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Sequence-based genotyping methods to assess the genetic diversity of *Riemerella anatipestifer* isolates from ducklings with tremor

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SUMMARY

The aim of this study was to investigate sequence-based genotyping methods to distinguish 27 *Riemerella anatipestifer* isolates from ducklings in South Korea. The 16S rRNA sequences of the 27 *R. anatipestifer* isolates showed 99–100% similarities to each other and to reference sequences from Genbank (AY871822.2, AY871834.2, CP002562.1, EU715016.1, EU016548.1, EU715000.1, EU715008.1 and EU715011.1). In addition, the *ompA* gene sequences of 25 of the 27 *R. anatipestifer* isolates were 100% identical to each other, and these sequences were also 100% identical to reference sequences (CP002562.1, GQ415419.1, DQ059079, FJ765034.1, AY606207.1, AF104937.1, and FJ765033.1). Alternatively, four housekeeping genes (*mdh, gdh, pgi,* and *rpoB*) and three virulence-associated genes (*prtC, hagA,* and *sspA*) were used for a multi-locus sequence typing (MLST) and a single-locus sequence typing (SLST) among *R. anatipestifer* isolates. Compared to 16S rRNA and the *ompA* gene, seven genes showed higher genetic divergence patterns, and the isolates were separated into three distinct groups in phylogenetic trees.

KEY WORDS: Housekeeping genes, MLST, Phylogenetic analysis, *Riemerella anatipestifer,* SLST, Virulence-associated genes.

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INTRODUCTION

Riemerella anatipestifer is a non-motile, Gram-negative, non-spore-forming rod-shaped bacterium (Segers *et al.,* 1993). This pathogen, which causes septicemic and exudative diseases in ducks, turkeys, chickens, and wild waterfowl, has considerable economic impact on poultry production (Graham *et al.,* 1938; Hendrickson and Hilbert, 1932). *R. anatipestifer* infection has been consistently reported since 1904, illustrating the chronic problems associated with this pathogen (Baba *et al.,* 1987; Hubalek, 2004; Leavitt and Ayroud, 1997). Mortality from *R. anatipestifer* infection varies from 5% to 75%, and morbidity is usually as high as 100% for ducklings <5 weeks of age (Zhong *et al.,* 2009).

Strategies aimed at preventing the spread of *R. anatipestifer* require a thorough knowledge of both the dissemination and the epidemiology of *R. anatipestifer* strains. A variety of band-based genotyping methods are available for classifying *R. anatipestifer* strains, such as restriction fragment length polymorphism, and pulsed-field gel electrophoresis (Kiss *et al.,* 2006; Rimler and Nordholm, 1998).

Recently, sequence-based approaches are becoming more frequently used. Compared to band-based methods, sequence-based genotyping methods have several advantages, such as ease of use, reproducibility, and comparability of results via the Internet, and thereby these methods become more frequently used (Alonso *et al.,* 2007). 16S rRNA sequences were commonly used for

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identification and differentiation of *R. anatipestifer* (Rubbenstroth *et al.,* 2011). Also, a gene *ompA* encoding for a 42-kDa outer membrane protein which seems to be a predominant and specific antigen of the species *R. anatipestifer* was reported as a potential target to examine the genetic diversity of *R. anatipestifer* (Huang *et al.,* 2002; Yu *et al.*, 2008).

In this study, four housekeeping genes (*mdh, gdh, pgi,* and *rpoB*) and three virulence-associated genes (*prtC, hagA,* and *sspA*) were used in a single-locus sequence typing (SLST) and a multi-locus sequence typing (MLST). The goal of this study was to investigate new sequence-based genotyping methods to analyze *R. anatipestifer* isolates from ducklings with tremor in South Korea.

MATERIAL AND METHODS

Bacterial isolation and identification

Moribund ducklings exhibiting clinical signs (listlessness, ataxia, tremors of the head and legs, and coma) were collected in South Korea during 2010-2011 (Table 1). Swabs from the brains and meninges were streaked onto tryptic soy agar (TSA; Difco, Sparks, MD, USA) and incubated for 20 h at 38°C. The isolates were kept frozen at -80°C in tryptic soy broth (TSB; Difco, USA) containing 20% glycerol until they were used.

Genomic DNA was extracted by suspending the bacteria in sterile water followed by boiling for 10 min. After 3 min of centrifugation at 10,000 × g, the supernatants were collected and 1:100 dilutions in sterile water were utilized as PCR templates.

The 16S rRNA sequence was amplified using 16SUNI-L/Rcol-2 primer pairs to identify the bacteria (Kuhnert *et al.,* 1996; Rubbenstroth *et al.,* 2011), and the *ompA* gene was amplified using the RAOMPA-F1/R3 primer pairs (Subramaniam *et al.,* 2000) (Table 2). The PCR reaction was performed in a final volume of 50 µl containing 100 ng of genomic DNA, 5 µl 10× Taq-Buffer, 4 µl 10 mM dNTP's, 1 U of Taq polymerase (Invitrogen, Carlsbad, CA, USA) and 2 pmol of each primer. After initial denaturation at 96°C for 1 min, 35 cycles of amplification was performed under the following conditions: 96°C for 15 s, 53°C for 1 min, and 72°C for 1 min. Cycling was followed by a final extension for 7 min at 72°C. Product lengths were 1460 bp for the 16S rRNA genes and 1022 bp for the *ompA* genes.

Direct sequencing was carried out by the Macrogen Genomic Division (Seoul, Korea) and analyzed using an ABI PRISM Big Dye TM Terminator Cycle Sequencing kit (Applied BioSystems, Foster City, CA, USA). Electrophoresis of the sequencing reactions was completed using an automated ABI PRISM 3730XL DNA Sequencing System (Applied BioSystems).

The sequences were subsequently analyzed with the AlignX tool in the Vector NTI program (Invitrogen). BLAST searches were carried out using both the blastn and blastx algorithms provided by the National Center for Biotechnology Information website (http.//www.ncbi.nlm.nih.gov).

Phylogenetic analysis based on 16S rRNA and *ompA* gene sequences

Phylogenetic analysis were conducted using Bioedit software and Molecular Evolutionary Genetics Analysis 5 software with bootstrap values calculated from 1,000 replicates based on the 16S rRNA sequences of the 27 *R. anatipestifer* isolates and the eight reference strains of *R. anatipestifer* (AY871834.2, AY871822.2, CP002562.1, EU715016.1, EU016548.1, EU715000.1 EU715008.1, and EU715011.1) (Tamura *et al.,* 2011). The neighbor-joining method was used to construct the phylogenetic tree. Additionally, a phylogenetic tree analysis was carried out based on the *ompA* nucleotide sequences of the 27 isolated strains and 12 reference strains (CP002562.1, GQ415419.1, DQ059079, FJ765034.1, AY606207.1, AF104937.1, FJ765033.1, FJ765036.1, AY606226.1, FJ765037.1, HQ701132.1, and EU118677.1).

Amplification of housekeeping genes sequences

Four housekeeping genes (*mdh, gdh, pgi*, and *rpoB*) were selected, and PCR primers (Table 2) were designed using primer3 software (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) based on the known sequences of *R. anatipestifer* strains (*R. anatipestifer* ATCC 11845, CP003388.1).

The PCR reaction was performed in a final volume of 50 µl containing 100 ng of genomic DNA, 5 µl 10× Taq-Buffer, 4 µl 10 mM dNTP's, 1 U of Taq polymerase (Invitrogen) and 2 pmol of each

TABLE 1 - *Riemerella anatipestifer strains used in this study.*

Name	*Organ*	*Year*	*Sequencing result (%) (Accession numbers)*	
			16S rRNA	*ompA gene*
SNUFPC- RA 1	Brain	2011	99 (AY871834.2)	100 (CP002562.1)
SNUFPC- RA 2	Brain	2011	99 (AY871834.2)	100 (CP002562.1)
SNUFPC- RA 3	Brain	2011	99 (AY871834.2)	100 (CP002562.1)
SNUFPC- RA 4	Brain	2011	99 (AY871834.2)	100 (CP002562.1)
SNUFPC- RA 5	Brain	2011	99 (AY871834.2)	100 (CP002562.1)
SNUFPC- RA 8	Brain	2011	100 (CP002562.1)	100 (CP002562.1)
SNUFPC- RA 9	Brain	2010	99 (AY871834.2)	100 (CP002562.1)
SNUFPC- RA 10	Brain	2010	100 (CP002562.1)	100 (CP002562.1)
SNUFPC- RA 11	Brain	2010	99 (AY871834.2)	100 (CP002562.1)
SNUFPC- RA 12	Brain	2010	100 (CP002562.1)	100 (CP002562.1)
SNUFPC- RA 13	Brain	2010	100 (CP002562.1)	100 (CP002562.1)
SNUFPC- RA 15	Brain	2011	100 (CP002562.1)	100 (CP002562.1)
SNUFPC- RA 16	Brain	2011	100 (CP002562.1)	100 (CP002562.1)
SNUFPC- RA 18	Brain	2011	100 (CP002562.1)	100 (CP002562.1)
SNUFPC- RA 19	Brain	2011	100 (CP002562.1)	100 (CP002562.1)
SNUFPC- RA 20	Brain	2011	100 (CP002562.1)	100 (CP002562.1)
SNUFPC- RA 23	Brain	2011	100 (CP002562.1)	100 (CP002562.1)
SNUFPC- RA 24	Brain	2011	100 (CP002562.1)	100 (CP002562.1)
SNUFPC- RA 25	Brain	2011	100 (CP002562.1)	100 (CP002562.1)
SNUFPC- RA 26	Brain	2011	100 (CP002562.1)	100 (CP002562.1)
SNUFPC- RA 27	Brain	2011	100 (CP002562.1)	100 (CP002562.1)
SNUFPC- RA 28	Brain	2011	100 (CP002562.1)	100 (CP002562.1)
SNUFPC- RA 29	Brain	2011	100 (CP002562.1)	100 (CP002562.1)
SNUFPC- RA 32	Brain	2011	100 (CP002562.1)	100 (CP002562.1)
SNUFPC- RA 33	Meninx	2011	100 (CP002562.1)	100 (CP002562.1)
KVCC-BA0001846	Brain	Unknown	99 (EU 715011.1)	95 (EU118677.1)
KVCC-BA0001842	Brain	Unknown	99 (EU 715011.1)	95 (EU118677.1)

TABLE 2 - *Primers used in this study.*

Name	*Sequences (5' to 3')*	*Target gene*	*Reference*
16S-UNI-L	AGA GTT TGA TCA TGG CTC AG	16S rRNA	Kuhnert *et al.* (1996)
Rcol-2	TGT TAC GAC TTA GCC CTA G	16S rRNA	Rubbenstroth *et al.* (2011)
RAOMPAF1 RAOMPAR3	GAC TGG CAA ACT TCA GTA GG GTA GCT TCA GCA GAA CCA AC	*ompA*	Subramaniam *et al.* (2000)
RAMDH1 RAMDH2	AGGAGCAGGAGCTGTAGGAG TAGCCGCAGTAGCGAATTTT	*mdh*	In this study
RAGDH1 RAGDH2	CAAGGGAAGTCGGATATGGA CGATGTTTGCTCCTTTCACA	*gdh*	In this study
RAPGI1 RAPGI2	TTGAGGGATTTTTCCGACAG GCTTCTCCCTCGGTTTTACC	*pgi*	In this study
RARPOB1 RARPOB2	TCAGCTTTAGGACCTGGTGGT ATGCCGAAATACCAGAAATCTGGT	*rpoB*	In this study
RAPRTC1 RAPRTC2	TCAGGCTGTGATTGCTTACG CGTCCACCATCATTTCAGTG	*prtC*	In this study
RAHAGA1 RAHAGA2	TGGGCAACAGATGACCAATA CATGCACCGTTACACTTTCC	*hagA*	In this study
RASSPA1 RASSPA2	TTTTTGCCTGGAGAAACAGG CCACGGTTGGAATTTTATGG	*sspA*	In this study

primer. After initial denaturation at 95°C for 30 s, 30 cycles of amplification was performed under the following conditions: 95°C for 30 s, 1 min at 54°C (57°C for the *rpoB* gene), and 1.5 min at 72°C. Cycling was followed by a final extension for 7 min at 72°C. Product lengths were 772 bp for the *mdh* gene, 895 bp for the *gdh* gene, 1016 bp for the *pgi* gene and 412 bp for the *rpoB* gene. Purified PCR products were sequenced as described previously.

Amplification of virulence associated genes sequences

Three virulence associated genes (*prtC*, *hagA*, and *sspA*) were selected, and PCR primers (Table 2) were designed using primer3 software based on the known sequences of *R. anatipestifer* strains (*R. anatipestifer* ATCC 11845, CP003388.1). The PCR reaction was performed in a final volume of 50 µl containing 100 ng of genomic DNA, 5 µl 10× Taq-Buffer, 4 µl 10 mM dNTP's, 1 U of Taq polymerase (Invitrogen) and 2 pmol of each primer. After initial denaturation at 95°C for 30 s, 30 cycles of amplification was performed under the following conditions: 95°C for 30 s, 1 min at 57°C, and 1.5 min at 72°C. Cycling was followed by a final extension for 7 min at 72°C. Product lengths were 839 bp for the *prtC* gene, 565 bp for the *hagA* gene, and 786 bp for the *sspA* gene. Purified PCR products were sequenced as described previously.

Sequence-based genotyping

Alignments of four housekeeping genes (*mdh, gdh, pgi* and *rpoB*) were trimmed to a uniform length (774 bp, 723 bp, 562 bp, and 351 bp, respectively), and alignments of three virulence associated genes (*prtC, hagA* and *sspA*) were also trimmed (744 bp, 486 bp, and 679 bp, respectively). To further analyze the genetic relationship between the isolates, sequences from the 27 isolates were compared to the available sequences of *R. anatipestifer* ATCC 11845 and RA-GD from Genbank (CP003388.1 and CP002562.1, respectively).

Allele sequences that differed from each other by one or more polymorphisms were attributed to

a unique allele number and each unique allelic profile, as defined by the allele number of the seven loci (four housekeeping genes combined with three virulence associated genes), was assigned a sequence type (ST) number.
In a SLST, a phylogenetic tree analysis was carried out based on the single locus alignment of the 27 isolated strains, but the trimmed alignments were used to construct a concatenated alignment in a MLST.

Nucleotide sequence accession numbers
Amplified sequences are available in the GenBank nucleotide sequence database with the following accession numbers: JQ339305, JQ339306, and JX069948 for the 16S rRNA sequences. JQ339307 and JX069949 for the *ompA* gene sequences. JX021740, JX021741 JX021742 JX021743 JX021744 JX021745 JX021746, JX021747, JX021748, JX021749, JX021750, and JX021751 for the *mdh* gene sequences. JX021752, JX021753, JX021754, JX021755, JX021756 JX021757, JX021758, JX021759, JX021760, JX021761, JX021762 JX021763, and JX021764 for the *gdh* gene sequences. JX021730, JX021731, JX021732, JX021733, JX021734, JX021735, JX021736 X021737, JX021738, and JX021739 for the *pgi* gene sequences., JX144362, JX144363, and JX144364 for the *rpoB* gene., JX032768, JX032769, and JX032770 for the *prtC* gene sequences. JX032771, JX032772, JX032773, and JX032774 for the *hagA* gene sequences. JX032775, JX032776, JX032777, and JX032778 for the *sspA* gene sequences.

RESULTS

Bacterial isolation and identification
Twenty-seven *R. anatipestifer* strains were isolated from ducklings with tremors in South Korea, between 2010 and 2011. These strains were identified as *R. anatipestifer* by PCR and sequencing of the 16S rRNA and *ompA* gene sequences (Table 1).

Analysis of 16S rRNA and *ompA* gene sequences
The 16S rRNA sequences were highly conserved among the isolated and reference strains (AY871834.2, AY871822.2, CP002562.1, EU715016.1, EU016548.1, EU715000.1 EU715008.1, and EU715011.1) from Genbank. The 16S rRNA sequences of the SNUFPC-RA1–5, RA9, and RA11 strains were 99% identical to those of AY871834.2 and AY871822.2. The 16S rRNA sequences of the SNUFPC-RA8, RA10, and RA12–33 strains were 100% identical to those of CP002562.1, EU715016.1, EU016548.1, EU715000.1, and EU715008.1. The 16S rRNA sequences of KVCC-BA0001846 and KVCC-BA0001842 were 99% identical to those of EU715011.1. A phylogenetic tree based on 16S rRNA sequences is shown in Figure 1.
The *ompA* gene sequences from 25 isolates were 100% identical to each other and also showed 100% sequence similarity to the known *ompA* genes of CP002562.1, GQ415419.1, DQ059079, FJ765034.1, AY606207.1, AF104937.1, and

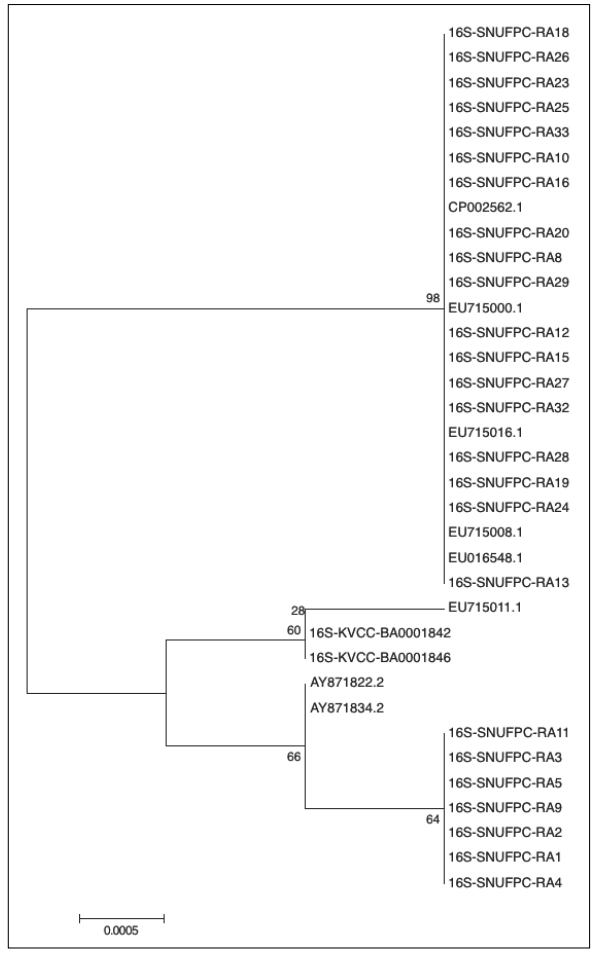

FIGURE 1 - *A neighbor-joining method tree based on the nucleotide sequences of the 16S rRNA sequences.*

FJ765033.1 and 99% similarity to the known *ompA* genes of FJ765036.1, AY606226.1, FJ765037.1, and HQ701132.1. A phylogenetic tree based on *ompA* genes sequences is shown in Figure 2.

Sequence-based genotyping

From the housekeeping gene sequences, 12 allele types in *mdh* loci, 13 allele types in *gdh* loci, 10 allele types in *pgi* loci, and three allele types in *rpoB* loci were found among isolates. From the virulence gene sequences, three allele types in *prtC,* and four allele types in *hagA* and *sspA* loci were found among the isolates. Based on this allelic profile, 24 STs were assigned ST1–ST24 (Table 3).

According to the MLST analysis, a tree revealed three major phylogroups, one of which (group 1) contained 17 isolates with 2 *R. anatipestifer* reference strains (ATCC 11845 and RA-GD), and the other 8 isolates belonged to the second group (group 2), whereas only 2 isolates (KVCC-BA0001846 and KVCC-BA0001842) were grouped together and highly distinguished (group 3). The phylogeny of the 27 *R. anatipestifer* isolates analyzed by the 4,319 bp concatenated sequences of seven loci is shown in Figure 3. According to the SLST analysis, trees from a single gene showed similar patterns with MLST analysis and were also separated into three distinct groups (data not shown).

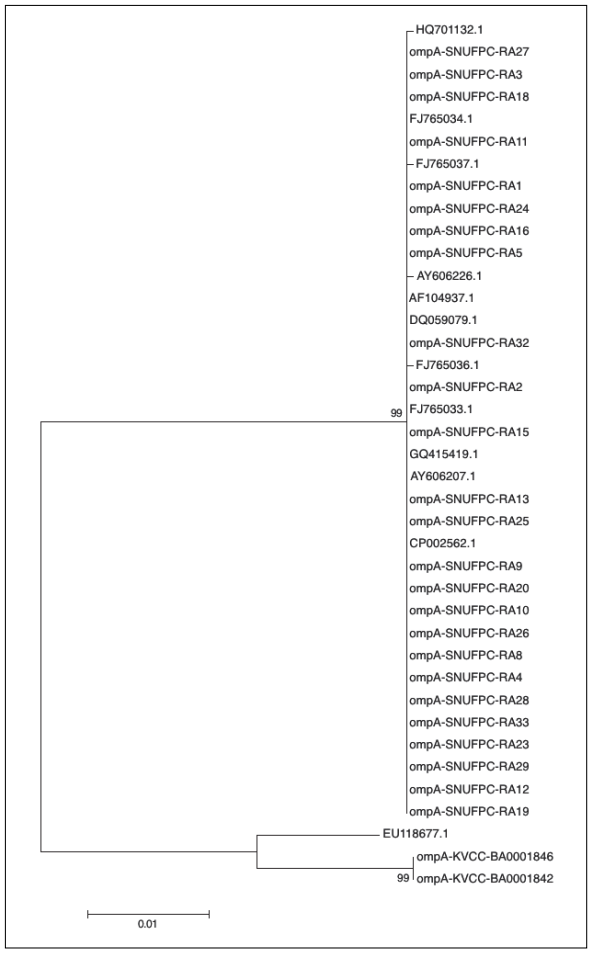

FIGURE 2 - *A neighbor-joining method tree based on the nucleotide sequences of the ompA gene sequences.*

DISCUSSION

Twenty-seven *R. anatipestifer* isolates were collected and identified from ducklings in South Korea, and new sequence-based genotyping methods were investigated for *R. anatipestifer* isolates.

Based on the 16S rRNA sequence analysis, *R. anatipestifer* belonged to the family Flavobacteriaceae in the rRNA superfamily V (Subramaniam *et al.,* 1997). The amplified 16S rRNA sequences were separated into three different groups in the phylogenetic tree but the scale bar (the number of nucleotide substitutions per site) was 0.0005, which did not support the existence of different strain lineages or serotypes (Figure 1). The *R. anatipestifer* 16S rRNA sequences seemed to be the best target to identify the isolates, but low genetic divergence patterns among the isolates made interpretation of genetic variation difficult.

Outer membrane proteins generally play an important role in virulence and induce a strong antibody response (Puohiniemi *et al.,* 1990; Weiser and Gotschlich, 1991). Additionally, the *R. anatipestifer ompA* gene is a new target to distinguish *R. anatipestifer* from other bacterial species and differentiation of *R. anatipestifer* (Subramaniam *et al.,* 1997; Subramaniam *et al.,* 2000; Yu *et al.,* 2008). However, the amplified *ompA* gene sequences of *R. anatipestifer* isolates were 100% identical to each other, except for two isolates (KVCC-BA0001846 and KVCC-BA0001842), and no genetic differences were found with those of reference strains (CP002562.1,

TABLE 3 - *Allelic profiles of R. anatipestifer isolates.*

Isolates	*Housekeeping genes*				*Virulence genes*			*Sequence types (STs)*
	mdh	*gdh*	*pgi*	*rpoB*	*prtC*	*hagA*	*sspA*	
SNUFPC- RA 1	1	1	1	1	1	1	1	ST1
SNUFPC- RA 2	1	1	2	1	1	1	1	ST2
SNUFPC- RA 3	2	1	1	1	1	1	1	ST3
SNUFPC- RA 4	3	1	1	1	1	1	1	ST4
SNUFPC- RA 5	3	1	1	1	1	1	1	ST4
SNUFPC- RA 8	4	1	1	1	1	1	1	ST5
SNUFPC- RA 9	5	2	6	2	2	2	2	ST6
SNUFPC- RA 10	6	2	3	2	2	3	2	ST7
SNUFPC- RA 11	7	3	4	2	2	2	2	ST8
SNUFPC- RA 12	8	1	5	1	1	1	1	ST9
SNUFPC- RA 13	8	1	1	1	1	1	1	ST10
SNUFPC- RA 15	6	2	6	2	2	2	2	ST11
SNUFPC- RA 16	6	2	6	2	2	2	2	ST11
SNUFPC- RA 18	6	4	6	2	2	2	2	ST12
SNUFPC- RA 19	7	5	6	2	2	2	2	ST13
SNUFPC- RA 20	6	2	7	2	2	2	2	ST14
SNUFPC- RA 23	6	2	6	2	2	2	2	ST11
SNUFPC- RA 24	6	6	8	2	2	2	2	ST15
SNUFPC- RA 25	6	7	6	2	2	2	2	ST16
SNUFPC- RA 26	9	8	6	2	2	2	2	ST17
SNUFPC- RA 27	10	2	6	2	2	2	2	ST18
SNUFPC- RA 28	11	2	8	2	2	2	2	ST19
SNUFPC- RA 29	6	9	9	2	2	2	2	ST20
SNUFPC- RA 32	6	10	6	2	2	2	2	ST21
SNUFPC- RA 33	6	11	6	2	2	2	2	ST22
KVCC-BA0001846	12	12	10	3	3	4	3	ST23
KVCC-BA0001842	12	13	10	3	3	4	4	ST24
No. of alleles	12	13	10	3	3	4	4	

GQ415419.1, DQ059079, FJ765034.1, AY606207.1, AF104937.1, FJ765033.1). Therefore this gene was unsuitable as a *R. anatipestifer* differentiation marker in the present study.

As an alternative, four housekeeping genes (*mdh, gdh, pgi,* and *rpoB*) and three virulence-associated genes (*prtC, hagA*, and *sspA*) were used in sequence-based genotyping methods. Among the 27 isolates, the developed typing scheme identified 24 STs and showed considerable divergence among the sequence of the seven genes. Sequence-based genotyping based on seven concatenated loci (MLST) revealed that 17 of the 27 isolates (group 1) were clustered together with two previously reported strain ATCC 11845 (CP003388.1) and strain RA-GD (CP002562.1) by Wang *et al.* (2012) and Yuan *et al.* (2011), but that the other isolates differed from these reference strains. Eight isolates (group 2) were branched together and somewhat related to group 1, whereas two isolates (group 3) were strongly distinguished from groups 1 and 2.

To demonstrate if there were important differences and to determine whether one of the seven

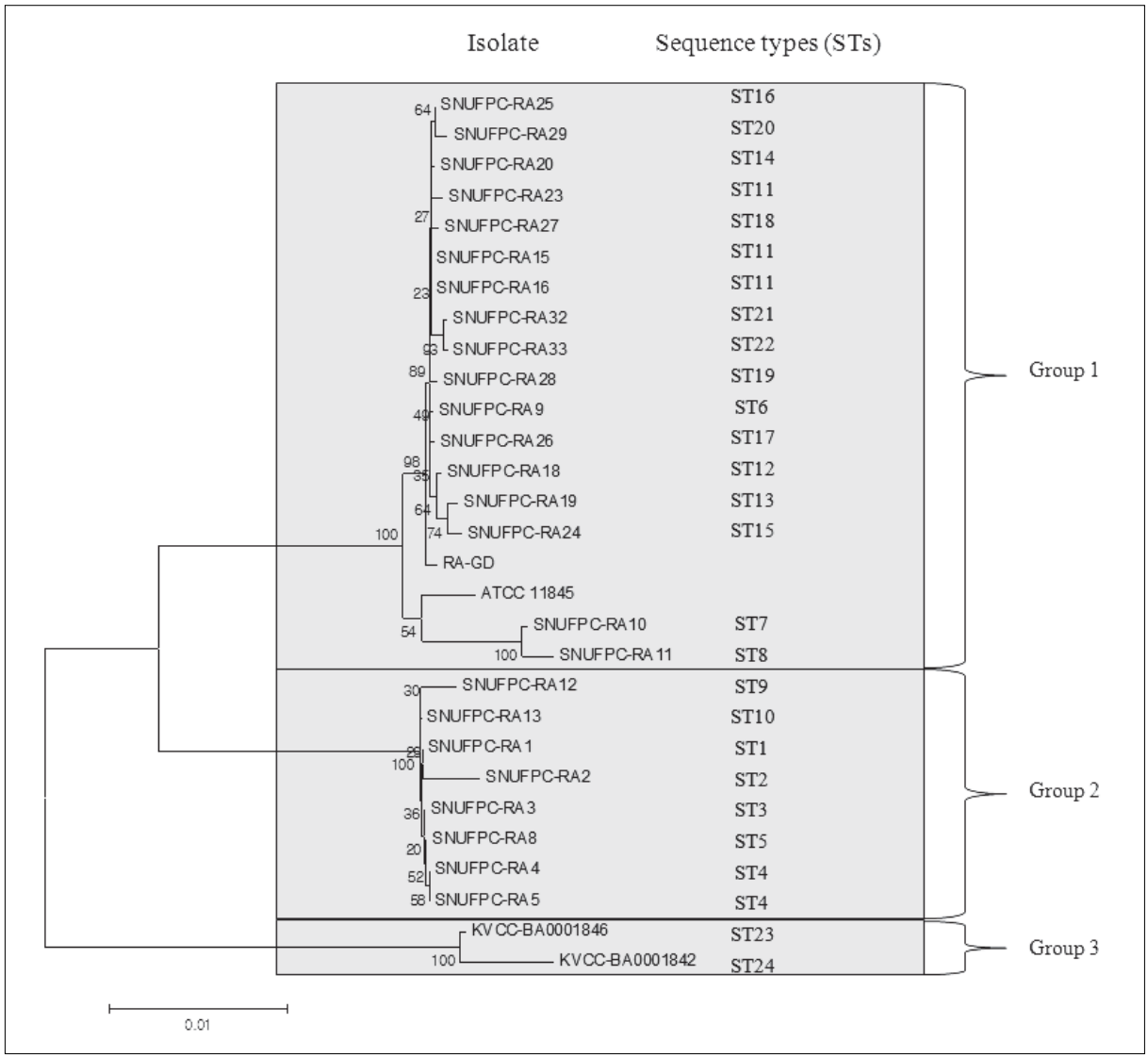

FIGURE 3 - *A tree based on concatenated nucleotide sequences of the four housekeeping (mdh, gdh, pgi, and rpoB) and the three virulence-associated (prtC, hagA, and sspA) genes.*

genes influenced this result, the phylogenetic tree from the concatenated sequence analysis (MLST) was compared to the seven independent trees constructed from each gene (SLST). As a result, the single gene based trees were very similar to the concatenated sequence tree (data not shown) and this comparison verified that the distribution of *R. anatipestifer* isolates into three groups did not result from the allelic diversity of a single gene but more likely from a general tendency of the whole genome.

Knowledge of the epidemiology of *R. anatipestifer* infections is limited by current genotyping methods and sequence-based typing investigated here will probably be the technique of choice for future global epidemiological studies. In addition, this study proposes sequence information for *R. anatipestifer*, because little work has been conducted on the molecular aspects of this organism.

In this study, 27 *R. anatipestifer* isolates were successfully identified and investigated by sequence-based genotyping methods. Four housekeeping genes (*mdh, gdh, pgi,* and *rpoB*) and three virulence-associated genes (*prtC, hagA*, and *sspA*) were used to classify the *R. anatipestifer* isolates in Korea. Sequence-based genotyping based on seven genes provided higher discriminatory power for the *R. anatipestifer* isolates than 16S rRNA or *ompA* gene-based analyses. In addition, according to the comparison of MLST and SLST, the result was not influenced by one of the seven genes and more likely by a general tendency of the whole genome.

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REFERENCES

ALONSO S., PETHE K., RUSSELL D.G., PURDY G.E. (2007). Lysosomal killing of Mycobacterium mediated by ubiquitin-derived peptides is enhanced by autophagy. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 6031-6036.

BABA T., ODAGIRI Y., MORIMOTO T., HORIMOTO T., YAMAMOTO S. (1987). An outbreak of *Moraxella (Pasteurella) anatipestifer* infection in ducklings in Japan. *Nippon Juigaku Zasshi.* **49**, 939-941.

GRAHAM R., BRANDLY C., DUNLAP G. (1938). Studies on duck septicemia. *Cornell Vet.* **28**, 1-8.

HENDRICKSON J., HILBERT K. (1932). A New and Serious Septicaemic Disease of Young Ducks with a Description of the Causative Organism, *Pfeifferella anatipestifer*. *Cornell Vet*. **22**, 239-252.

HUANG B., SUBRAMANIAM S., FREY J., LOH H., TAN H.M., FERNANDEZ C.J., KWANG J., CHUA K.L. (2002). Vaccination of ducks with recombinant outer membrane protein (OmpA) and a 41 kDa partial protein (P45N') of *Riemerella anatipestifer*. *Vet. Microbiol.* **84**, 219-230.

HUBALEK Z. (2004). An annoted checklist of pathogenic microorganisms associated with migratory birds. *J. Wildl. Dis.* **40**, 639-659.

KISS I., KARDOS G., NAGY J., TENK M., IVANICS E. (2007). DNA fingerprinting of *Riemerella anatipestifer* isolates from ducks. *Vet. Rec.* **160**, 26-28.

KUHNERT P., CAPAUL S.E., NICOLET J., FREY J. (1996). Phylogenetic positions of *Clostridium chauvoei* and *Clostridium septicum* based on 16S rRNA gene sequences. *Int. J. Syst. Evol. Microbiol.* **46**, 1174-1176.

LEAVITT S., AYROUD M. (1997). *Riemerella anatipestifer* infection of domestic ducklings. *Can. Vet. J.* **3**8, 113-113.

PUOHINIEMI R., KARVONEN M., VUOPIO-VARKILA J., MUOTIALA A., HELANDER I., SARVAS M. (1990). A strong antibody response to the periplasmic C-terminal domain of the OmpA protein of *Escherichia coli* is produced by immunization with purified OmpA or with whole *E. coli* or *Salmonella typhimurium* bacteria. *Infect. Immun.* **58**, 1691-1696.

RIMLER B.R., NORDHOLM G.E. (1998). DNA fingerprinting of *Riemerella anatipestifer*. *Avian Dis.* **42**, 101-105.

RUBBENSTROTH D., HOTZEL H., KNOBLOCH J., TESKE L., RAUTENSCHLEIN S., RYLL M. (2011). Isolation and characterization of atypical *Riemerella columbina* strains from pigeons and their differentiation from *Riemerella anatipestifer*. *Vet. Microbiol.* **147**, 103-112.

SEGERS P., MANNHEIM W., VANCANNEYT M., DE BRANDT K., HINZ K.H., KERSTERS K., VANDAMME P. (1993). *Riemerella anatipestifer* gen. nov., comb. nov., the causative agent of septicemia anserum exsudativa, and its phylogenetic affiliation within the *Flavobacterium-Cytophaga* rRNA homology group. *Int. J. Syst. Evol. Microbiol.* **43**, 768-776.

SUBRAMANIAM S., CHUA K.L., TAN H.M., LOH H., KUHNERT P., FREY J. (1997). Phylogenetic position of *Riemerella anatipestifer* based on 16S rRNA gene sequences. *Int. J. Syst. Evol. Microbiol.* **47**, 562-565.

SUBRAMANIAM S., HUANG B., LOH H., KWANG J., TAN H.M., CHUA K.L., FREY J. (2000). Characterization of a predominant immunogenic outer membrane protein of *Riemerella anatipestifer*. *Clin. Vaccine Immunol.* **7**, 168-174.

TAMURA K., PETERSON D., PETERSON N., STECHER G., NEI

M., KUMAR S. (2011). MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **28**, 2731-2739.

WANG X., ZHU D., WANG M., CHENG A., JIA R., ZHOU Y., CHEN Z., LUO Q., LIU F., WANG Y., CHEN X.Y. (2012). Complete Genome Sequence of *Riemerella anatipestifer* Reference Strain. *J. Bacteriol.* **194**, 3270-3271.

WEISER J.N., GOTSCHLICH E.C. (1991). Outer membrane protein A (OmpA) contributes to serum resistance and pathogenicity of *Escherichia coli* K-1. *Infect. Immun.* **59**, 2252-2258.

YU C.Y., LIU Y.W., CHOU S.J., CHAO M.R., WENG B.C., TSAY J.G., CHIU C.H., CHING WU C., LONG LIN T., CHANG C.C. (2008). Genomic diversity and molecular differentiation of *Riemerella anatipestifer* associated with eight outbreaks in five farms. *Avian Pathol.* **37**, 273-279.

YUAN J., LIU W., SUN M., SONG S., CAI J., HU S. (2011). Complete genome sequence of the pathogenic bacterium *Riemerella anatipestifer* strain RA-GD. *J. Bacteriol.* **193**, 2896-2897.

ZHONG C.Y., CHENG A.C., WANG M.S., ZHU D.K., LUO Q.H., ZHONG C.D., LI L., DUAN Z. (2009). Antibiotic Susceptibility of *Riemerella anatipestifer* Field Isolates. *Avian Dis.* **53**, 601-607.