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Molecular basis of arrested liver stage development of the gamma irradiated *Plasmodium yoelii* sporozoite

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Worldwide, about 270 million people are diagnosed with malaria annually and about a million deaths occur from this disease mostly in children and pregnant women in Africa. Despite enormous effort and resources directed towards malaria control and eradication, problems of resistance to chloroquine by the malaria parasite and resistance to insecticide by the mosquito vector have only contributed to worsening of the spread of the disease. These problems have led to the search for alternate and efficacious mode of treatments with one being vaccine. Previously, sporozoites isolated from mosquitoes exposed to 12,000 rads of radiation from a ^{137}Cesium source and injected into mice in laboratory studies were determine to invade hepatocytes where their development became arrested at the trophozoite stage, causing them to confer protection against subsequent challenge with live sporozoites. However, attempts to expand these studies in field trials on susceptible human in malaria endemic areas has been complicated by the problem of a need for large colonies of mosquitoes that can deploy the whole parasite-based vaccine. To circumvent this obstacle, peptide vaccines were developed. Unfortunately, only a few of the antigenic determinant on the surface of sporozoites were developed as peptide vaccines leading to an efficacy that is inferior to that of the whole parasite-based vaccine. Hence, to improve on the efficacy of the peptide vaccine, in this study, axenically-derived cultures of *P. yoelii* EEFs that were incubated as irradiation-attenuated sporozoites and as non-irradiated sporozoites were subjected to two rounds of suppression subtractive hybridization (SSH) along with dot blot analyses and southern blot (RT-PCR) to generate differentially expressed cDNA library clones from which peptide antigens will be identified for use in a multi subunit vaccine.

Key words: Peptide vaccines, suppression subtractive hybridization (SSH), Rhoptry bodies, Exo-erythrocytic Form (EEF), Gene-knockout, Parasitophorous vacuoles, dot blots hybridization.

INTRODUCTION

Malaria is one of the deadliest of human parasitic diseases and results in a high mortality and morbidity rate in tropical and sub-tropical areas of the world. It is estimated that worldwide, about 270 million new cases of malarial infections are diagnosed annually, of which 110 million cases proceed to develop the disease and about

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one to three million cases finally succumb to the infection (WHO, 2016).

Effort to mitigate the worldwide health burden that emanates from malarial infection has been complicated by development of resistance by the malaria parasite to chloroquine (Wellems 2004), which is a drug of choice for malaria treatment as well as development of resistance to insecticides by the Anopheline mosquito (Jones and Hoffman, 1994). These problems brought about concerted efforts in the scientific community for the identification and development of alternate and efficacious modes of therapies like vaccine development for use to prevent and treat malaria. The process of vaccine development for use to treat and prevent malaria started in 1945, with the first vaccine that was known as the x-irradiated sporozoites vaccine (Spitalny and Nussensweig 1972) that was tested in chicken (Taliaferro et al., 1945). The success of this first vaccine was later replicated in rodents in 1967 (Nussenzweig et al., 1967), primates in 1987 (Collins et al., 1989), humans first in 1973 (Clyde et al., 1973) and then in 1989 to 1999 (Hoffman et al., 2002). In each of these studies, mammalian hosts who were exposed to the irradiation-attenuated sporozoites developed a protective immunity that was elicited against subsequent challenges with homologous sporozoites that were fully infectious. Unfortunately, the positive results obtained in these studies to evaluate the vaccine potential of the irradiation attenuated sporozoites were possible because the studies were limited to laboratory-maintained animals. When it came to obtaining similar results in field studies where susceptible humans were exposed to bites from sporozoite infected mosquitoes that had been irradiated, the process proved to be extremely impractical. The rational being that it was easy to manipulate sporozoite infected mosquitoes within the confines of a research laboratory so that they can deliver whole parasite-based vaccine to susceptible laboratory-maintained animals that are kept in cages by way of feeding, than to do so with susceptible humans in field studies. This is because, in order to vaccinate susceptible humans by way of feeding, large colonies of infected mosquitoes that have been irradiated will be required to deliver whole parasite-based vaccines making this process to be cumbersome and very challenging for field studies. Equally challenging was the problem of how to successfully irradiate as well as transport large quantities of sporozoite infected mosquitoes to the field without resorting to creating the problems of breakthrough of the vaccines. Also problematic was the fact that, even if the whole parasite-based vaccine were to be dissected from the salivary gland of the irradiated sporozoite infected mosquitoes and cryopreserved for later use to vaccinate susceptible individuals by way of needles and syringes, large colonies of infected mosquitoes will still be required to be maintained. Consequently, to mitigate these challenges of the need for large colonies of infected mosquitoes for use in field studies to deliver the whole parasite-based vaccines, the feasible alternative was that antigenic targets on the surface of sporozoites could be identified, characterized, and then incorporated into multi-subunit vaccines that would provide a protection that is comparable to that of the whole parasite-based vaccine.

The concept to develop vaccines based on antigenic targets that are identified on the surface of sporozoites for use in multi-subunit vaccines stemmed from studies that looked at the developmental process of sporozoites in organelles, while they are inside mosquitoes. These organelles are known as micronemes dense body and rhoptry bodies (Mota et al., 2002). However, during a blood meal, in addition to saliva with anticoagulants that are inoculated into the skin of the host by infected mosquitoes, sporozoites are equally introduced into the skin of susceptible hosts. Immediately inside the skin of the host, the sporozoites release the peptides that they had synthesized and stored in micronemes, dense body and rhoptry bodies while they were inside the body of mosquitoes. The released peptides are then used to facilitate traversal motility through a wide variety of cell types during which there is no invagination of the plasma membranes of the affected skin cells. In this traversal process, the released peptides are translocated and redistributed backward to cover the surface of the body of sporozoites as well as deposited at the posterior end in such a manner that the peptides remain in continuous contact with substrates like integrins (Silvie et al., 2004; Dundas et al., 2018) to form a substrate-dependent type of movement characterize as gliding motility (Mota et al., 2002). This type of motility was confirmed by Yamauchi et al. (2007) who used video microscopy to document sporozoites as they actively breached intact plasma membrane of skin cells then enter and exit cells while at the same time leaving trails of secreted peptides that demarcate the migratory path that has been utilized by the sporozoites.

Incidentally, some of the secreted peptides from the micronemes, dense body and rhoptry bodies have been identified, their molecular structure determined, and their vaccine potential is currently being exploited. Some of the identified surface proteins include entities like: the circumsporozoite protein (CS) (Khan et al., 1992), thrombospondin-related anonymous protein (TRAP) (Bhanot et al., 2003; Garcia et al., 2006), sporozoite threonine-and asparagine protein (STARP), sporozoite microneme protein essential for cell traversal (SPECT) and sporozoite and liver-stage antigen (SALSA). Furthermore, the vaccine potential of some of the secreted peptides like the Sporozoite Microneme Protein Essential for Cell Traversal 1 & 2(SPECT1 & 2) (Silvie et al., 2008), the Circumsporozoite protein (CS) in the SPf66 (Goh et al., 2019) and the RTS, S vaccines (Moorthy et al., 2004) as well as the Thrombospondin-

Related Anonymous Protein (TRAP) in the ME-TRAP vaccine have been characterized and evaluated in field studies to ascertain their efficacies.

So far, results from field trials to monitor the efficacies of these peptide vaccines have been mixed in terms of their ability to provide long lasting protection against subsequent challenges with homologous sporozoites that were fully infectious. This limited protection has been attributed to the fact that of the many antigenic targets that are found on the surface of sporozoites, only a few of them have been characterized and incorporated into the peptide vaccines that are currently being monitored in field studies so as to determine their efficacies. Which implies that for a peptide vaccine to be efficacious, there is the need that it be constituted to include more than just the few peptides that are currently being used. In short, the peptide vaccines should be constituted to include most if not all of the secreted peptides that are involved in sporozoite traversal motility through the wide variety of cell types of the skin as well as the peptides that are used for attachment and invasion of hepatocytes (Pradel et al., 2002). Once such peptides are identified, characterized, and incorporated into multi-subunit vaccines, then will it be possible to obtain an efficacy that is comparable to that which is obtained from using the whole parasite-based vaccines (Goswami et al., 2020).

This need to develop vaccines with efficacies that are comparable to that obtained from the whole parasite-based vaccine is the rationale for studies, which include manipulation of certain genes in sporozoites. Some of the targeted genes identified and characterized for manipulation included genes that code for antigenic targets on the surface of sporozoites and the process was achieved by way of gene knockout as well as by way of genetically attenuated parasites (GAP) (Goh et al., 2019). The idea to modify sporozoites genetically was to abrogate some of those genes that code for peptides that are used by sporozoites for traversal motility through skin cells and most importantly that are used for attachment followed by invasion of hepatocytes. To produce the Gene knockout vaccine, the up-regulated in infective sporozoite gene-3 and 4 (*UIS3*and *UIS4*) were abrogated (Mueller, et al., 2005), while to generate the genetically attenuated parasites (GAP) vaccine, the targeted genes involved were *P52* and *P36* genes (VanBuskirk et al., 2009). Unfortunately, to develop these two types of vaccines, researchers were confronted with the reality that they were going to deal with the very same problem that they were trying to avoid, which was the need to produce large quantities of sporozoites that made field trial of the whole parasite-based vaccines on susceptible humans to be cumbersome and impractical.

Which is why the objective of this study was to contribute to vaccine development by the use of Suppression subtractive hybridization (SSH) (Matuschewski et al., 2002; Galbraith et al., 2004) to identify genes and their antigenic targets that can be incorporated into a multi-subunit peptide vaccine rather than manipulation of genes that code for antigenic determinants on the surface of sporozoites. This is significant because unlike in the case with the irradiated whole parasite-based vaccine or the genetically modified vaccine where a large colony of infected mosquitoes is required, with the peptide vaccines, there is no need for a large colony of mosquitoes to be maintained. On the contrary, to develop peptide vaccines, the requirement is for peptide genes to be identified and their sequences determined. The gene sequences can then be cloned onto vectors and expressed in bacteria to obtain several copies. Similarly, the sequences of antigenic targets on the surface of sporozoites can be determined and cloned to produce several copies of the peptides. This process can be achieved through a onetime use of a colony of infected mosquitoes from which sporozoites will be dissected from the salivary gland (Goh et al., 2019). Also, depending on the number of genes identified and their sequences characterized, peptide vaccines develop through this process will yield efficacies that are comparable to that of the irradiated whole parasite-based vaccine.

To identify genes in sporozoites whose peptides could be incorporated into multi-subunit vaccines in this study, a colony of *Plasmodium yoelii* (17XNL, clone 1.1) infected mosquitoes were divided into two batches. One batch of the sporozoite infected mosquitoes were expose to 12,000 rads of gamma radiation, while the second batch of sporozoite infected mosquitoes were not exposed to gamma-irradiation. Sporozoites dissected from the salivary glands of infected mosquitoes that were irradiated were identified as irradiated exo-erythrocytic form (IEEF), while sporozoites dissected from the salivary glands of infected mosquitoes that were not exposed to gamma-irradiation were identified as non-irradiated exo-erythrocytic form (NIEEF). The IEEF and the NIEEF were cultured axenically for 24 hours prior to RNA extraction. The reason being that, in previous studies where non-irradiated sporozoites were incubated with hepatocytes, there was attachment and invasion of hepatocytes by sporozoites through a process of invagination of plasma membranes to form parasitophorous vacuoles that were characterize by bulbous enlargements that progressively grew large because the sporozoites transformed to trophozoites (Meis et al., 1985) and then to schizonts that contain between 10,000 to 30,000 merozoites (Druilhe et al., 1998). This transformation by the non-irradiated exo-erythrocytic forms (NIEEF) resulted in parasitophorous vacuoles that measured between 40-45 µm in diameter (Scheller et al., 1995). The irradiation-attenuated sporozoites when incubated with hepatocytes were observed to also attach to and invade hepatocytes by a similar process of invagination of the plasma membrane to form parasitophorous vacuoles that only measure 4-8

µm in diameter (Scheller et al., 1995). The small size of the parasitophorous vacuoles was because the transformation of the irradiated exo-erythrocytic forms (IEEF) became arrested at the trophozoite stage. However, a major problem presented in these studies was that when attempts were made to isolate and characterize antigenic targets that belong solely to sporozoites the process became an exercise in futility as it was plagued by contamination of sporozoite peptides by peptides from hepatocytes (Kaiser et al., 2003).

To circumvent this problem of contamination by peptides from hepatocytes, axenically-derived cultures (Kaiser et al., 2003) of *P. yoelii* EEFs that were incubated as irradiation-attenuated sporozoites and as non-irradiated sporozoites were subjected to two rounds of suppression subtractive hybridization (SSH) (Galbraith et al., 2004) along with dot blot analyses and southern blot (RT-PCR) so as to generate differentially expressed cDNA library clones from which genes or peptide antigens that are uniquely of sporozoites will be identified. This experimental set up was also to decipher the molecular basis for the arrest in the development of the IEEF at the trophozoite stage in the liver. As result, cDNA clones that were randomly selected from the differentially expressed genes of the IEEF confirmed by displaying reactions that were characterized by up regulation of the tubulin tyrosine ligase gene, down regulation of the *yir* gene, abrogation of the erythrocyte membrane protein gene and no effect from the gene that codes for p235 kDa rhoptry protein, while cDNA clones that were randomly selected from differentially expressed genes of the NIEEF library confirmed by displaying reactions that were characterized by down regulation of the tubulin tyrosine ligase gene, up regulation of the *yir* gene, expression of the erythrocyte membrane protein gene and no effect on the gene that codes for the p235 kDa rhoptry protein.

MATERIALS AND METHODS

Sporozoite isolation from mosquito

Plasmodium yoelli (17XNL, clone 1.1) infected *Anopheles stephensi* were a gift from the Walter Reed Army Institute of Research (S.S., MD). Infected female *Anopheles stephensi* mosquitoes were exposed to 12,000 rad of gamma radiation from a ^{137}Cesium source at a dose rate of 833 rad per minute (Shepherd irradiator Mark 1, model 68; J.L. Shepherd and Associates, San Fernando, CA). Sporozoites were recovered from whole mosquitoes by grinding using a mortar and pestle. Sporozoite enrichment was done by centrifugation on a renografin-60 gradient (Squib Diagnostics, N.J.) for 20 min at 12,000 rpm to remove contaminating mosquito tissue and other debris. A 1:5 dilution of sporozoites was counted on a haemocytometer.

Total RNA and mRNA Isolation

Total RNA and later mRNA were obtained from sporozoites after homogenization in 1.0 ml of Trizol reagent (Life Technologies, Rockville, MD) (Promega, Madison, WI). Total RNA was isolated by chloroform and isopropanol precipitation from the aqueous phase followed by washing with 70% ethanol and dilution, using RNAse free water. RNA quantity was determined by spectrophotometry (A260/280), and RNA quality was determined by gel electrophoresis. Later, mRNA was isolated from the total RNA using the Poly (A) Purist (Ambion) according to the protocol of the manufacturer.

Suppression subtractive hybridization

Suppression subtractive hybridization analysis was performed using the SMART-PCR select cDNA hybridization kit, according to the manufacturer's recommendation (Clontech, Mountain View, CA, USA). Between 2 to 4 µl (2 µg) of mRNA was used for the synthesis of first strand cDNA from samples that were identified as gamma-irradiated and non-irradiated sporozoites. To each sample, 1.0 µl of cDNA synthesis primer was added and enough water to bring the final volume of each reaction mixture to 5.0 µl as indicated by the manufacturer of the SMART-PCR select cDNA hybridization kit (Clontech). The second strand cDNA was synthesized following the manufacturer's recommendation (Clontech). The samples were digested with Rsa I restriction enzyme to generate samples with blunt ends. A 1:5 dilution of digested cDNA pool was made. Each cDNA pool from IEEF and NIEEF was divided into two tester or experimental cDNA pool (1 and 2) and driver or reference cDNA pool. 2.0 µl of diluted tester cDNA pool identified as 1 and 2, were ligated with adaptors 1 and 2 that contain PCR priming sites. The adaptor ligated tester cDNAs were subjected to two cycles of subtractive hybridization using irradiated driver (ID) cDNA NIEEF and non-irradiated driver (NI-D) cDNA for irradiated tester (IT) cDNA to eliminate mRNAs that are common to both the irradiated (I) and non-irradiated (NI) cDNA pool. Meanwhile, because the driver cDNA was not ligated to adaptors or PCR priming sites, the tester-driver cDNA hybrids possessed only a single priming site that made them unavailable for subsequent PCR amplification. Hence, transcripts for housekeeping genes that are common to the irradiated and non-irradiated cDNAs were subtracted from the cDNA pools. In this way, in the final round of hybridization, tester 1 and 2 cDNA pools were combined so that the hybridization will produce subtracted irradiated and subtracted non-irradiated cDNA that contains only differentially expressed cDNA transcripts.

Differential Screening kits (Clontech Laboratories and Inc.) were used to enrich and identify differentially expressed genes in the driver and tester cDNA samples. Both forward and reverse subtraction were performed with the non-irradiated cDNA serving as driver and the irradiated cDNA serving as the tester (cDNA pools from which specific genes are to be identified) in one reaction while in the other reaction, the irradiated cDNA was the driver (cDNA pools used to selectively remove non-differentially expressed genes from the tester DNA) while the non-irradiated cDNA was the tester. Irradiated cDNA pool (2 µg) without adaptors was added to the non-irradiated sample as the driver while non-irradiated cDNA pool (2 µg) also without adaptors was added as driver to the irradiated cDNA tubes. These samples were subjected to two steps of subtractive hybridization to eliminate sequences that are common to both cDNA.

PCR amplification of differentially regulated genes

To amplify and enrich for differentially expressed mRNAs, primary PCR was used to exponentially increase the subtracted IEEF and the subtracted NEEF cDNA libraries that have different adaptor

sequences ligated to them. Later, 1.0 µl aliquot of the primary PCR product was used in nested PCR to further enrich for differentially expressed sequences as well as reduce background effect by using the SMART-PCR select cDNA hybridization protocol with a slight modification on the number of PCR cycles. The primary PCR reaction was increased to 35 cycles, while the nested PCR reaction was increased to 24 cycles rather than the manufacturers recommended 27 and 12 cycles. Primers for the following gene products were used as positive controls: MSP-1, AMA, 28S-rRNA, and Hep-17. An aliquot of the nested PCR product was purified on a 2% agarose gel with 1.0 µg/mL of ethidium bromide. Two micro liters of the nested PCR product was added to TOPO vector (Invitrogen, Carlsbad, CA) and incubated for 5 min at 24°C.

Cloning of differentially expressed cDNA

Two micro liters of the cloned product was used to transform TOP 10 One Shot™ Chemically Competent *E. coli* cells (Invitrogen, Carlsbad, CA). Transformed bacteria were selected by the blue (insert negative) and white (insert positive) colony selection criteria in combination with PCR amplification using vector specific T7 promoter primers to confirm successful transformation. Insert positive colonies were subcloned to agar plates with X-gal (50 mg/ml) and ampicillin (1.0 µl/ml) and incubated overnight at 37°C. Insert positive colonies were next incubated overnight at 37°C in 5.0 mL of Luria broth. Template plasmid was spin column purified with an SV miniprep kit (Promega, Madison, WI). Sequencing was done using dye terminator method on a 373 automated fluorescence sequencing system (Applied Bio systems, Foster City, CA). Sequencing products were analyzed for template contamination by using the MACVECTOR program (Accelrys, San Diego, CA). To look for similarity to known protein sequences in GENBANK database, DNA sequences for the IEEF and the NIEEF were compared against the public database using BLASTX. Genetic identity was ascertained based on an E (expect) value of ≤ 0.05 using a non-redundant Prot-ein tBlastX search.

Validation of differential gene expression by cDNA dot blots hybridization

To confirm differential gene expressions, candidate cDNA clones were subjected to differential screening, using the cDNA dot blot hybridization technique as recommended by the manufacturer (PCR select differential screening kit; Clontech). One microliter of each non redundant irradiated and non-irradiated cDNA clone was PCR amplified using the M13 forward and reverse primers. Five microliter aliquots of the PCR products were denatured in an equivalent volume of 0.6 N sodium hydroxide as per the recommendations of the manufacturer of the differential screening kit (Clontech). Two µL of the denatured PCR products were transferred to four identical positively charged nylon membranes. A set of four identical dot blots of irradiated and non-irradiated PCR products were transferred to each of four membranes. The four dot blot membranes were identified as: (a) clones hybridized to the forward subtracted probe; (b) clones hybridized to the reverse subtracted probe; (c) clones hybridize to the forward un-subtracted probe; and (d) clones hybridize to the reverse un-subtracted probe. Blots were neutralized for 2-4 min with 0.5 M Tris-HCL (pH 7.5) and washed with water before cross-linking the DNA to the membrane with a UV linker (Stratagene). Each membrane was placed in a separate 50 mL conical tube and incubated for 60 min. at 60°C in 10 ml of pre-hybridization buffer with continuous agitation. Membranes were later exposed overnight to subtracted and un-subtracted cDNA probes that were labeled with $\{\alpha\text{-}^{32}P\}$ d-ATP isotope. The following day, the membranes were washed to final stringency with 0.1% SDS and 0.1% SSC (0.1x of 3M sodium chloride, 300 mM sodium citrate) for 15 min (2x) at wash 60°. Washed membranes were exposed overnight to Kodak Biomax MS X-ray film -80°C and autoradiography done using the automated medical film processor, QX-130A Plus (Konica, NJ, USA).

Confirmation of differential gene expression by quantitative RT-PCR

To validate the differential expressed and investigate up regulation and down regulation of some gene, total RNA obtained from gene products of gamma-irradiated and non-irradiated sporozoites were confirmed by semi quantitative RT-PCR analysis. First strand cDNA was synthesized from 3 µg of DNase treated total RNA in a standard reverse transcription (RT) reaction using the Superscript Oligo (dT) first-strand synthesis system for RT-PCR (Invitrogen). PCR was done for 35 cycles with 94°C as the denaturing temperature, 50°C as the annealing temperature and 72°C as the elongation temperature. PCR product was analyzed on a 2% agarose gel containing 1.0 µg/ml of ethidium bromide.

RESULTS

Differential expression of subtracted IEEF and NIEEF

Table 2A shows a summary of matches between sequences from the subtracted NIEEF cDNA clones and those of known proteins in a non-redundant database. Similarly, Table 2B is a summary of matches between sequences from the subtracted IEEF cDNA clones and those of known proteins in the database to which they also exhibited homology. Most sequences from the subtracted IEEF and NIEEF cDNA clones produced no match to known protein sequences in the non-redundant database (data not shown).

RT-PCR analysis of in vitro derived EEFs

To evaluate how results of our sequences relate to differential gene expression in the IEEF and NIEEFs, primers were synthesized from sequences of 10 of the 104 randomly selected positive clones (Table 1) that displayed homology to those of the erythrocyte membrane protein gene, the *yir* gene, the tubulin tyrosine ligase gene and the p235 kDa rhoptry protein gene. These primers were used in a semi quantitative RT-PCR assay to evaluate total RNA from IEEF and NIEEFs that were axenically cultured at 37°C for 24 h. Figure 1 shows RT-PCR products from the IEEF and NIEEF samples. In each case, the amplified gene displayed a fluorescence intensity that was proportional to the amount of mRNA that was synthesized and is characterized by abrogation of the erythrocyte membrane protein (EMP) gene in the IEEF and up regulation of the said gene in the NIEEF. The fluorescence intensity of the *yir* gene was

Table 1. Clones with their Sense and Anti-sense primer sequences that were used in this Study.

Clone	Sense primers	Anti-sense primers
1NI 2-12	TAAGTTGGGTAACGCCAGG	TCGCTGTTTGCCTCATTG
2NI 2-12	TAAGTTGGGTAACGCCAGG	CGCACAAACAAAAACCGAC
DI 2-5	CGGTTGCTATGCTAAGAACAGC	TTCATTGTGCTCACCTCAGG
NI 2-19	TGTTGTGTGAAATGTGAGCG	TGACGGTTTTGATGGTAGAGG
DNI 2-6	CGTTACTCTTTTGGAGGCG	ATAGGTGGGAGGCAGTGAAG
4I 1-1	TTAGGACAGTCAGGAGGTTCAG	ATAAGCAATGAGCGATGTGG
NI 2-17	TAAGAGAGAGAGAGAGAGAGTC	GTTATGCGTGTAAAAGGCG

Table 2A. Randomly selected positive cDNA clones from subtracted NIEEF whose sequences were compared against the public database using BLASTX. Genetic identity based on an E value of ≤ 0.05 show ESTs with their corresponding contigs.

Non-irradiated clones	Contig	Identified protein
NI 1-5	Malpy02019	CCAAT-box DNA binding protein
NI 1-11	Malpy01430	235 kDa rhoptry protein
NI 1-12	Malpy00905	Putative bir1
NI 2-14	Malpy02672	Ribosomal protein S4
NI 2-10	Malpy00013	Uncharacterized protein family
NI 2-12	Malpy00271	Erythrocyte membrane protein
NI 2-2	Malpy00014	Putative uncharacterized protein family
NI 2-19	Malpy00954	Putative yir4 protein
NI 1-1	Malpy02241	Hypothetical protein
DNI 2-3	Malpy01423	Heat shock protein 60
DNI 2-7	Malpy00271	Uncharacterized protein family
NI 1-5	Malpy02019	Hypothetical protein

Table 2B. Randomly selected positive cDNA clones from subtracted IEEF whose sequences were also compared against the public database using BLASTX. Shown are ESTs with contigs that are based on an E value of ≤ 0.005.

Irradiated clones	Contig	Protein Identified
I 1-4	Malpy000471	Hypothetical protein
DI 2-2	Malpy01825	Hypothetical protein
DI 2-11	Malpy00500	Putative yir1 protein
DI 2-5	Malpy00137	Putative yir4 protein
DI 1-12	Malpy00664	Tubulin-tyrosine ligase family
DI 2-2	Malpy01557	Putative yir4 protein
I 1-6	Malpy01953	Hypothetical protein
DI 1-15	Malpy01115	Uncharacterized protein family
DI 2-15	Malpy00137	Putative yir3 protein
DI 2-9	Malpy00296	hypothetical protein
DI 2-13	Malpy00664	CCAAT-box DNA binding protein subunit

characterized by up regulation in the NIEEF and down regulation in the IEEF, while in the case of the tubulin tyrosine ligase gene, the fluoresce intensity was characterized by down regulation in the NIEEF and up regulation of the said gene in the IEEF. Amplicons for the gene that codes for p235 kDa rhoptry protein displayed a fluorescence intensity that was similar in the IEEF and the NIEEF despite the exposure of the IEEF sporozoites

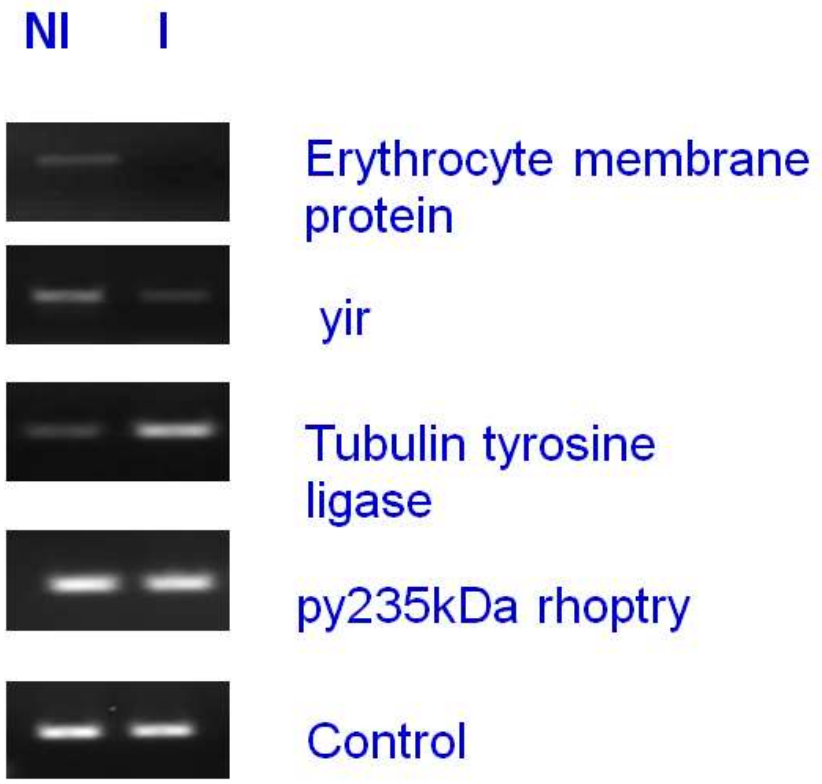

Figure 1. Semi-quantitative RT-PCR analysis of positive *P. yoelii* cDNA clones that were identified as differentially regulated because of gamma-irradiation. RNA from NIEEF and IEEF was used for first strand cDNA synthesis. Using clone-specific primers (Table 1) and cDNA as template, PCR amplification produced differential expressed gene products.

to 12 Krads of radiation.

Validation of differential expression

Figure 2, demonstrates a strong hybridization signal in panel B between the subtracted IEEF cDNA dot blots and the subtracted total cDNA probes, while in panel A where the subtracted IEEF cDNA dot blots were probed with un-subtracted total cDNA probes, a weak hybridization signal was observed in the two membranes. Between the subtracted IEEF cDNA dot blots and the subtracted total cDNA probes, 94 of 104 (90%) clones exhibited a strong hybridization signal, an indication of the high efficiency of the SSH technique.

Similarly, the set of four $\{\alpha\text{-}^{32}P\}$ d-ATP isotope labelled probes were used to probe 104 cDNA dot blots on four other separate nylon membranes as shown in Figure 3. These samples were randomly selected from subtracted NIEEF cDNA clones. Once more, like in Figure 2, a strong hybridization signal is observed in panel B between the subtracted NIEEF cDNA dot blots and the subtracted total cDNA probes while a weak hybridization signal is observed in panel A in the reaction between the subtracted NIEEF cDNA dot blots and the un-subtracted total cDNA probes.

RT-PCR analysis of in-vivo derived EEFs

No amplicons were observed when the same sets of primers were used to test blood that was infected with *P. yoelii* (Figure 4). Based on the differential display pattern of these results, we were curious as to whether there was any analogy of our results to that which was obtained with the *yir* gene by comparing the sequences from our five clones to those of known *Plasmodium* proteins in a non-redundant database. The result obtained showed homology but to proteins with unknown functions.

Next, primers that were designed from using randomly selected sequences that belong to (+) and (-) clones from axenically derived cultures of the IEEF were used in another RT-PCR assay to evaluate liver samples from Balb/c mice that were infected with gamma irradiated and

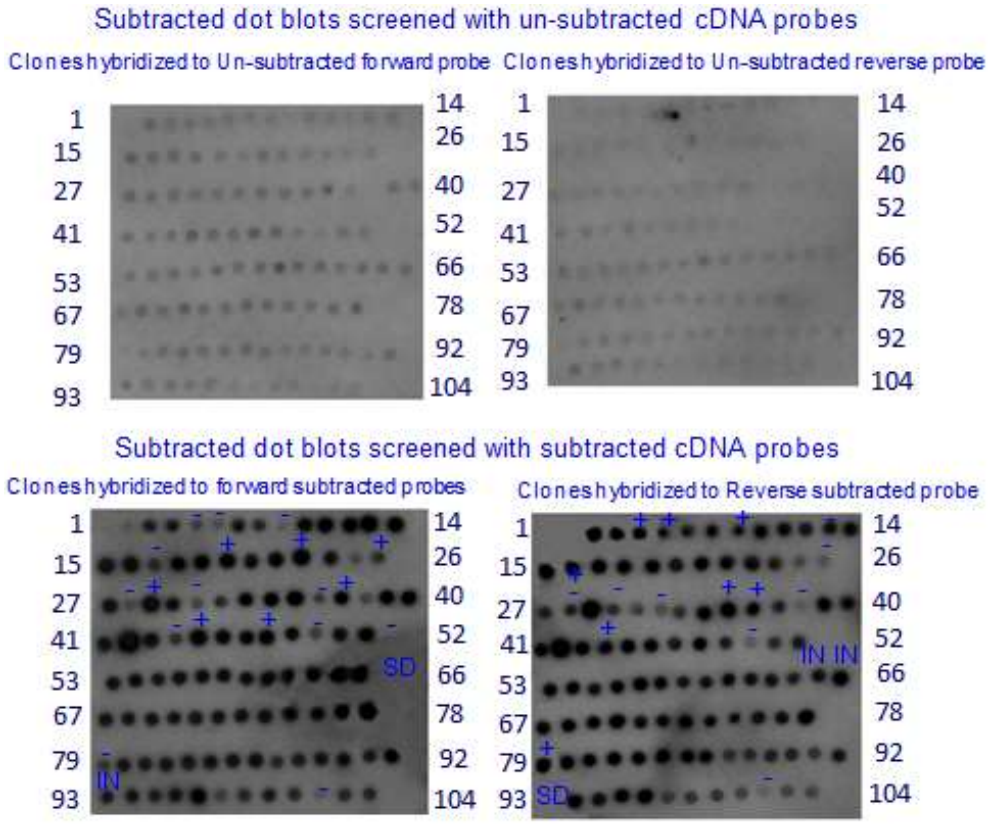

Figure 2. Validation of differential expression; PCR amplicons of insert positive clones that were denatured in 6N NaOH were analyzed on positively charged nylon membranes and probed with indicated probes. The plus (+) and minus (-) signs denote candidate cDNA clones of *P. yoelii* IEEF genes that were apparently up and down regulated in response to gamma-irradiation. SD and IN respectively denote shut down and induced.

non-irradiated sporozoites. At day five post Balb/c mice infection (Figure 4A) is a comparative analysis of the RT-PCR assay result, with “N” denoting amplicons from the liver of the Balb/c mice that was infected with non-irradiated sporozoites and “I” denoting amplicons from the liver of the Balb/c mice that was infected with sporozoites that were exposed to 12,000 rads of radiation. The conclusion from this study is that arrest in the development of the IEEF in hepatocytes is not an event that occurs suddenly. This can be explained by the fact that following attachment and invasion of hepatocytes by sporozoites, both the IEEF and the NIEEF undergo similar transformational changes from sporozoites to trophozoites. Subsequently, while the transformation of the NIEEF progresses to the trophozoite then the schizont stage, the transformation of the IEEF becomes arrested at the trophozoites stage. This explains why at day five post Balb/c mice infection, gene expression in the Balb/c mice inoculated with non-irradiated sporozoites and the Balb/c mice inoculated with irradiated sporozoites were the same following invasion of hepatocytes (Figure 4B).

Next, a third Balb/c mouse was infected with sporozoites that were dissected from the salivary glands of mosquitoes that were exposed to 12,000 rads of gamma radiation. Twelve days post infection, blood was drawn from the mice and RT-PCR assay was done on the sample with the primers that were designed from using randomly selected sequences that belong to (+) and (-) clones from axenically derived cultures of the IEEF. This time around, no bands were detected in the blood samples except in lane seven where Hep-17 (Wang et al., 2004) was used as positive control, confirming the fact that no breakthrough occurred following the adequate exposure of sporozoites to 12,000 rads of gamma radiations as the development of the IEEF does not proceed beyond the trophozoite stage (Figure 4C).

DISCUSSION

Suppression subtractive hybridization is a technique that

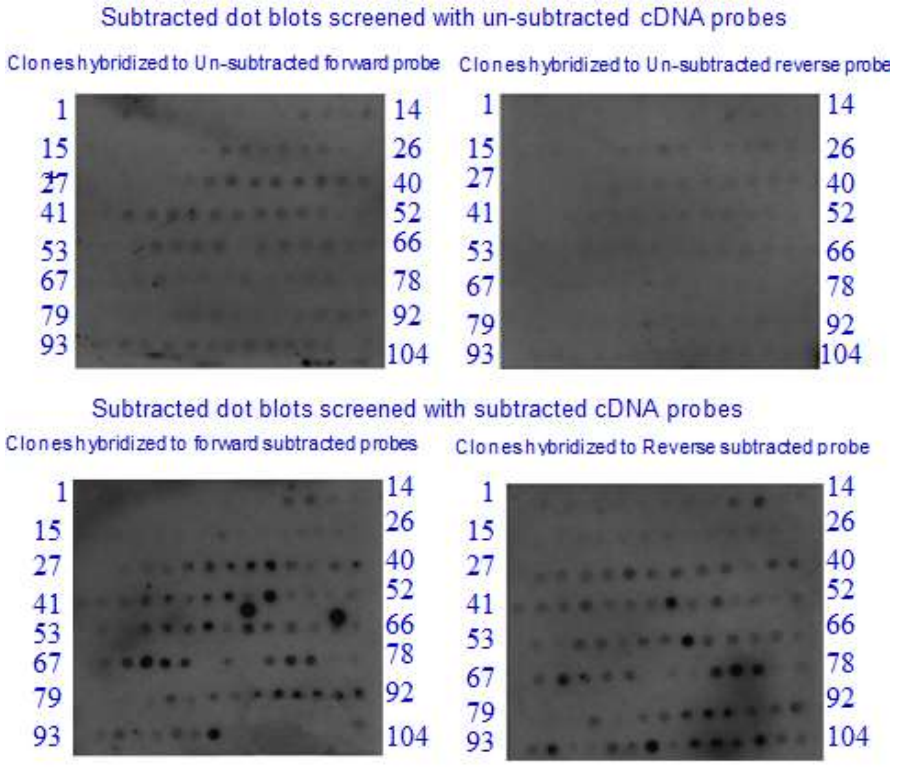

Figure 3. Validation of differential expression; PCR amplicons of insert positive clones denatured in 6N NaOH were arrayed on positively charged nylon membranes and probed with indicated probes. Lower two panels show successful enrichment of NIEEF differentially expressed genes.

is widely used to study differential gene expressions in cells. It was used in this study to identify cDNA clones that expressed genes or gene products that are uniquely of sporozoites that were axenically cultured at 37C for 24hrs as IEEF and NIEEF as well as to decipher the molecular basis for the arrest in the developmental of sporozoites at the liver-stage that were exposed to 12,000 rads of gamma radiation. Consequently, based on homology studies that resulted in the assigning of putative identity and function to our sequences, the data obtained revealed a modifying effect of irradiation on gene expression profiles of proteins in the IEEF in terms of immune response, mitosis, and receptor/epitope interaction (Figure 5C).

A putative immune response factor identified in this study based on homology studies is known as the *yir* multigene family. This gene family has been determined to be sequentially expressed as sets of genes during different developmental stages of the malaria parasite. The complexity of the *yir* multigene family is characterized by the fact that it exists as about 838 allelic forms that are homologous to the *vir* multigene family that is found in *Plasmodium vivax*. In terms of being expressed as different sets of genes, the process usually is initiated in the mosquito vector by the initial expression of different sets of *yir* genes by sporozoite, and then it is continued inside the mammalian host where different sets of *yir* genes are expressed during the liver and blood stages of the parasite. These different sets of *yir* genes that are expressed at different developmental stages of the sporozoites are for them to be used to fulfil unique functional roles. In the salivary gland of mosquitoes, the functional role of the expressed *yir* gene are for use to complement the activities of other stored proteins like those of the micronemes, rhoptry bodies and dense granules. When inoculated into the mammalian host, the role of *yir* gene products are to facilitate traversal motility as well as for attachment, invasion and development in hepatocytes. This expression of different sets of *yir* genes will continue with merozoites that are released from hepatocytes as YIR proteins have been identified on the surface of erythrocytes where they are associated with immune evasion. Demonstrating once more that, the set of *yir* genes that are being differentially expressed at each stage in the life cycle of sporozoites are for the purpose of fulfilling unique functions, key amongst which

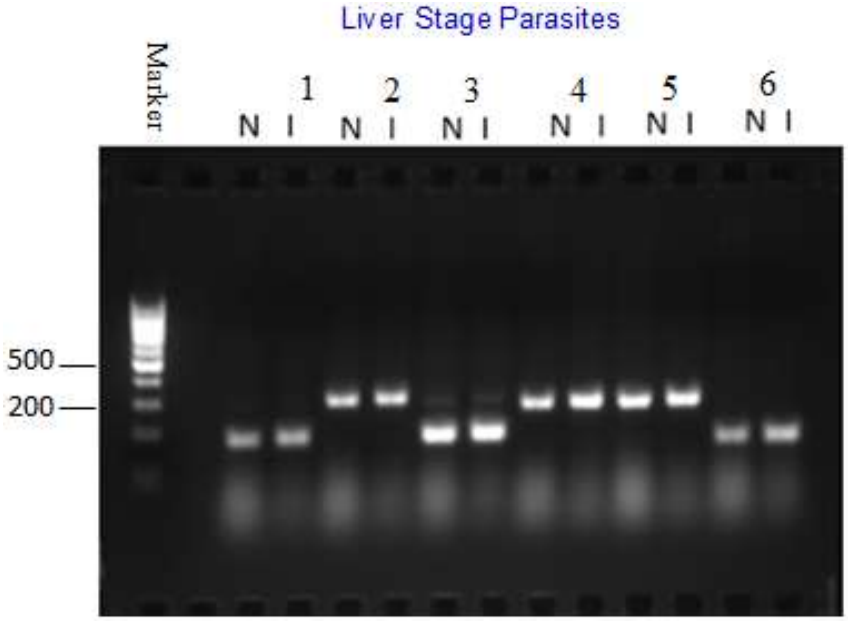

Figure 4A. RT-PCR assay with primers design from sequences of a random selection of the IEEF plus (+) and minus (-) clones were used to test liver samples infected with gamma-irradiated sporozoites (I) and non-irradiated (NI) sporozoites as well as blood samples infected with non-irradiated sporozoites. Amplicons are detected only in the liver samples.

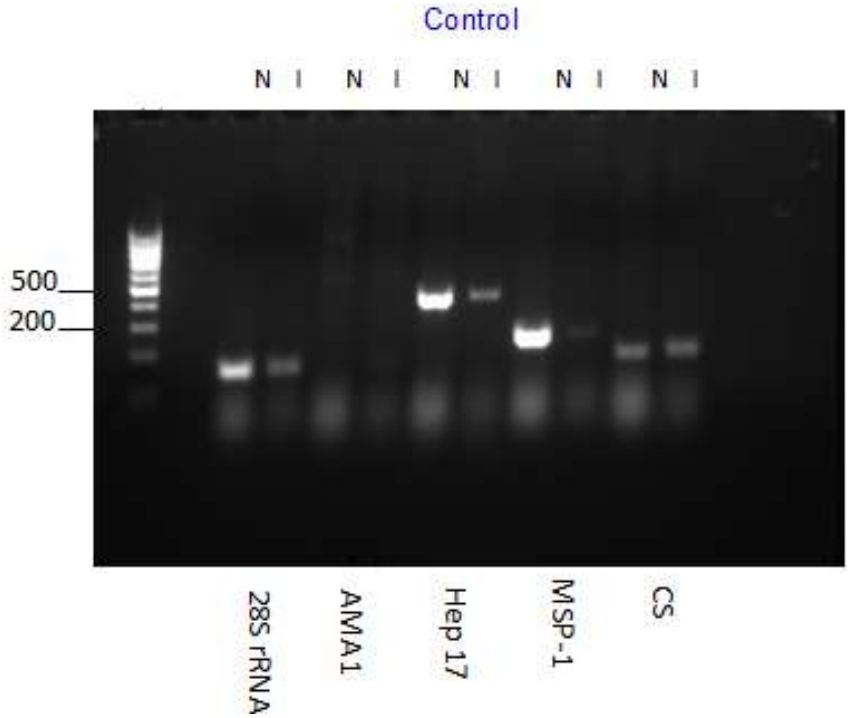

Figure 4B. Sequences from known antigens of EEF were used to generate primers that serve as control for the RT-PCR assay in Figure 4A. Noted here are amplicons that are differentially expressed in the irradiated and non-irradiated infected liver samples.

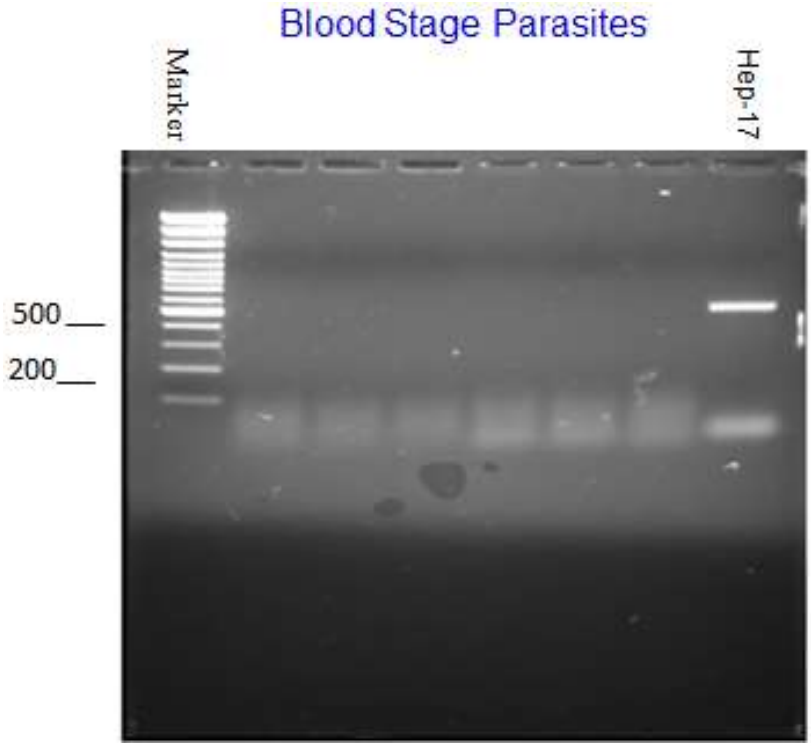

Figure 4C. No amplified PCR product is observed in the blood sample. Lane seven of the blood stage parasites is the positive control.

is the production of variant immune antibodies that are critical for used in immune evasion and for pathogenesis in the host. However, because there was down regulation of the *yir* gene in the IEEF as observed in this study, coupled with the fact that studies have shown irradiated sporozoites as having the propensity to invade hepatocytes during which their development becomes arrested at the trophozoite stage shows that *yir* multigene family has roles that are associated with sporozoite attachment and invasion of hepatocytes. The implication of this is that the product of the *yir* multigene family can be a suitable candidate for use in a multi-subunit vaccine to prevent malaria at the pre-erythrocytic stage.

Another putative immune response factor identified from homology studies of sporozoite sequences was the *py235* gene product that appears to be initiated at the trophozoite stage through to schizogony. The gene exits also as a multigene family with 11 – 50 allelic forms. Preiser et al. (1999 observed that each merozoite from schizonts formed inside the parasitophorous vacuoles of hepatocytes, expresses a *py235* gene that is unique from that of other merozoites With 10,000 – 30,000 merozoites per schizont (Druilhe et al., 1998) and the fact that there are literally thousands of schizonts that are formed inside infected hepatocytes of the liver, demonstrates that the products of the *py235* gene provides the EEF with means of protection against immune pressures from the host (Lyer et al., 2007). Moreover, expression characteristics suggest that transcriptional and translational machinery involve multiple members of the *py235* multigene family. And since each merozoite in a schizont expresses a unique *py235* phenotype the possibility of irradiation affecting all variants of the *py235* genes is minimal. As a result, genes not affected by sporozoite exposure to 12,000 rads of gamma radiation will be expressed later at the liver stage by both the IEEF and the NIEEF. This explains why expression of the py235 gene was similarly detected in the IEEF and the NIEEF because it will appear that transformation as a continuously evolving process gradually coalesces in the general shift in gene expression from the liver-stage-like to the blood-stage-like in the malaria parasite. Moreover, the fact that the IEEF following exposure to 12,000 rads of gamma radiation could still develop in parasitophorous vacoules that only measure 4-8 µm in diameter because sporozoite development has become arrested at the trophozoite stage in parasitophorous vacuoles means the *py235* multigene family codes for protein products with varied functions amongst which are protein products that may be utilize for attachment and invasion as well as for sporozoite development in hepatocytes. As such, this qualifies products of the *py235* multigene family as

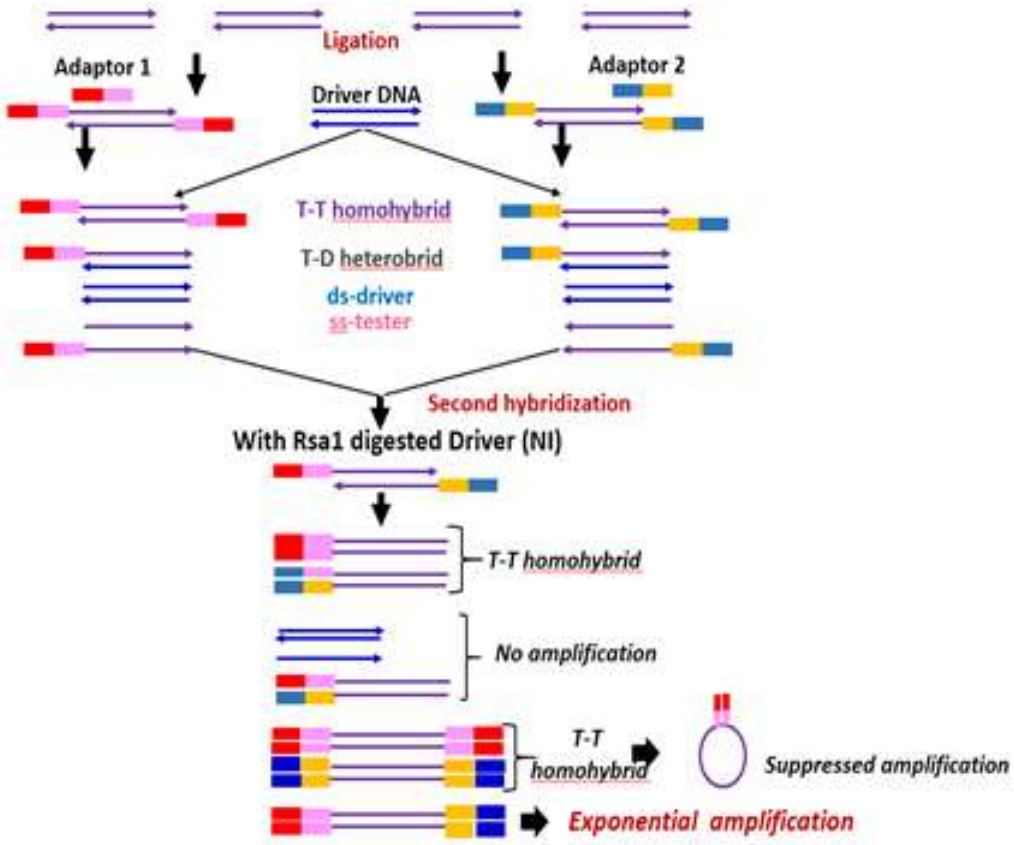

Figure 5. Schematic representation of the Suppression Subtractive hybridization technique.

possible candidates for use in a multi-subunit vaccine to prevent sporozoites from attaching and invading hepatocytes at the pre-erythrocytic stage.

Meanwhile, another putative immune factor identified from homology sequence study was that of the erythrocyte membrane protein 1 (EMP1) that is coded for by *var* multigene family. Two-third of this gene family is located at the ends of chromosome 14 and in the internal regions of chromosomes 4, 7, 8, and 12. The EMP1 is a variant protein in terms of its antigenicity and molecular size (200-350kDa). It is known to be compose of 2 to 7 Duffy-binding like (DBL) domains with each containing 5 cysteine-rich-interdomain regions (CIDR) (Claessens et al., 2014). Previously, the *var* multigene family was thought to be active mostly during the erythrocytic stage of malaria because they were found to be associated with pathogenicity and immune evasion (Senczuk et al., 2001). However, today, studies have shown that the *var* multigene family is expressed in mosquitoes, starting from the oocyst stage rather than from the stage when gametocytes are taken up by mosquitoes and then they transform into the ookinetes. During activation of these family of genes in oocysts, of a total of 60 known *var* genes, only one is activated to code for the EMP1, while the remaining 59 *var* genes are kept in an inactive state. This type of activation in the oocyst stage will remain in this manner through the sporozoite stage. This is significant because the oocyst is the stage in the mosquito during which sporozoites are formed and later released into the salivary gland in preparation for inoculation into a mammalian host during a blood meal. Which shows that the role played by *var* genes in terms of pathogenesis are only limited to the erythrocytic stage as the sporozoites stage is a silent phase of malaria during which no sign and symptom are manifested following inoculation of sporozoites into the mammalian host (Gomez-Diaz et al., 2017). It will appear that like in the case with the CS and TRAP proteins, the EMP1 is involve in traversal, attachment and successful invasion of hepatocytes especially as these processes require receptor/epitope interaction that involves ligands on the surface of sporozoites with several receptors on the surfaces of various cells on the mammalian host (Matuschewski et al., 2002). However, the unique receptor on the surface of hepatocytes to which sporozoites can bind using the EMP1 protein is still

controversial as while some studies conclude that *Plasmodium yoelii* and *Plasmodium falciparum* use the EMP1 ligand to bind to CD81receptor on the surface of hepatocytes, others conclude that the receptor used is the scavenger receptor BI (SR-BI) for invasion and development inside parasitophorous vacuoles of hepatocytes. These conclusions were arrived at partly because antibodies directed against the SR-BI and CD81 receptor prevented sporozoites from invading hepatocytes with the EMP1 ligand (Yalaoui et al., 2008). Later, with the release of merozoites and the initiation of the erythrocytic stage, the binding of EMP1 on the surface of erythrocytes to ICAM-1 and CD36 on the surface of host endothelial cells results in sequestration that causes the most life-threatening form of malaria known as cerebral malaria (Chen et al., 1998; Zanghi et al., 2018). The fact that in this study expression of the gene that codes for EMP1 was abrogated in cDNA clones from the libraries of the IEEF and the parasites could still invade hepatocytes where their development became arrested in parasitophorus vacuoles that only measure 4-8 µm demonstrates that when it comes to attachment and invasion of hepatocytes, sporozoites may be utilizing redundant mechanisms to achieve this process. This is because if the *var* gene peptides were the sole entities utilize by sporozoites for attachment and invasion of hepatocytes, then once abrogated as was the case in the IEEF, the sporozoites should not be able to enter hepatocytes to form parasitophorous vacuoles that only measure 4-8 µm in diameter (Scheller et al., 1995), demonstrating that the trophozoite phase gradually transitioned into the schizogony phase. And the fact that expression of the EMP gene was only observed in the cDNA clones of the NIEEF as oppose to the cDNA clones of the IEEF also corroborates the finding that gene expression in the malaria parasite shifts from liver-stage-like to blood-stage-like Robson et al. (1995).

The final gene identified in this study was that which codes for the enzyme tubulin tyrosine ligase. This enzyme shows preference for tubulin, which is a protein that has a genome that contains one gene that codes for β-tubulin subunit and two genes that code for α-tubulin subunit (Prota et al., 2013). When products from these genes are configured inside sporozoites, the result is the formation of a protein known as tubulin that serves as a basic building block for structures with different functionalities inside sporozoites. In one configuration involving the basic building blocks, the gene products are arranged end-to-end to form a tube shape heterodimer in which an alpha subunit and a beta sub-unit of the basic building block alternate with each other in such a manner that when one end terminates with the alpha sub-unit, the other begins with the beta sub-unit. In a second configuration involving the basic building block, the gene products are initially assembled to produce tube-like structures, in which globular structure that are compose of alternating α-tubulin and β-tubulin serve as walls of microtubules while the carboxy-terminal tails that protrude from the wall function as sites that can later be modified for attachment of microtubule associated proteins. In these configurations, the α/β tubulins are not only organized so that they become building blocks for microtubule, but they are organized in such manner that modifications can occur on the building blocks as well as on the carboxy terminal to generate a variety of microtubules with organelle-specific function and properties (Hammond et al., 2008). Featuring prominently in these changes that generate microtubules with different functionalities is post-translational modification of the wall that is compose of tubulin heterodimer by way of tyrosination/detyrosination (Gundersen et al., 1987) as well as modification of the carboxy terminal that will generate microtubules with very divers functions that include but are not limited to functions involving changes of cell shape, movement of vesicles with synthesized proteins from the rough endoplasmic reticulum to the golgi apparatus, movement of organelles and granules from one part of a cell to another, and chromosomes by way of specially attached proteins (Prota et al., 2013). The tyrosination/ detyrosination modification involves detyrosination which is the removal of a tyrosine residue from the c-terminal of α-tubulin by tubulin carboxypeptidase and occurs predominantly on polymeric tubulin while tyrosination involves the re-attached of tyrosine to unassembled α-tubulin by tubulin tyrosine ligase (Ersfeld et al., 1993; Hallak et al., 1977; Lafanechere et al., 1998). They are stimulated by microtubule-binding proteins to form microtubules. Shortly after the microtubules become stabilized, they accumulate as post-translationally modified tubulin better known as de-tyrosinated tubulin. Polymerized tubulins in microtubules are the main constituent of mitotic spindles, basal bodies of flagella because the α/β tubulins are organized in a manner that makes them to be building blocks for used to generate microtubule structures with organelle-specific function and properties (Masuda and Shibata 1996). The up regulation of the gene that codes for tubulin tyrosine ligase in the cDNA clones from the IEEF implies that exposure of sporozoites to 12,000 rads of radiation may have resulted in an increase in the synthesis of tubulin. This is significant because tubulin tyrosine ligase exclusively modifies unpolymerized tubulin whose increase in a cell results in unstable microtubules as oppose to depolymerized tubulin that results in stable microtubules Magiera and Janke (2014). As such, the arrest in the development of sporozoites inside hepatocytes can be explained by the fact that up regulation of the gene that codes for tubulin tyrosine ligase has resulted in an increase in the concentration of tyrosinated tubulin in sporozoites at the expense of detyrosinated tubulin thereby disrupting the tyrosination/ detyrosination phenomenon that is critical for a balance in

the development of sporozoites (Gundersen et al., 1987). For that the product of this gene can be a suitable vaccine candidate for use in a multi-subunit vaccine to prevent malaria during the silent phase of the disease. Arrested development having left the parasite in a perpetual trophozoite phase means the expression of the tubulin tyrosine ligase gene is constitutive due to the possible lack of genetic information that will signal the trophozoite to transition to the next developmental phase of the malaria parasite. Down regulation of the said gene in the cDNA clones of the NIEEF can be attributed to the fact that the trophozoite phase immediately transitioned into the schizogony phase after mitosis and cytokinesis that is marked by DNA replication and the formation of thousands of merozoites.

Meanwhile, to further validate our results from the suppression subtractive hybridization technique, do blot preparations from the original two *P. yoelii* cDNA libraries were applied onto nylon membranes. The templates were incubated with $^{\{\alpha\text{-}32}P^{\}}$ d-ATP labeled total cDNA probes that were subtracted as well as un-subtracted. Autoradiography demonstrated that a majority of the subtracted NIEEF cDNA clones exhibited up regulation as compared to those of the subtracted NIEEF cDNA clones. Next to understand the significance of the pattern of display of the PCR product in Fig. 5 Primers from selected clones were used in another RT-PCR analysis involving irradiated sporozoites, non-irradiated sporozoites and blood from mouse that was infected with *Plasmodium yoelii* parasites. Electrophoresis on a 2% agarose gel shows differential display of bands whose sequences would neither confirm yir, tubulin tyrosine ligase nor p235 rhoptry protein. This is an indication that more cDNA clones need to be selected for use to synthesize primers for the RT-PCR assay.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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