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Genetic Variation among *Plasmodium vivax* Isolates Adapted to Non-Human Primates and the Implication for Vaccine Development

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Abstract

Plasmodium vivax Duffy binding protein (DBP) is vital for parasite development, thereby making this molecule a good vaccine candidate. Preclinical development of a *P. vivax* vaccine often involves use of primate models prior to testing efficacy in humans, but primate isolates are poorly characterized. We analyzed the complete gene coding for the DBP in several *P. vivax* isolates that are used for experimental primate infections and compared these sequences with the Salvador I DBP isolate, which is being used for vaccine development. Our results affirm that primate-adapted isolates are genetically similar to *P. vivax* circulating in humans, but variability is greatest in the putative target of protective antibodies. In addition, some *P. vivax* isolates contain multiple genetically different clones. Testing a DBP vaccine may therefore be complicated by heterogeneity and diversity of the *P. vivax* isolates available for *in vivo* challenge.

INTRODUCTION

Plasmodium vivax is the most widely distributed malaria parasite of humans and the predominant species causing malaria in the Middle East, Central and Southeast Asia, Latin and South America, and Oceania. Malaria caused by *P. vivax* is less deadly than that caused by *P. falciparum*, but is a major contributor to morbidity and economic hardship in malaria-endemic regions.¹ The geographic distribution of *P. vivax* and the emergence of chloroquine-resistant *P. vivax* parasites emphasize the importance of developing long-term control strategies that can effectively prevent and substantially reduce the impact of this disease. An important part of any control strategy will be the implementation of a vaccine capable of inducing strain transcending immunity.

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The malaria asexual life cycle involves repeated cycles of parasite growth and subsequent destruction of host erythrocytes. Each cycle is characterized by production of merozoites, which recognize and invade new erythrocytes for survival and continuation of the parasite cycle.² Parasite invasion involves a cascade of events characterized by the deployment of a series of parasite surface and apical proteins that bind to erythrocyte surface molecules.^{3,4} In *P. vivax*, the Duffy binding protein (DBP) is generally considered to be critical for successful invasion of reticulocytes. Numerous studies have shown that populations lacking the Duffy antigen receptor for chemokines (DARC), which is the reticulocyte receptor on the surface bound by DBP, are refractory to *P. vivax* infection,⁵ thereby making the DBP-DARC interaction vital for *P. vivax* invasion of host erythrocytes. The essential nature of this interaction for *P. vivax* to infect human erythrocytes makes DBP a promising vaccine candidate for *P. vivax* malaria.

In *P. vivax* DBP, the cysteine-rich region II (DBPII) is a 330-amino acid residue binding motif (DBP amino acids 191–460) necessary for adherence to DARC and merozoite invasion of reticulocytes.⁶ Critical binding residues in DBPII have been mapped to a region between amino acids 191 and 460^{3,6–8} and are conserved among most isolates. Many other amino acids of the receptor-binding domain are highly polymorphic with a high ratio of non-synonymous to synonymous mutations.^{9–11} Genetic diversity within and between different populations is highest in DBPII compared with the rest of the gene. This pattern of polymorphisms suggests that allelic variation is an important mechanism of immune evasion. Although DBP represents an ideal target for a vaccine candidate, the allelic variation and the potential associated strain-specific immunity represent challenges for development of a broadly effective vaccine. Limited strain-specific immune response to the target antigen may be a confounding factor as for other vaccines.

Another problem confronting vaccine development and a major limitation for *P. vivax* research is the lack of a standardized *in vitro* culture method using human blood. Therefore, an important step in current pre-clinical development of vaccines against *P. vivax* is testing for efficacy in a non-human primate model system. Many of the vaccine candidates against *P. vivax* malaria are based on genes from the laboratory strain Salvador I. Although originally isolated from a human infection in El Salvador,¹² for research purposes it is now serially passed through different species of New World primates, such as *Aotus* spp. and *Saimiri* spp. However, the Salvador I strain is not yet fully adapted to generate reproducible courses of infection that obtain moderately high parasitemia in nonsplenectomized *Aotus* spp., which is typically desired for evaluating the efficacy of a vaccine in monkey models. In addition, not all nonhuman primate models of *P. vivax* will accurately mimic the invasion mechanism present in human infections because merozoite invasion of the erythrocytes of some primate species (e.g., *Saimiri* spp.) is not dependent upon recognition of the DARC receptor by DBP and may involve an alternative pathway for invasion.^{13,14}

The objective of this study was to evaluate several *P. vivax* isolates used for primate infections for sequence similarity to the DBP of Salvador I. Most of the diversity studied within the DBP has been limited to region II of isolates from human populations, but little is known about the genetic diversity outside this DBP region and among different parasite alleles in primate isolates. This study helps to identify potential families of DBP alleles in these isolates compared with human isolates in the field. In addition, our study examines DBP diversity in relation to alleles of several other important *P. vivax* vaccine candidates. The results obtained will help evaluate the strain selection and validity of primate model infections for testing human vaccines against *P. vivax* malaria.

MATERIALS AND METHODS

Plasmodium vivax strains and DNA extraction

Genomic DNA used in this work was extracted from seven *P. vivax* primate-adapted isolates (Palo Alto, Indonesia XIX, India VII, Belem, Brazil I, Chesson I, and North Korea) and *P. simium*, which are currently maintained at the Centers for Disease Control and Prevention (Atlanta, GA) for experimental research. Each isolate was originally obtained from various malaria-endemic regions. Blood from infected primates was lysed in 0.5% saponin, and parasites were obtained by centrifugation at $15,000 \times g$ for 2 minutes. The pellet was washed in TEN buffer, resuspended in TEN–2% sodium dodecyl sulfate, and parasite proteins were precipitated from the solution with 7.5 M ammonium acetate. Total genomic DNA was obtained by isopropanol precipitation of the supernatant and resuspension of the pellet in TE buffer after washing with 70% ethanol.

Gene amplification and cloning

The complete deduced amino acid sequences for DBP, apical membrane antigen-1 (AMA-1), and circumsporozoite protein (CSP) were each amplified by polymerase chain reaction (PCR) using the following gene-specific oligonucleotide primer pairs: DBPfd (5'-ATGAAAGGAAAAACCGCTCTTTATTG-3') and DBPrv (5'-TCATGAATAATCCAGGGGGTTGATGG-3'); CSPfd (5'-ATGAAGAAGCTTCATTCTCTGGCTGTTTCTTCC-3') and CSPrv (5'-CAGCGGATCCTTAATTGAATAATGCTAGG-3'), and AMA1fd (5'-ATGAATAAAATATAACATAATC-3') and AMA1rv (5'-GTAGTATGGCTTCTCCATCAGCAC-3'). Approximately 100 ng of genomic DNA from each isolate was used in a PCR amplification of various genes using specific oligonucleotides. Amplified DNA was gel purified and cloned into the pGEMT-Easy vector system (Promega, Madison, WI) according to manufacturer's protocols. Deduced nucleotide sequences were obtained from multiple sequenced fragments from two clones for each gene from all the isolates using the dideoxynucleotide chain termination method (Applied Biosystems, Foster City, CA) with specific overlapping primers in both directions.

Gene analysis

The deduced nucleotide and amino acid sequences were aligned using the ClustalW Multiple alignment editor (<http://jwbrown.mbio.ncsu.edu/BioEdit/bioedit.html>). The aligned amino acid residues were used for phylogenetic analysis. Cluster trees were constructed using the neighbor-joining method with 1,000 bootstraps as reported.^{15–17} Only regions common to all sequences from the respective genes were used for this analysis. For interspecies comparison, we included sequences from *P. falciparum*, *P. knowlesi*, and *P. cynomolgi*, as well as *P. vivax* human isolates obtained from field studies conducted in different geographic regions where *P. vivax* malaria is endemic. Field isolates used are all referenced sequences obtained from GenBank and their respective accession numbers are indicated on the phylogenetic trees. All primate sequences have been deposited in GenBank under accession nos. EU395587–94; EU551130–37, and EU551138–42 for DBP, EU395595–01 for AMA-1, and EU401923–32 for CSP.

RESULTS

Sequence polymorphisms in DBP

We analyzed the complete coding sequence of a single allele of DBP from seven *P. vivax* isolates and *P. simium*. The coding sequence of each isolate is comprised of 5 exons creating a single contiguous open reading frame of 3,210 basepairs coding for 1,070 amino acids, except

for the Brazil I and India VII isolates, which have an extra residue (477P). Only one *dbp* allele was identified in screening the PCR-amplified products from the genomic DNA for each isolate, and none of these alleles was identical to the Salvador I DBP. All sequences had a similarity matrix for pairwise alignment (BLOSUM62) > 98% when compared with the Salvador I strain. Of 1,070 amino acid residues, which make up the complete DBP coding sequence, 44 polymorphic sites (4.5% polymorphic rate) were observed ranging from 8 in the Chesson I and North Korea isolates to 17 in the Brazil I isolate. Most of the observed polymorphisms were located within region II, corresponding to the ligand domain between residues 191 and 460 (Figure 1A). The most common polymorphic residues (polymorphic residues that occur in at least 50% of the isolates) occurred at positions 308, 384, 385, 386, 417, 424, 437, 503, 655, and 981. Polymorphic ratios relative to Salvador I show the Chesson and North Korea strains to be the closest and Brazil I to be the furthest (Figure 2).

Similarity of sequence diversity of AMA-1 and DBP

To relate the diversity of DBP to another *P. vivax* vaccine candidate, we also studied the allelic diversity of AMA-1 in the same primate isolates. Only one *ama-1* allele was identified initially in the PCR-amplified products from the genomic DNA for each isolate, and none was identical to Salvador I. Analysis of the AMA-1 genes at the nucleotide level identified 48 mutations within the 1,686 nucleotide open reading frame, resulting in 35 amino acid polymorphisms and a similarity index of 96–98% when compared with Salvador I. The polymorphic sites are distributed throughout the coding sequence, although 33 (94%) of 35 polymorphisms are located in the ectodomain between residues 43 and 480 and mostly within subdomain I (Figure 1B). The Indonesia XIX isolate was found to have the maximum variation, including nine polymorphic residues not identified in any other allele.

Mixed clonal infections in primate isolates

In addition to representing a vaccine candidate, CSP is often used as a strain marker for molecular epidemiologic studies of *P. vivax*. The *csp* gene sequence consists of two flanking, conserved and non-repetitive regions, embracing a central region consisting of numerous tandem repetitive amino acid motifs with a number of residue replacements among the oligopeptides. Two types of amino acid repeat sequences were observed in the sequences: the VK210 type of repeat unit coding for Gly-Asp-Arg-Ala-Asp/Ala-Gly-Gln-Pro-Ala and the VK247 type coding for repeats of Ala-Asn-Gly-Ala/Asp-Gly-Asn-Gln-Pro-Gly. Of the eight strains we examined, six isolates (Belem, Brazil I, Chesson I, India VII, Indonesia XIX, and North Korea) were VK210, and only two isolates (*P. simium* and *P. vivax* Palo Alto) had VK210 and VK247, which clearly indicates a mixed clonal population in these strains, although this finding had not been evident in the initial screening of *dbp* genes. Our analysis was extended to the conserved regions flanking the repeat region. The N-terminal region had just one polymorphic site (N13G) in the Indian, Chesson, Indonesian, North Korean, and the VK247 types of the Palo Alto and *P. simium* isolates. Similarly, the C-terminal conserved region had only two polymorphic sites (N270D and E330K) in the Palo Alto VK247 gene type and one (V307E) in the Brazil I isolate. Unlike the DBP and AMA-1 sequences, size polymorphism that resulted from variation in the number of repeats within the central region was readily observed with the CSP sequences.

To further investigate the possibility of more DBP alleles in the *P. vivax* Palo Alto and *P. simium* isolates, we sequenced more clones from PCR-amplified DNA of these isolates. We identified eight variants from the Palo Alto isolate (70% were of the PA-1 type) and five from *P. simium* based on the highly polymorphic region II (Table 1).

Reflection of allelic diversity observed in natural parasite populations by primate isolates of *P. vivax*

Phylogenetic trees were constructed from aligned amino acid sequences of the full-length DBP, AMA-1, and CSP open reading frames based on the neighbor-joining method using Tamura's three-parameter distance (Figure 3). The Salvador I allele served as a reference for each alignment and for constructing phylogenetic trees. Separate trees were created to analyze only alleles of the primate isolates (Figure 3A, C, and E) and to compare these isolates to sequences available from public databases (Figure 3B, D, and F). Generally, the alleles from the primate isolates reflected the diversity of the observed alleles previously identified from human infections.

When the DBP of the *P. vivax* primate-adapted isolates were compared with sequences from other strains, three different clades were evident (Figure 3A and B). The N. Korea, Chesson I, Indonesia XIX, Palo Alto, and Belem isolates as well as human isolates from different regions clustered together as separate group, clade I. The India VII and Brazil I isolates and *P. simium* along with the DBP ortholog of *P. cynomolgi* made up clade II, and the *P. knowlesi* homologs constituted clade III. AMA-1 from the primate-adapted isolates are randomly scattered within different clades among the field isolates, and *P. knowlesi* and *P. falciparum* sequences are completely isolated from all *P. vivax* alleles (Figure 3C and D). Our phylogenetic analysis of CSP showed two separate clades corresponding to the VK210 and VK247 types (Figure 3E and F) and distant from the *P. knowlesi* sequence. The VK210 cluster is divided into two sub-clades based on the variations found within the gene type. Surprisingly, there is one sub-clade consisting of only the strains India VII, Indonesia XIX, Chesson, and Palo Alto, all Asian isolates, and the other of all American isolates (Figure 3F).

Mapping of DBP polymorphisms to the surface of the ligand domain

We next used the three-dimensional structure of region II of the *P. knowlesi* Duffy binding ligand (DBL) α , which shares 70% similarity with the *P. vivax* DBP region II, to map the location of polymorphic residues of the *P. vivax* primate strains. All the polymorphic residues observed, with the exception of W437R, which is buried within the helical structure (yellow residue, Figure 4Aii), are located on the surface of the molecule and clustered within region II, mostly in the central part and also in the C-terminal third. Some polymorphic sites (N417K and L424I) are very close to the portion of the ligand domain critical for receptor recognition. The *P. vivax* Palo Alto isolate is associated with a more consistent infection in *Aotus* spp. and invasion of the merozoite into *Aotus* spp. erythrocytes is Duffy dependent, making this strain a good model for experimental studies of the efficacy of a DBP vaccine or any other *P. vivax* vaccine. Analysis of polymorphisms in the Palo Alto strain shows that it has 16 of the 44 polymorphic sites (Figure 1A) and these polymorphic sites were concentrated on to the 3D structure (Figure 4B) in the critical receptor recognition zone of region II.

DISCUSSION

Duffy binding protein is an ideal vaccine candidate because it is vital for efficient *P. vivax* invasion of human reticulocytes.⁵ Receptor recognition occurs by a single DBL domain that is highly conserved among homologous *Plasmodium* spp. ligands, although it also is the most variable region among *P. vivax dbp* alleles.^{9,10,15,18} Previous studies have suggested that this pattern of polymorphic variability is consistent with immune selection, a mechanism used by the parasite to evade host immune pressure and this is similar to what occurs in adhesion domains of other microbial pathogens. Recent clinical testing of *P. falciparum* vaccines has highlighted the importance of strain-specific immunity as a major challenge for development of a broadly effective vaccine.^{19,20}

Vaccine development against *P. vivax* requires the use of non-human primate models for initial testing. Therefore, we focused our analysis on several primate isolates that may be used for these studies. Comparisons were made of the laboratory isolates to a limited number of human isolates and to the Salvador I allele, which is the reference strain used as the basis for vaccine development and for whole genome sequence analysis. Current vaccine development is directed towards inducing an inhibitory antibody response against the DBL domain because this region is critical for receptor binding. However, other regions of the ligand may also be important for its function, especially conserved regions such as the carboxyl cysteine-rich domain (region VI) and cytoplasmic domains. Thus, we analyzed the entire DBP coding sequence in each isolate. The extent of polymorphisms observed in the primate isolates was similar to previous results studying human isolates¹⁰ with the polymorphic sites concentrated within the central portion of the ligand domain critical for receptor recognition (Figure 1A). Similar patterns of polymorphisms have been identified in the *P. falciparum* erythrocyte binding antigen–175 DBL domains and other ligands and are interpreted to reflect immune selective pressures.^{21–25}

Phylogenetic analysis showed diversity in *P. vivax* primate isolates that is reflective of what has been observed in natural infections except for three of these isolates, *P. simium* and the India VII and Brazil I strains. Interestingly, these isolates represent a distinct clade that are more similar to the DBP of simian malaria species, *P. cynomolgi* and *P. knowlesi*, than to any of the other human *P. vivax* isolates. These two simian species that naturally infect Old World monkeys are also infective to humans.^{26–29} It is not clear whether the DBP in this *P. vivax* clade represents a random oddity or this has more significance as a subset of genes favored for growth in New World primates. For *P. vivax* blood-stage vaccine evaluation, the Palo Alto strain is a suitable choice because it leads to a relatively more virulent and reproducible infection than other *P. vivax* isolates adapted to monkeys. This type of infection will provide a robust evaluation of a vaccine's protective efficacy and importantly Palo Alto merozoite invasion in *Aotus nancymae* is Duffy blood group antigen dependent (McHenry AM, Adams JH, Barnwell JW, unpublished data), as are Belem strain merozoites.¹³ Most of the polymorphic sites in the Palo Alto strain are common in other isolates (Figure 4B), but variation in two residues, N417K and L424I, may alter sensitivity to immune antibodies induced by the Salvador I DBP.

The DBL domain of *P. vivax* shares approximately 70% sequence identity with its homolog *P. knowlesi* DBP α , which binds to Duffy antigen on human and rhesus erythrocytes.^{2,3} Structural features of *P. knowlesi* DBP α and *P. vivax* DBP required for binding host cell receptors, including essential contact residues, are located primarily in the central part of region II. Site-directed mutagenesis of many residues in this region abrogates DBP-DARC interaction.^{7,30} The recently determined crystal structure of the *P. knowlesi* DBP α DBL domain³¹ has proven suitable for modeling three-dimensional structures of other DBL domains, such as *P. vivax* DBP.^{32–34} All but one of the DBP polymorphic residues of the *P. vivax* primate isolates (Figure 1A) appear as surface-exposed residues, and although scattered over the entire three-dimensional structure, polymorphisms occur most frequently within the central portion of region II necessary for receptor recognition. Perhaps most importantly in terms of vaccine development are amino acid differences immediately adjacent to key residues implicated in receptor recognition (Figure 4) and different from Salvador I, which includes residues of Palo Alto (N417K, L424I). Previously, we have determined that the N417K conversion altered DBP Π sensitivity to inhibitory antibodies that were originally induced by the Salvador I DBP.³⁵

Mixed genotype infections in *P. vivax* primate isolates will further complicate their use for vaccine testing. The presence of *P. vivax* *csp* types, VK210 and VK247, in the Palo Alto strain and *P. simium* (Figure 3E and F),^{36–38} clearly indicates these isolates are composed of multiple

parasite clones of different genotypes. Additional heterogeneity of *dbp* in these isolates was shown by sequencing of more DBPII in these two isolates with DBP variants that represent distinct alleles (Table 1).

The extent of *dbp* allelic diversity is similar to that of *ama-1* and *csp* in these *P. vivax* primates. Phylogenetic analysis showed similar distribution of the AMA-1 alleles with those taken directly from human field isolates. Polymorphisms in AMA-1 were concentrated within the ectodomain, especially within subdomain I (Figure 1B). Amino acid substitutions in this region appear to be important for changing the antigenic character of AMA-1 and its sensitivity to antibody inhibition.^{25,39} The AMA-1 sub-domains I and II represent PAN-like ligand domains that may function as receptor recognition motifs similar to the DBL domains. Recent testing of a *P. falciparum* AMA-1 vaccine developed from the 3D7 strain demonstrated the potential to induce high level of antibodies, but these antibodies showed a strain-specific inhibitory effect on parasite growth *in vitro*.^{19,20}

In summary, our data shows that the diversity of DBP in *P. vivax* primate isolates is similar to what is observed within the parasites causing human infections. However, there are two potentially significant problems identified for the use of the Palo Alto isolate to test a DBP vaccine. The Palo Alto strain of *P. vivax* and *P. simium* contain more than one genotype of parasite clone and there are multiple amino acid differences in residues flanking a critical receptor recognition site on the DBP ligand domain, which may alter sensitivity to inhibitory antibodies. Although not ideal for measuring vaccine efficacy as a homologous challenge, the heterologous nature of the Palo Alto isolate combined with its relatively more virulent infection pattern may provide a realistic test of a DBP vaccine in the human population.

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A Sequence Polymorphisms of PvDBP

B Sequence polymorphisms of PvAMA-1

Figure 1. Amino acid sequence polymorphism of **A**, Duffy binding protein and **B**, apical membrane antigen-1 (AMA-1) from primate-adapted isolates showing positions of polymorphic residues. Alignment of sequences is based on the Salvador I sequence (**bold**). Dots indicate residues identical to the reference strain. Insertion site 477b is observed only in the Indian and Brazil isolates. Schematic below each table shows the regions where the respective polymorphic sites occur with respect to the various structural regions of the gene. Roman numerals designate the different regions/domains: SS = signal sequence; PS = pro-sequence; TM = transmembrane domain; CD = cytoplasmic domain. Various isolates are arranged according to their phylogenetic relatedness to the Salvador I strain. Sequence of AMA-1 from the Brazil I isolate was not included in the analysis.

Figure 2.

Numbers of polymorphic residues in each isolate shown as a ratio relative to the total number of polymorphic sites in the gene using the Salvador I as the reference strain. Isolates are arranged according to evolutionary distance of the respective isolates with respect to the reference strain.

Figure 3.

Phylogenetic trees. **A**, Duffy binding protein primate-adapted isolates alone and **B**, with human field isolates. **C**, *Plasmodium vivax* apical membrane antigen-1 primate-adapted isolates alone and **D**, with human isolates. **E**, Circumsporozoite protein (CSP) primate-adapted isolates alone and **F**, with human field isolates. Trees were constructed with the nearest neighbor-joining method¹⁷ based on pairwise differences. Percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. Trees are drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic trees. Evolutionary distances were computed using the maximum composite likelihood method.⁴⁰ All positions containing gaps and missing data were eliminated from the dataset. Phylogenetic analyses were conducted with MEGA4.⁴¹ * represents primate-adapted isolates, and ** in the CSP trees indicates primate-adapted isolate sequence with the VK247 gene type. All other CSP genes have the VK210 gene type.

Figure 4. Three-dimensional structure of *Plasmodium vivax* Duffy binding protein (DBP). A hypothetical molecule of DBP-II depicted as a space-filling molecule (i) and respective helices (ii) showing clusters of polymorphic residues. Structures are rotated in each representation by 90° along a horizontal axis to show all surfaces of the molecule (0° = front; 90° = top; 180° = back; 270° = bottom). Purple, blue, and light blue represent sub-domains 1, 2, and 3, respectively. **A**, Cluster of polymorphic residues from all eight isolates. Red, yellow (not exposed), orange, green, and gray represent polymorphic residues in at least 60%, 50%, 37%, 25%, and 12%, respectively, for all isolates. **B**, Cluster of polymorphic residues in the Palo Alto isolate alone. The area in white represents residues of the predicted binding pocket for receptor recognition. This figure appears in color at www.ajtmh.org.

Table 1
Different variants of Duffy binding protein II (DBPII) in the Palo Alto strain and *Plasmodium simium* based on the Salvador I sequence
(**bold**) *

		Variants of Palo Alto DBPII																			
Amino acid position	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Sal I	R	L	N	D	E	K	R	S	A	V	N	L	W	E	D	V	K	K	I	S	F
PA-1	S	F	D	G	K	N	H	.	.	.	K	I	R	.	.	.	.	.	K	.	.
PA-5	S	F	D	G	K	N	H	.	.	.	K	I	R	.	.	.	E	.	K	.	.
PA-13	S	F	D	G	K	N	H	P	T	.	K	I	R	.	.	.	.	.	.	.	.
PA-15	S	F	D	G	K	N	H	.	.	.	K	I	R	G	.	.	.	G	K	.	.
PA-18	S	F	D	G	K	N	H	.	.	.	K	I	R	.	G	.	.	.	K	.	.
PA-19	S	F	D	G	K	N	H	.	.	.	K	I	R	.	.	.	.	.	K	.	L
PA-21	S	F	D	G	K	N	H	.	.	.	K	I	R	.	.	.	.	R	K	.	.
PA-22	S	F	D	G	K	N	H	.	.	A	K	I	R	.	G	.	.	.	.	.	.
PA-00	S	F	D	G	K	N	H	.	.	.	K	I	R	.	.	.	.	.	K	.	.

Table 2
Variants of *P. simium* DBPII

		Variants of <i>P. simium</i> DBPII																			
Amino acid position	2	9	1	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Sal I	Y	C	N	R	R	L	D	E	K	R	T	K	L	C	W	I	A	V			
SM-1	H	.	Y	S	S	F	G	K	N	H	R	N	I	.	C	.	.	D			
SM-2	H	.	Y	S	S	F	G	K	N	H	R	N	I	.	.	.	.	D			
SM-3	H	.	Y	S	S	F	G	K	N	H	R	N	I	.	.	.	S	D			
SM-4	H	.	Y	S	S	F	G	K	N	H	R	N	I	W	.	.	.	D			

Variants of <i>P. simium</i> DBPII													
SM-6	R	.	.	.	.	.	.	.	.	.	.	.	.
SM-00	H	.	.	.	.	.	.	.	.	.	.	K	.

* Dots indicate residues identical to the reference strain and PA-00 and SM-00 represent primary sequences of the Palo Alto strain and *P. simium*, respectively