HHS Public Access

Author manuscript

Parasitol Int. Author manuscript; available in PMC 2019 August 09.

Published in final edited form as:

Parasitol Int. 2019 February ; 68(1): 87–91. doi:10.1016/j.parint.2018.10.006.

Antibodies against a *Plasmodium falciparum* RON12 inhibit merozoite invasion into erythrocytes

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Abstract

Proteins coating *Plasmodium* merozoite surface and secreted from its apical organelles are considered as promising vaccine candidates for blood-stage malaria. The rhoptry neck protein 12 of *Plasmodium falciparum* (PfRON12) was recently reported as a protein specifically expressed in schizonts and localized to the rhoptry neck of merozoites. Here, we assessed its potential as a vaccine candidate. We expressed a recombinant PfRON12 protein by a wheat germ cell-free system to obtain anti-PfRON12 antibody. Immunoblot analysis of schizont lysates detected a single band at approximately 40 kDa under reducing conditions, consistent with the predicted molecular weight. Additionally, anti-PfRON12 antibody recognized a single band around 80 kDa under non-reducing conditions, suggesting native PfRON12 forms a disulfide-bond-mediated multimer. Immunofluorescence assay and immunoelectron microscopy revealed that PfRON12 localized to the rhoptry neck of merozoites in schizonts and to the surface of free merozoites. The biological activity of anti-PfRON12 antibody was tested by in vitro growth inhibition assay (GIA),

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Conflict of interest disclosure

The authors declare no commercial or financial conflict of interest.

and the rabbit antibodies significantly inhibited merozoite invasion of erythrocytes. We then investigated whether PfRON12 is immunogenic in *P. falciparum*-infected individuals. The sera from *P. falciparum* infected individuals in Thailand and Mali reacted with the recombinant PfRON12. Furthermore, human anti-PfRON12 antibodies affinity-purified from Malian serum samples inhibited merozoite invasion of erythrocytes in vitro. Moreover, *pfron12* is highly conserved with only 4 non-synonymous mutations in the coding sequence from approximately 200 isolates deposited in PlasmoDB. These results suggest that PfRON12 might be a potential blood-stage vaccine candidate antigen against *P. falciparum*.

Keywords

Blood-stage vaccine; Malaria; Mali; *Plasmodium falciparum*; Rhoptry; Thailand

Plasmodium merozoites are invasive forms which contain specialized secretory apical organelles, termed rhoptries, micronemes, and dense granules. These organelles secrete proteins that play crucial roles in a erythrocyte invasion process, and subsequent infection [1]. Therefore, proteins stored in the apical organelles are generally considered as promising vaccine candidates for blood-stage malaria. Rhoptries are the largest among the three apical organelles and secrete two classes of proteins, rhoptry neck proteins (RONs) and rhoptry bulb proteins. Among the RONs, RON2 is well known to form a protein complex with RON4, that further associate with apical membrane antigen 1 (AMA1) during the formation of tight junction together with [2]. Furthermore, AMA1-RON2 complex was demonstrated have potent blood-stage vaccine efficacy [3,4]. We recently reported that human antibodies against RON2 and RON4 significantly associate with clinical protection, suggesting the blood-stage malaria vaccine candidacy of these RONs [5]. We also demonstrated the other rhoptry neck protein, RALP1, as a potential blood-stage vaccine candidate [6]. Therefore, we hypothesized that, in addition to the current candidates, novel *Plasmodium* RONs are potential blood-stage vaccine candidates.

A novel *Plasmodium falciparum* rhoptry neck protein 12 (PfRON12) was recently reported by Knuepfer et al. [7]. The protein is mostly retained within the rhoptry neck and only released into the parasitophorous vacuole after completion of invasion. Some of the protein is however detected at the moving junction, suggesting that PfRON12 has important roles in erythrocyte invasion. Although RON12 seems not essential for parasite growth, both *P. falciparum* and *P. berghei* Δ*ron12* conditional knockout parasites exhibited sluggish invasion and growth rates. Combination of these characteristics prompted us to further investigate the blood-stage vaccine candidacy of PfRON12.

To characterize PfRON12, we first generated a recombinant GST-fused PfRON12 as described [8]. Briefly, a fragment encoding PfRON12 but lacking the signal peptide (PF3D7_1017100: amino acid positions [aa] 26–310) was amplified by PCR from cDNA obtained from schizontrich *P. falciparum* 3D7 parasites using a sense primer with *Xho*I site and an antisense primer with *Not*I site (PfRON12-F1: 5′-ctcgagAAGACACA AAAAAATGAAGGAATTATTATTG-3′, PfRON12-R1: 5′-gcggccgcCTAT TCTTGTGTTAAATCGGAAACGTTCTC-3′). The amplified DNA fragment was cloned in

pEU-E01-GST-TEV-MCS-N2 plasmid (CellFree Sciences, Matsuyama, Japan) and GST-PfRON12 was expressed using the wheat germ cell-free translation system (CellFree Sciences). Expressed GST-PfRON12 (Fig. 1A, lane 1 arrowhead, around 70 kDa) was affinity purified using glutathione-Sepharose 4B column (GE Healthcare, Ca-marillo, CA, USA) and, the purified PfRON12 fraction was eluted by on-column cleavage with AcTEV protease (Invitrogen, Carlsbad, CA, USA) (Fig. 1A, lane 5 arrow, around 40 kDa). To generate PfRON12 antisera, 250 μg of purified PfRON12 with Freund's complete adjuvant was used to subcutaneously immunize a Japanese white rabbit. This was followed by two booster immunizations of 250 μg of the PfRON12 with Freund's incomplete adjuvant at 3-week intervals. Antisera was collected 14 days after the last immunization. The animal work was conducted by Kitayama Labes (Ina, Japan) in compliance with the guidelines based on "Charter for Laboratory Animal Welfare" (Japanese Society for Laboratory Animal Resources). The specificity of anti-PfRON12 antibodies were examined by Western blotting using 10^6 schizont-rich parasite extracts from cultured *P. falciparum* 3D7 parasites [9]. Immunoblot analysis detected a single band at approximately 40 kDa under reducing condition (Fig. 1B, lane R arrowhead), consistent with the predicted molecular weight. In contrast, the anti-PfRON12 antibodies recognized a single band at approximately 80 kDa under non-reducing condition (Fig. 1B, lane NR arrow), suggesting the native PfRON12 forms a disulfide-bond-mediated multimer. These results indicated that the rabbit anti-PfRON12 antibodies specifically recognize native PfRON12.

In order to determine subcellular localization of the PfRON12 in merozoites, immunofluorescent assay (IFA) was performed on mature schizonts and released merozoites. Briefly, infected blood smears were fixed on glass slides with ice-cold acetone for 5 min and blocked with PBS containing 5% non-fat milk at 37 °C for 1 h. The slide was then incubated with rabbit anti-PfRON12 (1:500) and mouse anti-RON2 (PF3D7_1452000) antibody (1:100) as rhoptry neck marker [2] at 37 °C for 1 h, followed by incubation with Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen, Carlsbad, CA) and Alexa Fluor 546-conjugated goat anti-mouse IgG (Invitrogen) (1:500) at 37 °C for 30 min. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; 2 μg/ml). The slides were mounted in ProLong Gold Antifade (Invitrogen) and observed using a confocal scanning laser microscope (LSM710; Carl Zeiss MicroImaging, Thornwood, NY). Free merozoites were isolated and fixed with 4% paraformaldehyde – 0.0075% glutaraldehyde in PBS as described [6] then double-stained with rabbit anti-PfRON12 (1:500) and mouse anti-myosin A tail domain interacting protein (MTIP; PF3D7_1246400) antibody (1:100) as inner membrane complex marker in the presence or absence of pre-treatment with Triton-X100. In schizonts, the fluorescent signals of anti-PfRON12 were detected as a punctate pattern in each merozoite which partially overlapped with those of anti-RON2 (Fig. 1C, upper panels). In addition, permeabilized released merozoites showed peripheral distribution of both PfRON12 and MTIP (Fig. 1C, middle panels). Unpermeabilized merozoites showed negligible signal of MTIP due to its intracellular localization (Fig. 1C, lower panels). Among the unpermeabilized merozoites, 63% ($n = 22$) showed the surface fluorescence signal of anti-PfRON12 (Fig. 1C, lower panels), and 37% ($n = 10$) showed faint signal of anti-PfRON12 (data not shown). These results clearly demonstrate that PfRON12 is translocated to the surface of released merozoites. Rabbit pre-immune serum did not show

any signal with both parasite specimens (data not shown). To confirm the precise localization of the PfRON12 in merozoites, we performed immunoelectron microscopy (IEM). Cultured parasites were fixed, embedded in LR White resin (Polysciences, Warrington, PA), and ultrathin sections were immunostained as described [10]. Specifically, rabbit anti-PfRON12 was used at 1:4000 dilution. Samples were examined with transmission electron microscope (JEM-1230; JEOL, Tokyo, Japan). Gold particles corresponding to PfRON12 localization were detected on the rhoptry neck portion of the merozoite (Fig. 1D). These findings clearly demonstrate that PfRON12 is stored in rhoptry neck of merozoites prior to egress and translocated to the released merozoite surface. Although PfRON12 was clearly proved as a secreted protein [7], elucidating how it localizes on the surface of merozoites requires further investigation.

The merozoite surface localization of PfRON12 strongly prompted us to evaluate growth inhibitory activity of the anti-PfRON12 antibody. In order to test whether antibodies to PfRON12 could block parasite invasion, rabbit total IgG to PfRON12 was tested for inhibition of parasite growth over one cycle of replication by flow cytometry [6,11]. When anti-PfRON12 IgGs were tested at a final concentration of 20, 10, and 5 mg/ml, they inhibited invasion by (mean ± SEM) 27.5% ± 3.3%, 11.6% ± 1.9%, and − 1.0% ± 1.8%, respectively (Fig. 2A). Anti-EBA175_R3–5 (PF3D7_0731500) (70.7% ± 1.3%) and anti-GST (3.7% ± 1.9%) rabbit IgGs were used as positive and negative controls at a final concentration of 20 mg/ml. The invasion-inhibitory activity of anti-PfRON12 IgG was dose dependent and was significantly higher than that of the anti-GST at the 20 mg/ml final concentration (Kruskal-Wallis test followed by Dunn's multiple-comparison test, $P < .05$).

We then investigate whether PfRON12 is immunogenic in humans. Human serum samples from Thailand were collected from asymptomatic *P. falciparum* carriers, adults with uncomplicated symptomatic *P. falciparum* malaria, and malaria naïve individuals as described [12]. Human serum samples were also collected from healthy immune Malian adults [13,14]. Ethics approvals for the Thai and the Mali studies were previously described [12,14]. Measurements of antibodies by enzyme-linked immunosorbent assays (ELISAs) against recombinant PfRON12 in the Thai or Mali samples were performed as described [6]. Seropositive samples were defined by a cut-off threshold of mean + 2 standard deviations (SD) of OD values to anti-PfRON12 in Thai malaria-naïve sera ($n = 10$). More than half (51.9%) of asymptomatic Malian adults ($n = 52$), 76.5% asymptomatic Thai adults ($n = 17$), and 50% symptomatic Thai patients ($n = 22$), showed positive reactivity to PfRON12 (Fig. 2B). There was however, no statistical difference between the groups, probably due to the limited sample size.

We further evaluated whether anti-PfRON12 antibodies induced by natural infections in humans actually have functional activity. Serum samples collected from 4 Malian immune adults were pooled (a total of 16 ml), and antigen-specific IgG was isolated using a NHS-activated Sepharose 4 Fast Flow column (GE Healthcare) coupled with recombinant PfRON12 protein. Approximately 50 μg of anti-PfRON12-specific IgG was isolated from the 16 ml of sera. The GIA reaction mixture was incubated for 40 h in the presence of test IgG to allow most of the invading parasites to develop to the schizont stage, and then parasite growth was determined by a biochemical assay specific for parasite lactate

dehydrogenase as described [15]. The PfRON12 specific human IgG showed 41.7% inhibition at 1.27 mg/ml (at the highest concentration could be tested) in GIA. In further evaluations, genetic polymorphism of *pfron12* obtained from PlasmoDB indicated that *pfron12* is highly conserved with only 4 non-synonymous mutations in the coding sequence from approximately 200 isolates deposited to date. Importantly, 99% of them were identical amino acid sequence to that of the reference 3D7 strain. Taken together, these results suggest that PfRON12 is highly conserved and immunogenic during malaria infection in humans and their anti-PfRON12 antibodies contribute at least in part to naturally acquired immunity.

RON12 of *P. falciparum* was previously found to be specifically expressed in schizont stages and localized to the rhoptry neck of merozoites on the basis of IFA and IEM. It has also been refractory to gene knockout attempts, suggesting that PfRON12 is not essential for blood-stage parasite survival. However, PfRON12 has not yet to be experimentally characterized as a blood-stage vaccine candidate. Here, we aimed to characterize PfRON12 as a blood-stage vaccine candidate by using recombinant PfRON12 expressed in the wheat germ cell-free system. Our Western blotting results under reducing-conditions (Fig. 1B) are in agreement with previous report [7]. In addition, native PfRON12 formed a putative multimer under non-reducing conditions (Fig. 1B, lane NR). In addition, our IEM results (Fig. 1D) have re-confirmed the previous study that PfRON12 is indeed localized in the merozoite rhoptry neck. Furthermore, our IFA results obtained with released merozoites demonstrated for the first time that PfRON12 translocates to the merozoite surface (Fig. 1C, lower panels). These differences of antibody reactivities in this study and the previous one [7] may be partially explained by the difference of the folding of the recombinant proteins. We used the wheat germ cell-free eukaryotic expression system, but Knuepfer, et al. [7] used an *Escherichia coli*-based prokaryotic system. Since the amino acid sequence of PfRON12 contains four cysteine residues, the conformational epitopes formed by disulfide bonding and putative disulfide-dependent multimerization may be better recreated by the eukaryotic than the prokaryotic expression systems.

Immunoreactive antigens involved in erythrocyte invasion are considered to be potential candidates for a blood-stage malaria vaccine [16]. We confirmed that the PfRON12 is immunogenic in the two populations (Fig. 2B). Since PfRON12 translocates to the merozoite surface similar to known micronemal proteins such as AMA1 and GAMA [17], we expected that PfRON12 might be involved in erythrocyte invasion. In this study, we demonstrate that not only rabbit antibodies against PfRON12 have invasion-inhibitory activity (Fig. 2A), but the PfRON12 specific human IgG from Malian adults also inhibited parasite growth in vitro. These findings suggest that PfRON12 contains the merozoite invasion-inhibitory epitope(s) and anti-PfRON12 human antibody against the epitope(s) develops following natural infection in malaria endemic areas.

Overall, our findings indicate that PfRON12 is highly conserved and an immunogenic antigen in humans, and anti-PfRON12 antibodies may contribute at least in part to naturally acquired immunity. Therefore, PfRON12 might be a potential blood-stage vaccine candidate antigen of *P. falciparum*. The recombinant PfRON12 protein not only elicited functional antibodies in rabbit, but was also successfully used to isolate antigen-specific, functional antibodies from human sera. The results again prove that the wheat germ cell-free system

can be an optimal recombinant protein expression system for basic malaria research and vaccine candidate discovery [18].

Acknowledgments

We thank volunteers in Thailand and Mali who participated in the epidemiology study. We also thank the Japanese Red Cross Society for providing human erythrocytes and human plasma. We appreciate Masachika Shudo at the Division of Analytical Bio-Medicine, the Advanced Research Support Center (ADRES), Ehime University for the technical assistance. We also thank Bernard N. Kanoi for critical reading of the manuscript. This work was supported in part by JSPS KAKENHI (JP26253026, JP15H05276, JP16K15266) in Japan to TT, and Thailand Research Fund (5TMU45H1) to RU. GIA work performed at NIAD was supported in part by the Intramural Research Program of NIAID, NIH and by the United States Agency for International Development (USAID). The funding source was not involved in any part of the study design, analysis, or interpretation of the data.

Abbreviations:

RON	rhoptry neck protein

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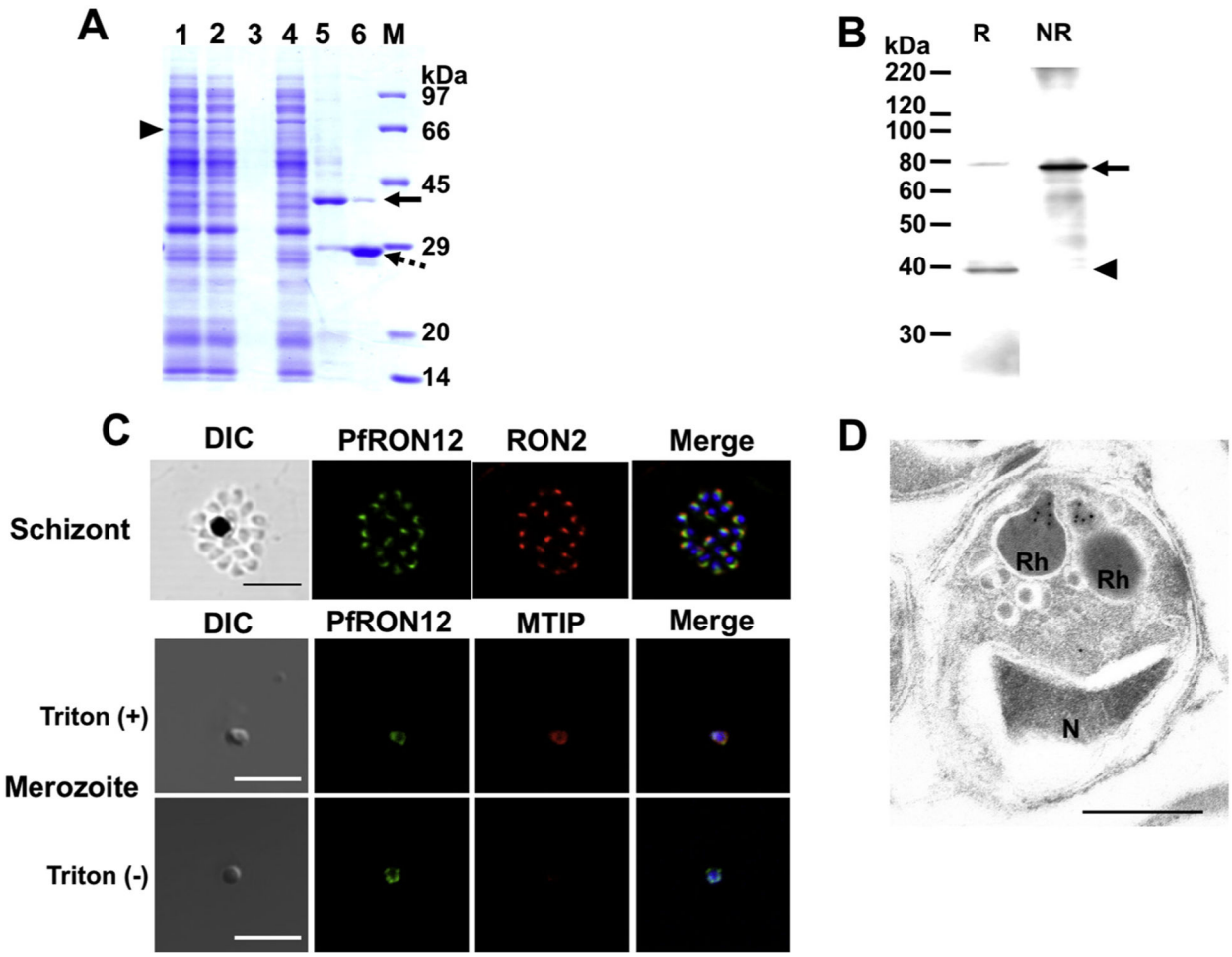

Fig. 1.

(A) SDS-PAGE analysis of the proteins expressed by the wheat germ cell-free system. Protein mixture was separated by 12.5% SDS-polyacrylamide gels (SDS-PAGE) under reducing conditions and stained with Coomassie brilliant blue. Samples in each lane were as follows: total reaction mixture (lane 1), supernatant and precipitated fractions after brief centrifugation (lanes 2 and 3, respectively), unbound and affinity-purified proteins (lanes 4 and 5, respectively), adsorbed protein left on the affinity matrix (lane 6), and protein molecular weight marker (lane M). The GST-fused PfRON12 products and purified proteins with AcTEV protease digestion are indicated by arrowhead and arrow, respectively. Cleaved GST remained on the affinity matrix is indicated by dashed arrow. (B) Western blot analyses using antisera against PfRON12. In each lane, proteins extracted by SDS-PAGE loading buffer from approximately 10^6 *P. falciparum* 3D7 schizonts were separated either under reducing (lane R) or non-reducing (lane NR) condition. A single band of approximately 40 kDa under reducing conditions (arrowhead) is consistent with the predicted molecular weight of PfRON12. A single band at approximately 80 kDa under non-reducing condition (arrow) represents the native PfRON12 that forms a disulfide-bond-mediated multimer. (C) Subcellular localization of PfRON12 in schizonts and free merozoites by indirect immunofluorescence assay. PfRON12; staining with rabbit anti-PfRON12 polyclonal antibodies, RON2; staining with mouse anti-RON2 polyclonal antibodies, MTIP; staining

with mouse anti-MTIP polyclonal antibodies, Merge; merged image including DAPI stained nucleus, DIC; differential interference contrast microscopy image. Triton (+); permeabilized with 0.1% Triton X-100. Bars 5 μm. (D) Subcellular localization of PfRON12 in *P. falciparum* merozoite in schizont stage by immunoelectron microscopy. PfRON12 localization was indicated by15-nm gold particles observed on the neck portion of the rhoptry. Rh; Rhoptry, N; nucleus. Bar 500 nm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

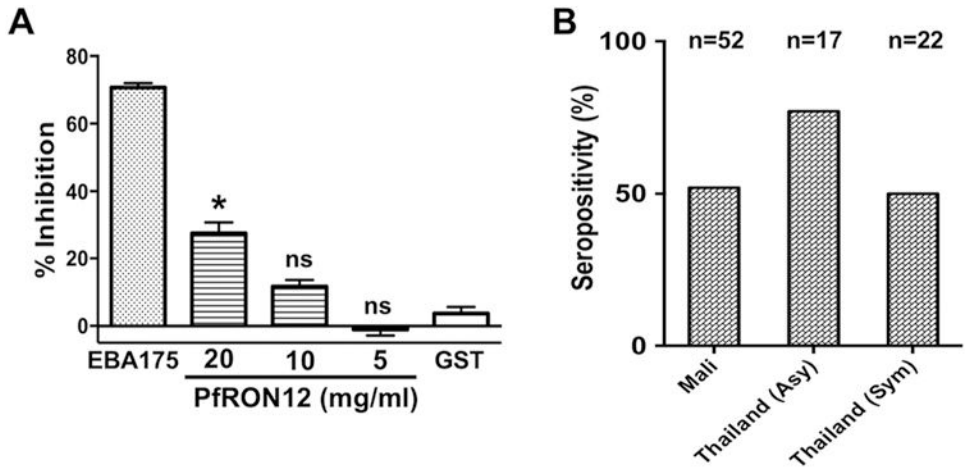

Fig. 2.

(A) Anti-PfRON12 rabbit antibodies have invasion-inhibitory activity in vitro. The ability of the anti-PfRON12 antibodies to inhibit the parasite invasion of erythrocytes was tested in a one-cycle GIA. Anti-EBA175 region 3–5 (EBA175) and anti-GST (GST) antibodies were used as positive and negative controls, respectively. The error bars represent the standard errors of the means of three independent experiments performed in triplicate. The Kruskal-Wallis test was performed, followed by Dunn's multiple-comparison test, to compare percent inhibition by anti-GST and anti-PfRON12 antibodies in three different IgG concentrations. *; statistically significant. ns; not significant. (B) Human sera from malaria endemic areas in Mali and Thailand recognize PfRON12 in ELISA. The seropositivities were determined by the threshold of mean + 2 standard deviations (SD) in OD values against PfRON12 from Thai malaria-naïve sera ($n = 10$). The PfRON12 antigen was probed with samples from immune Malian adults (Mali), and from *P. falciparum*-infected asymptomatic and symptomatic Thai adults (Thailand (Asy) and Thailand (Sym), respectively). The number (n) of samples analyzed are shown.