Published in final edited form as:

J Struct Biol. 2008 June ; 162(3): 460–467.

The avian malaria parasite *Plasmodium gallinaceum* causes marked structural changes on the surface of its host erythrocyte

Eriko Nagao^{a,1}, Takayuki Arie^{a,*1}, David W. Dorward^b, Rick M. Fairhurst^a, and James A. Dvorak^{a,2}

^a Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892

^b Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, MT 59840

Abstract

Using a combination of atomic force, scanning and transmission electron microscopy, we found that avian erythrocytes infected with the avian malaria parasite *Plasmodium gallinaceum* develop ~60 nm wide and ~430 nm long furrow-like structures on the surface. Furrows begin to appear during the early trophozoite stage of the parasite's development. They remain constant in size and density during the course of parasite maturation and are uniformly distributed in random orientations over the erythrocyte surface. In addition, the density of furrows is directly proportional to the number of parasites contained within the erythrocyte. These findings suggest that parasite-induced intraerythrocytic processes are involved in modifying the surface of host erythrocytes. These processes may be analogous to those of the human malaria parasite *P. falciparum*, which induces knob-like protrusions that mediate the pathogenic adherence of parasitized erythrocytes to microvessels. Although *P. gallinaceum*-infected erythrocytes do not seem to adhere to microvessels in the host chicken, the furrows might be involved in the pathogenesis of *P. gallinaceum* infections by some other mechanism involving host-pathogen interactions.

Keywords

Plasmodium gallinaceum; avian malaria; surface changes; atomic force microscopy

1. Introduction

Historically, from the 1890s to the 1940s, avian malarias were commonly used as models for human malaria physiology and chemotherapy (*e.g.*, see Slater, 2005). Although rodent malarias replaced avian malarias as the most productive model following World War II (*e.g.*, see Aviado, 1969), the relative ease of maintenance of *Plasmodium gallinaceum*, an avian malaria parasite, has resulted in its continued use in basic malaria research. Its abilities to infect a variety of

* Corresponding author: Takayuki Arie, Ph.D. Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Twinbrook III, Room 2W-09, 12735 Twinbrook Parkway, Rockville, MD 20852, Phone: 301-496-4880, Fax: 301-480-1438, Email: TArie@niaid.nih.gov.

¹These authors contributed equally to this work.

²Dedicated to the memory of the author, James A. Dvorak, who passed away in February, 2007.

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avian hosts and produce high parasitemias are important factors in its ongoing use in a wide range of cell biological and biochemical studies. Although *P. gallinaceum*-infected erythrocytes have been widely used as a research tool, very little is known about their surface structural characteristics.

While ultrathin sections and freeze-fracture replicas were used in the 1960s and 1970s to visualize by transmission electron microscopy (TEM) the fine structure of *P. gallinaceum*-infected erythrocytes (Ristic and Kreier, 1964; Seed et al., 1971; Lushbaugh et al., 1976), the structural details of infected erythrocyte surfaces were not reported. In a TEM study, Mackenstedt et al. (1989) described morphological alterations in infected erythrocytes induced by various parasite species including *P. gallinaceum*. In parasite-infected mammalian erythrocytes they described knobs, invaginations, and caveola-vesicle complexes on the erythrocyte surface, and clefts, microvesicles, and small vesicles in the erythrocyte cytoplasm. However, they did not describe or discuss changes in the surface of *P. gallinaceum*-infected erythrocytes although erythrocyte surface alterations appeared to be present (*c.f.* Fig. 18). They concluded that “*P. gallinaceum* induced only slight alterations in host cells; however, invaginations on the surface of the erythrocyte were not induced.”

In contrast to *P. gallinaceum*, the human malaria parasite *P. falciparum* has been studied extensively using biochemical and biophysical methods including TEM, scanning electron microscopy (SEM) and atomic force microscopy (AFM) (Binnig et al., 1986). The mature stages of *P. falciparum* parasites may induce the formation of knob-like protrusions on the surface of their host erythrocyte (Trager et al., 1966). Knobs play a crucial role in the pathogenesis of malaria by mediating the sequestration of parasitized erythrocytes in the microvessels of the brain and other critical organs such as the kidney, lung, and liver, which may contribute to host death (MacPherson et al., 1985; Aikawa et al., 1990; Sherman et al., 2003). While *P. gallinaceum* is reported to be evolutionarily closely related to *P. falciparum* (Waters et al., 1991; Waters et al., 1993b; Waters et al., 1993a; McCutchan et al., 1996), detailed structural-biological comparisons between these mammalian and avian *Plasmodium* species have not been described. In this report, we present the first study of the surface topography of *P. gallinaceum*-infected erythrocytes using a combination of SEM, AFM and TEM.

2. Materials and Methods

2.1 Parasite cultivation

The 8A strain of *P. gallinaceum* was used for this study. The *P. falciparum* ITO/A4 clone (Roberts et al., 1992) was cultured in complete medium as described previously (Nagao et al., 2000) with the exception that 10% human serum (Type O+) was used in place of AlbuMAX I. Cultures were initiated with a mixture of freshly thawed, cryopreserved parasites and fresh erythrocytes at a 2% hematocrit in T75 culture flasks (Corning, Cambridge, MA) and incubated at 37 °C.

2.2 AFM sample preparation and imaging

P. gallinaceum-infected erythrocytes were diluted to 1×10^7 cells/ml with complete medium, and 200 μ l portions were plated onto 22 mm-diameter coverglasses (Becton Dickinson Labware, Lincoln Park, NJ), previously coated with silane or poly-L-lysine (Sigma, St. Louis, MO). The erythrocytes were allowed to settle and attach to the surface of the coated coverglasses for 30 min at 37 °C, after which the culture medium was carefully removed. The erythrocytes were fixed with osmium tetroxide vapor for 2 min at room temperature, washed with 0.1 M sodium cacodylate buffer (pH 7.4) (Electron Microscopy Sciences, Fort Washington, PA), post-fixed with 4% paraformaldehyde (Electron Microscopy Sciences) in

0.1 M sodium cacodylate buffer for 1 h at room temperature, washed and stored in 0.1 M sodium cacodylate buffer at 4 °C until processed for AFM imaging.

For AFM studies, erythrocytes were further fixed with methanol, incubated with 1 U/μl DNase I, (RNase-free, Roche Molecular Biochemicals, Germany) in 50 mM Tris-HCl buffer (pH 7.5) containing 14 mM NaCl and 10 mM MgCl₂ for 2 h at 37 °C in a moist chamber, and subsequently stained with 2.5 μM YOYO-1 (Molecular Probes, Inc, Eugene, OR) in 0.1 M sodium cacodylate buffer for 1 h at room temperature. The cells were washed with deionized (DI) water, serially dehydrated for 5 min each in ethanol/water mixtures (30–90 %) and two changes of absolute ethanol, hexamethyldisilazane (HMDS) (Electron Microscopy Sciences) for at least 10 min, and then air-dried. In parallel light microscopy (LM) experiments, infected erythrocytes were smeared on 1" x 3" microscope slides, dried, fixed with methanol and stained with Giemsa.

Prior to AFM scanning, bright field (BF) and YOYO-1 fluorescence (FL) images of infected erythrocytes were collected with a Zeiss Plan APOCHROMAT 20x/0.75 objective, a 4 x projector and a chilled CCD video camera (Model C5985, Hamamatsu Photonic Systems, Bridgewater, NJ). All images were passed into a DT3155 video frame grabber (Data Translation, Marlboro, MA), and captured with Image-Pro Plus version 4 or 5.1 (Media Cybernetics, Silver Spring, MD).

We used a Bioscope AFM (Digital Instruments, Santa Barbara, CA) mounted on a Zeiss Axiovert (Carl Zeiss, Thornwood, NY) inverted LM and a Nano-ScopeTM III controller (Digital Instruments) as described previously (Nagao and Dvorak, 1998). A modified Bioscope AFM equipped with an nPoint X-Y stage (nPoint, Inc, Madison, WI) was used to collect some of the images. Silicon probes (Model TESP, Digital Instruments) on single beam cantilevers (125 μm in length and 30–40 μm in width) with a nominal spring constant of 20–80 N/m and tuned to a resonant frequency of 250–320 kHz were used to image the cells. Topographic and amplitude (error signal) images were collected simultaneously at a tapping force (A_{sp}/A_0) of 0.8 (Magonov et al., 1997; Nagao and Dvorak, 1999) and scan rate of 0.5 Hz as 512 x 512 data arrays in the trace direction using tapping mode in air.

2.3 SEM and TEM sample preparation and imaging

Erythrocytes were plated onto 6 mm coverglasses and fixed as described for AFM. For SEM imaging, the cells were washed twice for 15 min each in 0.1M sodium cacodylate buffer, pH 7.2, post-fixed in 1% osmium tetroxide in cacodylate buffer for 1 h, washed once in cacodylate buffer, twice in DI water and dehydrated in a graded series of ethanol (30–100 %). The cells were dried through liquid carbon dioxide in a model CPD-30 critical point dryer (Bal-Tec, Balzer, Liechtenstein), lightly coated with chromium, and visualized in a Hitachi S5200 field emission scanning electron microscope (Hitachi High Technologies America, Pleasanton, CA). Digital images were captured with built-in hardware and PC-SEM software (Hitachi High Technologies America).

For TEM imaging, the cells were washed twice for 30 min each in 0.1M sodium cacodylate buffer, pH 7.2, post-fixed for 2 h in a mixture of 1% osmium tetroxide, 0.8% potassium ferrocyanide and 0.1M sodium cacodylate buffer, pH 7.2, washed once in cacodylate buffer, twice in DI water, and stained with 1% aqueous uranyl acetate for 1 h. The cells were subsequently washed twice in DI water, dehydrated in a graded series of ethanol (30–100 %), and embedded in Spurr resin (Ted Pella Inc, Redding, CA). Thin sections were cut parallel to the layer of cells on the coverglass. The sections were stained in 1% uranyl acetate, 1% lead citrate, and visualized with a Hitachi H7500 transmission electron microscope (Hitachi High Technologies America) operating at 80 kV. Digital images were captured with an Advantage HR camera (Advanced Microscopy Technologies, Danvers, MA).

2.4 LM imaging and data collection

For a detailed comparison between YOYO-1-induced fluorescence and Giemsa-stained *P. gallinaceum*-infected erythrocytes, differential interference contrast (DIC) and YOYO-1 reacted images of erythrocytes were made with a Leica DMI-RE2 inverted microscope (Leica Microsystems, Bannockburn, IL) and a Hamamatsu C5985 video camera (Hamamatsu Photonic Systems, Bridgewater, NJ). Images were collected with a PC containing a DT 3155 capture card (Data Translation, Marlboro, MA) controlled by ImagePro (Media Cybernetics) ver. 4.1. Giemsa-stained erythrocytes were imaged using a Leica DMI-6000 inverted microscope (Leica Microsystems) equipped with a Hamamatsu Orca ER camera (Hamamatsu Photonic Systems, Bridgewater, NJ) in combination with red, green and blue color filters. True color images were produced in ImagePro Plus ver. 5.1.

3. Results

3.1 Surface topography of *P. gallinaceum*-infected avian erythrocytes

While SEM can be used to study the surface topography of avian erythrocytes, it is incapable of elucidating the internal structures that may be responsible for any intererythrocytic differences in surface topography. This problem is exemplified by the SEM images shown in Figure 1. A noninfected avian erythrocyte is shown in Fig. 1a. *P. gallinaceum*-infected erythrocyte is shown in Figs. 1b and 1c. The difference in surface topography of the two cells is obvious, but the reason for the difference can not be determined. In addition, in this example of an infected erythrocyte, neither the number nor developmental stage of the parasite(s) can be determined. We therefore used AFM to study the topography of *P. gallinaceum*-infected erythrocytes.

3.2 Identification of *P. gallinaceum*-infected erythrocytes

In order to correlate the surface topography to the intraerythrocytic parasite stage, we used the nucleic acid staining reagent YOYO-1 to identify infected erythrocytes as described previously (Nagao et al., 2000; Arie et al., 2005). In contrast to *P. falciparum*-infected erythrocytes stained with YOYO-1 (Fig. 2a), however, a problem arises when nucleated avian erythrocytes are infected with *P. gallinaceum* (Fig. 2b). The intense staining of the erythrocyte nucleus makes it difficult to unambiguously determine whether intracellular parasite(s) are present. When the infected erythrocytes are pretreated with DNase I, however, only the remaining parasite RNA stains with YOYO-1. This procedure reveals the presence and identifies the developmental stage of the parasite (Fig 2c) since RNA synthesis ceases during avian erythrocyte maturation (Cameron and Prescott, 1963).

Figure 3 shows a detailed comparison between conventional Giemsa (Figs. 3a–d) and YOYO-1 (Figs. 3e–h) staining of *P. gallinaceum*-infected erythrocytes to identify the developmental stages of the parasite. The combination of DNase I pretreatment and RNA staining with YOYO-1 resulted in the unambiguous identification of the parasite developmental stage, and the number and position of parasites within the avian erythrocytes.

3.3 AFM image analyses of *P. gallinaceum*-infected erythrocytes

Infected erythrocytes at various stages of parasite development were identified based on YOYO-1-induced fluorescence patterns and imaged by AFM. Figure 4a (arrow) shows the characteristic lesions resulting from the recent invasion of an erythrocyte by a merozoite. Such lesions are still visible at the trophozoite stage of parasite development (Figs. 4b and 4c, arrows). Ring stage-infected erythrocytes showed no additional surface structural changes on the erythrocyte surface (not shown). Furrows in the form of long, slender perturbations of the erythrocyte surface begin to appear at the early trophozoite stage of development (Fig. 4b).

The furrows are uniformly distributed, multi-directional, often with two or three furrows overlapping. Once furrows appeared on the erythrocyte surface, there were no additional changes in their features or density at the later stages or the mature gametocyte stage (Figs. 4c–e). In addition, doubly infected erythrocytes show a markedly higher density of furrows (Fig. 4f) although their general features were indistinguishable from those on singly infected erythrocytes.

3.4 Quantitative analyses of furrows

The number of furrows on an infected erythrocyte ranged from 4.3 to 4.7 / μm^2 from the early trophozoite to the schizont stage (Fig. 5a). Doubly infected erythrocytes showed more than a two-fold increase in the density of furrows with a mean of 11.7 / μm^2 . In addition, although mean furrow width (57.3 nm) and height (7.6 nm) did not vary between the different parasite stages, furrow length (mean 428 nm) varied from 225 nm to 750 nm (Fig. 5b). We found that mean furrow dimensions of AFM data analyses differed slightly from those of SEM data analyses (42.6 ± 5.6 (mean $\pm$ S.D.) nm in width and 391 ± 134 nm in length). These discrepancies may be due to the fact that the accuracy of SEM analyses is influenced by the variable geometry of the sample on the goniometry stage relative to the scanning beam. For this reason, and because SEM sample preparation techniques preclude the identification of either the number or developmental stage of intracellular parasites, AFM was favored to quantify the furrows' dimensions.

3.5 Internal structural imaging of *P. gallinaceum*-infected erythrocytes

In contrast to AFM and SEM imaging techniques, TEM can be used to visualize intracellular details. TEM images of noninfected (Fig. 6a) and trophozoite-stage infected erythrocytes (Figs. 6b–d) confirm and extend AFM and SEM images by further characterizing the profound ultrastructural changes that occur in the membrane of *P. gallinaceum*-infected erythrocytes. The noninfected erythrocyte presents a smooth membrane surface (Fig. 6a). In contrast, the furrows seen by AFM and SEM appear in cross section as spike-like projections of the infected erythrocyte membrane (Figs. 6b–d, black arrows). In addition, vesicles in the erythrocyte cytosol were frequently visualized (Fig. 6d, white arrow).

4. Discussion

Recently, AFM has markedly improved our understanding of many biological processes involving changes in cell surfaces. Using AFM in cell biological studies is potentially advantageous in that samples can be imaged fixed or unfixed, in an air or liquid, and in combination with other microscopy techniques. Although erythrocytes are too deformable to be imaged unfixed, YOYO-1 staining and wide-field fluorescence microscopy can be used to unambiguously identify the stage of malaria parasites in fixed human erythrocytes being scanned by AFM (Nagao et al., 2000; Arie et al., 2005). In contrast to human erythrocytes, however, avian erythrocytes are nucleated. The fluorescence intensity of the avian erythrocyte nucleus is so intense in comparison to that of the parasite that it is impossible to reliably determine either the presence or developmental stage of the parasite within the erythrocyte. To unambiguously identify and correlate by AFM the parasite stage to the furrow formation on *P. gallinaceum*-infected erythrocytes, we treated samples with DNase I and stained only the parasite RNA with YOYO-1.

While the surface morphology of *P. falciparum*-infected erythrocytes has been studied extensively by SEM and TEM (Langreth et al., 1978; Gruenberg et al., 1983) as well as AFM (Nagao et al., 2000; Arie et al., 2005), the surface morphology of *P. gallinaceum*-infected avian erythrocytes has not been reported. By using a combination of SEM, TEM and AFM, we found that *P. gallinaceum* infection induced the formation of marked furrow-like structures on the

erythrocyte surface. The width and distribution of *P. gallinaceum*-induced furrows were similar to those of *P. falciparum*-induced knobs. Also, avian erythrocytes multiply-infected with *P. gallinaceum* showed an increase in the density of furrows that was reminiscent of increased knob densities on the surface of human erythrocytes multiply-infected with *P. falciparum* (Nagao et al., 2000).

Our data suggest that *P. gallinaceum* and *P. falciparum* modify the architecture of their host erythrocyte membrane by analogous processes. In a *P. falciparum*-infected erythrocyte, knobs consist of parasite-derived proteins whose synthesis begins at the early ring stage of the parasite's development (Kilejian, 1979). These knob proteins are transported to the erythrocyte surface membrane via Maurer's clefts and other vesicular compartments and incorporated into knobs during the trophozoite and schizont stages (Wickham et al., 2001; Haeggstrom et al., 2004; Knuepfer et al., 2005b; Knuepfer et al., 2005a). The cytosolic vesicle-like structures that we frequently observed in *P. gallinaceum*-infected erythrocytes may be similarly involved in the transport of parasite-derived furrow proteins. Compared to singly-infected erythrocytes, doubly-infected erythrocytes have twice the number of furrows present on their surface. Once furrows appear, however, their density remains constant throughout *P. gallinaceum* development. By contrast, the density of knobs increases as *P. falciparum* matures. These observations indicate that *P. gallinaceum* furrow formation is analogous to *P. falciparum* knob formation, but also suggest that the trafficking and/or placement of furrow components may be completed more rapidly than knob components.

Knob-like protrusions have been observed not only on erythrocytes infected with *P. falciparum*-type malaria parasites (*P. falciparum*, *P. coatneyi* and *P. fragile*) but also on erythrocytes infected with other primate malaria parasites such as *P. malariae*-type (*P. malariae* and *P. brasilianum*) and *P. ovale*-type (*P. ovale*, *P. simiovale* and *P. fieldi*) malaria parasites (Table). By contrast, we found that furrow-like structures were present on *P. gallinaceum*-infected erythrocytes. Based on the nucleotide sequence of small subunit rRNA genes (Waters et al., 1991; Waters et al., 1993b; Waters et al., 1993a) and the cross-species sequence comparison and phylogenetic analysis of thrombospondin-related anonymous protein genes (Templeton and Kaslow, 1997) and circumsporozoite protein genes (McCutchan et al., 1996), it has been proposed that *P. falciparum* is evolutionarily closely related to *P. gallinaceum*. Therefore, we propose that the surface projections may represent an ultrastructural motif that is common to erythrocytes infected with these malaria parasites. The erythrocyte surface modifications induced by different malaria parasites may assume different phenotypic roles, presumably involving interactions between parasite proteins and those of the respective host erythrocyte membrane and cytoskeleton.

Knobs are believed to mediate the adherence of *P. falciparum*-infected erythrocytes to microvascular endothelial cells in the brain and other organs, thereby enabling them to avoid clearance from the bloodstream by the spleen. During their life cycle, a small proportion of *P. falciparum* parasites develop into gametocytes that transmit the infection to *Anopheles* mosquito vectors. Erythrocytes infected with mature gametocytes lack knobs presumably to facilitate their uptake from the circulation by mosquitoes (Smalley et al., 1980; Day et al., 1998). In contrast, we found that erythrocytes infected with *P. gallinaceum* gametocytes also showed furrows on their surface. This finding and our observations of freely circulating asexual blood stages and sexual gametocyte stages of *P. gallinaceum* in chickens suggest that *P. gallinaceum*-infected erythrocytes do not sequester efficiently in the chicken.

It was recently reported that bovine erythrocytes infected with the apicomplexan parasite *Babesia bovis* show 200 nm-long ridge-like protrusions on their surface (Hutchings et al., 2007). A proposed hypothesis is that these structures represent parasite-derived proteins associating and aligning themselves along the length of spectrin molecules. These structures

were shown to mediate the adherence of *B. bovis*-infected erythrocytes to brain capillaries, causing severe pathologic sequelae in cattle (Aikawa et al., 1992). Although these structural alterations in *B. bovis* appear to be analogous to those in *P. gallinaceum* (Table), they are unlikely to mediate similar cytoadherence interactions in *P. gallinaceum*-infected chickens. Further studies will investigate the function of these structures and their potential role in the pathogenesis of *P. gallinaceum* infections.

Acknowledgements

We thank Thomas E. Welles, LMVR, NIAID for helpful discussions. The authors also thank Thomas F. McCutchan and Jianbing Mu, LMVR, NIAID for discussions on malaria parasite phylogeny, Fuyuki Tokumasu and Crina Frincu-Mallos, LMVR, NIAID for constructive comments on the manuscript, and Andre Laughinghouse and Kevin Lee for technical help with *P. gallinaceum*-infected chickens. The authors are grateful to Carol C. Cunnick, NIAID, for her expert technical assistance. This work was supported by the Intramural Research Program of the National Institutes of Health, National Institute of Allergy and Infectious Diseases.

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Fig. 1. Typical SEM images contrasting the surface topography of noninfected (a) and *P. gallinaceum*-infected erythrocytes (b, c). Noninfected erythrocytes have a smooth surface. In contrast, the furrow-like surface structures are seen on infected erythrocytes. Bars in (a) and (b) represent 1 μm , in (c) 200 nm.

Fig. 2.

Composite bright field and epifluorescence microscopy images of (a) *P. falciparum*-infected erythrocytes, (b) *P. gallinaceum*-infected erythrocytes, and (c) *P. gallinaceum*-infected erythrocytes treated with DNase I prior to staining with YOYO-1. Bar in (a) represents 10µm.

Fig. 3.

Representative example of *P. gallinaceum*-infected erythrocytes stained with Giemsa (a–d) or YOYO-1 following DAase I treatment (e–h). A typical early stage of development is shown in (a) and (e). As the parasite develops from the trophozoite stage (b, f) to the schizont stage (c, g), merozoite maturation occurs. Unstained erythrocyte nuclei enable us to unambiguously identify the developmental stage, number and position of the parasites as shown in (e–g). Merozoites are visible in the schizont stage of development. Mature gametocytes are shown in (d) and (h). Bar in (d) represents 5 μ m.

Fig. 4. AFM images of *P. gallinaceum*-infected erythrocytes at various stages of development. The insert shows the same cell imaged by combined LM and YOYO-1 fluorescence following DNase I treatment. The cell surface lesion resulting from merozoite invasion (arrows in (a–c)) persist throughout parasite development. Recently invaded erythrocyte (a); ring stage of the parasite with no apparent furrowing of the erythrocyte surface (not shown); early trophozoite stage showing marked furrow development (b); late trophozoite stage showing both furrows and erythrocyte deformation resulting from parasite growth (c); schizont stage showing furrows similar to those on trophozoite-infected erythrocytes (d); mature gametocyte stage showing both furrows and increased erythrocyte deformation (e); doubly infected erythrocyte showing markedly increased numbers of furrows on the surface (f). The bars in (a) represent 3 μ m for composite LM-fluorescence images and 1 μ m for AFM images.

Fig. 5.

Graphical representation of (a) furrow density on *P. gallinaceum*-infected erythrocytes at various developmental stages as well as on doubly-infected erythrocytes at the trophozoite stage, and (b) height, width and length of furrows on *P. gallinaceum*-infected erythrocytes at the early trophozoite, late trophozoite and gametocyte stages of development. Data in the graph are expressed as the mean $\pm$ S.E.M. Note that the feature of furrows on the surface remains constant even at the gamete stage.

Fig. 6. TEM images of *P. gallinaceum*-infected erythrocytes. Noninfected (a) and trophozoite stage (b–d) of parasite development showing marked modification of the infected erythrocyte surface membrane (arrows in (b–d)). A food vacuole and pigment are also visible. Note that vesicles can be visualized within the infected erythrocyte cytosol (d). Bars represent 500 nm.

Table 1Morphological changes on erythrocytes infected with *Plasmodium* species and *Babesia bovis*.

Parasite	Changes	Size	Cytoadherence	
<i>P. falciparum</i>	Knob-like	83 nm wide and 22 nm	Yes	Nagao et al., 2000 (AFM)
<i>P. coatneyi</i>		high		Rudzinska and Trager, 1968
<i>P. fragile</i>		ND		Fremount and Miller, 1975
<i>P. malariae</i>	Knob-like	ND	No	Smith and Theakston, 1970
<i>P. brasillianum</i>		ND		Cochrane et al., 1988
<i>P. ovale</i>	Knob-like	ND	No	Matsumoto et al., 1986
<i>P. simiovale/P. fieldi</i>				Aikawa et al., 1977
<i>B. bovis</i>	Ridge-like	70 nm wide, 11 nm	Yes	Hutchings et al., 2007 (AFM)
		high, and 200 nm long		
<i>P. gallinaceum</i>	Furrow-like	60 nm wide, 8 nm high, and 430 nm long	ND	In this report (AFM)

ND, not determined.