding 1.0, 1.5, 2.0, 2.5, or 3.0 m*M* Mg, and 2.0 and 2.5 m*M* produced similar intensities for their respective targets (data not shown). The dNTP concentration was increased stepwise from 50 to 800 m*M*, and the best results were obtained for 200 and 400 m*M* of each dNTP. Different concentrations of e*Taq* DNA polymerase were tested from 0.5 to 2.5 U in a 25-μL reaction; 1.0 U was optimal, and higher concentrations decreased reaction sensitivity. Different combinations of primer ratios were subsequently tested, and the primer combination that was optimal for mPCR was 0.3 μ*M* each for *P. multocida*, 0.35 μ*M* each for *S. enterica*, 0.4 μ*M* each for *R. anatipestifer*, and 0.15 μ*M* each for *E. coli*. The optimal annealing temperature was 55°C to amplify these 4 targets in the multiplex mixtures, and a 2-min extension was used to complete synthesis of all products (data not shown).

Analytical Specificity and Sensitivity of mPCR

The mPCR was applied to other bacteria that can also be found in ducks to detect the cross-reactivity between mPCR primer sets except those of *P. multocida*, *S. enterica*, *R. anatipestifer*, and *E. coli*. As a result, no amplicons were produced with *Staphylococcus aureus*, *Streptococcus pyogenes*, *Clostridium perfringens*, *Mycoplasma gallinarum*, *Mycoplasma synoviae*, or *Mycoplasma gallisepticum* (Table 1). The PCR assay sensitivity evaluation was carried out using a series of target pathogen genomic DNA at a 10-fold dilution. The mPCR detection limits were 10 pg of DNA template for the 4 bacterial pathogens (Figure 2).

mPCR for the Field Samples

To evaluate the discriminating power of the method even further and to test its applicability, 123 flocks

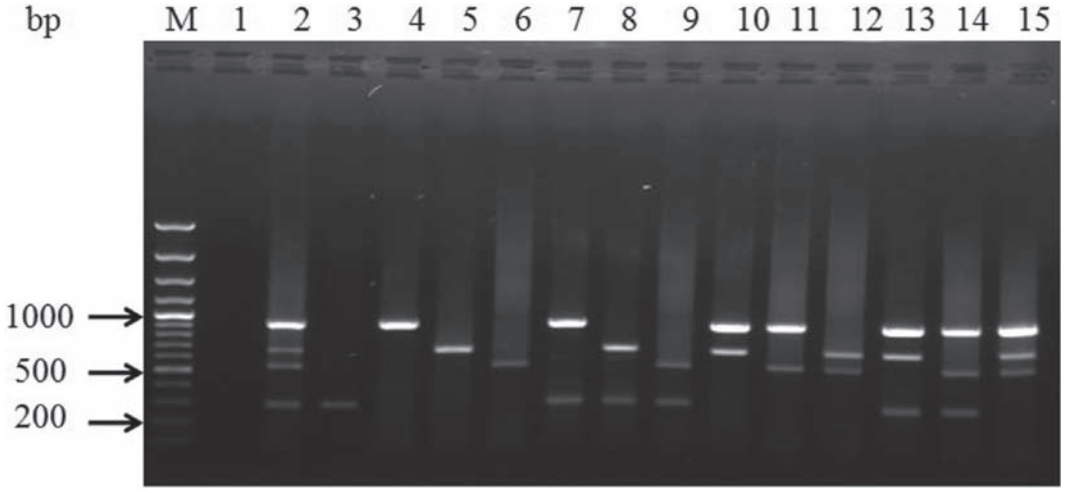

Figure 1. Agarose gel electrophoresis of multiplex PCR amplified products. M, 100-bp DNA marker; lane 1, negative control; lane 2, mixture of *Pasteurella multocida*, *Salmonella enterica*, *Riemerella anatipestifer*, and *Escherichia coli*; lane 3, *P. multocida*; lane 4, *E. coli*; lane 5, *R. anatipestifer*; lane 6, *S. enterica*; lane 7, mixture of *P. multocida* and *E. coli*; lane 8, *P. multocida* and *R. anatipestifer*; lane 9, *P. multocida* and *S. enterica*; lane 10, *E. coli* and *R. anatipestifer*; lane 11, *E. coli* and *S. enterica*; lane 12, *R. anatipestifer* and *S. enterica*; lane 13, *E. coli* and *R. anatipestifer*; lane 14, *P. multocida*, *E. coli*, and *S. enterica*; lane 15, *E. coli, R. anatipestifer*, and *S. enterica*.

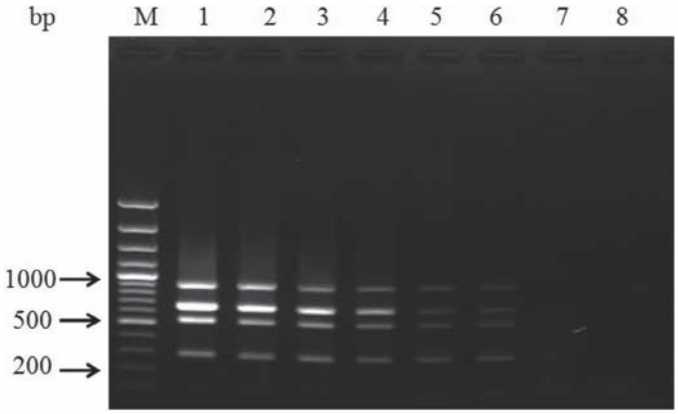

Figure 2. Sensitivity for detecting *Pasteurella multocida*, *Salmonella enterica*, *Riemerella anatipestifer*, and *Escherichia coli* by multiplex PCR. M, 100-bp DNA marker; lane 1, 1,000 ng of DNA for each of *P. multocida*, *S. enterica*, *R. anatipestifer*, and *E. coli*; lane 2, 100 ng; lane 3, 10 ng; lane 4, 1 ng; lane 5, 100 pg; lane 6, 10 pg; lane 7, 1 pg; lane 8, 0.1 pg.

of duck were tested for *P. multocida*, *S. enterica*, *R. anatipestifer*, and *E. coli* using the mPCR and confirmed by routing PCR with the same 4 sets of primers. The mPCR results were the same as the routing PCR results (date not shown). *Pasteurella multocida*, *S. enterica*, *R. anatipestifer*, and *E. coli* were detected in 0, 63, 48, and 106 flocks, respectively. According to the results shown in Table 3, 111 flocks were positive for one or more of these organisms by mPCR assay, and coinfection with 2 or more bacteria was demonstrated in 82 flocks.

DISCUSSION

Multiplex PCR is a powerful tool in clinical microbiology and has been widely applied to detect bacteria and genes of interest (Kong et al., 2002). A novel mPCR protocol was developed to simultaneously detect and distinguish *P. multocida*, *S. enterica*, *R. anatipestifer*, and *E. coli*, which are the most important bacterial pathogens of ducks, based on the problem that similar clinical signs and coinfections with 2 or more than 2 pathogens often occur in duck flocks. Numerous PCR tests have been described for detecting all 4 of these bacterial pathogens, but no information is available for detecting these 4 pathogens in a single mPCR tube, and these pathogens are currently detected separately (Tiong, 1990; Townsend et al., 1998; Kong et al., 1999; Hu et al., 2011). The mPCR primer pairs must be specific to the genes of interest, and the PCR products should be of different sizes (Chang et al., 2008). Target gene fragments were specifically amplified by conventional PCR of KMT1 for *P. multocida*, invasion protein gene (***invA***) for *S. enterica*, 16s rDNA for *R. anatipestifer*, and alkaline phosphatase gene (***phoA***) for *E. coli*. The KMT1 gene, which is a specific chromosomal region confirmed by a genomic subtraction study, is unique to *P. multocida* (Townsend et al., 1998). In this study, detection of the *P. multocida* KMT1 gene was based on KMT1-T7 and KMT1-SP6 primers described previously (Townsend et al., 2001). The forward primer was redesigned to modulate PCR product size. The *invA* gene was used as the target gene for *S. enterica*, consistent with other studies (Upadhyay et al., 2010; Akiba et al., 2011; Jeyasekaran et al., 2011) but was redesigned to suit the present mPCR. For *R. anatipestifer*, primers for amplifying 16S rDNA as a candidate to detect *R. anatipestifer* were explored because the 654-bp product specifically detects *R. anatipestifer* in almost all the serotypes (Qu et al., 2006). Primer sets from a housekeeping gene, *phoA*, which is universal in *E. coli* strains, were used for detecting *E. coli* (Kong et al., 1999). No cross-amplification of the 4 targets was observed with the DNA from other pathogens (*Staphylococcus aureus*, *Clostridium perfringens*, *Streptococcus pyogenes*, *Mycoplasma gallinarum*, *Mycoplasma synoviae*, and *Mycoplasma gallisepticum*). In this study, the mPCR detection limits were as low as 10 pg DNA for the 4 duck bacterial pathogens. This was the same as the 10 pg used for *E. coli*, *Listeria monocytogenes*, and *Salmonella* Typhimurium DNA templates from artificially inoculated wheat using another mPCR method (Kim et al., 2006). This result suggests that the present mPCR assay may be useful in detecting bacterial diseases. We also showed that the mPCR had a similar sensitivity to that of a single PCR assay, and this was confirmed in clinical specimens.

The performance of the mPCR in field studies with infected flocks further validates its use as an optimal tool for *P. multocida*, *S. enterica*, *R. anatipestifer*, and *E. coli* surveillance. Samples positive for all of these bacterial pathogens produced clear agarose gel bands,

Table 3. Frequency of bacterial pathogens alone or in combination in duck flocks from liver organs

	2011		2012		Total	
Pathogen	No. of positive	Positive rate (%)	No. of positive	Positive rate (%)	No. of positive	Positive rate (%)
Escherichia coli	10	25.6	14	16.7	24	19.5
Riemerella anatipestifer	1	2.6	1	1.2	2	1.6
Salmonella enterica	0	0.0	3	3.6	3	2.4
E. coli + *S. enterica*	6	15.4	30	35.7	36	29.3
S. enterica + *R. anatipestifer*	0	0.0	1	1.2	1	0.8
E. coli + *R. anatipestifer*	9	23.1	13	15.5	22	17.9
E. coli + *S. enterica* + *R. anatipestifer*	2	5.1	21	25.0	23	18.7

although there was no *P. multocida*-positive result in the clinical samples. The mPCR results showed that 111 flocks were positive for one or more of these organisms, which was same result obtained by single PCR, suggesting that the mPCR is sensitive enough to apply for clinical evaluations (Nguyen et al., 2010; Liu et al., 2011). Thus, our investigation showed that the present mPCR assay is as useful as single PCR and that mPCR could be a rapid and cost-effective diagnostic tool to survey these 4 bacteria pathogens in ducks.

In this study, 90.2% of the flocks were positive for bacterial infection, and 48 (39.0%), 63 (51.2%), and 106 (86.2%) flocks were positive for *R. anatipestifer*, *S. enterica*, and *E. coli* respectively. Furthermore, 82 (66.7%) flocks were infected with 2 or more pathogens simultaneously, confirming a previous study that ducks can be infected with 2 or more bacterial pathogens simultaneously. Coinfection with 2 or more pathogens increases the risk of being unable to distinguish the diseases using clinical signs (Tiong, 1990; Hoque et al., 2010, 2012). These multiple infections are difficult to diagnose because the clinical signs can be variable and may not be pathogen-specific (Tiong, 1990). Thus, in comparison with the standard single PCR to detect these 4 pathogens, the mPCR is more cost-effective and rapid, rendering it more useful for clinical diagnosis and epidemiological surveillance (Benson et al., 2008).

Pasteurella multocida was not detected in the clinical samples processed in the current study, indicating that *P. multocida* infection might be absent in the regions of the samples collected, as no *P. multocida* outbreak has occurred in domestic ducks (Kwon and Kang, 2003; Woo and Kim, 2006). However, it is generally accepted that mature birds are more susceptible than young ducks (Mbuthia et al., 2008), based on the low incidence of this pathogen in poultry from South Korea (Woo and Kim, 2006). Another possibility could be the role of antibiotics. The *P. multocida* strains isolated from chickens and wild birds are sensitive to amoxicillin, which is used widely on domestic duck farms (Kwon and Kang, 2003; Woo and Kim, 2006).

In conclusion, we demonstrated that a mPCR assay can be used as a rapid and cost-effective diagnostic tool to detect the presence of *P. multocida*, *S. enterica*, *R. anatipestifer*, and *E. coli* from clinical samples. Furthermore, this assay will be useful to diagnose, screen, and survey these 4 bacterial pathogens in duck flocks.

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