A retrospective study of the prevalence of *cpa*, *cpb*, *cpe*, and *cpb2* toxin genes among *Clostridium perfringens* field isolates obtained from pigs with gastrointestinal diseases using PCR genotyping

Boh Chang Lin, DVM, MS, PhD
MVP Laboratories, Inc., Ralston, Nebraska

Abstract

Clostridium perfringens plays an important role in causing gastrointestinal (GI) diseases in pigs. The virulence of this pathogen comes from its ability to produce toxins. The *C. perfringens* isolates of pig origin have been found to be able to produce at least four different toxins (i.e., the alpha toxin, beta toxin, enterotoxin, and beta 2 toxin). The knowledge about the prevalence of these four toxin genes among the field strains of *C. perfringens* is very limited. In this study, a total of 113 field strains were isolated from pigs with GI diseases and examined with PCR genotyping. Of these, 46.0% were PCR positive for the *cpb2* gene, 18.6% were positive for the *cpe* gene, 98.2% were positive for the *cpa* gene, and 31.0% were positive for the *cpb* gene. Of the 113 field isolates, 69.0% were type A, 29.2% were type C, and 1.8% were untypeable due to missing the *cpa* gene. The isolates that only carried the *cpa* gene (29.2%) and those that carried both *cpa* and *cpb2* genes (29.2%) were most prevalent and were followed by those that carried both *cpa* and *cpb* genes (11.5%). Only a small portion of the 113 isolates carried both cpe and cpb2 genes (7.1%). Based on the results obtained by PCR genotyping, a subtyping system was established to help distinguish the difference due to the presence of *cpe* and *cpb2* genes among field strains that are traditionally classified as type A or type C.

Introduction

Clostridium perfringens is a gram-positive, anaerobic bacterium that can cause clostridial enteric disease in both human and domestic animals[1]. The virulence of *C. perfringens* comes from its ability to produce many different exotoxins. The commonly used classification system for this bacterium divides this species into five types (types A to E) on the basis of the ability of an isolate to produce four major lethal toxins (i.e., the alpha, beta, epsilon, and iota toxins). *C. perfringens* isolates of pig origin have been found to be able to produce at least four toxins (i.e., the alpha toxin, beta toxin, enterotoxin, and beta2 toxin). The alpha toxin is a phospholipase and can be hemolytic, necrotizing and potentially lethal[1,2]. The beta toxin is a trypsin-sensitive toxin and can cause mucosal necrosis and central nervous system signs in domestic animals. It has been reported that about 5% of all *C. perfringens* type A isolates of human origin carry the enterotoxin gene (*cpe)* encoding the *C. perfringens* enterotoxin (CPE). The CPE-positive type A isolates are important causes of food-poisoning and non-food-borne cases of diarrhea in humans, and are also responsible for GI disease in domestic animals[1,2]. Recently, the CPE-positive *C. perfringens* type A isolates have been found to carry the *cpe* gene on either the chromosome or a plasmid. But it was also found that *cpe* is present on the chromosome of most of type A food-poisoning isolates and on the plasmid in most of the non-food-borne human GI diseases or veterinary isolates[3]. A newly identified beta2 toxin, encoded by *cpb2* gene, was found to be produced by *C. perfringens* isolated from piglets with necrotic enteritis and from horses with enterocolitis[4].

C. perfringens has long been recognized as an important cause of clostridial enteric diseases in pigs. However, the knowledge about the prevalence of the above-mentioned four toxin genes among the *C. perfringens* field isolates in this country is very limited. The aim of this study is to perform a retrospective study of the prevalence of *cpa, cpb, cpe*, and *cpb2* genes among 113 *C. perfringens* isolates obtained from clinical cases submitted to MVP Laboratories. The test results may help us

obtain some insight about the enteropathogenicities of the *C. perfringens* isolates surveyed in this study.

Materials and methods

Bacterial strains: A total of 113 *C. perfringens* isolates that had caused GI diseases in pigs were selected for genotyping by a multiplex PCR assay. The ATCC type strains of C. perfringens (ATCC 13124, ATCC 14809, and ATCC 3624) were used as controls. ATCC 3624 was previously shown to be cpe negative and can be used as a negative control[5].

DNA isolation: Genomic DNA was extracted by suspending one colony of the test isolate from a solid medium in 200 ul of HPLC grade water in a microcentrifuge tube and boiling for 20 minutes, and then centrifuging for 2 minutes at 15,000 xg as described[6]. The supernatant was used as DNA template.

PCR assay: A multiplex PCR assay was adopted to type all of the isolates in this study as described[4,6]. Briefly, the PCR reaction mix for one test sample contains 5 ul of 10x PCR buffer, 2.4 ul of dNTP (each at a concentration of 40 uM), 4.0 ul of 25 mM magnesium chloride, 1.0 ul of Taq DNA polymerase (5U/ul), 21.3 ul of deionized water, 10.0 ul of DNA template, and multiplex primers (0.625 ul of CPAF, 0.625 ul of CPAR, 0.450 ul of CPBF, 0.450 ul of CPBR, 0.550 ul of ETXF, 0.550 ul of ETXR, 0.650 ul of IAF, 0.650 ul of IAR, 0.425 ul of CPEF, 0.425 ul of CPER, 0.450 ul of CPB2F, and 0.450 ul of CPB2R, each at a concentration of 40 uM). The oligonucleotide primers for this PCR assay are listed in Table 1.

The PCR conditions are as follows: Thirty five cycles at 94°C for one minute, 53°C for one minute, and 72°C for two minutes. Then one cycle at 72°C for ten minutes. The PCR amplicons are analyzed by electrophoresis in 1.5% agarose gel stained with ethidium bromide and recorded by using UV transilluminantion and Polaroid film.

A new PCR assay that can rapidly determine if a *cpe*-positive *C. perfringens* isolate carries a chromosomal or a plasmid borne *cpe* gene was recently reported[7] and adopted in this study to examine all of the cpe-positive isolates.

Results and discussion

A total of 113 *C. perfringens* field strains isolated from cases of swine enteritis were genotyped by PCR assays to detect *cpa, cpb, cpe,* and *cpb2* genes in each isolate. Approximately 18.6% were PCR positive for the *cpe* gene, and 46.0% were PCR positive for the *cpb2* gene. About 15.4% of the *C. perfringens* type A isolates carried the *cpe* gene, while 27.3% of the *C. perfringens* type C isolates carried the *cpe* gene. About 50.0% of the type A isolates carried the *cpb2* gene, while 39.4% of the type C isolates carried the *cpb2* gene. The majority of these 113 isolates were classified as *C. perfringens* type A that either carried only the *cpa* gene or carried both *cpa* and *cpb2* genes. Only a small portion of these isolates carried both *cpe* and *cpb2* genes (see Tables 2, 3, and 4).

In this study the higher rate (46.9%) of *cpb2*-positivity among the 113 field strains isolated from cases of swine enteritis supports some recent reports which claim that *cpb2* toxin gene may play an important role in the pathogenesis of porcine necrotic enteritis[1, 4, 8]. On the other hand the rate of *cpe*-positivity among these 113

Table 1: Oligonucleotide primers for PCR amplification of *cpa, cpb, etx, iA, cpe*, and *cpb2* toxin genes of *C. perfringens* toxins.

Toxin gene		Oligonucleotide sequence	Fragment length
cpa	CPAF	5′ -GCT AAT GTT ACT GCC GTT GA-3′	324 bp
	CPAR	5′ - CCT CTG ATA CAT CGT GTA AG-3′	
cpb	CPBF	5′ - GCG AAT ATG CTG AAT CAT CTA-3′	196 bp
	CPBR	5′ - GCA GGA ACA TTA GTA TAT CTT C-3′	
etx	ETXF	5′ - GCG GTG ATA TCC ATC TAT TC-3′	655 bp
	ETXR	5′ - CCA CTT ACT TGT CCT ACT AAC-3′	
iA	IAF	5′ - ACT ACT CTC AGA CAA GAC AG-3′	446 bp
	IAR	5′ - CTT TCC TTC TAT TAC TAT ACG-3′	
cpe	CPEF	5′ - GGA GAT GGT TGG ATA TTA GG-3′	233 bp
	CPER	5′ - GGA CCA GCA GTT GTA GAT A-3′	
cpb2	CPB2F	5′ - AGA TTT TAA ATA TGA TCC TAA CC-3′	567 bp
	CPB2R	5′ - CAA TAC CCT CCT TCA CCA AAT ACT C-3′	

Table 2: Prevalence of *cpa, cpb, cpe,* and *cpb2* toxin genes of *C. perfringens* among 113 field isolates obtained from pigs with enteric diseases.

Toxin gene	Number of isolates	Positivity (%)
cpa	111	111/113 (98.2%)
cpb	35	35/113 (31.0%)
cpe	21	21/113 (18.6%)
cpb2	52	52/113 (46.0%)

isolates is lower (18.6%) than that of *cpb2*-positivity, yet it is still much higher than the previous reports which ranged from 0% to 8%[1]. In addition to *cpa* and *cpb* genes, both *cpe* and *cpb2* genes could be two of the several toxin genes that contribute to the pathogenesis of the GI diseases in pigs. But the recent discovery of the *cpb2* gene also brings into question whether the entire repertoire of *C. perfringens* toxins has yet been established.

It has been reported that *cpe*-positive type A isolates are important causes of GI diseases in domestic animals, and the *cpe* gene is present on the chromosomes of most human type A food-poisoning isolates or on the plasmids in most veterinary isolates[3]. In this study, a duplex PCR genotyping assay was adopted to determine if the *cpe*-positive isolates were carrying a plasmid-borne *cpe* gene or chromosomal *cpe* gene. The plasmid-borne *cpe* gene is found in 6 *cpe*-positive isolates in this study. The rest of the *cpe*-positive isolates are negative by this PCR assay. The presence of a plasmid-borne cpe gene in 6 isolates also brings into the question whether plasmid-borne cpe genes can be transferred *in vivo* to naturally cpe-negative isolates via conjugation. Further *in vivo* study is needed to answer this important question.

The present classification system for *C. perfringens* genotyping is based on the production of the four major toxins, alpha, beta, epsilon, and iota. Type A is defined as strains producing alpha toxin, type B as strains producing alpha, beta, and epsilon toxins, type C as strains producing alpha and beta toxins, type D as strains producing alpha and epsilon toxins, and type E as strains producing alpha and iota toxins. However, this classification does not include the two important toxins, the enterotoxin and the newly recognized beta 2

Table 3: Prevalence of *cpe* and *cpb2* toxin genes among 78 *C. perfringens* type A isolates, 33 *C. perfringens* type C isolates and 2 untypeable isolates obtained from pigs with enteric diseases.

Genotype	Number of isolates carrying *cpe* gene (positivity, %)	Number of isolates carrying *cpb2* gene (positivity, %)	Number of isolates carrying *cpe* and *cpb2* (positivity, %)
A (n=78)	12 (15.4%)	39 (50.0%)	6 (7.7%)
C (n=33)	9 (27.3%)	13 (39.4%)	2 (6.1%)
Untypeable (n=2)	0	1 (50.0%)	0

n = number of isolates belong to that type of *C. perfringens*.

Table 4: Ten subtypes have been determined within 111 strains of *C. perfringens* type A and type C and 2 strains of untypeable based on the presence *cpa, cpb, cpe,* and *cpb2* genes in each isolate.

Subtype	*cpa*	*cpb*	*cpe*	*cpb2*	Number of isolates
Abeb2 (A)	+	–	–	–	33 (29.2%)
AbEb2 (A)	+	–	+	–	6 (5.3%)
AbeB2 (A)	+	–	–	+	33 (29.2%)
AbEB2 (A)	+	–	+	+	6 (5.3%)
ABeb2 (C)	+	+	–	–	13 (11.5%)
ABEb2 (C)	+	+	+	–	7 (6.2%)
ABeB2 (C)	+	+	–	+	11 (9.7%)
ABEB2 (C)	+	+	+	+	2 (1.8%)
aBeb2 (UT)	–	+	–	–	1 (0.9%)
aBeB2 (UT)	–	+	–	+	1 (0.9%)

* A: type A, C: type C, UT: untypeable.

toxin. In fact, an increasing number of reports have indicated a significant association between cpb2-positive *C. perfringens* isolates and diarrhea in pigs[4,8]. Some reports also indicated that there is an association between enterotoxigenic strains of type A *C. perfringens* and the etiology of diarrhea in pigs[1, 2, 3]. In order to better describe the presence or absence of these four toxin genes in a single *C. perfringens* isolate, a new nomenclature system was used in the genotyping of the 113 isolates in this study as listed on Table 4. For example, the capitalized letter A stands for the presence of *cpa* gene and the small letter a represents the absence of *cpa* gene of *C. perfringens*. The four letters A, B, E, and B2 were used to represent the presence of *cpa, cpb, cpe*, and *cpb2* genes in a single isolate. Ten subtypes can be found among the 113 *C. perfringens* isolates of pig origin. However, there are also two isolates that can not be typed by the traditional typing system due to the lack of the *cpa* gene as shown in Table 4.

References

1. Songer, J. G. 1996. Clostridial enteric diseases of domestic animals. Clin. Microbiol. Rev. 9:216-234.

2. Kokai-Kun, J. F., J. G. Songer, J. R. Czeczulin, F. Chen, and B. A. McClane. 1994. Comparison of Western immunoblots and gene detection assays for identification of potentially enterotoxigenic isolates of *Clostridium perfringens*. J. Clin. Microbiol. 32:2533-2539.

3. Brynestad, S., M. R. Sarker, B. A. McClane, P. E. Granum, and J. I. Rood. 2001. Enterotoxigenic plasmid from *Clostridium perfringens* is conjugative. Infect. Immun. 69:3483-3487.

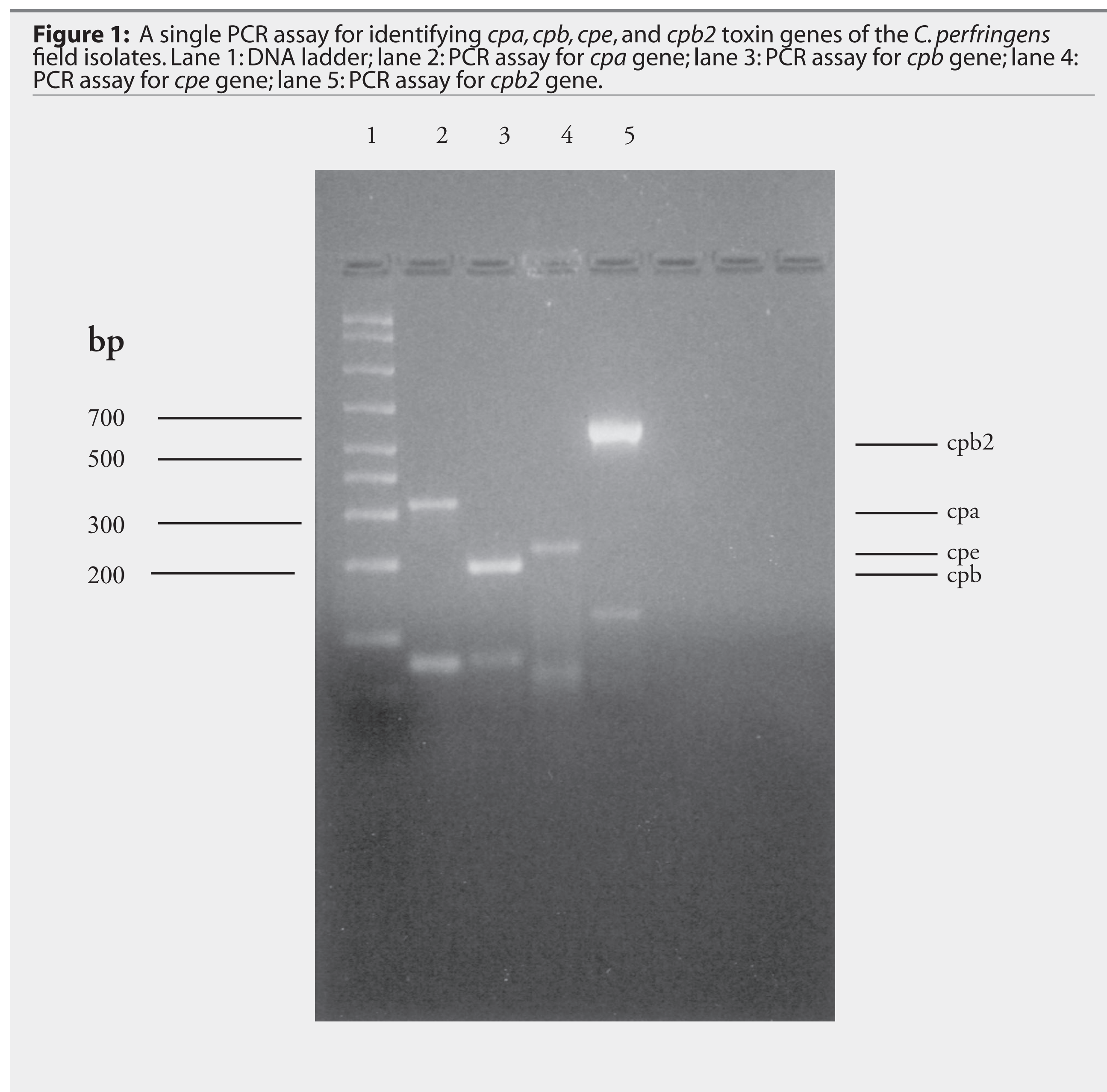
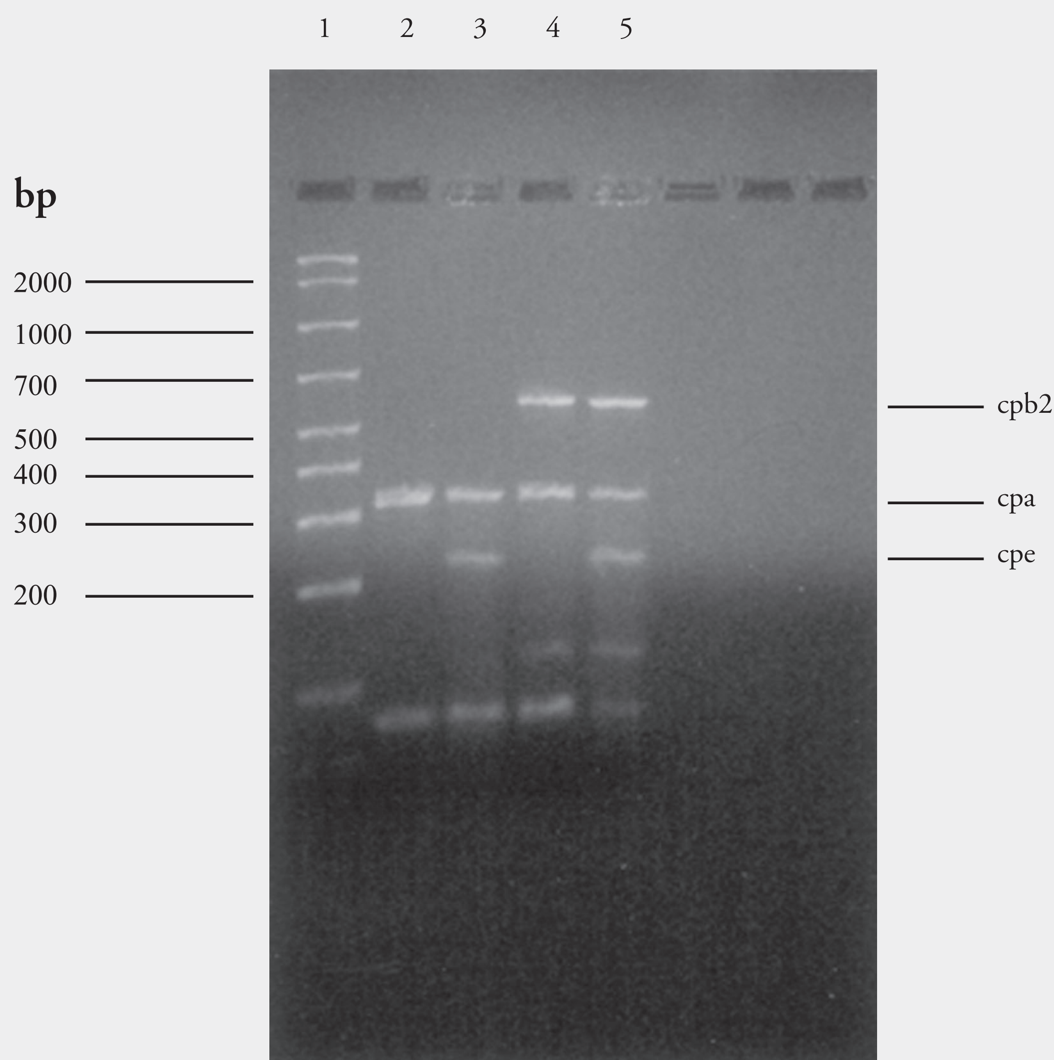


Figure 1: A single PCR assay for identifying *cpa, cpb, cpe*, and *cpb2* toxin genes of the *C. perfringens* field isolates. Lane 1: DNA ladder; lane 2: PCR assay for *cpa* gene; lane 3: PCR assay for *cpb* gene; lane 4: PCR assay for *cpe* gene; lane 5: PCR assay for *cpb2* gene.

Figure 2: Multiplex PCR assay for genotyping *C. perfringens* field isolates. Lane 1: DNA ladder; lane 2: a field isolate of subtype Abeb2; lane 3: a field isolate of subtype AbEb2; lane 4; a field isolate of subtype AbeB2; lane 5: a field isolate of subtype AbEB2.



4. Gilbert, M., C. Jolivet-Reynaud, and M. R. Popoff. 1997. Beta 2 toxin, a novel toxin produced by *Clostridium perfringens.* Gene 203:56-73.

5. Czeczulin, J. R., R. E. Collie, and B. A. McClane. 1996. Regulated expression of *Clostridium perfringens* enterotoxin in naturally cpe-negative type A, B, and C isolates of *C. perfringens*. Infect. Immun. 64:3310-3309.

6. Songer, J. G., and D. Bueschel. 1998. *Clostridium perfringens* multiplex PCR assay for genotyping. P 14-15. In: Lloyd H. Lauderman (ed), Nucleic Acid Amplification Assays for Diagnosis of Animal Diseases. Life Technologies and American Association of Veterinary Laboratory Diagnosticians.

7. Wen, Q., K. Miyamoto, and B. A. McClane. 2003. Development of a duplex PCR genotyping assay for distinguishing *Clostridium perfringens* type A isolates carrying chromosomal enterotoxin (cpe) genes from those carrying plasmid-borne enterotoxin (cpe) genes. J. Clin. Microbiol. 41:1494-1498.

8. Waters, M., A. Savoie, H. S. Garmory, D. Bueschel, M. R. Popoff, J. G. Songer, R. W. Titball, B. A. McClane, and M. R. Sarker. 2003. Genotyping and phenotyping of beta2-toxigenic *Clostridium perfringens* fecal isolates associated with gastrointestinal diseases in piglets. J. Clin. Microbiol. 41:3584-3591.

Figure 3: Multiplex PCR assay for genotyping *C. perfringens* field isolates. Lane 1: DNA ladder; lane 2: a field isolate of subtype ABeb2; lane 3: a field isolate of subtype ABEb2; lane 4: a field isolate of subtype ABeB2; lane 5: a field isolate of subtype ABEB2; lane 6: a field isolate of subtype aBeb2; lane 7: a field isolate of subtype aBeB2.