Genetic diversity and immunogenicity of the merozoite surface protein 1 C-terminal 19-kDa fragment ($MSP1_{19}$) of *Plasmodium ovale* imported from Africa to China

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Abstract

Background

Merozoite surface protein 1 (MSP1) plays an essential role in erythrocyte invasion by malaria parasites. The C-terminal 19-kDa of MSP1 has long been considered as one of the major candidate antigens for malaria blood-stage vaccine in *Plasmodium falciparum*. However, there are limited information on the C-terminal 19-kDa region of *Plasmodium ovale* merozoite surface protein 1 (PoMSP1$_{19}$). This study, therefore, aims to analyse genetic diversity and immunogenicity of PoMSP1$_{19}$.

Methods

A total of 37 clinical *P. ovale* isolates including *P. ovale curtisi* (Poc) and *P. ovale wallikeri* (Pow) imported from Africa to China and collected between 2012 and 2016 were used. Genomic DNA were used to amplify *pocmsp1$_{19}$* and *powmsp1$_{19}$* genes by polymerase chain reaction (PCR). Genetic diversity of *pomsp1$_{19}$* was analyzed using the MegAlign and GeneDoc v.6 programs. Recombinant PoMSP1$_{19}$-GST proteins were expressed in an *Escherichia coli* expression system and analyzed by Western blot. Immune responses in BALB/c mice immunized with rPoMSP1$_{19}$-GST were determined using enzyme-linked immunosorbent assays (ELISA). In addition, antigen-specific T-cell responses were performed by lymphocyte proliferation assays. A total of 49 serum samples from *P. ovale* infections and healthy people were used to evaluate natural immune responses through protein microarray assays.

Results

Sequences of *pomsp1$_{19}$* were found thoroughly conserved in all clinical isolates. Recombinant PoMSP1$_{19}$ proteins were efficiently expressed and purified as ~ 37 kDa proteins. High antibody responses in immunized mice with rPoMSP1$_{19}$-GST were observed. The rPoMSP1$_{19}$-GST induced high avidity indexes with an average of 92.57% and 85.32% for Poc and Pow, respectively. Cross-reactivity between rPocMSP1$_{19}$ and rPowMSP1$_{19}$ was observed. Cellular immune responses to rPocMSP1$_{19}$ (69.51%) and rPowMSP1$_{19}$ (52.17%) induced in rPocMSP1$_{19}$- and rPowMSP1$_{19}$-immunized mice were found during splenocyte proliferation assays. The sensitivity and specificity of rPoMSP1$_{19}$-GST proteins for natural immune responses detection in patients infected with *P. ovale* were 89.96% and 75%, respectively.

Conclusions

This study revealed high conservation in sequences of *pomsp1$_{19}$* and high immunogenicity of rPoMSP1$_{19}$. The rPoMSP1$_{19}$ proteins detected humoral immune responses in patients with *P. ovale*

infection. Such informative results advance our understanding of natural immunity to *P. ovale* infection and contribute to the knowledge base for the development of a PoMSP1$_{19}$-based vaccine.

Background

Malaria is one of the most severe infectious diseases threatening human health. According to the World Health Organization (WHO), malaria caused 229 million clinical cases and 490,000 deaths in 2019 [1]. *Plasmodium ovale* is one of the five species of *Plasmodium* that regularly infect humans and, is geographically distributed in sub-Saharan Africa and the Western Pacific [2]. *Plasmodium ovale* (*P. ovale*) was classified into two subspecies including *P. ovale curtisi* and *P. ovale wallikeri* in 2010 [3]. *Plasmodium ovale* shares similar morphology and life-cycle with *P. vivax* [2]. The prevalence of *P. ovale* malaria is underestimated because of the low density of parasites in infected subjects, mild clinical symptoms, and mixed infections with other species of *Plasmodium* [2, 4–5]. However, *P. ovale* infection could evolve in cases of severe malaria, especially in areas where malaria is endemic [6–7]. Although massive research achievements have been obtained for *falciparum* malaria vaccines [8–10], no widely effective vaccine exists for *P. ovale* malaria. It is therefore essential to pay more attention to *P. ovale* in malaria detection and to engage in studies for the development of effective ovale vaccines. Since 2017, there have been no local malaria cases reported for nearly four consecutive years in China, which have reached the WHO's malaria elimination standard [11]. Meanwhile, with economic growth and deepening of the global trade, the number of imported malaria cases to China has increased in recent years and, cases of *P. ovale* infection have been proven to be no exception [12–14].

During the invasion of human erythrocytes by malaria parasites, merozoites surface proteins (MSPs) are exposed to the immune system with the coat of surface proteins shedding from the merozoite surface, in which the merozoite surface protein 1 (MSP1) is predominant [15]. Moreover, antibodies targeting MSP1 have been observed in individuals from malaria-endemic areas and have been shown to confer immunity [16–18]. The molecular weight of MSP1 is approximately 200 kDa [19]. The MSP1 protein undertakes two proteolytic cleavage steps during the invasion of erythrocytes. In a first processing step, MSP1 is cleaved into four polypeptides (83 kDa, 30 kDa, 38 kDa, and 42 kDa). Subsequently, the 42 kDa-fragment is cleaved into 33 kDa (MSP1$_{33}$) and 19 kDa (MSP1$_{19}$) fragments which remains attached to the merozoite surface and enters into erythrocyte [20–22]. In particular, the limited sequence polymorphism of the C-terminal 19 kDa-region of *Plasmodium falciparum* merozoite surface protein 1 (PfMSP1$_{19}$) has been identified in some of the earlier studies [23–25]. Several studies have investigated that sequences polymorphism of the C-terminal 19-kDa region of *P. vivax* and also found limited genetic diversity [26–27]. Limited genetic polymorphism is advantageous for vaccine development to ensure the inability to escape from the host immune response [27–28]. Moreover, antibodies against *P. falciparum* MSP1$_{19}$ (PfMSP$_{19}$) can prevent invasion of merozoites into erythrocytes [29]. Antibodies to PfMSP1$_{19}$ have been linked with protection in pregnant women, infants and older children from clinical malaria cases [30–32]. These finding have an implication that MSP1$_{19}$ is a promising candidate antigen for blood stage vaccine.

Although genetic polymorphisms of MSP1 in Thailand *P.ovale* isolates has been previously analyzed and showed low level in sequence diversity [33]. Little is known on genetic diversity and immunogenicity of PoMSP1$_{19}$. In this study, sequences of *pomsp1*$_{19}$ in clinical *P. o. curtisi* and *P. o. wallikeri* isolates from cases of *ovale* malaria patients imported into China from Africa were investigated. In addition, immunogenicity of PoMSP1$_{19}$ was assessed in mice model, as well as levels of immune responses against PoMSP1$_{19}$ in serum samples of patients infected with *P. ovale*.

Methods

Study site and sample collection

Blood samples of *P. ovale*-infected febrile patients who had returned from work in malaria endemic areas of sub-Saharan Africa between 2012 and 2016, were obtained from local hospitals in Jiangsu Province, China [34–35]. PCR was used to identify the isolates and parasite species were differentiated by real-time Taq Man PCR [13]. Genomic DNA was extracted from the blood samples to serve as template for PCR. For protein microarray assays, serum samples of 29 *P. ovale*-infected patients who returned from Africa to China between 2012 and 2016 were used. In addition, serum samples of 20 healthy individuals from China were also obtained from local hospitals of Jiangsu Province for use.

PCR amplification and sequencing of the pomsp1$_{19}$

A total number of 37 clinical *P. ovale* isolates (*P. o. curtisi*, n = 20 and *P. o. wallikeri*, n = 17) were randomly selected for PCR amplification (Additional file 1: Table S1). The gene sequences of *pocmsp1* (KC137343) and *powmsp1* (KC137341) from the GenBank database of National Centre for Biotechnology Information (NCB1) were used as reference sequences [33–34]. The 258 bp- sequences of *pomsp1*$_{19}$ were identified via matching with similar sequences was as previously reported [36–37], and were amplified by nested PCR. The first round primers were designed as: *pomsp1*$_{19}$ forward (5'-AGT AAG GAA AAA GAT TTG ACA A -3') and *pomsp1*$_{19}$ reversed (5'-AAG TAA GTT AAA TAG GAT GAT-3'). The primers for nested PCR were as follows: *pomsp1*$_{19}$ forward (5'-ATG GGA TCT AAA CAT AAA TGT – 3') and *pomsp1*$_{19}$ reversed (5'-GAA AAC ACC TTC GAA GAA TGG – 3'). Both amplification reactions used the same reaction conditions as follows: 98°C for 3 minutes, followed by 35 cycles of 98°C for 10 seconds, 45°C for 1 minute, and 72°C for 1 minute, and final extension at 72°C for 5 minutes. PCR products were electrophoresed on a 1.2 % agarose gel, analyzed under an ultraviolet transilluminator (Bio-Rad ChemiDoc MP), and sequenced by GENEWIZ (Suzhou, China). PCR amplified fragments were cloned into pUC57 vector and sequenced by Genewiz using universal primers (M13F: 5'-TGT AAA ACG ACG GCC AGT-3', M13R: 5'-CAG GAA ACA GCT ATG AC-3'). To evaluate the diversity within sequences of different isolates, the *pocmsp1*$_{19}$ and *powmsp1*$_{19}$ sequences were aligned using GeneDoc v.2.7.0.

Protein expression and purification

The genes of *pomsp1*$_{19}$ were subcloned into pGEX-6p-1 expression vector which contained a GST-tag fusion protein (Talen-bio Scientific). *Escherichia coli* strain BL21 pLysS cells were used to express recombinant pGEX-6p-1$^{pomsp1}{}_{19}$ plasmids, which were cultured in Luria Bertani (LB) containing ampicillin 50 μg/ml by shaking at 250 rpm, 37°C, until optical density (OD) at 600 nm reached 0.6–0.8. To induce the expression of rPoMSP1$_{19}$ proteins, isopropyl β-D-1-thiogalactopyranoside (IPTG, 0.1 mM) (TransGen Biotech, Beijing, China) was added and the culture were allowed to grow for another eight hours. Proteins were purified by Tanlen-bio Scientific (Wuxi, China). The rPoMSP1$_{19}$-GST proteins were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) and detected via Western Blot and Coomassie brilliant blue staining (Beyotime Biotech, China). For Western blot analysis, the separated proteins from SDS-PAGE were electrophoresed on polyvinylidene difluoride (PVDF) membrane (Immobilon, Millipore Sigma). Thereafter, nonspecific binding was blocked by incubating with 5% skimmed milk in Tris-buffered saline supplemented with 0.1% Tween-20 (TBST) at room temperature for two hours. The membranes were incubated with anti-GST rabbit monoclonal antibody (CWBio Biotech) as primary antibody at 1:2000 dilution overnight at 4°C, following by three times wash with 0.1% TBST. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (CWBio Biotech) was used as secondary antibody for incubation at 1:5000 dilution for one hour. Finally, the membranes were analyzed with a ChemiDoc MP imaging system (Bio-Rad) for detection.

Mouse immunizations

Female BALB/c mice (Cavens, Changzhou, China) at six to eight-week-old were intraperitoneally injected with 50 μg of rPocMSP1$_{19}$-GST, rPowMSP1$_{19}$-GST, GST, or phosphate-buffered saline (PBS) mixed with Freund's complete adjuvant (Sigma, San Francisco, USA) as primary immunization. The same amount of antigen was mixed with incomplete Freund's adjuvant then injected on days 21 and 42 after the initial injection as booster immunization. Mouse serum samples were collected and stored at -80 ℃ on days 0, 7, 14, 28, 35, and 49 after the initial injection.

To detect antibodies directed against rPoMSP1$_{19}$-GST from immunized mice, Western blot analysis was carried out. Concretely, rPoMSP1$_{19}$–GST and GST proteins were transferred from SDS-PAGE onto PVDF membranes. The membranes were incubated with serum from mice immunized with rPoMSP1$_{19}$-GST as primary antibody, GST immunized group or PBS as negative control group, and then incubated with HRP-conjugated goat anti-mouse IgG (Cowin Biotech) at 1:5000 dilution.

Enzyme-linked immunosorbent assay (ELISA)

Levels of mice immunoglobulin (Ig)G antibodies against rPoMSP1$_{19}$-GST and GST were investigated through enzyme-linked immunosorbent assays (ELISA) as previously described [35, 38]. For the assays, 50 ng of rPoMSP1$_{19}$-GST and GST were immobilized on 96-well plates overnight at 4°C in coating buffer (15 mM sodium carbonate and 35 mM sodium bicarbonate in distilled water) and blocked with 5% (w/v) non-fat milk in TBST at room temperature for two hours. Thereafter, 100 μl of a twofold serial dilution (1:10000 to 1:5120000) of anti-rPoMSP1$_{19}$-GST and anti-GST mouse sera was added to each well and

incubated for one hour at room temperature. The plates were washed three times with PBS containing 0.1% of Tween-20 (PBST) then, incubated with HRP-conjugated goat anti-mouse IgG antibodies (Southern Biotech) at 1:5000 dilution for one hour at room temperature. Finally, the plates were washed again and incubated with 100 µl of 3,3',5,5'- tetramethylbenzidine (TMB, Invitrogen) substrate for height minutes in the dark and the reaction was stopped with 50 µl 2 M H_2SO_4 in each well. The absorbance was read at 450 nm. All samples were tested in duplicate and the mean absorbance was calculated.

Affinity test for anti-rPoMSP1$_{19}$-GST IgG antibody was carried out following the ELISA test as described above, except that here, the test was duplicated in 96-well plates coated with same antigens. Then, sera were incubated for 90 minutes at room temperature, following by washing of one of the plates with PBST while the other was incubated with 100 µl of TBST containing 6 M urea for 10 minutes at room temperature before final washing with PBST. Finally, all the plates were incubated with HRP-conjugated goat anti-mouse IgG antibodies at 1:5000 dilution for one hour at room temperature, the reaction was stopped, and the absorbance was measured at 450 nm. Avidity index (AI) was calculated as follows [39]:

AI = (OD450 of a sample treated with 6 M urea/OD450 of a sample not treated with 6 M urea) × 100.

Lymphocyte proliferation assays

Assays for lymphocyte proliferation were performed using the Cell Counting Kit-8 (CCK-8, Beyotime Biotech) as previously described [35, 40]. Briefly, lymphocytes from mice immunized with rPoMSP1$_{19}$-GST, GST and PBS (5×10^5 cells/well) were treated with 10 µL of rPocMSP1$_{19}$-GST (5 µg/mL), 10 µL of rPowMSP1$_{19}$-GST (5 µg/mL), 10 µL of GST (5 µg/mL) or 10 µL of concanavalin A (Con A, 2 µg/mL) as positive control in 96-well flat bottom microtiter plates then incubated for 72 h at 37°C with 5% CO_2. Thereafter, 10 µL of CCK-8 was added to each well and the plates were incubated at 37°C for two hours. Finally, cells proliferation was measured at 450 nm through a microplate reader.

Serum screening using arrays

Sera from 29 cases of *P. ovale*-infected and 20 healthy individuals were screened by well-type amine arrays. The microarray screening was performed as previously described [41–42]. Briefly, modified glass slides (75 × 25 mm,) were prepared for protein arrays (CapitalBio, Beijing, China) and warmed at room temperature before use. Teflon tapes with holes were pasted on the glass slides to make well-type amine arrays. One microliter of rPocMSP1$_{19}$-GST, rPowMSP1$_{19}$-GST and GST solution in PBS (100 ng/µl) were spotted to each well of the arrays and incubated for two hours at 37°C. This was followed by three times wash with PBST (PBS containing 0.1% Tween 20) for 10 minutes and the arrays were blocked with 5% of BSA in PBST at 37°C for two hours. Thereafter, the arrays were washed again and probed with 1 µl of serum samples from *P. ovale*-infected patients or healthy individuals at 1:200 dilution. Finally, 1 µl of Alexa Fluor 546 goat anti-human IgG (10 ng/µl, Invitrogen) in PBS-T was added to the arrays for detection. The intensity of serological responses on the arrays was measured in a fluorescent microarray scanner (CapitalBio, Beijing, China). The positive cut-off value was calculated as the mean plus two

standard deviations of mean intensity of the negative controls. Mann-Whitney U test was performed to compare differences in mean fluorescence intensity (MFI) between groups.

Statistical analysis

The GraphPad Prism software version 5.0 was used for statistical analysis and graphing (GraphPad Software Inc.). Unpaired, two-tailed Students t-test was used and differences were considered statistically significant if *p*-values < 0.05.

Result

Genetic description and PCR analysis of $pomsp_{19}$

The predicted full length of PocMSP1 (KC137343) and PowMSP1 (KC137341) were 1727 and 1672 amino acids, respectively [34]. Given that MSP1 undergoes proteolytic processing during merozoite invasion process, the schematic diagram of PocMSP1 and PowMSP1 were divided into seven domains as follows: signal peptide (SP), 83 kDa; 30 kDa; 38 kDa; 33 kDa; 19 kDa; and glycolphosphatidylinositol (GPI) (Fig. 1a and 1b). Alignment of the amino acid sequences between $PocMSP1_{19}$ and $PowMSP1_{19}$ showed that only one amino acid was different. Two different amino acids located at positions 1640 and 1585 in PocMSP1 and PowMSP1, respectively (Fig. 1c). Clinical *P. o. curtisi* and *P. o. wallikeri* isolates (n = 20 and n = 17, respectively) were used as source of genomic DNA for PCR amplification (Additional file 1: Table S1) and, the 258 bp-size of $pomsp1_{19}$ genes was successfully amplified. The PCR products were tested by electrophoresis and showed a single band on the electropherograms for $pomsp1_{19}$ (Fig. 2a). Alignment of $pomsp1_{19}$ sequences in all isolates showed that no amino acid mutation occurred (Additional file 2: Fig S1). This suggested that $pomsp1_{19}$ was completely conserved across the isolates.

Expression, purification, and analysis of $rPoMSP1_{19}$-GST proteins

The molecular weight of $rPoMSP1_{19}$-GST was estimated at approximately 35 kDa (including the molecular weight of $PocMSP1_{19}$/$PowMSP1_{19}$ estimated at approximately 9 kDa and that of the pGEX-6p-1 expression vector which contained a GST-tag fusion protein at approximately 26 kDa). Recombinant proteins were productively expressed and purified as shown in Fig. 2b. The rPoMSP19-GST protein presented in SDS-PAGE as a single band of approximately 37 kDa. Western Blot analysis that used anti-GST tag antibody confirmed expression of $rPoMSP1_{19}$-GST (Fig. 2c).

Micederived antibodies against $rPoMSP1_{19}$-GST recognized the recombinant proteins

To verify whether mice immunized could generate anti-$rPoMSP1_{19}$-GST antibodies and also recognize the $rPoMSP1_{19}$-GST proteins, we performed an immunoblot for a specific ~ 37 kDa band of purified proteins.

Obviously, sera from mice immunized with rPocMSP1$_{19}$-GST and rPowMSP1$_{19}$-GST detected rPocMSP1$_{19}$-GST and rPowMSP1$_{19}$-GST, respectively, as expected (Fig. 3a and 3b). In addition, sera from mice immunized with GST could also recognize the recombinant proteins (Fig. 3c). No band was found the recombinant proteins were treated with sera from mice immunized with PBS (negative control). These results indicated that rPoMSP1$_{19}$-GST could induce anti–rPoMSP1$_{19}$-GST antibodies in mice.

Moreover, in addition to the detection of rPocMSP1$_{19}$-GST, rPowMSP1$_{19}$-GST, and GST proteins by specific antisera (anti-rPocMSP1$_{19}$, anti-rPowMSP1$_{19}$, and anti-GST sera, respectively) from immunized mice, the antisera also cross-reacted with the rPoMSP1$_{19}$ proteins and GST as well (Fig. 3a, 3b, and 3c). The sera from mice immunized with rPocMSP1$_{19}$-GST could recognize rPowMSP1$_{19}$-GST and GST as shown in Fig. 3a. The sera from mice immunized with rPowMSP1$_{19}$-GST could also recognize rPocMSP1$_{19}$-GST and GST (Fig. 3b). All these observations showed that anti–rPocMSP1$_{19}$-GST and anti–rPowMSP1$_{19}$-GST antibodies had the ability to cross-react with PocMSP1$_{19}$ and PowMSP1$_{19}$ antigens.

Immune response in mice immunized with rPoMSP1 $_{19}$ -GST To measure levels of immune responses against rPoMSP1$_{19}$-GST or PBS (negative control) in mice, ELISA were performed using the rPoMSP1$_{19}$-GST and GST proteins as the coating antigens. The results demonstrated that both rPocMSP1$_{19}$-GST and rPowMSP1$_{19}$-GST were immunogenic (Fig. 4). The average of serum antibody titers were determined by ELISA at 49 days after the first intraperitoneal injection. The rPocMSP1$_{19}$-GST and rPowMSP1$_{19}$-GST proteins induced comparable antibody responses with end-point titers ranging from 1:10000 to 1:2560000 (Fig. 4a). After three consecutive immunizations, the high IgG antibody responses were induced in mice immunized with rPoMSP1$_{19}$-GST (Fig. 4b and 4c). Meanwhile, the titration curves of the GST were indicative of lower responses compared to those of the rPoMSP1$_{19}$-GST proteins. In addition, mice immunized with GST or PBS failed to induce high or no IgG antibody responses, respectively, compared to those immunized with the rPoMSP1$_{19}$-GST proteins. This result suggested that mice immunized with rPoMSP1$_{19}$-GST induced high-avidity IgG antibodies (rPocMSP1$_{19}$-GST mean: 92.57%, $p = 0.0024$; and rPowMSP1$_{19}$-GST mean: 85.32%, $p = 0.014$) compared to mice immunized with GST (mean: 37.84%) (Fig. 4d). However, there was no statistically significant difference of immune responses between rPocMSP1$_{19}$-GST and rPowMSP1$_{19}$-GST mice groups ($p > 0.05$).

Cellular immune responses induced by rPoMSP1$_{19}$-GST were assessed through spleen lymphocyte proliferation assays. The effects of the splenocyte proliferative in vitro under rPocMSP1$_{19}$-GST, rPowMSP1$_{19}$-GST, GST, and ConA (positive control) stimulations were determined. The rPocMSP1$_{19}$-GST protein-induced cell proliferation was 69.51% and that induced by the rPowMSP1$_{19}$-GST protein was 52.17%. Collectively, the rPoMSP1$_{19}$-GST proteins induced a stronger proliferation effect on spleen cells (rPocMSP1$_{19}$-GST: $p = 0.0471$ and rPowMSP1$_{19}$-GST: $p = 0.026$) when compared to ConA, and more importantly, GST failed to induced cell proliferation ($p < 0.00001$; Fig. 4e).

Cross-reactivity of rPoMSP1 $_{19}$ -GST proteins with anti-rPoMSP1 $_{19}$ -GST antibodies Because of the similarity in the amino acid sequences of PocMSP1$_{19}$ and PowMSP1$_{19}$, we examined cross-reactivity between rPocMSP1$_{19}$-GST and rPowMSP1$_{19}$-GST through ELISA. The rPocMSP1$_{19}$-GST could recognize and combine IgG of sera from mice immunized with rPowMSP1$_{19}$-GST protein (Fig. 5a and 5d) and, no significant difference was observed in cross-reactivity and avidity indices of cross-reaction ($p > 0.05$). However, IgG of anti-GST protein showed significantly lower level of cross-reactivity and avidity indices of cross-reaction with rPocMSP1$_{19}$-GST protein compared with reaction of rPocMSP1$_{19}$-GST to IgG of antirPocMSP1$_{19}$-GST ($p < 0.001$, Fig. 5a and 5d). In addition, cross-reaction and affinity of anti-rPocMSP1$_{19}$-GST and rPowMSP1$_{19}$-GST showed no significance difference compared with reaction of rPowMSP1$_{19}$-GST to anti-rPowMSP1$_{19}$-GST sera (Fig. 5b and 5e). Unexpectedly, rPocMSP1$_{19}$-GST and rPowMSP1$_{19}$-GST showed significantly higher cross-reaction and affinity than GST to anti-GST sera ($p < 0.05$, Fig. 5c and 5f). These results suggested that either rPocMSP1$_{19}$-GST or rPowMSP1$_{19}$-GST had the ability to recognize and bind with high affinity the antibodies induced by mice immunized with rPoMSP1$_{19}$-GST.

Humoral immune responses to rPoMSP1 $_{19}$ -GST in Plasmodium ovale **infections**To further assess humoral immune responses against rPoMSP1$_{19}$-GST in patients with *P. ovale*, we used protein microarray technology to screen antibodies against rPoMSP1$_{19}$-GST protein. Antibody responses against rPoMSP1$_{19}$-GST were analyzed from 29 patients infected with *P. ovale* and 20 serum samples from healthy individuals (Additional file 3: Table S2). We observed that *P. ovale*-infected patients showed significantly higher MFI of total IgG against rPocMSP1$_{19}$-GST and rPowMSP1$_{19}$-GST than that from healthy individuals (Fig. 6a and 6b, $p < 0.0001$). The prevalence for anti-rPocMSP1$_{19}$-GST antibodies showed a sensitivity of 89.96% (MFI value of 26 out of 29 patient samples > cut-off value 5752.4) and specificity of 75% (MFI value of 15 out of 20 healthy samples < 5752.4). In addition, the prevalence for anti-rPowMSP1$_{19}$-GST antibodies showed a sensitivity of 89.96% (MFI value of 26 out of 29 patient samples > cut-off value 7092) and specificity of 75% (MFI value of 15 out of 20 healthy samples < cut-off value 7092). However, no significance was observed in MFI of total IgG against GST protein between *P. ovale*-infected or healthy individuals ($p = 0.5964$, Fig. 6c). These results showed that both rPocMSP1$_{19}$-GST and rPowMSP1$_{19}$-GST are targets of signatures of exposure and immunity.

Discussion

The complex life cycle of malaria parasites is a major challenge for the development of an effective malaria vaccine. Malaria vaccines targeting attractive blood-stage molecules of the parasites is therefore a research priority [43]. Several merozoite surface proteins have been regarded as promising malaria vaccine candidates given they are targets of host immune system and play essential roles in erythrocyte invasion [28, 44]. Antigenic diversity of malaria vaccine targets increased the complexity of the prediction effect as it helps parasites escape from host immune responses [45]. We blasted amino acid sequences between PocMSP1$_{19}$ and PowMSP1$_{19}$ as template and analyzed sequences of C-terminal of *pomsp1*

(pomsp1$_{19}$) in clinical samples. Interestingly, we found that sequences of *pomsp1$_{19}$* were completely conserved in 20 *P. o. curtisi* and 17 *P. o. wallikeri* isolates. From the perspective of reference amino acid sequences from PocMSP1$_{19}$ and PowMSP1$_{19}$, the 20th amino acid of PocMSP1$_{19}$ is Serine, whereas that of PowMSP1$_{19}$ is Proline. These results were consistent with previous reports [33, 36–37]. Immune-mediated selection pressure is an important mechanism that may cause antigenic diversity [46]. This study found the C-terminal region of PoMSP1$_{19}$ under relatively less selective pressure compared with the N-terminal of PoMSP1 which showed limited genetic diversity [34].

Antibody play an important role in clinical protection against blood-stage malaria parasites. Early studies suggested that antibody responses to PfMSP1$_{19}$ might protect children from high levels of blood-stage parasitemia and clinical malaria [32]. In this study, cross-reaction between rPocMSP1$_{19}$-GST and rPowMSP1$_{19}$-GST was detected. In addition, sera from mice immunized with GST protein as nonspecific control, could detect rPocMSP1$_{19}$-GST and rPowMSP1$_{19}$-GST considering that rPoMSP1$_{19}$-GST contained GST tag. These evidences indicated that rPocMSP1$_{19}$ and rPowMSP1$_{19}$ shared similar antigenic determinants and, therefore, PoMSP1$_{19}$ might possess species-specific efficacy for a vaccine candidate. Of the five classes of immunoglobulins, IgG is well-known in playing a critical role in malaria immunity [47]. The significantly higher levels of IgG antibodies response to rPocMSP1$_{19}$-GST and rPowMSP1$_{19}$-GST compared to GST (Fig. 4), showed that rPoMSP1$_{19}$-GST induced immune responses in mice. Avidity indices of anti–rPocMSP1$_{19}$-GST IgG and anti–rPocMSP1$_{19}$-GST IgG were not significantly different in cross-reactivity, whereas avidity indices of rPoMSP1$_{19}$-GST IgG were higher than those of GST group either in crossreactivity or in auto-antigen binding test (Fig. 5). Such findings suggest that antibodies produced by mice immunized with rPoMSP1$_{19}$-GST could bind tightly to antigens and tend to mature. Although antibodies were induced in mice immunized with GST, it could not promote affinity maturation. High-affinity antibodies are advantageous in a number of biological functions [48]. A better understanding of cellular responses can help develop more effective blood-stage malaria vaccine candidates. Lymphocytes play key roles in the immune system given that they have been shown implied in the specificity of immune responses to infectious micro-organisms and other foreign substances [49]. To determine T cell immune responsiveness to an antigenic stimulus, Lymphocyte proliferation assays are widely being used to determine T cell immune responsiveness to an antigenic stimulus. Results of the current study showed that rPocMSP1$_{19}$-GST could stimulate proliferation of T cells compared to GST (Fig. 4f). Such an instructive result proved that rPocMSP1$_{19}$-GST could induce cellular immune responses in mice and more importantly, demonstrated the immunogenicity of rPocMSP1$_{19}$-GST in mice model.

Furthermore, serologic analyses that investigated IgG antibodies against *P. falciparum* and *P. vivax* specific antigens have been reported in a number of studies to assess infection incidence and immunity levels [17, 50–51]; however, the question of response specificity has not been fully explored. In this study, we also analyzed humoral immune responses in *P. ovale* infections and most of the samples with ovale infection showed positive antibody responses to rPoMSP1$_{19}$-GST. The rPoMSP1$_{19}$-GST showed high sensitivity (89.96%) and specificity (75%). This significant difference in immune responses was not

related to the presence of GST protein because no significance was observed in MFI of total IgG against GST protein between *P. ovale*-infected or healthy individuals (Fig. 6). These data confirmed the antigenicity of rPoMSP1$_{19}$-GST, which may indirectly reflect or contribute to protection against ovale malaria infection, because humoral immune responses are partly involved in preventing from clinical malaria [52]. Sero-epidemiological studies have been particularly effective in areas of low transmission where the sensitivity of surveys for prevalence of parasite was impacted by the number of parasite-infected individuals [53]. Previous studies used PfMSP1$_{19}$ and *P. vivax* MSP1$_{19}$ as serological markers to detect the prevalence of malaria parasites [54–55]. Due to the limitation in number of serum samples, this study was not able to compare differences in signal of serological responses following other criteria such gender and parasite density. This study characterized immune responses to PoMSP1$_{19}$ which has proven to be immunogenic. Investigation on PoMSP1$_{19}$ as biomarker of exposure is worthy of further study.

Conclusions

This study demonstrated that *pomsp1$_{19}$* sequences from clinical *P. ovale curtisi* and *P. ovale wallikeri* isolates imported from Africa to China were completely conserved. Furthermore, high immunogenicity of rPoMSP1$_{19}$-GST was observed in mice model and *P. ovale* infections. In addition, cross-reactivity between rPocMSP1$_{19}$-GST and rPowMSP1$_{19}$-GST was observed and indicated that these proteins shared similar antigenic determinants. Collectively, these findings advanced our knowledge of the immunogenicity of MSP1$_{19}$ in ovale infections and more importantly, contributed to the basis for the rational design of PoMSP1$_{19}$-based vaccine.

Abbreviations

MSP1: Merozoite surface protein 1; PCR: Polymerase chain reaction; ELISA: Enzyme-linked immunosorbent assays; SP: signal peptide; GPI: glycophosphatidylinositol.

Declarations

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Availability of data and materials:

The data supporting the conclusions of this article are included within the article and its additional file. New sequences identified in this study are deposited in GenBank with the accession numbers MZ766552-MZ766553.

Authors' contributions

YC and JC conceived and designed the study. YBL, GDZ, JC and QBW collected the samples. QWX, SHL, BY and JCL conducted the laboratory work. QWX and KK wrote the manuscript. YFS reviewed the manuscript. WLZ and MSZ analysed the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate:

This study was approved by the Ethics Committee, Jiangsu Provincial Key Laboratory on Parasite and Vector Control Technology, Jiangsu Institute of Parasitic Diseases (JIPD) (IRB00004221), Wuxi, China. Informed consent was obtained from all of the participants, and the animal trial was approved by the Animal Ethics Committee, Jiangnan University (JN. No20180615t0900930[100]).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

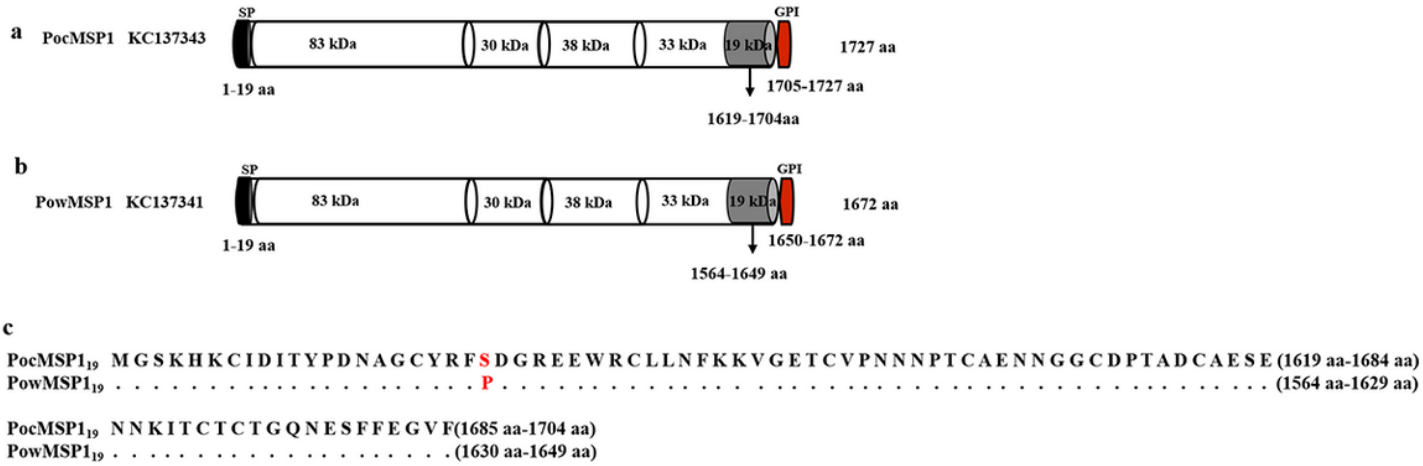

Figure 1

Schematic diagram of protein structure and alignment of PoMSP119 amino acid sequences. (a and b) Black, red and gray colors indicate SP, GPI and expression area of proteins, respectively. (c) Alignment of amino acid sequences between PocMSP119 and PowMSP119. Abbreviations: aa: amino acid; kDa, kilodalton; SP, signal peptide; GPI, glycophosphatidylinositol.

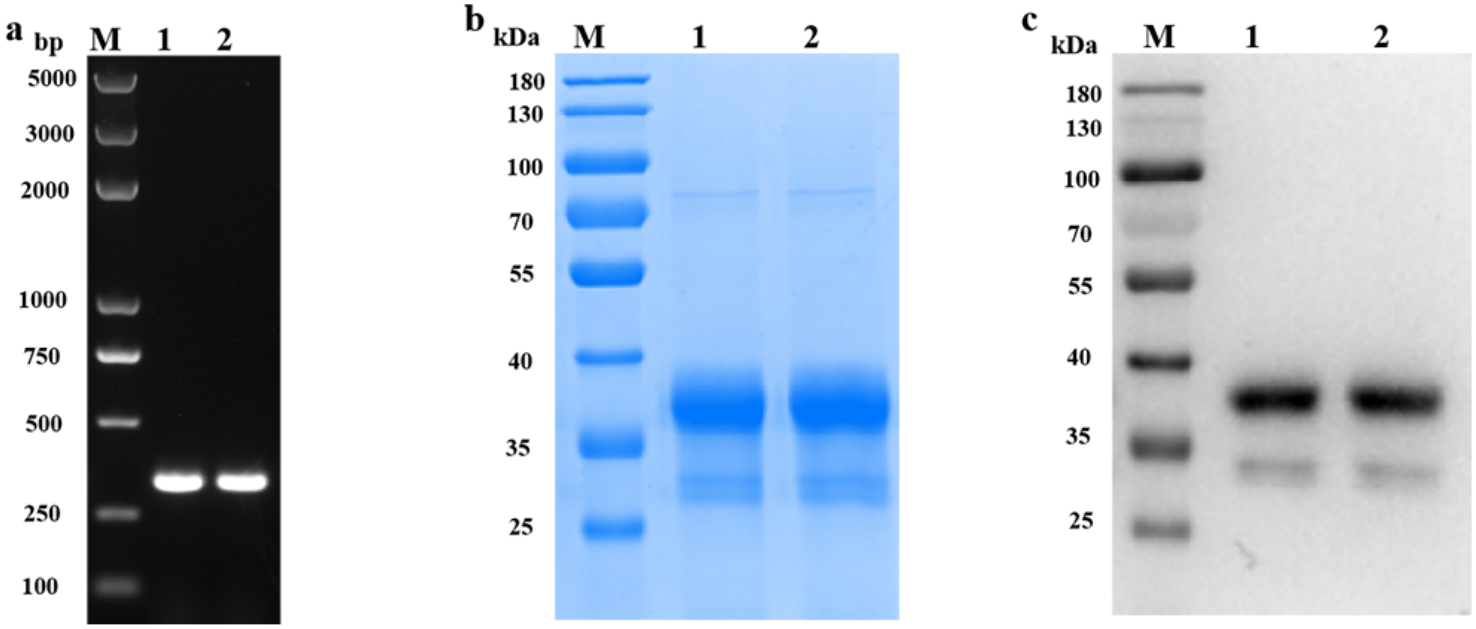

Figure 2

Polymerase chain reaction amplification (PCR) of pomsp119 and expression, purification of recombinant PoMSP119-GST proteins. (a) The pocmsp119 and powmsp119 were amplified by PCR. (b) Purified rPoMSP119-GST (~37 kDa) was resolved by 10% SDS-PAGE. (c) Western blot analysis of rPoMSP119-GST using an anti-GST rabbit monoclonal antibody. 1: purified rPocMSP119-GST protein; 2: purified rPowMSP119-GST protein.

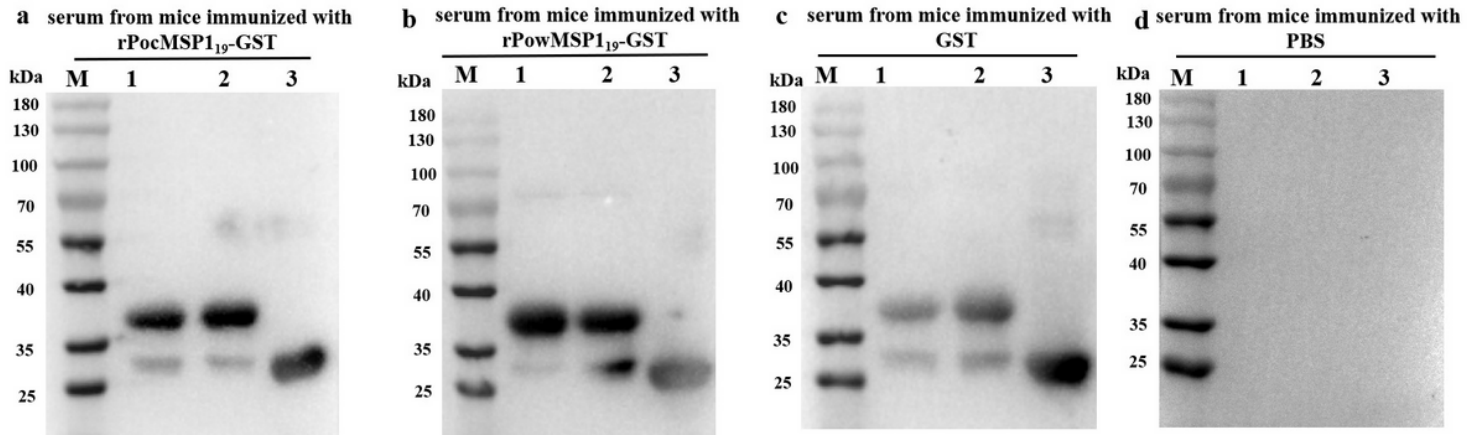

Figure 3

Detection of antibodies in the serum of mice immunized with rPoMSP119-GST. (a, b and c) Western blot analyses for the detection of rPoMSP119-GST proteins by using antibodies from serum of mice immunized with rPoMSP119-GST and GST proteins. (d) Western blot analysis using antibodies from serum of mice immunized with PBS. 1: purified rPocMSP119-GST protein; 2: purified rPowMSP119-GST protein; 3: purified GST protein.

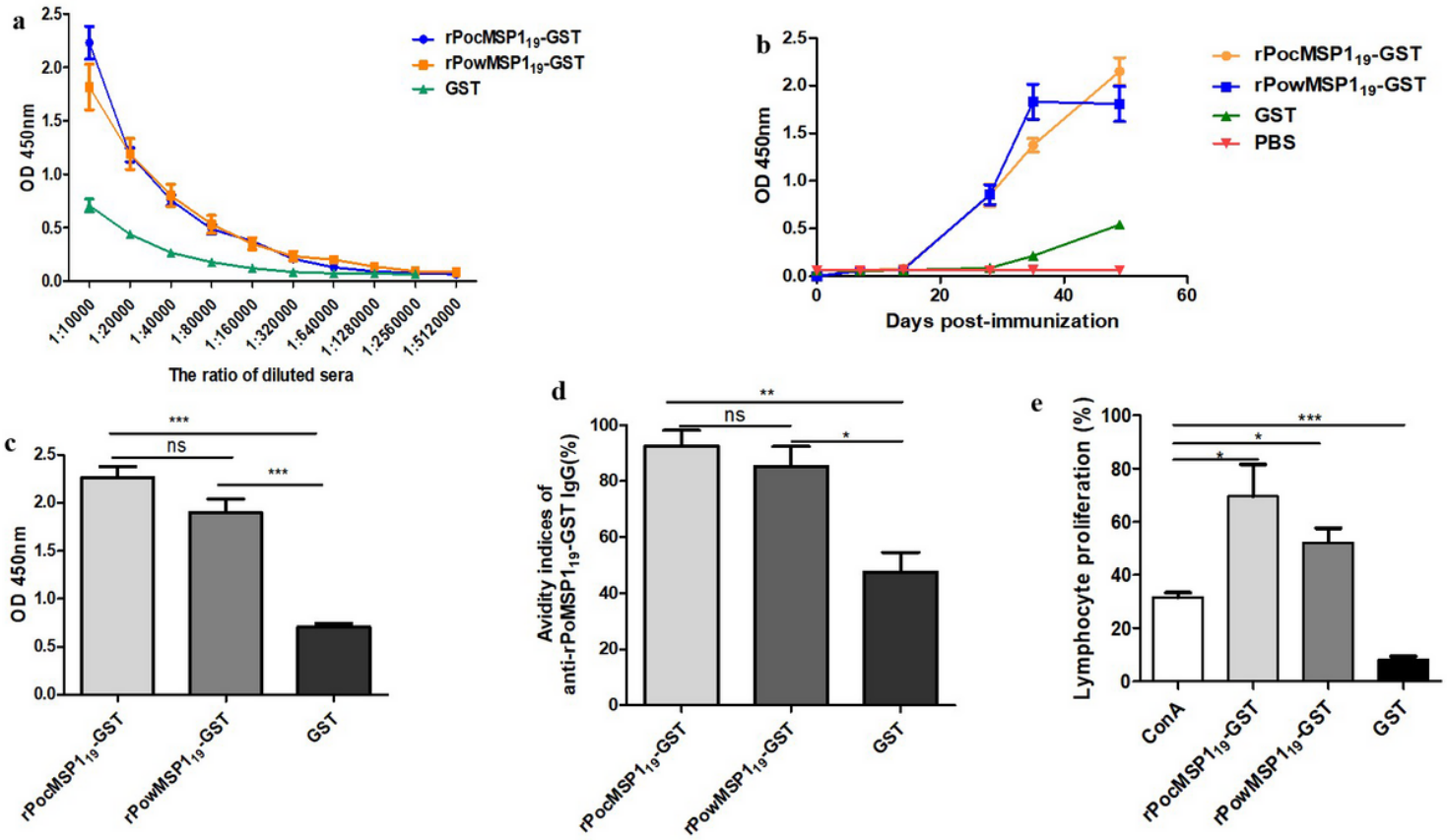

Figure 4

Immune responses in mice immunized with rPoMSP119-GST. (a) Sera from immunized mice were diluted to different multiples (1:10,000 to 1:5,120,000) and the data were expressed by average OD value. (b) IgG rising trend detected through ELISA. (c) Specific immune responses in sera from mice immunized with different proteins confirmed by testing the interaction of antibodies with rPocMSP119-GST, rPowMSP119-GST and GST. The mean OD was shown as the strength of specific immune responses. (d) Avidity indices of anti-rPoMSP119-GST IgG through ELISA. (e) Solenocytes from mice immunized were stimulated with recombinant proteins and concanavalin A (ConA) as positive control. Significant differences between groups are marked on the chart: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Nonsignificant (ns) differences ($P > 0.05$) are shown.

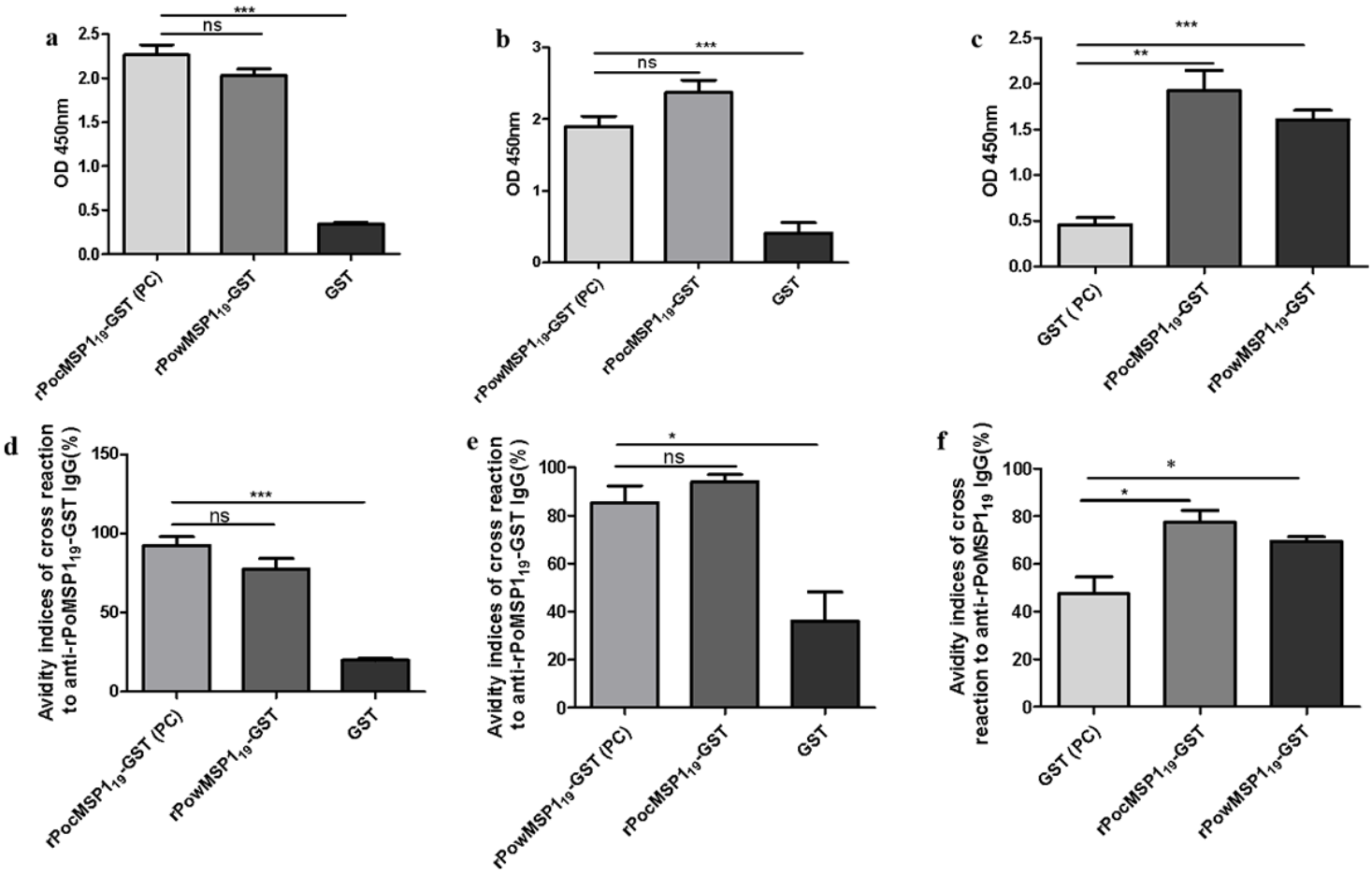

Figure 5

Cross-reaction of rPoMSP119-GST proteins with antisera from mice immunized with rPoMSP119-GST. (a) Cross-reactivity of rPowMSP119-GST and GST with serum of mice immunized with rPocMSP119-GST protein. (b) Cross-reaction of rPocMSP119-GST and GST with serum of mice immunized with rPowMSP119-GST protein. (c) Cross-reactivity of rPoMSP119-GST with serum from mice immunized with GST protein. (c, d and e) Avidity indices of cross-reaction to anti-rPoMSP119-GST IgG. (PC, positive control). The data are shown as the mean OD expressed which reflects the magnitude of the immune responses. Significant differences between groups are marked on the chart: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Nonsignificant (ns) differences ($P > 0.05$) are shown.

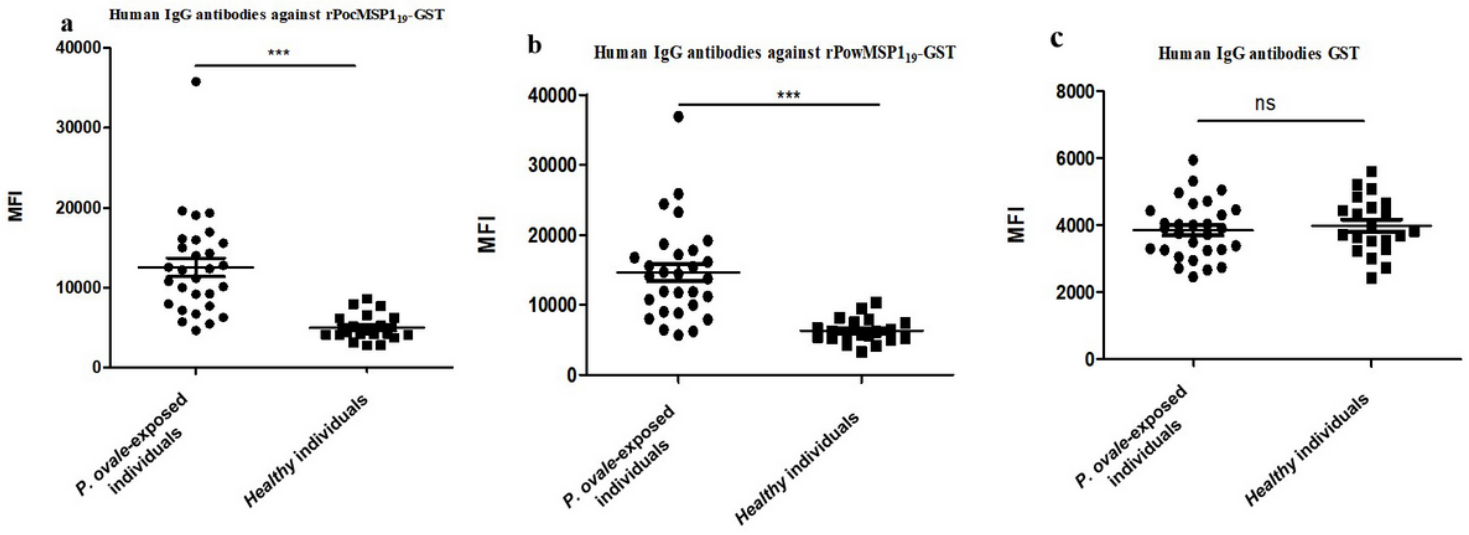

Figure 6

Analysis of humoral immune responses to rPoMSP119-GST in Plasmodium ovale infections. (a) IgG responses to rPocMSP119-GST in patients infected with P. ovale and healthy people. (b) IgG responses to rPowMSP119-GST in patients infected with P. ovale and healthy people. (c) IgG responses to GST in P. ovale-infected patients and healthy people. Significant differences between groups are marked on the chart: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Nonsignificant (ns) differences ($P > 0.05$) are shown.

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