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Inverse PCR for subtyping of *Acinetobacter baumannii* carrying IS*Aba*1

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Acinetobacter baumannii **has been prevalent in nosocomial infections, often causing outbreaks in intensive care units. IS*Aba1* is an insertion sequence that has been identified only in *A. baumannii* and its copy number varies among strains. It has been reported that IS*Aba1* provides a promoter for $bla_{\text{OXA-51-like}}$, $bla_{\text{OXA-23-like}}$, and bla_{ampC}, which are associated with the resistance of *A. baumannii* to carbapenems and cephalosporins. The main purpose of this study was to develop a novel inverse PCR method capable of typing *A. baumannii* strains. The method involves three major steps: cutting of genomic DNA with a restriction enzyme, ligation, and PCR. In the first step, bacterial genomic DNA was digested with *Dpn*I. In the second step, the digested genomic DNAs were ligated to form intramolecular circular DNAs. In the last step, the ligated circular DNAs were amplified by PCR with primers specific for IS*Aba1* and the amplified PCR products were electrophoresed. Twenty-two clinical isolates of *A. baumannii* were used for the evaluation of the inverse PCR (iPCR) typing method. Dendrogram analysis revealed two major clusters, similar to pulsed-field gel electrophoresis (PFGE) results. Three IS*Aba1*-associated genes – bla_{ampC}, $bla_{\text{OXA-66-like}}$, and *csuD* – were amplified and detected in the clinical isolates. This novel iPCR typing method is comparable to PFGE in its ability to discriminate *A. baumannii* strains, and is a promising molecular epidemiological tool for investigating *A. baumannii* carrying IS*Aba1*.**

Keywords: *Acinetobacter baumannii*, IS*Aba1*, molecular epidemiology, inverse PCR, PFGE

Introduction

Acinetobacter baumannii is reported as a major pathogen responsible for nosocomial infections and frequently causes outbreaks and high mortality rates with symptoms of septicemia and pneumonia in intensive care units (Chen *et al.*, 2001; Garnacho-Montero *et al.*, 2003; Levin *et al.*, 2003). Most strains responsible for such outbreaks are highly resistant to multiple antimicrobial agents including carbapenems, which have been drugs of choice in severe infections caused by this organism (Perez *et al.*, 2007; Zarrilli *et al.*, 2009).

Insertion sequences (ISs) are among the smallest and most abundant transposable elements that are able to transpose genes independently in microbial genomes, including that of *A. baumannii* (Mugnier *et al.*, 2009). ISs cause insertion mutations and genome rearrangements, thus enhancing the spread of resistance and virulence determinants within species (Ravasi *et al.*, 2011). IS*Aba*1, an *A. baumannii*-specific IS, has been reported to mobilize antibiotic resistance genes and provides promoter sequences that enhance the expression of 5′-flanked resistance genes in *A. baumannii*. IS*Aba1* is a 1,180-bp element that belongs to the IS*4* family and has been identified only in *A. baumannii* thus far, and its copy number varies among strains. It provides a promoter sequence for $bla_{\text{OXA-51-like}}$, $bla_{\text{OXA-23-like}}$, and bla_{ampC}, which are associated with the resistance of *A. baumannii* to carbapenems and cephalosporins (Heritier *et al.*, 2006; Segal *et al.*, 2007; Chen *et al.*, 2010; He *et al.*, 2011). Nosocomial infections caused by *A. baumannii* have been increasing in developed nations. Therefore, this unique IS has been investigated to understand its role in acquired antibiotic resistance of *A. baumannii*.

For the molecular epidemiological studies on *A. baumannii*, pulsed-field gel electrophoresis (PFGE) or repetitive element-based polymerase chain reaction has been performed to determine the genomic relationships among isolates (Wang *et al.*, 2007; Lu *et al.*, 2009; He *et al.*, 2011). Many clinical microbiologists have tried to develop more efficient and easier molecular epidemiological methods to investigate genomic relationships among bacteria. In particular, studies on *A. baumannii* have focused on the role of IS*Aba1* in antibiotic resistance of this bacterium, and efforts have been made to determine the exact locations of IS*Aba1* in its genome (Segal *et al.* 2007; Chen *et al.* 2010; He *et al.*, 2011).

In this study, first we report an inverse PCR (iPCR) method as a novel molecular epidemiological tool for investigating *A. baumannii* based on the insertion sites and the IS*Aba1* copy number. The results of this inverse PCR method could not only aid in the clonal relationship and clustering analysis, but also the insertion sites of IS*Aba1*. We also report a novel insertion site of IS*Aba1* in the *csu* operon in multidrug-resistant *A. baumannii* isolates.

Materials and Methods

Bacteria

Twenty-two clinical isolates of *A. baumannii* were used in this study. Among these isolates, 12 isolates carried $bla_{\text{PER-1}}$ and showed higher biofilm formation abilities than the other 10 isolates, which did not carry $bla_{\text{PER-1}}$ (Lee *et al.*, 2008).

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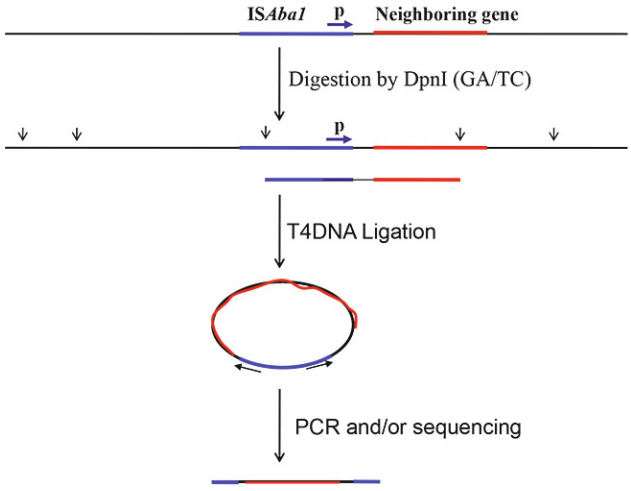

Fig. 1. Schematic diagram of iPCR. Blue and red lines stand for IS*Aba1* and a neighboring gene, respectively. Vertical arrows are *Dpn*I restriction sites and 'p' is the outward promoter of IS*Aba1*. The two PCR primers are indicated by arrows in the circularized DNA.

IS*Aba1*-specific iPCR

We developed an IS*Aba1*-specific iPCR method based on a previously published method (Ochman *et al.*, 1988) (Fig. 1). Genomic DNAs (gDNAs) from *A. baumannii* isolates were purified using a Genomic DNA Prep Kit (Promega A1120) according to the manufacturer's instructions. The purified gDNAs were digested with *Dpn*I restriction enzyme (NEB) for 90 min at 37°C, followed by heat inactivation for 20 min at 80°C. The *Dpn*I target sequence is 5′-GATC-3′, which is located at the 980–983 nucleotide region of total 1,180 bp-size IS*Aba1* (GenBank Accession: AY758396). Digested gDNAs (150 ng each) were ligated using T4 DNA ligase (NEB) for 4 h at 16°C, during which random intra- and inter-strand DNA ligation occurs. PCR was performed with CCL-F and CCL-R primers (Table 1) using these ligated gDNAs as templates. For the PCR experiments, an AccuPower PCR PreMix kit (Bioneer) was used. The PCR conditions were 5 min at 95°C for the initial denaturing step, 32 cycles of 1 min at 95°C, 30 sec at 50.6°C, and 1 min at 72°C, and then 7 min at 72°C for the final extension step. The PCR products were resolved in 1% agarose gel electrophoresis for 30 min at 100 V (Mupid Mini Kit) and visualized by staining with ethidium bromide.

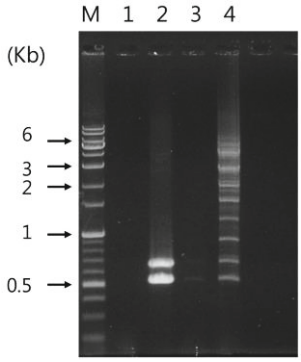

Fig. 2. PCR controls and iPCR. PCR results using distilled water (lane 1), intact genomic DNA (lane 2), *Dpn*I-digested genomic DNA (lane 3), and *Dpn*I-digested and ligated genomic DNA (lane 4) as templates with CCL-F/CCL-R primers. Genomic DNA of *A. baumannii* isolate 1656-2 was used in this experiment. M indicates molecular size markers.

PFGE of *A. baumannii* gDNA

The preparation of agarose plugs containing bacterial gDNA and in-gel digestion with a restriction enzyme were performed according to a previous study (Pournaras *et al.*, 2006). The gDNAs were digested in agarose gel plugs using restriction enzyme *Apa*I (Boehringer Mannheim) at 30°C for 16 h and resolved using a PFGE CHEF-DRIII system (Bio-Rad Laboratories). The PFGE running conditions were as follows: 5 sec for the initial time pulse, 30 sec for the final time pulse, 6 V/cm, 14°C running temperature, and a 20-h run time. The macro-restricted DNA fragments were visualized by staining with ethidium bromide and photographed using an SI-20 DNA Image Visualizer system (Seoulin Scientific).

Southern analysis

Southern probes specific for *csuD*, $bla_{\text{OXA-66-like}}$, and bla_{ampC} were amplified by PCR (ExTaq, TaKaRa) using specific primers (Table 1), and digoxigenin-labeled probes were prepared with a DIG DNA Labeling Kit (Boehringer Mannheim). iPCR products were analyzed in 1% agarose gels and then sequentially denatured, neutralized, and blotted according to the manufacturer's instructions. Products were transferred onto positively charged nylon membranes (Roche) and fixed using a UV-Stratalinker 1800 (Stratagene). Probes were hybridized with the membrane-bound iPCR products using ExpressHyb Hybridization Solution (Clontech, Mountain View) according to the manufacturer's instructions. Detection of hybridized Southern probes was performed using Anti-Digoxigenin-Alkaline phosphatase Fab fragments and NBT/BCIP solution (Roche Diagnostics).

Table 1. Primers used in this study

Primer	Sequence (5′–3′)	Target gene(s)	Amplicon size (bp)	Purpose of PCR
CCL-F	ATG TGT CAT AGT ATT CGT CG	IS*Aba1* neighboring genes	various	iPCR
CCL-R	CTT AAC GGG TGA ATG GCA AC			
AmpC-F	TGAATAACACCCACAGCC	bla_{ampC}	568	Southern probe
AmpC-R	GCATGATGAGCGCAAAGC			
OXA66-F	AGCATTTTGAAGGTCGAAGC	$bla_{\text{OXA-66}}$-like	337	Southern probe
OXA66-R	GTCATAGTATTCGTCGTTAG			
CsuD-F	TTACGGTTATAGCTGTATCC	*csuD*	889	Southern probe
CsuD-R	GTCGCTCAGTCTGGTATAGG			

Clustering analysis

PFGE and iPCR patterns were analyzed using BioNumerics software version 4.0 (Applied Maths). Dendrograms were produced by using the Dice coefficient for similarity and unweighted pair method with arithmetic mean (UPGMA) clustering.

Results and Discussion

To evaluate the effectiveness of the iPCR method for the molecular epidemiological study of *A. baumannii*, PCR was performed using distilled water, undigested, *Dpn*I-digested, and *Dpn*I-digested and ligated gDNA of the *A. baumannii* 1656-2 isolate (Park *et al.*, 2011). As shown in Fig. 2, iPCR did not produce any DNA amplicon from the distilled water, but two DNA bands were detected for the reaction with the intact gDNA from the 1656-2 isolate. These two PCR products were undetectable in the reaction with the *Dpn*I-digested gDNA. The digested and ligated gDNA produced ladder-like bands. Taken together, these results suggest that iPCR could specifically amplify IS*Aba1* according to our experimental design. If the gDNA could be completely digested by the *Dpn*I restriction enzyme, non-specific iPCR products could be avoided. The ladder-like DNA bands in lane four

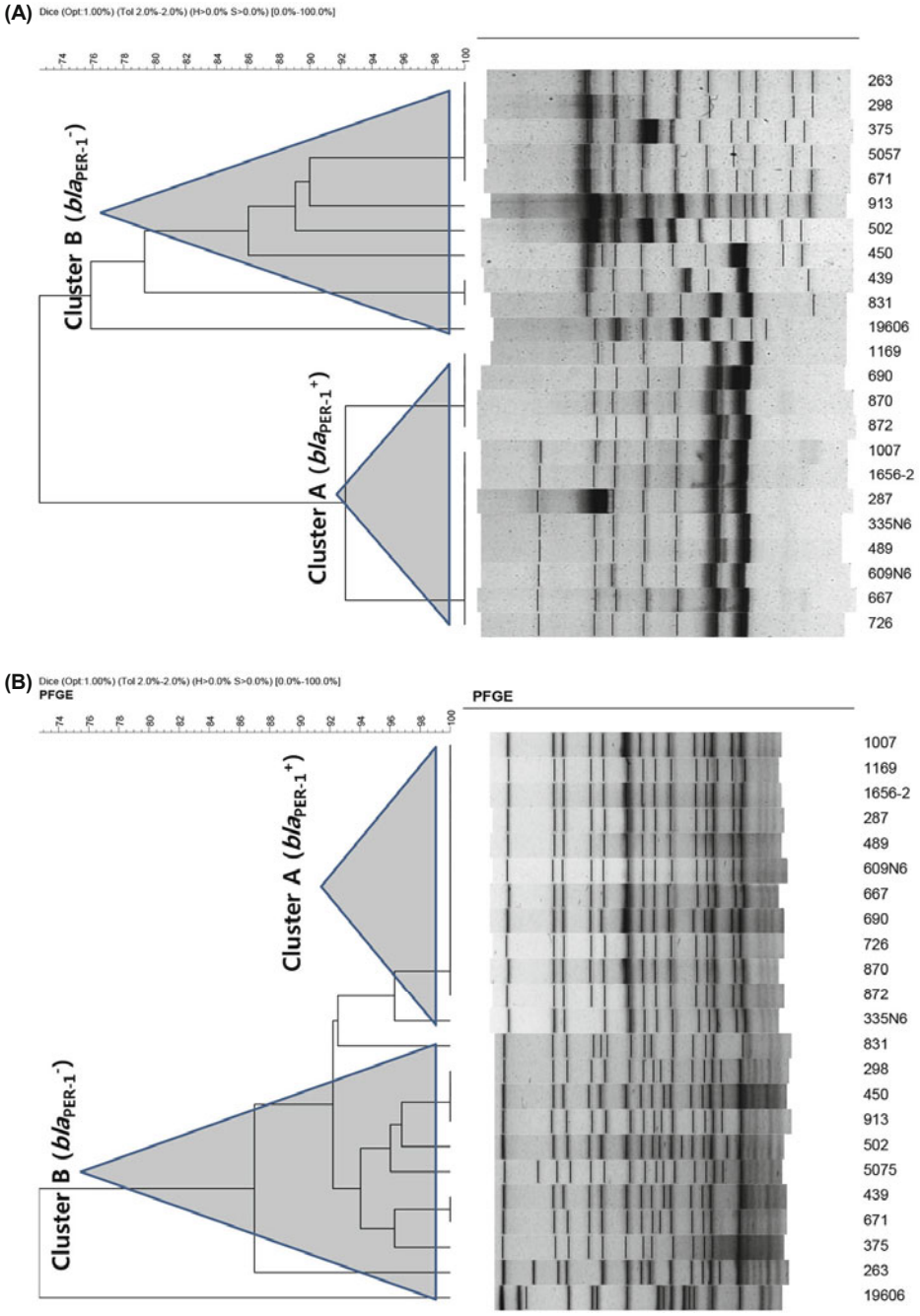

Fig. 3. Dendrograms of iPCR (A) and PFGE (B).

were expected, as IS*Aba1*-specific PCR amplicons are produced from the intramolecular ligation that results in DNA circularization (Collins and Weissman, 1984). Subsequently, we used iPCR for 22 *A. baumannii* clinical isolates and one *A. baumannii* ATCC 19606 strain, which showed PCR positive results using IS*Aba1*-specific primers (data not shown).

After the iPCR results were obtained for the 22 *A. baumannii* clinical isolates, we observed six to eleven discrete DNA bands in the 1% agarose gel ranging from 250 bp to 6 kb (Fig. 3A). According to the dendrogram analysis, two major clusters could be differentiated. Interestingly, one of the genotypic differences between cluster A and B isolates was the existence or absence of $bla_{\text{PER-1}}$; cluster A consisted of 12 isolates that harbored $bla_{\text{PER-1}}$ and cluster B consisted of 10 isolates that were $bla_{\text{PER-1}}$ negative. Among the cluster A isolates, eight isolates in cluster A1 and four isolates in cluster A2 produced indistinguishable patterns. However, the isolates in cluster B showed more heterogeneous patterns than those of the isolates in cluster A. The number of detectable bands of an isolate theoretically indicates the IS*Aba1* copy number of the isolate: e.g., an A1 pattern consisting of seven detectable bands indicates seven copies of IS*Aba1* in the isolates. According to our full sequence information for the 1656-2 isolate, it has 15 copies of IS*Aba1* in its genome (GenBank Accession: CP001921.1) and two copies in its plasmid (GenBank Accession: CP001922.1) (Park *et al.*, 2011). Thus, these results show that iPCR amplification of the 1656-2 isolate did not reveal all genes linked to IS*Aba1*.

*Apa*I-PFGE of the *A. baumannii* isolates showed 13 to 16 bands ranging from approximately 50 kb to approximately 700 kb (Fig. 3B). Bands smaller than 50 kb were ignored for the dendrogram analysis. The PFGE patterns could be clustered into two major groups, similar to the iPCR results, and the two major clusters were differentiated on the basis of the presence or absence of $bla_{\text{PER-1}}$. Furthermore, the isolates carrying $bla_{\text{PER-1}}$ showed identical or highly similar patterns both in PFGE and iPCR, and the isolates without $bla_{\text{PER-1}}$ showed heterogeneous band patterns in a major respective cluster in PFGE and iPCR. This result indicates that the iPCR method could efficiently subtype *A. baumannii* and is a viable molecular epidemiological tool. PFGE showed nine different patterns, indicating that it had greater discriminatory power than iPCR in this study, which displayed seven different patterns.

These multidrug-resistant *A. baumannii* isolates were previously characterized, and those carrying $bla_{\text{PER-1}}$ had greater capacity to form biofilm and to adhere to respiratory epithelial cells than those without $bla_{\text{PER-1}}$ (Lee *et al.*, 2008). Taken together with our PFGE and iPCR results, these two isolate groups were revealed to have completely different genetic backgrounds, and the multidrug-resistant isolates harboring $bla_{\text{PER-1}}$ showed high clonality.

According to full genomic sequence information for the 1656-2 isolate, we chose three genes, bla_{ampC}, $bla_{\text{OXA-66-like}}$, and *csuD*, to assess the iPCR method by performing Southern hybridization. These genes are linked to IS*Aba1* present in the genome of the 1656-2 isolate. Among these genes, bla_{ampC} and $bla_{\text{OXA-66-like}}$ are known to be highly expressed by the outward promoter of IS*Aba1* (Heritier *et al.*, 2006; Figueiredo *et al.*, 2009). *csuD* is a strong candidate gene whose expression could be enhanced by the outward promoter of IS*Aba1* according to our genetic analysis (data not shown).

Eleven out of 22 isolates, including the 1656-2 isolate, were selected for Southern blot analysis with the three gene-specific probes (Fig. 4A), and the results are shown in Fig. 4B. Using the bla_{ampC}-specific probe, Southern blotting showed an approximately 570-bp band in all isolates, indicating that the bla_{ampC} gene is adjacent to IS*Aba1* in all the isolates and that the iPCR works well. According to the theoretical calculation of the iPCR product from the 1656-2 genome, assuming complete digestion of the genomic DNA by *Dpn*I there should be an approximately 220-bp band detectable

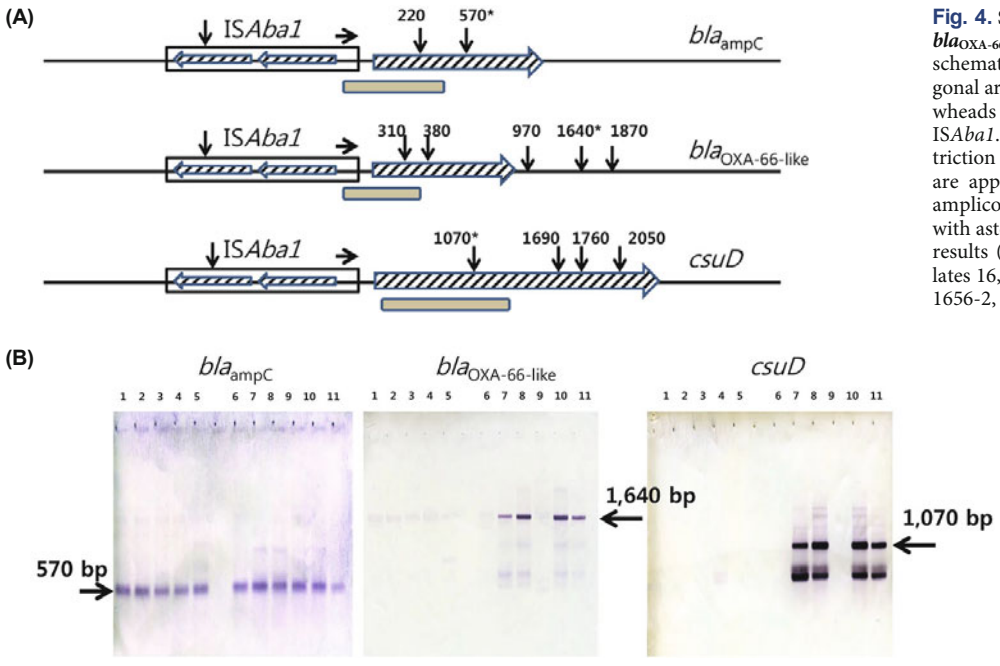

Fig. 4. Southern blot analysis with bla_{ampC}-, $bla_{\text{OXA-66-like}}$-, and *csuD*-specific probes. A schematic diagram of the three genes (diagonal arrows) and IS*Aba1* (open box). Arrowheads indicate the outward promoter of IS*Aba1*. Vertical arrows indicate *Dpn*I restriction sites and the numbers on the arrows are approximate sizes of theoretical iPCR amplicons (A). The theoretical amplicons with asterisks were detected in the Southern results (B). Lanes 1–11: *A. baumannii* isolates 16, 26, 32, 298, 439, 450, 489, 667, 913, 1656-2, and 335N6, respectively.

with the *bla*$_{ampC}$-specific probe. The 570-bp signal could have been obtained from the digestion of the second nearest *Dpn*I site from IS*Aba1* and intramolecular circularization. Self-ligation of the 220 bp-DNA fragment may be difficult because it is too short to circularize or that the circularization is sequence dependent (Vologodskaia and Vologodskii, 2002). *bla*$_{OXA-66-like}$- and *csuD*-specific Southern blot analysis showed 1,640 bp and 1,070 bp signals, respectively, which are consistent with the theoretical sizes of the iPCR amplicons. There were additional *csuD* Southern signals below the 1,070-bp-signals in lanes 7, 8, 10, and 11 that were unexpected and uninterpretable. Those four isolates that showed *bla*$_{OXA-66-like}$ and *csuD* Southern signals were clustered as an indistinguishable pattern in the same group for both iPCR and *Apa*I-PFGE. To our knowledge, this is the first report of the novel insertion site for IS*Aba1* in the *csu* operon, and its effect on biofilm formation should be further investigated.

In this study, we developed a novel molecular epidemiological method for *A. baumannii* carrying IS*Aba1*. Our iPCR results with 22 *A. baumannii* clinical isolates showed discriminatory power with efficiency similar to that of *Apa*I-PFGE and specific amplification of IS*Aba1*-neighboring genes, as demonstrated by Southern blot analysis.

To realize its full potential, some aspects of our iPCR method should be optimized. For example, the final output may not be consistent according to the choice of *Taq* polymerase and PCR conditions, which is a common problem with subtyping methods using PCR technique. In addition, incomplete *Dpn*I digestion and discrepancies between iPCR band number and IS*Aba1* copy number currently limit its applicability.

Nonetheless, our novel iPCR method is a promising molecular epidemiological tool to understand genomic structural variation and to assess the *A. baumannii* IS*Aba1* copy number. IS*Aba1*-specific iPCR is simpler, more rapid, and more economical than PFGE for the molecular epidemiological investigation of *A. baumannii*. Furthermore, this method will facilitate determining the exact integration sites of IS*Aba1* by directly sequencing the iPCR amplicons in future trials.

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