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Sequencing of the *Chlamydophila psittaci ompA* Gene Reveals a New Genotype, E/B, and the Need for a Rapid Discriminatory Genotyping Method

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Twenty-one avian *Chlamydophila psittaci* isolates from different European countries were characterized using *ompA* restriction fragment length polymorphism, *ompA* sequencing, and major outer membrane protein serotyping. Results reveal the presence of a new genotype, E/B, in several European countries and stress the need for a discriminatory rapid genotyping method.

Chlamydophila psittaci is classified into 6 serovars, designated A to F. Serovar A is endemic among psittacine birds and causes zoonotic disease in humans. Serovar B is endemic among pigeons, has been isolated from turkeys, and was the cause of abortion in a dairy herd. Serovar C isolates (GD, MT1 and 91/1264, 91/1301, CT1 and Par1) were obtained from a German, Bulgarian and Belgian duck, a white swan, and a Californian turkey and a partridge, respectively. Serovar D has mainly been isolated from turkeys but also from a seagull, a budgerigar, and from humans. Serovars C and D are known occupational hazards for poultry workers. Serovar E isolates, known as Cal-10, MP, or MN (meningopneumonitis), were isolated during an outbreak of human pneumonitis in the late 1920s and early 1930s. Subsequently, MN isolates have been obtained from a variety of birds worldwide, including ducks, pigeons, ostriches, and rheas. A single serovar F isolate, VS225, was obtained from a parakeet (1–3, 8, 10, 11, 21).

Serotyping is performed with a panel of serovar-specific monoclonal antibodies (MAbs) in an immunofluorescence test. MAb-secreting hybridomas were previously screened by immunofluorescence staining using whole organisms (1, 2) and, as for *Chlamydia trachomatis*, were believed to recognize the major outer membrane protein (MOMP) (4). Only the serovar A- and D-specific MAbs were characterized using Western blotting and a radioimmunoprecipitation assay (RIPA), and those MAbs indeed recognized MOMP (22). However, the other serovar-specific MAbs still needed to be characterized. Consequently, the first objective of the present study was the characterization of the complete panel of serovar-specific MAbs. The second objective was to compare MOMP serotyping, *ompA* restriction fragment length polymorphism (RFLP), and *ompA* sequencing for characterizing *C. psittaci* strains from *Psittaciformes*, *Columbiformes*, *Anseriformes*, and *Galliformes*. MOMP serotyping and *ompA* RFLP have been compared previously (15, 18, 21) and sometimes gave incongruent results. Therefore, *ompA* sequencing was included as the reference typing method.

Twenty-one *C. psittaci* isolates from Belgium ($n = 7$), Germany ($n = 6$), Italy ($n = 7$), and The Netherlands ($n = 1$) were characterized (Table 1). Additionally, serovars A to F reference strains VS1, CP3, GD, NJ1, MN, and VS225 were used (2) (Table 2). Bacteria were grown in Buffalo Green Monkey (BGM) cells as described previously (21, 23).

Serovar-specific MAbs VS1/7A7/E8, CP3/4C6/G2, GD/2H9, NJ1/2GB/C2, MN/4A10/A4, and VS225/D6/36 (2) were characterized using immunoblotting and a RIPA. Isotypes were determined using the Hbt Mouse Monoclonal Isotyping kit (Sanbio, Uden, The Netherlands). Immunoblotting was performed as previously described (24). An irrelevant monoclonal antibody of the same isotype as well as phosphate-buffered saline (PBS) was used as negative control, while a family-specific MAb (7B6III) served as positive control. RIPA cell lysates were prepared using a modification of the method described by Caldwell and Perry (6). Briefly, reference strains were cultured in cycloheximide-treated BGM cells using minimum essential medium without methionine (Sigma-Aldrich, St. Louis, Mo.) supplemented with 25 μCi (35S) L-methionine (TRAN^{35}S-LABEL; MP Biomedicals, Irvine, CA) per ml. Three days postinoculation (PI), infected and noninfected control monolayers were washed with PBS (pH 7.4) and disrupted with RIPA lysis buffer (150 mM NaCl, 50 mM Trizma base, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 0.1% sodium dodecyl sulfate [SDS], 0.1% NaN_3, 1% Triton X-100, and 10% sodium deoxycholate) at 37°C for 30 min. Labeled chlamydial suspensions were centrifuged (10,000 × *g*, 10 min, 4°C). Supernatants were recovered and used as test antigen for immunoprecipitation. Immunoprecipitation was performed as

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TABLE 1. European *C. psittaci* isolates

Isolate no.	*C. psittaci* isolate characteristics				
	Year	Country	Host	Clinical signs	Reference
89/1291	1989	Belgium	*Melopsittacus undulatus*	Mortality with respiratory signs	20
90/1051	1990	Belgium	*Amazona* spp.	Mortality with respiratory signs	20
91/0154	1991	Belgium	*Melopsittacus undulatus*	Mortality with respiratory signs	20
91/0237	1991	Belgium	*Poicephalus senegalus*	Mortality with respiratory signs	20
5051A06	2001	Belgium	*Meleagris gallopavo*	Nasal discharge	This study
41A12	2001	Belgium	*Meleagris gallopavo*	Nasal discharge	This study
7778B15	2001	Belgium	*Meleagris gallopavo*	Nasal discharge	This study
WS/RT/E30	2001	Germany	*Anas platyrhynchos*	No clinical signs	This study
2/290300	2000	Germany	*Anas platyrhynchos*	No clinical signs	This study
3/20901	2001	Germany	*Anas platyrhynchos*	No clinical signs	This study
4/20901	2001	Germany	*Anas platyrhynchos*	No clinical signs	This study
5/20901	2001	Germany	*Anas platyrhynchos*	No clinical signs	This study
18/290800	2000	Germany	*Anas platyrhynchos*	No clinical signs	This study
99	1995	Italy	*Agapornis* spp.	Not available	This study
3005	1999	Italy	*Neophema elegans*	Mortality with respiratory signs	This study
61/8	1998	Italy	*Columba livia*[a]	No clinical signs	This study
3759/2	1999	Italy	*Columba livia*[a]	No clinical signs	This study
6098	1998	Italy	*Columba livia*[a]	No clinical signs	This study
7344/2	1997	Italy	*Columba livia*[a]	No clinical signs	This study
8615/1	1997	Italy	*Columba livia*[a]	No clinical signs	This study
92/1293	1992	The Netherlands	*Meleagris gallopavo*	Dyspnoea, mortality	20

[a] These birds were free-living urban pigeons.

described by Sambrook et al. (13) using the same controls as for immunoblotting. Samples were electrophoretically analyzed on a 12% SDS-polyacrylamide vertical slab gel (Mini Protean 3; Bio-Rad, Hercules, CA). Subsequently, the gel was washed in 7% acetic acid solution (5 min), double-distilled water (15 min), and finally Amplify (30 min) (Amersham Biosciences, Piscataway, N.J.). Afterwards, the gel was dried in a slab gel dryer (Hoefer; Amersham Biosciences) and analyzed 24 h later using the Typhoon 9200 phosphoimager (Amersham Biosciences).

TABLE 2. Serotyping and genotyping results for laboratory reference strains and European field strains

Animal order	Serotyping and genotyping results				
	Isolate	*ompA* sequencing	*ompA* RFLP	MOMP serotyping	Sequencing reference
Psittaciformes	VS1	A	A	A	This study
	99	A + E/B	A + E	B	This study
	3005	A	A	–[a]	This study
	89/1291	A	A	A	This study
	90/1051	A	A	A	This study
	91/0154	A	A	A	This study
	91/0237	A	A	A	This study
Columbiformes	CP3	B	B	B	5
	61/8	A + E/B	A + E	A + B	This study
	3759/2	E	E	E	This study
	6098	B	B	–[a]	This study
	7344/2	B + D	B + D	B	This study
	8615/1	B + E/B	B + E	B	This study
Anseriformes	GD	C	C	C	5
	WS/RT/E30	E/B	E	E + B	This study
	2/290300	E/B	E	E + B	This study
	3/20901	E/B	E	E + B	This study
	4/20901	E/B	E	E + B	This study
	5/20901	E/B	E	B	This study
	18/290800	E/B	E	E + B	This study
Galliformes	NJ1	D	D	D	5
	92/1293	D	D	D	22
	5051A06	A	A	A	This study
	41A12	B	B	B	This study
	7778B15	B + F	B + F	B	This study
Human	MN	E	E	E	This study
Parrot	VS225	F	F	F	5

[a] Two isolates could not be serotyped.

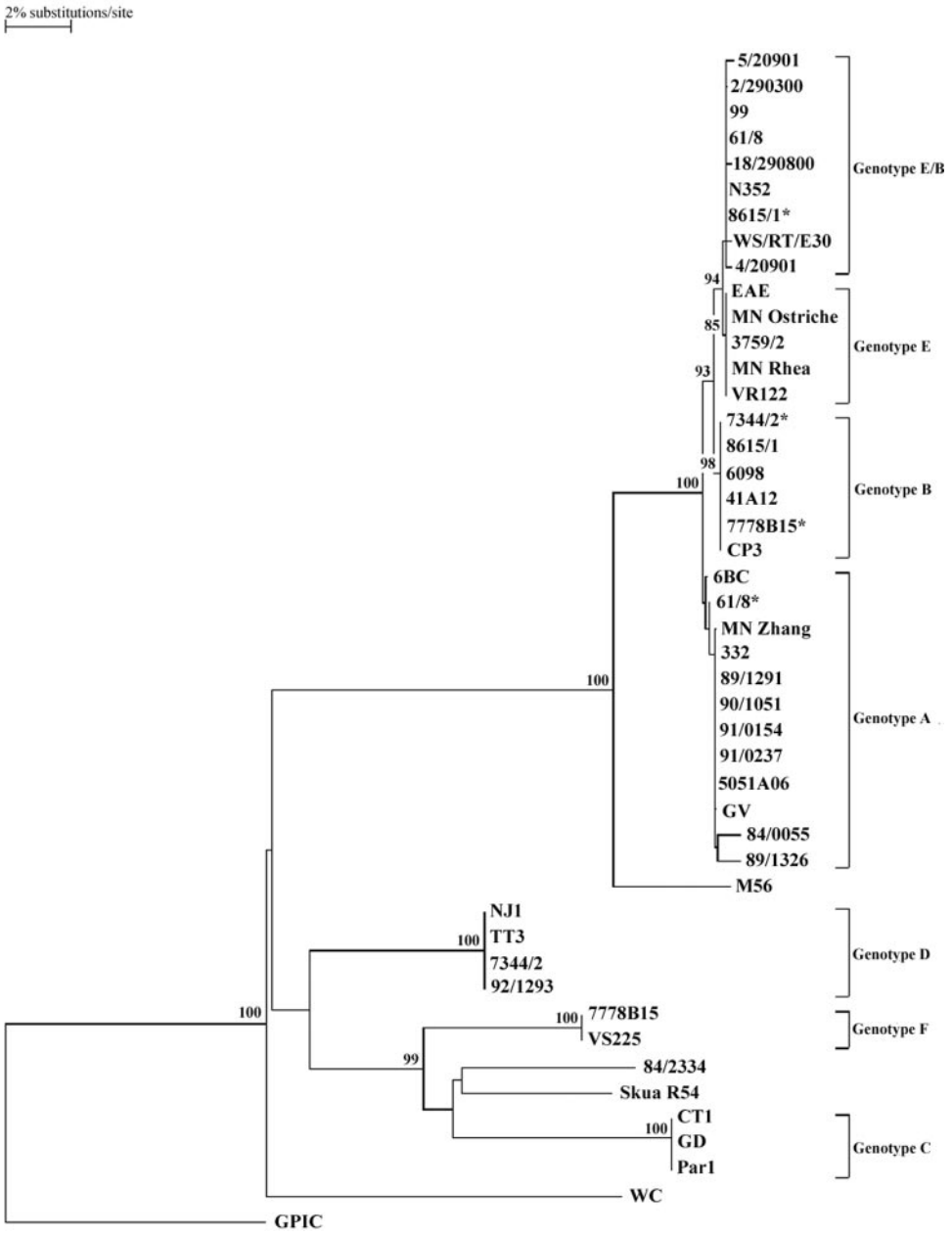

FIG. 1. *ompA* phylogenetic tree. An asterisk indicates that an additional sequence was found in the isolate. Bootstrap values are shown if the reliability is equal to or greater than 85%.

Genomic DNA for *ompA* sequencing was prepared as described by Wilson et al. (25). *ompA* was PCR amplified as described previously (22), resulting in a fragment of 1,065 to 1,098 bp depending on the strain. PCR products were purified using QIAGEN spin columns (Westburg, Leusden, The Netherlands) and cloned into pGemT (Promega, Madison, WI) following the manufacturer's protocol. Heat shock-transformed (30 s, 42°C) JM109-competent cells (Promega) were grown on Luria-Bertani (LB) plates in the presence of carbenicillin (30 μg/ml; Duchefa, Haarlem, The Netherlands). Five clones were selected and subsequently grown in liquid LB medium for plasmid purification (QIAGEN Tip 100 Westburg). *ompA* sequence analysis was performed by the VIB Genetic Service Facility (University of Antwerp, Belgium) and the Laboratory of Physiology and Immunology of Domestic Animals (KULeuven, Belgium) using vector associated T7 and SP6 priming sites.

Obtained *ompA* sequences were submitted to GenBank, aligned with previously published *ompA* sequences using ClustalX software (16), and subsequently imported into TreeconW (17) using the Jukes and Cantor algorithm and the Neighbor-Joining method (12) for designing the *ompA* phylogenetic tree (Fig. 1). Two thousand bootstraps were calculated (9). The tree was rooted with the *Chlamydophila caviae* (GPIC) *ompA* sequence (26) (GenBank AF269282).

AluI-based RFLP analysis was performed as previously described (21). Fragments were separated on a 2% LSI MP agarose gel (Life Science International, Zellik, Belgium). As control, sequences of the reference strains were pasted into Webcutter 2.0 (http://www.firstmarket.com/cutter/cut2.html), and the in silico-obtained bands were compared with the obtained gel-based calculation (Magelan Demo Software; Incogen, Williamsburg, VA).

Serotyping was performed by use of an indirect immunofluorescence staining on *C. psittaci*-infected BGM monolayers grown on 13-mm-diameter coverslips on the bottom of Chlamydia Trac Bottles (CTB; International Medical, Brussels, Belgium). Each isolate was inoculated in 8 CTBs, and 72 h postinfection (hpi) monolayers were fixed in methanol (10 min, −20°C), where after nonspecific binding sites were blocked with PBS (pH 7.3) plus 5% FCS (1 h, room temperature). All dilutions were prepared in PBS plus 1% bovine serum albumin (BSA), and all incubations were performed in a moist chamber at 37°C for 1 h. Following blocking, 25 μl of either PBS, the family-specific control MAb 7B6III, or the differentiating dilution of the six serovar-specific MAbs A to F was added. The differentiating dilution of the serovar-specific MAbs was designated as the dilution at which the serovar-specific MAb reacted only with the corresponding reference strain. Differentiating dilutions were 1/5,000 for all MAbs, except for the serovar D-specific MAb, where a dilution of 1/500 had to be used. The monolayers were incubated and washed three times (5 min) in PBS plus 1% BSA, and subsequently 25 μl of fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulin (IgG H+L; DakoCytomation; diluted 1/200) was added. CTBs were incubated and washed two times (5 min) in PBS followed by two times (30 s) in bidest. Coverslips were removed, mounted on microscopic glass slides, and examined with a confocal laser scanning microscope (×600; Radiance 2000; Bio-Rad).

None of the MAbs, except for the family-specific control MAb 7B6III, recognized MOMP in immunoblotting, whether reducing or nonreducing conditions were used. However, all MAbs recognized MOMP in the RIPA, indicating they recognize conformational epitopes, which is rational since 90% of antigen epitopes are predicted to be discontinuous and highly conformational (19). B-cell epitopes are considered to contain 15 to 20 residues derived from two to five peptide segments, occupying a surface of 70 to 90 nm (7). Strangely, the serovar A- and D-specific MAbs recognized MOMP in former immunoblottings and RIPAs and now failed to react in immunoblotting (22). Looking for an explanation, we found out that present and formerly used serovar A- and D-specific MAbs originated from different clones (A. A. Andersen, personal communication). Recognition by the formerly used MAbs was probably less highly conformation dependent, or MAbs showed a higher affinity for the serovar-specific epitope. However, maybe the *C. psittaci* MOMP, like in *C. trachomatis*, can carry more than one serovar-specific epitope (4), and present and formerly used MAbs were against different serovar-specific epitopes on MOMP. Epitope mapping could provide an answer. Isotyping revealed the following results: IgG1 (7B6III), IgG2b (VS1/7A7/E8, MN/4A10/A4, VS225/D6/36), and IgG3 (CP3/4C6/G2, GD/2H9, NJ1/2GB/C2).

ompA sequencing of 21 isolates revealed eight genotypes A, five genotypes B, two genotypes D, and one genotype E and F (Table 2). Remarkably, in nine birds, mostly ducks, genotyping discovered a new *ompA* sequence, designated E/B. The E/B *ompA* showed high amino acid homology with the *ompA* of genotypes A, B, and E (97.3%, 98.6%, and 99.5%, respectively) and was characterized by a unique combination of a guanosine on positions 1,006 and 1,021 and a cytidine on position 1,022 resulting in an A instead of S on position 341 in the variable segment 4 of MOMP. In 94% of the cases, bootstrap values in the *ompA* phylogenetic tree supported the creation of an E/B branch starting from the genotype E group (Fig. 1). The latter justified the proposition of the new genotype E/B, closely related to genotypes A, B, and E. Interestingly, BLAST search revealed one *ompA* sequence (N352) (GenBank L04980) 100% identical to our E/B sequence (5). N352 was isolated from an English duck and classified as serovar E using *ompA* RFLP (14). E/B strains were isolated in three different countries (Italy, Germany, and recently also Belgium) and in different hosts (urban pigeons, commercial fattening Pekin ducks, and turkeys). Genotype E/B was highly prevalent, occurring mostly in ducks, while the closely related genotype E was only discovered in one pigeon. RFLP cannot distinguish between genotype E/B and E. Perhaps the 12 duck strains (including N352) formerly classified by Sayada et al. (14) using *ompA* RFLP were actually genotype E/B instead of genotype E, leading to the conclusion that we may have underestimated the occurrence of genotype E/B in the past. Five out of 21 birds (3 urban pigeons, a lovebird, and a turkey) were simultaneously infected with two different genotypes, which is in accordance with previous studies showing the presence of mixed infections (20, 21).

Only 19 out of 21 isolates could be serotyped (Table 2), revealing six mixed infections. The two remaining isolates only reacted with the family-specific MAb 7B6III; RFLP was successful in all cases, and like sequencing, revealed five mixed infections. Differentiating restriction patterns were obtained for genotypes A to F when performing an in silico AluI digest (Fig. 2), but the new genotype E/B could not be distinguished from genotype E. Genotypes A and B generated characteristic bands of 110 and 117 bp, respectively. Genotype E could be distinguished from genotypes A and B because the restriction pattern lacked the 110 and 117 bands. Genotype C generated a specific band of 438 bp. Genotypes D and F were characterized by 471-bp and 222-bp bands, respectively (Fig. 2). Due to inefficient restriction digests, the gel-determined and theoretically calculated RFLP restriction patterns sometimes differed, although all isolates could be genotyped by looking at characteristic bands.

All of the known genotype C isolates except CT1 (2) were isolated from European waterfowl, leading to the hypothesis that genotype C is preferentially associated with waterfowl (21). In the present study, six duck isolates were included in order to examine this hypothesis. Unexpectedly, all ducks were infected with genotype E/B, resulting in RFLP restriction pattern E. Using serotyping, the E/B genotype was detected as serovar B (1 of 6) or a mixed serovar E plus B (5 of 6) infection, indicating the possibility of the occurrence of both serovar B and E epitopes on genotype E/B. Sequencing revealed genotype A in all psittacines and, additionally, genotype E/B in a lovebird (99). Expectantly, the E/B strain found in this lovebird

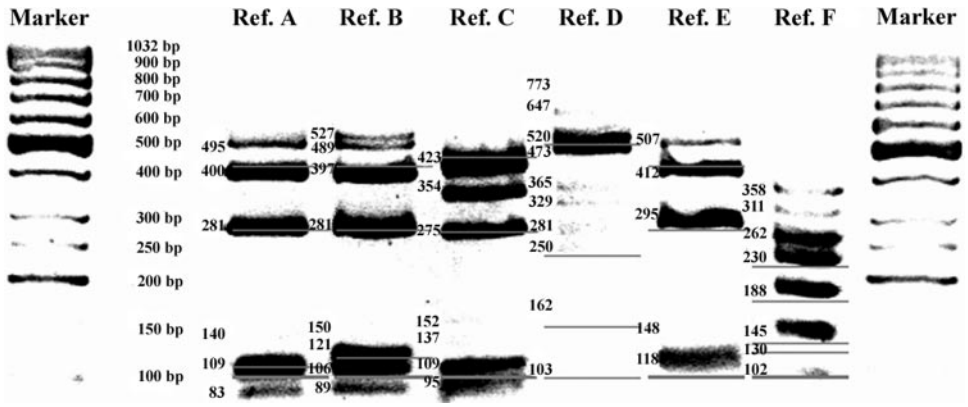

FIG. 2. Gel-based and in silico-based *ompA* RFLP using AluI. Thin lines represent the in silico-calculated lengths of the restriction fragments.

generated the RFLP restriction pattern E and was probably detected by the serovar B-specific MAb. In the lovebird, genotype A was undetected by serotyping. In pigeons, sequencing revealed genotypes A, B, D, E, and E/B, with genotype B being most prevalent. Three pigeons showed a mixed infection. RFLP could confirm this, with the exception of genotype E/B being identified as genotype E. Serotyping identified genotype E/B as serovar B (61/8 and maybe 8615/1) and could not detect one genotype D (7344/2) and B (6098) strain and maybe also not the genotype B strain present in 8615/1. In turkeys, sequencing and RFLP revealed genotypes A, B, D, and F. Serotyping gave the same results, except that genotype F (7778B15) was undetected.

Serotyping cannot always be performed directly on clinical samples, as there may be insufficient antigen, requiring culture. However, culture is expensive and labor-intensive, requires a special biosafety level, and postpones serotyping for at least 3 to 6 days. Interpretation of the immunofluorescence staining is time-consuming and needs the interpreter's keen eye. At present, serotyping cannot conclusively detect genotype E/B. Theoretically, *ompA* RFLP analysis is more sensitive and specific, less expensive, and much faster. However, amplification of the full-length *ompA* gene directly from clinical samples is sometimes impossible without former culture. RFLP produces distinct restriction patterns, leading to conclusive typing results when looking at the presence of known specific bands except for the new genotype E/B, which generates pattern E. Like for RFLP analysis, *ompA* sequencing sometimes requires former culture to obtain the full-length PCR product for subsequent cloning. Sequencing is more expensive and more time-consuming than RFLP. However, at present, *ompA* sequencing is the only typing method capable to identify all known genotypes, including genotype E/B.

As formerly observed, certain genotypes were preferentially, though not strictly, associated with a specific bird species (21). Genotype A occurred in all psittacines, genotype B occurred in three of five pigeons, and genotype E/B was present in all ducks. Interestingly, so far, genotype F was only found in an American parakeet (VS225) and a German parrot (84/2334) (2, 18). Presently, we isolated an additional genotype F strain on a Belgian turkey farm dealing with respiratory disease.

Serotyping was historically important but has been replaced by *ompA* RFLP. However, RFLP cannot distinguish genotype E from the newly discovered genotype E/B. *ompA* sequencing is at present the only way to discover all known avian *ompA* genotypes and additional new genotypes. We clearly showed that new genotypes cannot always be discovered by RFLP nor by serotyping, as single-nucleotide polymorphisms did not generate another restriction pattern (unless they appear at the restriction site) and base mutations did not influence the conformation of the serovar-specific epitope(s). The present study emphasizes the need for a discriminatory, more rapid genotyping technique for examining both birds and humans given the zoonotic risk of *C. psittaci* infections. For this purpose, a combinatory species- and genotype-specific real-time PCR is currently being evaluated.

Nucleotide sequence accession numbers. The ompA gene sequences for the A, B, D, E, F, and E/B genotypes were submitted to GenBank and assigned accession numbers AY762608, AY762609, AY762610, AY762611, AY762612, and AY762613, respectively.

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