

Novel Antigen Identification Method for Discovery of Protective Malaria Antigens by Rapid Testing of DNA Vaccines Encoding Exons from the Parasite Genome

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We describe a novel approach for identifying target antigens for preerythrocytic malaria vaccines. Our strategy is to rapidly test hundreds of DNA vaccines encoding exons from the *Plasmodium yoelii yoelii* genomic sequence. In this antigen identification method, we measure reduction in parasite burden in the liver after sporozoite challenge in mice. Orthologs of protective *P. y. yoelii* genes can then be identified in the genomic databases of *Plasmodium falciparum* and *Plasmodium vivax* and investigated as candidate antigens for a human vaccine. A pilot study to develop the antigen identification method approach used 192 *P. y. yoelii* exons from genes expressed during the sporozoite stage of the life cycle. A total of 182 (94%) exons were successfully cloned into a DNA immunization vector with the Gateway cloning technology. To assess immunization strategies, mice were vaccinated with 19 of the new DNA plasmids in addition to the well-characterized protective plasmid encoding *P. y. yoelii* circumsporozoite protein. Single plasmid immunization by gene gun identified a novel vaccine target antigen which decreased liver parasite burden by 95% and which has orthologs in *P. vivax* and *P. knowlesi* but not *P. falciparum*. Intramuscular injection of DNA plasmids produced a different pattern of protective responses from those seen with gene gun immunization. Intramuscular immunization with plasmid pools could reduce liver parasite burden in mice despite the fact that none of the plasmids was protective when given individually. We conclude that high-throughput cloning of exons into DNA vaccines and their screening is feasible and can rapidly identify new malaria vaccine candidate antigens.

The year 2002 saw the publication of the genomic sequences of the human malaria parasite *Plasmodium falciparum* (12) and the rodent parasite *Plasmodium yoelii yoelii* (5). The hope is that this information will bring insights into parasite biology and lead to the development of new vaccines and drugs. However, novel research approaches are required to efficiently study the thousands of genes. This paper describes the development of a high-throughput technique for the identification of vaccine target antigens among newly annotated malaria genes. Our method rapidly produces large numbers of DNA vaccines carrying *P. y. yoelii* exons and measures their ability to reduce parasite load in mice. We call this screening technique the antigen identification method.

The novelty and efficiency of the antigen identification method come from a combination of rapid production of DNA vaccines and sensitive measurement of parasite killing. With the annotated *P. y. yoelii* genomic sequence, we identify *P. y. yoelii* genes expressed during the sporozoite stage by comparison with expressed sequence tags (ESTs) generated from a cDNA library of *P. yoelii* sporozoites (20). PCR primers for these sporozoite *P. y. yoelii* genes are synthesized to be com-

patible with the Gateway cloning system, which allows rapid production of DNA vaccine plasmids. Mice are immunized with the DNA vaccines and challenged with *P. y. yoelii* sporozoites, and parasite burden in the liver is assessed by quantitative reverse transcription-PCR based on *P. y. yoelii*-specific 18S rRNA. Any *P. y. yoelii* vaccine that reduces the liver-stage parasite burden becomes an antigen of interest, and the *P. falciparum* orthologs are identified by reference to the *P. falciparum* genomic sequence. Antibodies from immunized mice are used for studies of gene expression in the *P. y. yoelii* parasite.

We believe that target antigen discovery in the *P. y. yoelii* mouse malaria model system is relevant for human malaria vaccine development. *P. y. yoelii* infection of mice is an established model in malaria vaccine research (8). DNA vaccination with *P. y. yoelii* antigens protects mice against infection with sporozoites (10, 27), indicating that the immune responses induced by plasmid vaccines can kill *P. y. yoelii* parasites. The protein coding regions of *P. y. yoelii* genes show significant homology with those of *P. falciparum* (5), and several *P. y. yoelii* sporozoite and liver-stage antigens (circumsporozoite protein [CSP], SSP2, and HEP17) which protect mice from *P. y. yoelii* infection have *P. falciparum* orthologs that are being developed as human vaccines (8, 15). Thus, we believe that any *P. y. yoelii* antigen that protects mice against malaria infection should have its *P. falciparum* counterpart investigated as a human vaccine candidate.

This paper describes a strategy for the rapid cloning of 192

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identified *P. y. yoelii* exons and their expression by DNA vaccines and a pilot study with 19 of these vaccines to compare immunization approaches for single plasmids and plasmid pools.

MATERIALS AND METHODS

Identification of *P. y. yoelii* genes expressed during the sporozoite stage. With the annotated genome sequence of *P. y. yoelii*, complete genes or exons of genes identified as being expressed during the sporozoite stage were selected as follows: 5,687 *P. y. yoelii* contigs were searched for homology to 1,923 *P. y. yoelii* ESTs from a sporozoite cDNA library (20) with the algorithm BLAST (21). The 571 contigs identified as having a significant match to an EST (>90% identity over 100 bp) were analyzed for the position of the EST within a predicted gene model. A final set of 192 genes or exons (Supplement 1 at <http://www.nmrc.navy.mil/pages/supplementaldata.xls>) were chosen with a set of criteria such as length of the gene model (>200 bp to <4,000 bp) and lack of overlap into noncoding regions. One hundred eight were single-exon genes, and the remainder were single exons from multiple-exon genes.

Gateway cloning of *P. y. yoelii* genes. Gateway technology (Invitrogen Inc., Carlsbad, Calif.) was used for cloning of *P. y. yoelii* malaria genes into DNA vaccines. This system has been used extensively in a variety of studies of novel proteins, such as those investigating protein interaction in *Caenorhabditis elegans* (31), protein localization (28), and recombinant protein expression (4, 14). The Gateway system is designed to clone large numbers of DNA sequences into plasmids by exploiting the bacteriophage lambda DNA recombination process, with specific integration and excision sites (*attB* × *attP*) to clone genes into entry plasmids. Once cloned into an entry plasmid, these genes are rapidly moved into Gateway-compatible expression vectors following a similar recombination reaction with specific sites (*attL* × *attR*). A two-step PCR approach is used to amplify the malaria genes integrating the *attB* sites required for cloning. *P. y. yoelii* genomic DNA was used as the template in the first PCR. The final PCR products were visualized on a 1% agarose gel and purified with a PCR purification kit (Millipore, Bedford, Mass.). The BP cloning reactions were carried out with the pDONR207 vector (Invitrogen) according to the manufacturer's specifications. Screening for recombinants clones was done by PCR on two to eight colonies from each transformant, with plasmid-specific primers.

PCR primer design. For our preliminary experiments, exon-specific primers for the first 50 exons were designed manually. These primers were 20 to 26 bases in length, with melting temperatures (T_m) between 50 and 60°C. In subsequent experiments, 192 exons (including the first 50) were designed automatically with a customized Perl script controlling Primer3 primer design software (http://www.genome.wi.mit.edu/ftp/distribution/software/README.primer3_0_9_test) (Whitehead Institute, Cambridge, Mass.). The script was designed to identify primers with T_m of 60 to 65°C and lengths of 18 to 35 bases which would amplify for each exon in an in-frame product of maximum length, but which would avoid strings of four or more homopolymers, common in the *Plasmodium* genome.

A 5' start and/or a 3' stop codon was added if not already included in the selected exon sequences. The forward primer contained the recombination sequence GCAGGCTCCACC, and the reverse primer has the sequence AGAAAGCTGGGT immediately preceding the start and stop codons, respectively. For the second-step PCR, a set of forward (attB1, GGGGACAAGTTTGTACAAAAAGCAGGCTCCACC) and reverse (attB2, GGGGACCACTTGTACAAGAAAGCTGGGT) primers were used. These sequences overlapped the 12 bases in the primers used in the first-step PCR, and together they added the phage recombination site.

PCR amplification of *P. y. yoelii* exons for Gateway cloning. For the amplification of the *P. y. yoelii* exons in the first-step PCR, we used 150 to 250 ng of *P. y. yoelii* genomic DNA and 0.2 μM exon-specific primers. Pfu Turbo DNA polymerase (Stratagene, La Jolla, Calif.) was used for all amplifications. Extension times and temperatures for the first PCR were varied to maximize products (see Results). Second-step PCR amplifications were carried out according to the manufacturer's protocol, with the exception that extension times were increased to match optimal times in the first PCR step. All PCR amplifications were performed in 96-well plates with a 9700 thermal cycler (Perkin Elmer, Wellesley, Mass.). PCR products were visualized under UV light on a 1% agarose gel stained with ethidium bromide with a ChemiImager 4400 (Alpha Innotech Corporation, Imgen Technologies, Alexandria, Va.).

Plasmids. Donor vector pDONR207 was used for recombinational cloning of the PCR-amplified fragment to generate an entry clone. The mammalian plasmid immunization or destination vector was VR1012-DV (J. Aguiar et al., unpublished data), a modified version of VR1012 (Vical Inc., San Diego, Calif.).

This vector was adapted to the Gateway system by insertion of a recombination cassette (19) with a blunt site in the multiple cloning sites as instructed by Invitrogen (Aguiar et al., unpublished data).

Bacterial transformation with entry or destination plasmids. Heat shock transformation of *Escherichia coli* DH5α library efficient bacteria (Invitrogen) was performed as described in the Gateway instruction manual (19).

Analysis of cloned exons. A PCR for confirmation of the size of an entry clone was performed directly on individual bacterial colonies (30). Primers (5'-TCGC GTTAAACGCTAGCATGGATCTC-3' and 5'-GTAACATCAGAGATTTTG AGACAC-3') specific for the recombination sites in the pDONR207 plasmid were used for insert amplification. If inserts were of the predicted size, they were moved into the VR1012-DV immunization vector. The VR1012-DV clones were analyzed by PCR for size and were sequenced with vector-specific primers (5'-GCAGTGTAGTCTGAG-3' and 5'-GGCAAACAACAGATGGCTGGCAA-3'), with the BigDye terminator sequencing protocol (Applied Biosystems, Foster City, Calif.) and a 377 automated fluorescence sequencing system (Applied Biosystems).

Plasmid production. Plasmids from each entry clone were prepared in 96-well format with the REAL prep plasmid kit (Qiagen, Valencia, Calif.). Plasmids for immunization were manufactured by Aldevron (Fargo, N.D.) and had endotoxin levels less than 0.1 EU/μg of DNA and were 60% to 80% supercoiled.

Immunization of mice. All animal experiments were approved by the Walter Reed Army Institute of Research, Naval Medical Research Center Institutional Animal Care and Use Committee and were conducted according to the Guide for the Care and Use of Laboratory Animals 1996 (18).

Female BALB/c mice (Jackson Laboratories, Bar Harbor, Maine) 4 to 6 weeks old were used. For intramuscular immunization of mice with single plasmids, 25 μg of DNA was mixed with 30 μg of plasmid VR1701 encoding murine granulocyte-macrophage colony-stimulating factor as an adjuvant (34) in a total volume of 100 μl of phosphate-buffered saline (PBS). For intramuscular immunizations with pools of six or seven plasmids, 25 μg of DNA from each plasmid was mixed with 30 μg of plasmid VR1701 in a total volume of 100 μl of PBS. Half of each dose (50 μl) was injected into each gastrocnemius muscle with a 29-gauge needle. Mice were injected three times at 4-week intervals.

Gene gun immunization. DNA vaccine plasmids were prepared for gene gun injection as described in the Bio-Rad Gene Gun instruction manual and elsewhere (24, 33). Briefly, 60 μg of each plasmid was precipitated onto 0.5 to 1 mg of gold particles (0.5 to 2 μm) (Degussa, Washington, Pa.). Mice were injected intradermally with the Helios Gene Gun system (400-lb/in² helium pressure; Bio-Rad, Hercules, Calif.). Each mouse received three bullets containing 2 μg of DNA each into nonoverlapping areas of the shaved abdomen skin. Mice were injected three times at 4-week intervals.

Immunofluorescence. Sera from all immunization groups were collected 2 weeks after the second and third DNA doses and used to assess expression of the exon sequence in *P. y. yoelii* sporozoites and blood stages by indirect immunofluorescence assay (6). Slides containing sporozoites or blood stages were fixed by air-drying, and the procedure was carried out without membrane permeabilization. As a positive control for the sporozoite indirect immunofluorescence assay, a monoclonal antibody (NYS1) reactive with the surface of the sporozoite (6) was used, while for the blood-stage-parasite indirect immunofluorescence assay, we used a polyclonal anti-*P. y. yoelii* MSP-1 antiserum (kind gift of M. Sedegah). Normal mouse serum was used as a negative control in all indirect immunofluorescence assay tests.

Sera from gene gun-vaccinated mice collected 2 weeks after the third immunization were further tested against liver stages. Briefly, slides containing liver stages were prepared for indirect immunofluorescence assay from sporozoite-infected mice (*P. y. yoelii* 17X NL clone 1.1). Livers were removed 40 h after infection, fixed in zinc solution for 48 h, and placed in 37°C paraffin (Sigma-Aldrich). Five-micrometer sections were placed on Superfrost Plus slides (Menzel-Glaser, Braunschweig, Germany). Slides were washed with ethanol to remove paraffin, hydrated in PBS, blocked with 5% milk in PBS, and incubated with mouse serum samples diluted in 0.05% PBS-Tween 20. After washing with the same buffer used for sample dilution, slides were stained with goat anti-mouse immunoglobulin-Alexa Fluor 488 (A-11029; Molecular Probes) in Evans blue counterstain. Slides were washed once again, mounted in 30% glycerol in PBS, and observed under UV light at x400 magnification.

Sporozoite challenge and quantitative measure of liver-stage *P. y. yoelii* parasites. Four weeks after the third DNA dose, the ability of the vaccinated mice to withstand a sporozoite infection was assessed by quantitative PCR (35). This method measures stage-specific *P. y. yoelii* 18S rRNA in mouse liver. Briefly, mice were challenged intravenously with 50,000 *P. y. yoelii* 17 XL clone 1.1 sporozoites. Livers were removed 42 h after challenge, placed in RNAlater (Ambion, Inc., Austin, Tex.), homogenized in Trizol (Life Technologies, Gaith-

ersburg, Md.) with an Omni TH polytron homogenizer (Omni International), and stored at -80°C until analysis. Total RNA was extracted from each liver homogenate, and synthesis of total cDNA was performed with a robot Biomek 2000 (Applied Biosystems, Foster City, Calif.). Real-time fluorescence detection and measurement of *P. y. yoelii* 18S and mouse glyceraldehyde-3-phosphate dehydrogenase PCR products were performed with an ABI 7700 sequence detector (PE Applied Biosystems).

Blood-stage expression of 19 selected *P. y. yoelii* exons. Blood-stage-*P. y. yoelii*-infected BALB/c mice served as a source for parasite material. Parasitized blood was collected and leukocytes were removed with Plasmodipur filters (Euro-Diagnostica, Arnhem, Netherlands). Total RNA was extracted with Trizol (Invitrogen) following standard protocols. cDNA was prepared with the Super-script One-Step reverse transcription-PCR for long templates, following the manufacturer's instructions (Invitrogen) with the same PCR primers (except for one new forward primer, GATGTACCAGATGTACCAGAATATGTACC, corresponding to PY01499) and conditions as used for initial amplification from genomic DNA.

Bioinformatic analysis. The amino acid sequence of each *P. y. yoelii* open reading frame was analyzed with the Simple Modular Architecture Research Tool (SMART) (<http://smart.embl-heidelberg.de>) to identify structural and functional domains. Within SMART, transmembrane segments are predicted by the TMHMM2 program, signal peptides are determined by the SignalP program, and regions containing repeats are detected by the Prospo program. The orthologous sequences of the *P. y. yoelii* open reading frames in other *Plasmodium* spp. were detected with BLAST and the genome database for all *Plasmodium* open reading frames of ≥ 50 bp found in Plasmodb (www.plasmodb.org). The BLAST program used was Wash U-BlastP 2.0 and the BLAST parameters applied were the most stringent that are available in the Plasmodb database with an expected value (E) of 0.0001 and the cutoff value (S) of 110. Preliminary sequence data for *P. vivax* were provided by the Institute for Genomic Research through the website at <http://www.tigr.org>.

Statistical analysis. Statistical tests were conducted between individual vaccines and only their associated controls with one-way and two-way unbalanced analysis of variance. The second factor in the two-way analysis of variance was the date of analysis for vaccines that were studied on multiple days. Since we were interested in selecting only vaccines that were significantly better than no treatment at all, F-tests were treated as T2 in order to obtain one-sided tests. The possible range of adjusted *P* values was indicated by presenting both the Bonferroni familywise adjustment and the testwise values (2). Results were calculated and graphically presented with R (17).

RESULTS

Selection of exons for cloning. We randomly selected 192 *P. y. yoelii* exons expressed in a *P. y. yoelii* sporozoite cDNA library (20) for cloning into DNA vaccine plasmids (Supplement 1 at <http://www.nmrc.navy.mil/pages/supplemental-data.xls>). Of these, 108 were predicted to be single-exon genes, and the remainder were exons from multiple-exon genes. The selected exons varied in size from 0.2 kb to 3.8 kb.

Cloning of exons. Preliminary studies with the Gateway cloning method showed that *P. y. yoelii* genomic DNA was a more reliable PCR template than parasite cDNA (data not shown). Thus, all *P. y. yoelii* exons in this study were amplified from *P. y. yoelii* genomic DNA.

Primer design. Primers for amplification of all 192 selected *P. y. yoelii* exon sequences were designed with a Perl-customized script controlling the Primer3 software. This program selects primers to amplify the longest possible fragment from each DNA sequence but excludes certain configurations of bases, such as runs of repeated nucleotides, predicted to cause nonspecific annealing in the PCR. Based on these criteria, it is rare that full-length exons are selected for PCR. On average, our PCR primers amplified products that were 48 bp shorter than the exon sequences selected. A 5' start and/or a 3' stop codon was added if not already included in the selected primer sequences. The sequences corresponding to the 192 primer

pairs are attached in Supplement 1 (<http://www.nmrc.navy.mil/pages/supplementaldata.xls>). In our preliminary experiment, manually designed primer sets for 50 *P. y. yoelii* exons were produced with similar criteria (sequences not shown).

Optimization of PCR conditions for Gateway cloning. In our preliminary cloning experiment, we used parameters for the first-step PCR as follows: incubation at 95°C for 2 min and 20 cycles of 94°C for 30 s, 55°C for 30 s, and 68°C for 3 min. The second PCR step used incubation at 94°C for 1 min, five cycles of 94°C for 15 s, 45°C for 30 s, 68°C for 3 min, and 35 cycles of 94°C for 15 s, 55°C for 30 s, and 68°C for 3 min. With these PCR conditions, we obtained 37 of 50 visible PCR products, where only 29 amplicons were of the correct size. The size range of these 29 amplicons was between 0.5 and 2.2 kb, while all 13 nonamplified exons ranged in size from 2.2 to 3.8 kb.

To improve PCR efficiency, we tried altering the annealing temperatures, number of cycles, and the extension times. Altering annealing temperatures in the PCR protocol did not improve our amplifications (data not shown). However, reducing the number of cycles and increasing the extension times (36) yielded a larger number of correctly sized PCR amplicons.

Figure 1A shows the products of PCRs with exons 97 to 192. These 96 exons have sizes from 0.2 to 3.0 kb. We used 15 cycles in the first-step PCR and 25 cycles in the second-step PCR with an extension time of 5 min in each. These conditions successfully amplified 83 out of 96 exons, where 57 of 60 (90%) exons were between 0.2 and 2 kb, and 16 of 36 (44%) exons from 2 to 3.0 kb showed bands of the predicted size. This set of 96 exons (97 to 192) was separated by size into three different groups, each subjected to PCR with increasing extension times depending on exon length (Fig. 1B). Exons between 0.2 and 1.5 kb received 3 min of extension time on each cycle; exons between 1.5 and 1.8 kb received 5 min; and exons between 1.8 and 3 kb received 10 min. Such graded extension times resulted in successful amplification of 92 of 96 exons, 47 of 48 from 0.2 to 1.5 kb, 12 of 12 from 1.5 to 1.8 kb, and 33 of 36 from 1.8 to 3 kb.

Exons 1 to 96 (0.5 to 3.8 kb) were amplified with this PCR protocol with increasing extension times. A total of 95 exons were amplified, the sole exception being an exon of 2,270 kb (data not shown). In summary, a total of 188 of 192 exons (98%) were successfully PCR amplified in the two-step PCR (95 of 96 and 93 of 96 from the first and second plates, respectively).

Cloning of PCR products into the Gateway donor and vaccine plasmid. We used the 29 *P. y. yoelii* amplicons from our preliminary PCR for our first cloning into Gateway plasmids. With the manufacturer's protocol for the BP reaction, 300 ng of donor plasmid pDONR207 was mixed with 100 to 500 ng of amplification product. A maximum of four bacterial colonies were analyzed for each PCR product. At least one correctly sized entry clone was found for 19 of 29 (65%) of the *P. y. yoelii* exons. One colony containing a correct entry plasmid was chosen from each of the 19 *P. y. yoelii* exons, and all (19 of 19) exons were successfully transferred in a single reaction to the VR1012-DV immunization plasmid. Single colonies from each of the 19 VR1012-DV constructs were analyzed by PCR for size and by terminal DNA sequencing, and all (19 of 19) positive bacterial colonies contained the correct *P. y. yoelii*

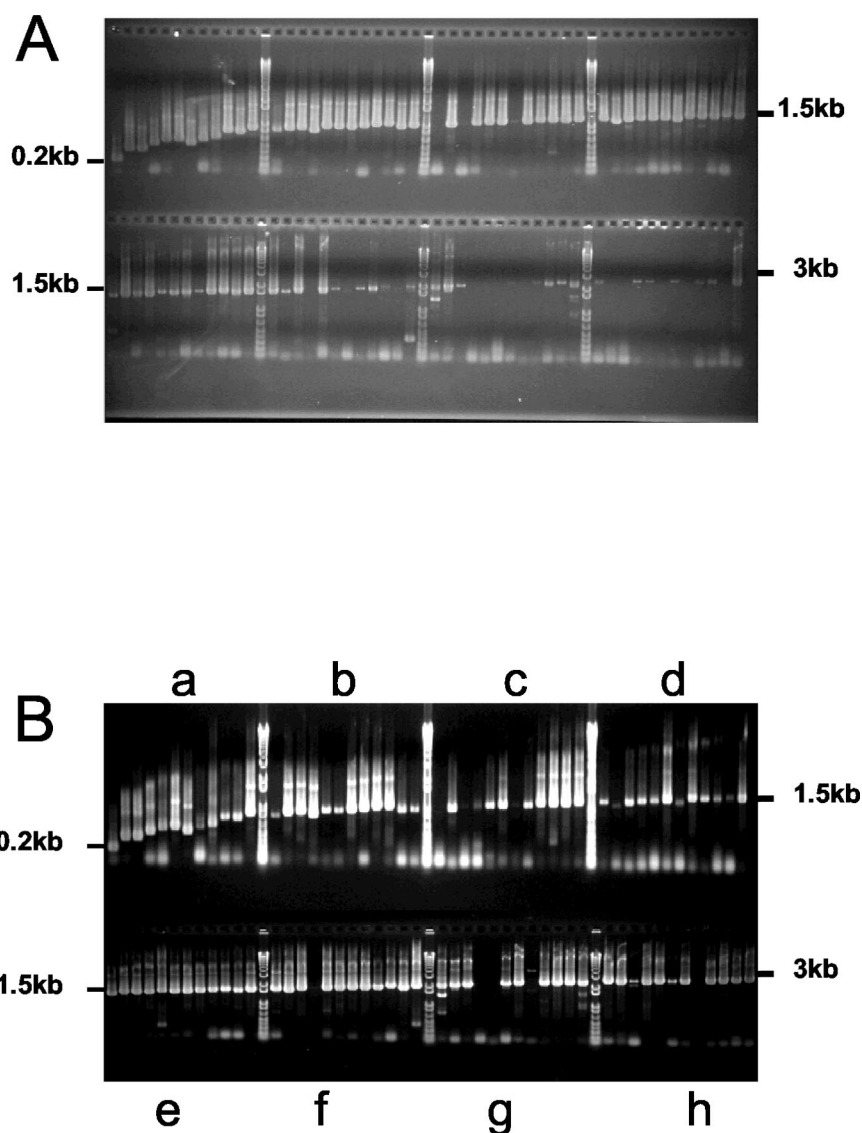


FIG. 1. Optimization of a PCR for the amplification of 96 *P. yoelii* exons of different sizes for use in the Gateway cloning system. The relevant adjustment in this PCR is the extension time. (A) Amplification of 96 exons between 0.2 and 3 kb in size with a 5-min extension time in the first and second step of the PCR. (B) Amplification of the 96 exons shown above by applying different extension times according to exon size. Exons ranging in size between 0.2 and 1.5 kb (a to d), 1.5 and 1.8 kb (e), and 1.8 and 3 kb (f to g) were amplified with extension times of 3 min, 5 min, and 10 min, respectively. Amplicons were loaded onto a 1% agarose gel according to size, starting with the smallest products. Shown in A and B are PCR products obtained after the second PCR.

exon sequences (data not shown). These 19 DNA vaccines were used for our immunization studies.

To clone the 188 *P. yoelii* amplicons from the PCR optimization study into Gateway plasmids, we used the same conditions described above. After the BP reaction, two bacterial colonies were picked for PCR analysis from each transformation. At least one of these colonies was found by PCR to contain a plasmid of the predicted length in 143 of 188 cases. An additional four colonies were analyzed from the remaining 45 transformations, which yielded an additional 13 colonies with correctly sized plasmids, for an overall success rate of 156 of 188 (83%). For the 32 uncloned PCR amplicons, we modified the BP reaction by using equimolar ratios of the PCR amplicon and pDONR207. This led to successful cloning of 26

of 32 additional amplicons after screening of two to eight bacterial colonies from each transformant. In total, 182 of 188 (97%) correctly sized entry clones were produced by these modifications to the BP reaction.

All 182 entry clones were successfully transferred in one step to the VR1012-DV immunization vector following the manufacturer's indications for the LR reaction. Single bacterial colonies from each transformant were analyzed by PCR, and all 182 destination clones showed the predicted insert size. The *P. yoelii* exon inserts from 96 of these VR1012-DV clones were sequenced, and all had the expected nucleotide sequence in the correct reading frame (data not shown). Based on the lack of errors, we did not consider it cost-effective to confirm the sequence of the remaining 86 plasmid vaccines.

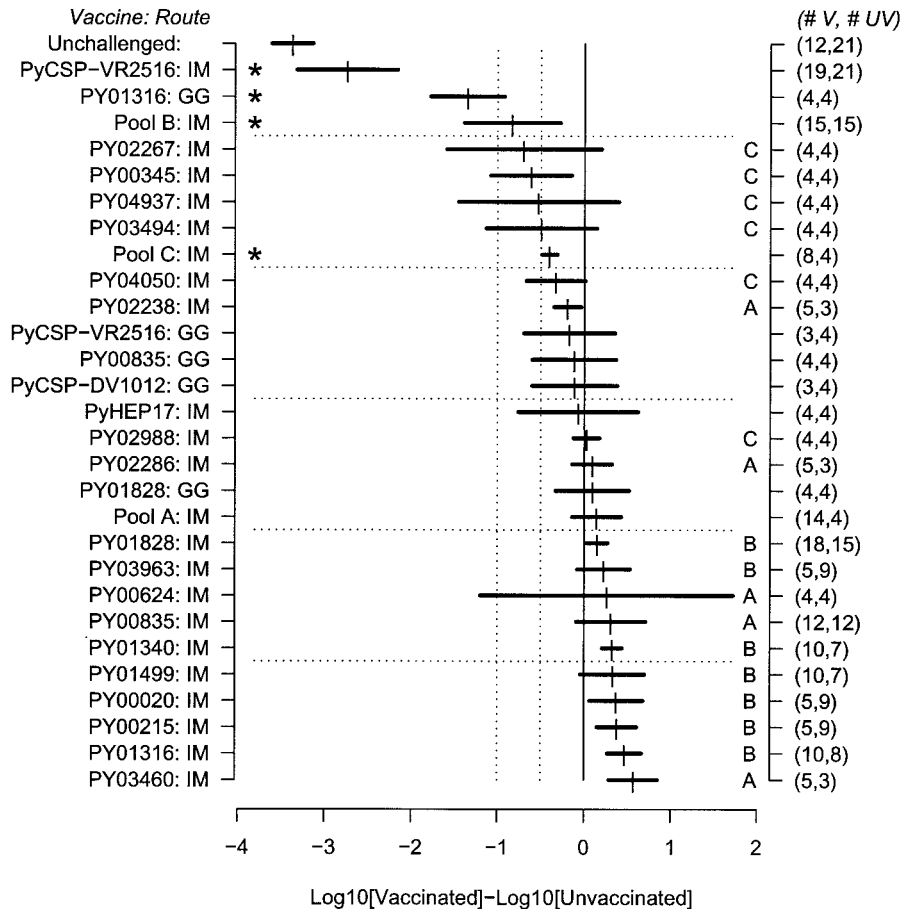


FIG. 2. Median differences between real-time PCR measurements of liver parasite burden in unvaccinated challenged mice and vaccinated mice challenged with *P. y. yoelii* sporozoites. Vaccination groups are shown on the y axis. Mice were immunized with single DNA vaccine plasmids or with pools of plasmids. Single-plasmid immunizations were either intramuscular (IM) or intradermal with a gene gun (GG). Plasmid pools A, B, and C containing six to seven different single plasmids were injected intramuscularly only. The x axis shows changes in liver stage parasite burden on a log scale, with zero representing the parasite load in challenged nonvaccinated mice. #V/#UV, number of vaccinated/number of unvaccinated mice used to calculate each vaccine effect. Vertical lines indicate median effects, with horizontal lines showing 95% confidence for an individual vaccine. *, parasite burden significantly different at the $\alpha < 0.05$ level adjusted for all 30 tests. Data are a summary of results from multiple experiments.

Screening strategy: intramuscular immunization with plasmid pools or single plasmids. Pooling and coinjecting several DNA vaccines is an attractive way to rapidly screen vaccine antigens and is the basis of expression library immunization (1, 29). However, pooling DNA vaccines limits the amount of plasmid administered and could lead to positive or negative interactions between immunogens. To evaluate plasmid pooling as a screening strategy, we randomly grouped the 19 *P. y. yoelii* plasmids into three pools, A, B, and C, containing six or seven plasmids each. No specific feature was taken into account in grouping these plasmids into pools. Twenty-five micrograms of each plasmid was used, and the total dose of 100 μ l was split between two intramuscular injection sites. For comparison, we injected groups of mice with 25 μ g of each of the 19 *P. y. yoelii* individual plasmids. As a positive control, mice were immunized with the *P. y. yoelii* CSP plasmid, known to give protection against *P. y. yoelii* sporozoite infection. Additional controls included were unchallenged unvaccinated mice, unvaccinated but challenged mice, and mice vaccinated

with noncoding plasmids and challenged. As an adjuvant, 30 μ g of a plasmid encoding murine granulocyte-macrophage colony-stimulating factor was mixed with the *P. y. yoelii* plasmids or with the different controls. We immunized mice three times at 1-month intervals and challenged with infectious *P. y. yoelii* sporozoites (50,000 sporozoites per mouse) 1 month after the last immunization. Each individual plasmid or pool was tested from one to four times.

We measured the protective effect of the vaccines with quantitative real-time PCR to measure parasite load in mouse livers removed 42 h after sporozoite challenge. Figure 2 summarizes the results of over a dozen immunization experiments. *P. y. yoelii* CSP plasmid injected intramuscularly was able to reduce parasite burden by almost three logs or over 99%, with 95% confidence intervals overlapping those for uninfected mice. *P. y. yoelii* HEP17 plasmid, which is a weak immunogen in BALB/c mice (10), did not decrease parasite burdens when injected intramuscularly. None of the newly cloned *P. y. yoelii* vaccines tested approached the efficacy of *P. y. yoelii* CSP when

TABLE 1. Comparison of routes of immunization

Vaccine	Route	Relative parasite burden ^a	Immunofluorescent antibody titer		
			Sporozoite ^b	Blood stage ^b	Liver stage ^c
PY00835	Gene gun	-0.1	2,560	20	ND
	IM	0.3	Neg	Neg	ND
PY01316	Gene gun	-1.3*	2,560	20	Neg
	IM	0.5	Neg	Neg	ND
PY01828	Gene gun	0.1	2,560	640	Pos
	IM	0.1	Neg	Neg	ND
CSP	Gene gun	-0.1	2,560	Neg	Neg
	IM	-2.7*	2,560	Neg	ND

^a Parasite burden in liver is in comparison with that in challenged unvaccinated mice and is expressed as log₁₀. *, parasite burden significantly different ($P < 0.05$) versus that in challenged unvaccinated mice.

^b Shown are the antibody titers from pooled antisera. Values are the last dilution at which fluorescence was detected. Neg, negative; Pos, positive; ND, not done.

^c Sera tested at a single dilution (1:80).

injected intramuscularly. However, four single-plasmid vaccines injected intramuscularly decreased the mean parasite load by more than 0.5 log, which represents a 68 to 79% reduction in parasite burden (Fig. 2).

Intramuscular vaccination with plasmid pools A, B, or C produced various levels of protection, which were difficult to reconcile with the data from single-plasmid vaccinations. Plasmid pool B reduced the parasite burden of immunized mice by 0.8 log or 85%, while pool C had a smaller, 0.4-log or 60% protective effect and pool A did not protect. Despite the protection afforded by pool B, none of the seven individual plasmids making up this pool was able to reduce parasite burden. The response to pool B may represent either additive or synergistic effects from immune responses to single plasmids. However, we doubt that the summation of small protective responses to individual plasmids is responsible for the protection rendered by pool B, as addition of several single plasmids that had a protective response did not increase the protection by pool C. Our results with pool B show that pooling plasmid vaccines may lead to false-positive results in screening for individual protective antigens.

To investigate inhibitory effects caused by a mixture of plasmids, an immunization experiment was carried out where the *P. y. yoelii* CSP plasmid was included in plasmid pool A, B, or C. We did not observe any suppression of the protective effect of as little as 2 µg of *P. y. yoelii* CSP plasmid when mixed with 150 to 175 µg of total DNA from plasmid pool A, B, or C (data not shown).

Gene gun versus intramuscular immunization. As an alternative to intramuscular injection, we immunized mice in the skin by gold particle bombardment (gene gun immunization). Only four vaccines were included in this experiment, *P. y. yoelii* CSP, PY00835, PY01316, and PY01828.

Table 1 compares gene gun with intramuscular immunization for these four plasmids. In contrast to intramuscular needle injection, gene gun immunization with *P. y. yoelii* CSP did not reduce parasite burden in mice. Conversely, the PY01316 vaccine substantially reduced parasite burden when given by gene gun but had no effect when given by intramuscular injection. We believe that intramuscular and gene gun immunization may activate different arms of the immune system and that different antigens may protect by distinct immune effector mechanisms. Thus, it appears that for plasmid vaccination

there is no single route which can produce the best protective response for all antigens (24, 33).

Detection of antibody reactivity to parasite stages with indirect immunofluorescence assay. Sera from all intramuscular-immunization groups were tested individually against sporozoites and blood stages by indirect immunofluorescence assay. Besides the positive control plasmid *P. y. yoelii* CSP, only two constructs (PY00835 and PY01828) elicited antibodies against sporozoites at titers equal to or higher than 10 (Table 1). None of the constructs elicited antibodies against blood stages.

To test whether their immunogenicity would be improved, three plasmid vaccines and *P. y. yoelii* CSP as a control were used for gene gun immunization. Sera from animals administered all four vaccine plasmids gave positive reactivity against sporozoites and blood stages (Table 1). The antibody reactivity to sporozoites was dramatically increased from barely detectable to 2,560, while reactivity to blood stages was now detectable. The fluorescent staining of either sporozoites or blood stages from each sample was indistinguishable, as was the localization of the staining (i.e., surface or cytoplasmic).

Sera from mice immunized with plasmids *P. y. yoelii* CSP, PY01316, and PY01828 by gene gun were further tested as a pool at a 1:80 dilution against liver stages. Sera from mice immunized with *P. y. yoelii* CSP and PY01316 gave no detectable staining on *P. y. yoelii* liver-stage schizonts, although the same sera strongly labeled *P. y. yoelii* sporozoites. In contrast, the PY01828 sera brightly stained the parasitophorous vacuole of *P. y. yoelii* liver-stage schizonts (data not shown).

Expression of *P. y. yoelii* exons in *P. y. yoelii* blood-stage parasites. Expression of the 19 cloned *P. y. yoelii* exons in *P. y. yoelii* blood-stage parasites was examined with blood-stage-parasite cDNA. All 19 exons were detected in blood-stage-parasite cDNA (data not shown).

The presence of these 19 exons in *P. y. yoelii* liver stages was investigated by examining a *P. y. yoelii* liver-stage-parasite cDNA library database (Peter L. Blair, unpublished data). Five genes were identified (PY00624, PY02988, PY03494, PY04050, and PY04937).

Annotation of 19 *P. y. yoelii* genes used for immunization. With the bioinformatics tools provided in SMART (<http://smart.embl-heidelberg.de>) searches were conducted for each translated *P. y. yoelii* protein to identify protein domains and possible biological function. To search for orthologs in other

TABLE 2. Annotation of 19 *P. y. yoelii* exons cloned into DNA vaccine vectors and screened for their capacity to protect against a challenge with sporozoites

Locus	GenBank accession no.	Length (bp)	Signal sequence	T_m domain	Domain homology	<i>Plasmodium</i> spp. with orthologs ^a
PY00345	EAA23004.1	654	—	—	Unconventional myosin PfM-B related	P.f., P.b., P.k., P.v.
PY00398	EAA15582.1	1,328	—	—	58-kDa phosphoprotein (heat shock-related protein)	P.f., P.b., P.c., P.k., P.v.
PY00624	EAA17354	2,271	—	—	CTP synthase, glutamine amidotransferase class-I	P.f., P.b., P.k., P.v.
PY02238	EAA21673	1,443	—	—	Xeroderma pigmentosum G N&I region, helix-hairpin-helix class	P.f., P.k., P.b., P.v.
PY02286	EAA21722	696	—	—	Hypothetical protein	P.f., P.b., P.k., P.v.
PY03460	EAA23034	516	—	—	Hypothetical protein	P.f., P.b.
PY03458	EAA23032	909	—	—	Hypothetical protein	P.f., P.b., P.c., P.k., P.v.
PY03494	EAA15212	906	—	—	K-homology (KH domain) RNA-binding protein	P.f., P.v.
PY03963	EAA15758	1,422	—	—	K-homology (KH domain) RNA-binding protein	P.f., P.b., P.k., P.v.
PY00020	EAA21217	1,346	—	—	Hypothetical protein	P.f., P.b.
PY00215	EAA21530	1,452	—	—	Hypothetical protein	P.f., P.b., P.k.
PY01316	EAA20613	1,224	—	Yes	Topoisomerase II (TOP2c) domain	P.k., P.v.
PY01340	EAA20643	1,446	—	Yes	Hypothetical protein	P.f., P.b., P.k., P.v.
PY01499	EAA20836	3,378	—	Yes	Von Willebrand factor type A domain, thrombospondin 1	P.f., P.b., P.v.
PY01828	EAA21199	1,235	—	—	Porphobilinogen deaminase, putative	P.f., P.b., P.k., P.v.
PY02267	EAA21703	804	—	—	<i>Dictyostelium discoideum</i> HSPC148, 10/100, putative	P.f., P.b., P.c., P.k., P.v.
PY02988	EAA22511	1,383	—	—	Leu/Phe-tRNA protein transferase, 1st eukaryotic member	P.f., P.b., P.k., P.v.
PY04050	EAA15863	499	—	—	Hypothetical protein	P.b.
PY04937	EAA16880	1,174	—	Yes	Hypothetical protein	P.b., P.c.

^a P.b., *P. berghei*; P.c., *P. chabaudi*; P.f., *P. falciparum*; P.k., *P. knowlesi*; P.v., *P. vivax*.

Plasmodium species, the database for all *Plasmodium* open reading frames of ≥ 50 bp available in Plasmodb (<http://www.plasmodb.org>) and the Wash U-BlastP 2.0 BLAST program were used (Table 2). More than half of these genes appear to be involved in metabolic processes. Interestingly, most genes have orthologs in *P. falciparum* and other *Plasmodium* species.

DISCUSSION

The last 5 years have seen the publication of the genomic sequences of the parasite *Plasmodium falciparum* (13, 3, 12), the mosquito vector *Anopheles gambiae* (16), and the human host (30). A full understanding of malaria biology seems within reach and, with that, the hope of new drugs and vaccines. But the complexity is daunting. The *Plasmodium* genome alone is predicted to contain at least 5,000 genes. In response to this wealth of data, new methods of studying large numbers of genes have been developed, including expression library immunization (1, 29), microarray analysis (7, 25, 26), and multi-dimensional protein identification technology (32, 11, 23). All these methods facilitate the screening and analysis of large amounts of genomic data.

In this paper we describe our antigen identification method, a new method for screening genes for their vaccine potential. The antigen identification method can rapidly produce data on hundreds of gene products to identify new candidate antigens as vaccine targets. The method relies on cloning of individual gene sequences and plasmid vaccination. We want to contrast the antigen identification method with expression library immunization, which is based on cloning of random DNA fragments and immunization with very large plasmid pools. We reasoned that DNA immunization solely with protein coding regions would improve screening sensitivity and specificity compared with expression library immunization. Our choice of name emphasizes that this method focuses on single genes and individual immune responses and avoids the difficulties of com-

plex immunizations that can be difficult to deconstruct. Our data on false-positive screening with small pools of plasmids support our interest in screening with single-plasmid immunization.

We used the BALB/c mouse-*P. y. yoelii* DNA vaccination model for our antigen identification method screening system because experience suggested that this would give a high probability of success. BALB/c mice are easily protected with the *P. y. yoelii* irradiated sporozoite vaccine (8), and we reasoned that they would have strong immune responses to many preerythrocytic target antigens. DNA vaccination with the known *P. y. yoelii* antigens CSP and HEP17 protects mice against infection with sporozoites (27, 10), indicating that the immune responses induced by plasmid vaccines can kill *P. y. yoelii* parasites. Finally, we chose our screening end point to be parasite burden as a direct measure of parasite killing instead of surrogate markers such as gamma interferon production, as we wanted to avoid all assumptions about immune mechanisms of parasite killing.

The approach we describe in this paper is intended as a screening method. By definition, screening systems rapidly give a small amount of information about a large number of items. Screening is an exercise in efficiency. Screening also implies a defined objective. In our case, our goal is to find new malaria parasite proteins for which targeting by the immune system will be lethal to the parasite. Any screening method may give false-negative results, and thus, we may miss some good vaccine targets. This could be because of poor antigen production by a plasmid, lack of epitopes recognized by BALB/c mice, or improper folding of conformational epitopes. In addition, only 63% of the annotated *P. y. yoelii* genes have orthologs in *P. falciparum* (5), so only a subset of potential human vaccine targets can be evaluated by our method. Finally, our present study focuses on preerythrocytic immunity and does not pretend to identify candidate antigens for blood-stage-parasite or transmission-blocking malaria vaccines. While we are explor-

ing ways to overcome these limits to antigen identification method screening, it is obvious that there will always be deficiencies in any screening algorithm. However, our screening method will be a success if even a small number of new protective target antigens which are useful in vaccine development are identified. The important point is that screening methods can be useful without being perfect.

The technical methods we used in the antigen identification method are not novel: computer algorithms for exon prediction, the Gateway cloning system, DNA vaccination, and measurement of malaria parasites by real-time PCR. Our challenge has been in adapting these elements to our *P. y. yoelii*-BALB/c mouse model system and improving the efficiency of each step. Our results show the successes made in these areas, and we continue to improve all aspects of this screening method.

Our goal is to find single protective antigens. Difficulties with pooling plasmids for multiantigen immunization have been our biggest concern, and it appears that plasmid pooling can lead to both false-positive and false-negative results. In an ideal screening situation, if a pool was composed of vaccine plasmids, none of which could individually kill substantial numbers of parasites, immunization with that pool should not protect mice. However, our experience with plasmid pool B shows exactly this type of false-positive result. Mice immunized with pool B had substantial decreases in their parasite burden, while each of the individual plasmids in pool B was unable to kill parasites on its own. From a screening perspective, false-positive pools cause inefficiencies because the work spent re-screening will not yield protective antigens. From an immunological perspective, we do not yet know how pools generate protective immune responses. From a malaria vaccine perspective, the fact that multiantigen vaccines may work better than their individual components is encouraging (9, 22).

If plasmid interactions lead to false-positive plasmid pools, can there be false-negative plasmid pools? If plasmids encoding protective antigens would not kill parasites when mixed with other plasmids, screening strategies with pools might miss important discoveries. In experiments with the *P. y. yoelii* CSP plasmid, we were unable to find evidence of decreased protection when this highly protective vaccine was mixed with other plasmids. However, other researchers have found plasmid pools where strong negative interactions occur (M. Sedegah, submitted for publication). In terms of planning a screening strategy, the importance of false-positive and false-negative plasmid pools depends on their frequency, which is not yet known.

Interestingly, the route of DNA immunization had a great effect on the protective efficacy of several plasmids. While the *P. y. yoelii* CSP DNA vaccine protected only when given intramuscularly and not by gene gun, the reverse was true for the PY01316 vaccine, which only killed parasites when delivered by gene gun immunization and not when given intramuscularly. Antibody responses were also higher for three of four plasmids when vaccines were given by gene gun. It may be that different parasite antigen targets may be successfully attacked by different immune effector mechanisms. This has important implications for the immunizations used in our DNA screening strategies, as well as for malaria vaccine development in general.

Immunization of mice with single plasmids identified five which decreased liver parasite burdens by 68 to 95%. Addi-

tional studies to confirm these protective effects are in progress. For the most protective antigen, PY01316, comparison with genome sequence data from other malaria parasites shows clear orthologs in *P. knowlesi* and *P. vivax* but none in *P. falciparum*. Structural analysis of the PY01316 protein sequence shows that it has a predicted transmembrane anchor but no predicted signal sequence. There are no other obvious structural features and no homology to known proteins that would have led us to predict that this gene would be a good target antigen. PY01316 is expressed not only in *P. y. yoelii* sporozoites, but also in blood stages. Thus, we would never have selected this gene for screening if we had been looking at proteins expressed only in exo-erythrocytic parasites. We believe that the identification of PY01316 confirms the need to cast a wide net and to screen large numbers of malaria parasite gene products as vaccine targets without preconceptions as to what structure those targets must have. The antigen identification method will be a useful tool in this search.

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