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First Characterization of Fluoroquinolone Resistance in *Streptococcus suis*▽

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We have identified and sequenced the genes encoding the quinolone-resistance determining region (QRDR) of ParC and GyrA in fluoroquinolone-susceptible and -resistant *Streptococcus suis* clinical isolates. Resistance is the consequence of single point mutations in the QRDRs of ParC and GyrA and is not due to clonal spread of resistant strains or horizontal gene transfer with other bacteria.

Streptococcus suis is a gram-positive bacterium distributed worldwide that causes meningitis, endocarditis, septicemia, septic arthritis, pneumonia, and abortion in humans and pigs (32). In intensive swine industry, *S. suis* infections are one of the major causes of bacterial infections and economic loss. Sporadically, human cases are described, being considered by the World Health Organization an occupational disease due to infection through direct contact with pigs or pig products (34). In Southeastern Asia transmission to humans usually occurs in a constant rate, but sudden outbreaks have also recently been reported (31). During the summer of 2005, 215 people were infected by *S. suis* in the Sichuan province, China; 39 (18%) of these infections led to a fatal outcome (35). Although deaths were also caused by meningitis, they were mainly due to a novel form of invasive toxic shock syndrome (29). In all cases, *S. suis* from pigs was the origin of the outbreak.

Along with aminopenicillins, quinolones (fluoroquinolones), such as enrofloxacin in pigs or ciprofloxacin in humans, were the preferred treatment for *S. suis* infections (17). Most importantly, these antimicrobials are currently used against gram-negative pathogens in pigs that are frequently carriers of *S. suis*. In recent years, we have observed an emergence of fluoroquinolone-resistant strains among clinical swine *S. suis* isolates. The emergence of this resistance in a zoonotic pathogen such as *S. suis* has unpredictable consequences for pig production and public health (1, 33).

Resistance to fluoroquinolones in streptococci is mainly due to specific point mutations in the quinolone resistance-determining regions (QRDRs) of the GyrA subunit of the DNA gyrase and in the ParC subunit of the DNA Topoisomerase IV, enzymes that control DNA topology (6). Acquisition of mutations in the coding genes of these subunits, *gyrA* and *parC*, has been related to the appearance of single amino acid substitutions at positions S79 and D83 in ParC or S81 and E85 in GyrA. Furthermore, the genes or gene fragments containing these mutations may be transferred to other streptococci, rendering them resistant to fluoroquinolones by recombining their susceptible gene with the resistant homologue (3, 18).

(An initial report of this study has been presented at the 16th European Congress for Clinical Microbiology and Infectious Diseases [J. A. Escudero, et al., Abstr. p1251, 2006].).

The Veterinary Health Surveillance Group (VISAVET) at the Veterinary School in Madrid diagnoses bacterial diseases in Spanish pig farms. Since 2003, 992 samples have been identified through the commercial biochemical Rapid ID32 system as being *S. suis*. From these samples, ~1.2% (12 isolates) were highly resistant to enrofloxacin, a fluoroquinolone currently used for the treatment of insidious infections in pig farms as an alternative to aminopenicillins. To characterize fluoroquinolone resistance in these strains, a more complete quinolone and fluoroquinolone resistance profile with MIC determination of all resistant bacteria was assessed (Table 1). Microdilutions with incubations at 37°C for 24 h with antimicrobials supplied by Sigma Aldrich (Sigma Chemical Co., St. Louis, MO) were performed. The breakpoints used for enrofloxacin were those recommended by the Clinical and Laboratory Standards Institute (formerly National Committee for Clinical Laboratory Standards) in 2002 (24) for veterinary gram-positive microorganisms. All isolates were highly resistant to the three quinolones tested (nalidixic acid, flumequine, and oxolinic acid), with an MIC of ≥64 μg/ml. Further, all isolates resistant to enrofloxacin were highly resistant to ciprofloxacin, levofloxacin, and norfloxacin, with MICs ranging from 16 to ≥64 μg/ml. Six fluoroquinolone-susceptible strains, including type strain ATCC 43765, a clinical isolate from South America, and four epidemiologically unrelated isolates from our collection, were also analyzed for their antimicrobial profiles. All susceptible strains had MICs to ciprofloxacin of 0.5 μg/ml and were highly resistant to quinolones such as flumequine, oxolinic acid (data not shown), and nalidixic acid (MIC ≥ 16 μg/ml).

Before continuing characterization of these *S. suis* isolates, species identification was confirmed. For this purpose, the recently described PCR technique based on amplification of a 688-bp fragment from the glutamate dehydrogenase gene

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TABLE 1. Susceptibility to selected quinolones and fluoroquinolones of *S. suis* strains

Strain	MIC (μg/ml)[a]						Country or region	Source or reference
	CIP	CIP+R	ENR	NOR	LVX	NAL		
ATCC 43765	0.5	0.5	0.5	4	1	>64	England	Collection
BB1001	0.5	0.5	0.5	2	1	>64	Spain	This study
BB1002	0.5	0.5	0.5	2	1	>64	South America	This study
BB1003	0.5	0.5	0.5	4	0.5	>64	Spain	This study
BB1004	0.5	0.5	0.5	2	1	>64	Spain	This study
BB1005	0.5	0.5	0.25	4	0.5	>64	Spain	This study
BB1006	2	1	1	32	1	>64	Spain	This study
BB1007	8	4	4	16	4/2	>64	Spain	This study
BB1008	64	32	32	>64	16	>64	Spain	This study
BB1009	32	16	16	32	16	>64	Spain	This study
BB1010	32	16	32	>64	32	>64	Spain	This study
BB1011	16	16	16	32	16	>64	Spain	This study
BB1012	32	16	8	>64	16	>64	Spain	This study
BB1013	64	16	8	>64	16	>64	Spain	This study
BB1014	64	16	16	>64	32	>64	Spain	This study
BB1015	32	16	16	>64	16	>64	Spain	This study
BB1016	32	16	8	>64	32	>64	South America	This study
BB1017	64	16	16	>64	64	>64	Spain	This study

[a] CIP, ciprofloxacin; ENR, enrofloxacin; LVX, levofloxacin; NAL, nalidixic acid; NOR, norfloxacin; R, reserpine (10 μg/ml).

(*gdh*) from *S. suis* was used (25) (Table 2). All isolates biochemically identified in our laboratory as *S. suis* gave a positive PCR signal (data not shown), showing that biochemical identification together with experienced laboratory personnel is a reliable method for the identification of *S. suis* (17). To assess the genetic variability of these strains, the 12 fluoroquinolone-resistant and the 6 fluoroquinolone-susceptible isolates were subjected to pulsed-field gel electrophoresis (PFGE) essentially as described previously (30). All isolates showed different PFGE patterns, confirming the high genetic diversity of *S. suis* shown in previous studies (5, 30) and implying that fluoroquinolone resistance in *S. suis* is not due to the clonal spread of a resistant isolate but rather to a characteristic independently acquired by each isolate (Fig. 1).

As stated above, the products of the *parC* and the *gyrA* genes are the primary and secondary targets of ciprofloxacin in other streptococci (2, 23). However, these genes have not been identified in *S. suis*. To determine the QRDRs of *parC* and *gyrA* in this species, the genes in the six unrelated fluoroquinolone-susceptible *S. suis* isolates were amplified and sequenced (Table 2). The predicted amino acid sequence of the *parC* and *gyrA* genes revealed, in all six fluoroquinolone-susceptible isolates, no single amino acid difference in the QRDRs, showing the high degree of conservation of this regions, even in geographically and epidemiologically unrelated strains (Fig. 2 and 3).The QRDR of ParC in *S. suis* is homologous to the ParC sequences of other streptococci, with identities ranging from 97% with the QRDRs from *S. oralis* and *S. mitis* to 88% with the QRDRs from *S. agalactiae* and *S. mutans* (Fig. 2). Analogously, the QRDRs of GyrA presented an identity from 95% with *S. pyogenes* to 92% with *S. equi* subsp. *equi* and *S. equi* subsp. *equisimilis*.

To assess the involvement of GyrA and ParC of *S. suis* in fluoroquinolone resistance, the QRDRs of the *gyrA* and *parC* genes were determined in 12 fluoroquinolone-resistant clinical isolates (Table 3). In all cases, an amino acid change in a position known to be related to fluoroquinolone resistance in other streptococci (S79 and D83 in ParC or S81 and E85 in GyrA) was detected. Most isolates had one or two modifications in both GyrA and ParC, indicating that, as in many other species, both proteins are involved in fluoroquinolone resistance in *S. suis*. One isolate, BB1006, had a single nucleotide substitution in the *parC* gene (TCC→T**T**C), giving rise to an S79F replacement, implying that one mutation in *parC* is sufficient to confer resistance to these antimicrobials. The finding suggests that topoisomerase IV is a primary target of fluoroquinolones in *S. suis*, as in other gram-positive bacteria such as *S. aureus* (9) and *S. pneumoniae* (23). However, it has been shown that mutations in either gyrase or topoisomerase IV can occur depending on the structure of the fluoroquinolone (26). The isolates analyzed in the present study were probably selected through treatment with enrofloxacin. Studies from our

TABLE 2. Primer sets used to amplify the *gdh*, *gyrA*, and *parC* genes of *S. suis*

Gene	Primer	Sequence (5′–3′)	Position	GenBank accession no.
gdh	JP4	GCAGCGTATTCTGTCAAACG	1177–1196	AF229683
	JP5	CCATGGACAGATAAAGATGG	508–527	
gyrA	*gyrA*-F	CGCCGTATTTTGTATGGGATG	130–150	DQ832724
	gyrA-R	GTTCCGTTAACCAGAAGGTT	487–507	
parC	*parC*-F	AAGGACGGCAACACTTTTGAC	151–171	DQ832742
	parC-R	AGTGGGTTCTTTTTCCGTATC	442–462	

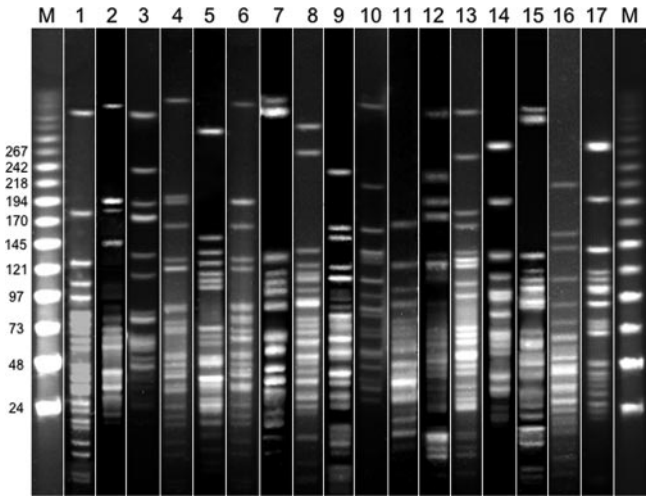

FIG. 1. PFGE fingerprint patterns of *S. suis* isolates used in the present study using the ApaI endonuclease. Lanes 1 to 6, fluoroquinolone-susceptible strains; lanes 7 to 17, fluoroquinolone-resistant isolates. The first and last lanes are bacteriophage lambda ladder PFGE markers (Boehringer Mannheim, Germany); lane 1, *S. suis* ATCC 43765; lane 2, BB1001; lane 3, BB1002; lane 4, BB1003; lane 5, BB1004; lane 6, BB1005; lane 7, BB1006; lane 8, BB1007; lane 9, BB1008; lane 10, BB1009; lane 11, BB1011; lane 12, BB1012; lane 13, BB1013; lane 14, BB1014; lane 15, BB1015; lane 16, BB1016; lane 17, BB1017. Analysis was performed with QuantityOne software (Bio-Rad, Richmond, CA).

laboratory with other fluoroquinolones show that single mutations in GyrA, but not in ParC, may be selected in vitro, indicating that the primary target of fluoroquinolones in *S. suis* may also depend on the type of molecule selecting resistance (data not shown). Ten other isolates carried identical amino acid substitution at the same codon of ParC, S79Y, albeit combined with either a single (S81Y, S81K, S81I, or S81F) or double (S81K E85D) amino acid change in GyrA. One strain, BB1007, presented a unique combination, with substitutions D83H in ParC and E85K in GyrA (Table 3). Nonetheless, it is worth mentioning that these data do not fully characterize fluoroquinolone resistance in *S. suis*. The reduction of the MIC down to 16 μg/ml in three resistant isolates when the pump inhibitor reserpine was added to ciprofloxacin shows that active efflux pumps, such as PmrA in *S. pneumoniae* (11) or Lde in *Listeria monocytogenes* (12), may play a role in resistance in *S. suis*. Further, mutations in GyrB or ParE may also contribute to resistance to fluoroquinolones in *S. suis*, as is the case in viridans group streptococci (13) and pneumococci (15).

Horizontal gene transfer has been shown to be responsible for the development of resistance to fluoroquinolones in *S. pneumoniae*, which acquires *gyrA* or *parC* with resistance-conferring mutations from viridans streptococci (3, 8, 27), and in *S. pyogenes*, which can acquire resistance from a mutated *parC* gene from *S. dysgalactiae* (28). This would be especially problematic in the case of *S. suis*, which could serve as donor of fluoroquinolone resistance for other pathogenic bacteria more widely distributed in humans than *S. suis*. In our strains,

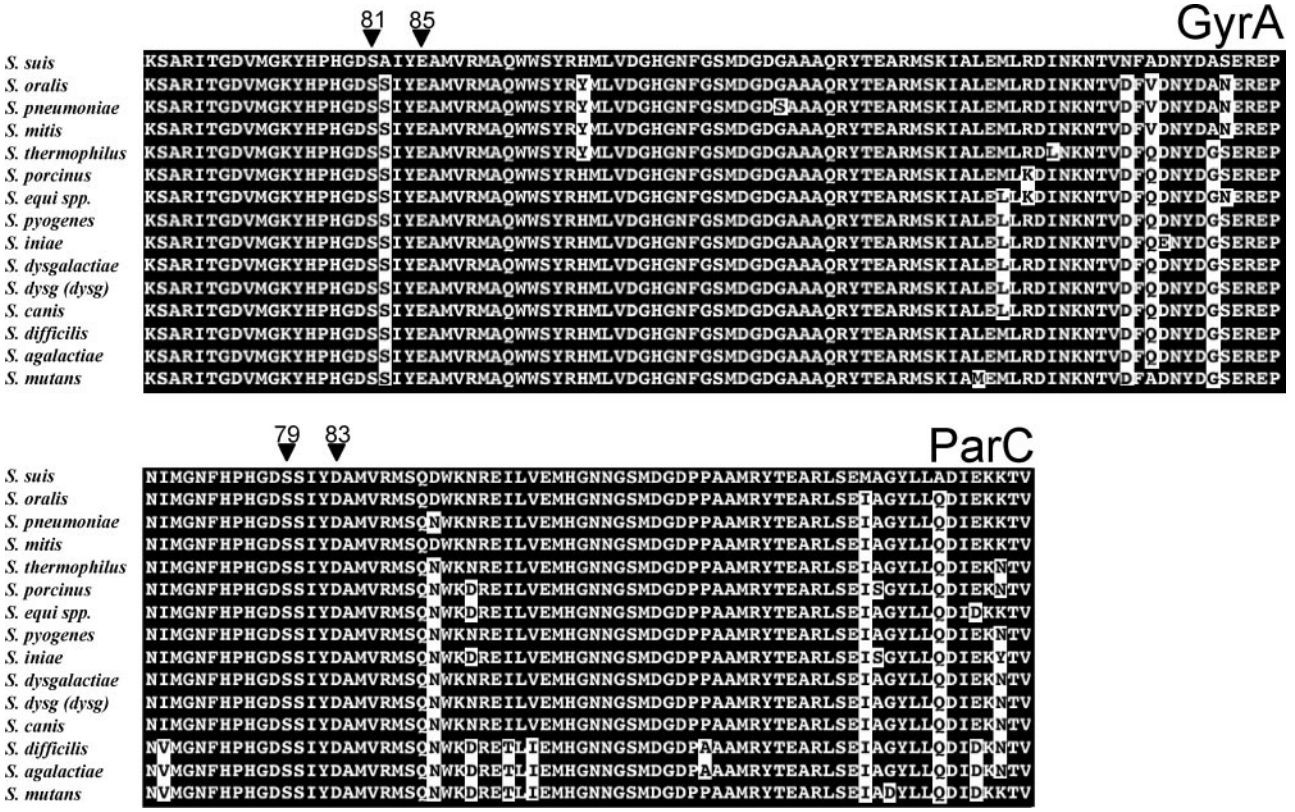

FIG. 2. Amino acid sequence alignment of the QRDRs of GyrA and ParC in streptococci. Amino acids critical for fluoroquinolone resistance are marked with an arrowhead and standard *E. coli* numbering. White shading denotes different amino acids in relation to the *S. suis* sequence.

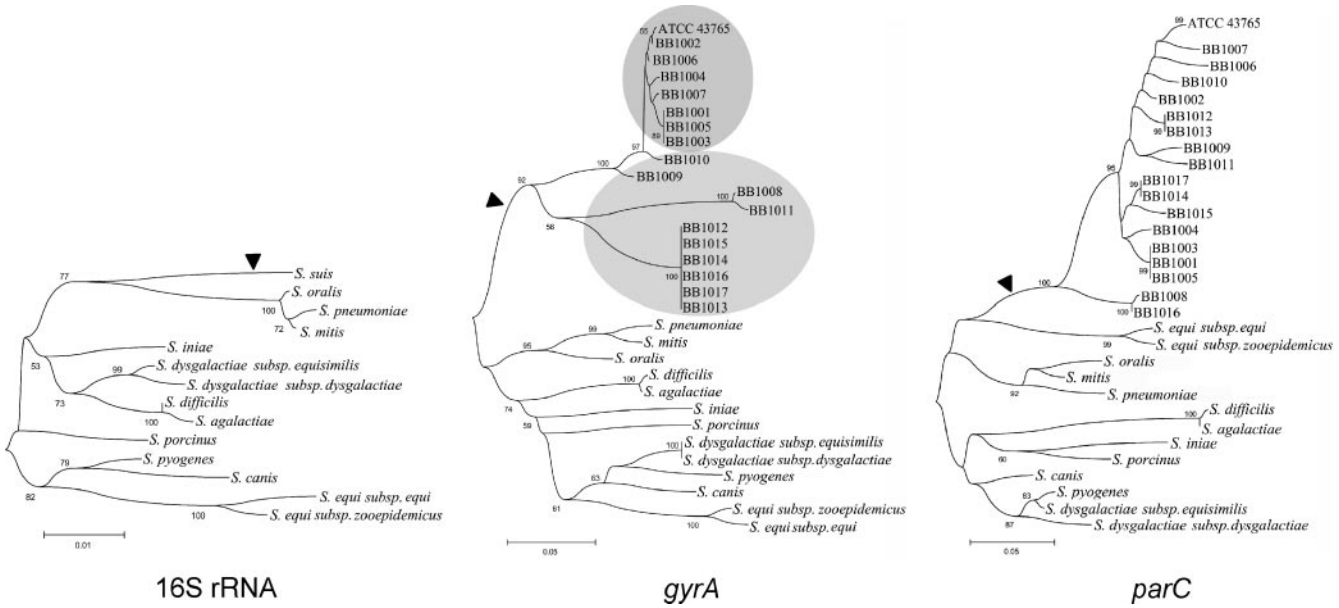

FIG. 3. Dendrograms from the nucleotide sequences of 16S rRNA gene and the QRDRs of *gyrA* and *parC* of streptococci. Note that in the 16S rRNA gene tree the branch of *S. suis* (arrowhead) contains in the corresponding *gyrA* and *parC* trees all of the *S. suis* strains analyzed in the present study (arrowheads), implying that these genes from *S. suis* were not acquired from other streptococci by horizontal gene transfer. Further, the *gyrA* dendrogram clusters fluoroquinolone-susceptible and low-level resistant strains (upper oval) and high-level resistant strains (lower oval), whereas in the *parC* tree the strains are dispersed, indicating that *parC* is more prone to accumulating mutations than *gyrA*. In line with this, the 16S rRNA gene dendrogram has no significant differences with the *gyrA* tree, whereas streptococci are clustered differently in the dendrogram constructed with the *parC* sequences. The dendrograms were constructed with MEGA software version 3.1 (21) by using the neighbor-joining grouping procedure with the Kimura two-parameter distance measure (19). The bar denotes genetic distance. Bootstrap values are the result of 1,000 iterations.

gyrA and *parC* showed an identity of ≥96% and no trace of non-*S. suis* DNA in either gene, as revealed by the dendrograms (Fig. 3). The GenBank database was screened for the presence of DNA sequences of *gyrA* and *parC* from *S. suis* in other bacterial species. This was performed by launching sequential 10-bp-overlapping 30-bp fragments of the *parC* or *gyrA* genes encoding fluoroquinolone resistance against the GenBank database, estimating 100% identity as horizontal gene transfer. Neither acquisition nor donation of *gyrA* or *parC* fragments was observed, and resistance is due to the emergence of mutations in each isolate, as previously shown in enterococci (7, 16) and staphylococci (10, 22). Thus, these

TABLE 3. Amino acid and codon changes in critical positions of the *gyrA* and *parC* QRDRs in *S. suis*[a]

Strain	CIP MIC (μg/ml)	*gyrA*				*parC*			
		Position 81		Position 85		Position 79		Position 83	
		Codon	Aa	Codon	Aa	Codon	Aa	Codon	Aa
ATCC	0.5	AGT	Ser	GAA	Glu	TCC	Ser	GAT	Asp
BB1001	0.5	-	-	-	-	-	-	-	-
BB1002	0.5	-	-	-	-	-	-	-	-
BB1003	0.5	-	-	-	-	-	-	-	-
BB1004	0.5	-	-	-	-	-	-	-	-
BB1005	0.5	-	-	-	-	-	-	-	-
BB1006	2	-	-	-	-	**T**TC	Phe	-	-
BB1007	8	-	-	**A**AA	Lys	-	-	**C**AT	His
BB1008	64	**TA**T	Tyr	-	-	TA**C**	Tyr	-	-
BB1009	32	A**AG**	Lys	-	-	TA**C**	Tyr	-	-
BB1010	32	A**T**T	Ile	-	-	TA**C**	Tyr	-	-
BB1011	16	**TT**T	Phe	-	-	TA**C**	Tyr	-	-
BB1012	32	A**AG**	Lys	G**A**C	Asp	TA**C**	Tyr	-	-
BB1013	64	A**AG**	Lys	G**A**C	Asp	TA**C**	Tyr	-	-
BB1014	64	A**AG**	Lys	G**A**C	Asp	T**AT**	Tyr	-	-
BB1015	32	A**AG**	Lys	G**A**C	Asp	TA**C**	Tyr	-	-
BB1016	32	A**AG**	Lys	G**A**C	Asp	TA**C**	Tyr	-	-
BB1017	64	A**AG**	Lys	G**A**C	Asp	T**AT**	Tyr	-	-

[a] CIP, ciprofloxacin. -, identical to the ATCC strain. Sequence differences are indicated in boldface. Aa, amino acid.

genes from *S. suis* do not represent an antimicrobial resistance reservoir for other animal or human pathogens, at least in our strains. This analysis also revealed that evolution of *gyrA* in *S. suis* elicits a clear clustering of bacteria highly resistant on one branch and susceptible or low-level resistant to fluoroquinones in another branch (Fig. 3). This indicates that gyrases are genetically more stable than topoisomerases IV and reflect species microevolution (14), as shown by the recent use of these genes in multilocus sequence typing protocols in *Yersinia* and *Acinetobacter* spp. (4, 20). The analysis of *gyrA* and *parC* in more *S. suis* isolates would be a significant step forward to further understand the mechanisms and spread of fluoroquinolone resistance in this emerging zoonotic pathogen.

Nucleotide sequence accession numbers. The nucleotide sequences for the *gyrA* and *parC* QRDRs have been deposited in GenBank under the following respective accession numbers: ATCC 43765, DQ832724 and DQ832742; BB1001, DQ832725 and DQ832743; BB1002, DQ832726 and DQ832744; BB1003, DQ832727 and DQ832745; BB1004, DQ832728 and DQ832746; BB1005, DQ832729 and DQ832747; BB1006, DQ832730 and DQ832748; BB1007, DQ832731 and DQ832749; BB1008; DQ832732 and DQ832750; BB1009, DQ832733 and DQ832751; BB1010, DQ832734 and DQ832752; BB1011, DQ832735 and DQ832753; BB1012, DQ832736 and DQ832754; BB1013, DQ832737 and DQ832755; BB1014, DQ832738 and DQ832756; BB1015, DQ832739 and DQ832757; BB1016, DQ832740 and DQ832758; and BB1017, DQ832741 and DQ832759.

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