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ABSTRACT

Background: Malaria in pregnancy is associated with significant morbidity in pregnant women and their offspring. *Plasmodium falciparum* infected erythrocytes (IE) express VAR2CSA that mediates binding to chondroitin sulphate A (CSA) in the placenta. Two VAR2CSA-based vaccines for placental malaria are in clinical development. The purpose of this study was to evaluate the robustness and comparability of binding inhibition assays used in the clinical development of placental malaria vaccines.
Methods: The ability of sera from animals immunised with different VAR2CSA constructs to inhibit IE binding to CSA was investigated in three *in vitro* assays using 96-well plates, petri dishes, capillary flow and an *ex vivo* placental perfusion assay.
Results: The inter-assay variation was not uniform between assays and ranged from above ten-fold in the flow assay to two-fold in the perfusion assay. The intra-assay variation was highest in the petri dish assay. A positive correlation between IE binding avidity and the level of binding after antibody inhibition in the petri dish assay indicate that high avidity IE binding is more difficult to inhibit. The highest binding inhibition sensitivity was found in the 96-well and petri dish assays compared to the flow and perfusion assays where binding inhibition required higher antibody titers.
Conclusions: The inhibitory capacity of antibodies is not easily translated between assays and the high sensitivity of the 96-well and petri dish assays stresses the need for comparing serial dilutions of serum. Furthermore, IE binding avidity must be in the same range when comparing data from different days. There was an overall concordance in the capacity of antibody-mediated inhibition, when comparing the *in vitro* assays with the perfusion assay, which more closely represents *in vivo* conditions. Importantly the ID1-ID2a protein in a liposomal formulation, currently in a phase I trial, effectively induced antibodies that inhibited IE adhesion in placental tissue.

1. Introduction

Infection with *Plasmodium falciparum* in pregnancy is associated with maternal anemia, low birth weight and stillbirths [1,2]. The infected erythrocytes (IE) adhere to chondroitin sulphate A (CSA) in the placenta [3]. The binding is mediated by the *P. falciparum* erythrocyte membrane protein 1 VAR2CSA [4]. After exposure to *P. falciparum* during pregnancy, women develop VAR2CSA specific antibodies protecting them during subsequent pregnancies [5,6]. Substantial evidence suggests that antibodies inhibiting the VAR2CSA-CSA binding convey protection against placental malaria [7,8]. Although opsonising antibodies might contribute to immunity [9], activated monocytes and macrophages might cause pathology [10]. Therefore, the induction of anti-adhesive VAR2CSA IgG is the focus for vaccine design. Two VAR2CSA based vaccines are currently in phase I clinical trials [11,12]. The large full-length VAR2CSA protein may not be feasible as a vaccine, and identification of a construct that elicits protective antibodies has been a

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central goal for vaccine development. The ability of VAR2CSA constructs to elicit anti-adhesive antibodies in animals has been evaluated using different binding inhibition assays. Commonly used binding inhibition assays in vaccine development are: (i) a high-throughput 96-well assay [13], (ii) a petri dish assay [14] and (iii) a flow assay [15]. The 96-well and petri dish assays are static assays where IEs bind to chondroitin sulphate proteoglycan (CSPG) coated onto plastic surfaces, where after non-adherent cells are washed off. The flow assay use channels or chambers coated with CSA [15], CSA-expressing cells such as BeWo [16] or primary trophoblast [17], or placental cryosections [18]. A weakness of *in vitro* models is the difficulty of translating IE binding inhibition to the *in vivo* situation and to which degree they represent correlates of immunity.

We recently described adhesion of *P. falciparum* IEs in a more biologically relevant placental perfusion model, where IE circulate through the intervillous space of a non-fixed intact human placental cotyledon [19].

It has not been established to which degree results from binding inhibition studies can be translated between assays. Laboratories involved in vaccine development have used different assays complicating interpretation and comparison of vaccine candidate performance. Identification of potential surrogate measures of protection could reduce duration of clinical trials to measure the efficacy of a placental malaria vaccine. Therefore, an understanding of performance of the different assays will be important for the further clinical development of VAR2CSA based placental malaria vaccines. The objective of this study was to evaluate the robustness and read-outs of binding inhibition assays used for clinical development of placental malaria vaccines.

2. Materials and methods

2.1. Parasite cultures

Parasite isolates were cultured *in vitro* as previously described [20,21]. FCR3 parasite cultures were selected for binding to BeWo cells (purchased from American Type Culture Collection (http://www.lgcpromochem-atcc.com) [22,23], producing an isolate exclusively expressing VAR2CSA [20]. Cultures were regularly analysed for mycoplasma infection and genetic identity verified by PCR [24,25].

2.2. Protein production

The recombinant full-length VAR2CSA antigen and VAR2CSA constructs were produced as described (Table 1) [26–28]. All proteins were quality controlled by reduced and non-reduced SDS page, ELISA and western blot [27,29].

2.3. Immunizations

Rat (Wistar (inbred), Taconic, Denmark) antisera were produced as previously described [30]. Rabbit (New Zealand White (outbred), HB Lidköping Kaninfarm, Sweden) FV2 antisera were produced by subcutaneous injection and collected 10–14 days after the final boosting injection. The proteins for rat and rabbit immunisations were adjuvanted with Freund's adjuvant (Sigma-Aldrich) or Alhydrogel (Statens Serum Institut, Denmark). Mice ID1-ID2a antisera were produced in adult mice (C57bl/6 (inbred), Taconic, Denmark) by intra-muscular injection. The protein was adjuvanted with a liposomal formulation (Infectious Disease Research Insititute, Seattle, USA) [31]. Groups of 8 mice were immunized and sera were collected 7 days after the final boosting injection and pooled.

An overview of constructs and immunizations is shown in Table 1.

2.4. Binding inhibition assays

2.4.1. 96-well assay

A high-throughput binding assay was performed as described previously [13]. Briefly, 2×10^5 tritium-labelled late-stage IE and immune-sera or control pre-immunization serum from each species at 1:10, 1:100, 1:1000 and 1:2000 in were added in triplicates to wells coated with 2 μg/ml Decorin (D8428; Sigma-Aldrich). Background binding was evaluated in BSA coated wells. After incubation for 90 min at 37 °C, unbound IE were washed off by resuspension. The number of adhering IE was determined by liquid scintillation counting. Three individual experiments were performed.

A binding ratio was obtained by dividing the median number bound IE in each sample by the median number bound IE in wells with IE incubated with pre-immunization sera from the same species and at the same concentration.

The inter-assay variation of FCR3-CSA binding was determined using the mean binding on each day to calculate a coefficient of variation (CV-standard deviation of the day means/mean of the day means).

The proportion of IE binding in a well was estimated after determining the counts per minute/IE in wells with a known number of IE.

2.4.2. Petri dish assay

The assay was performed as described by Saveria et al. [14] with some modifications. 22 spots in a Petri dish (Falcon 351029) were

Table 1
Protein production and immunisations.

Sera	VAR2CSA construct	Expression system	Species	Adjuvant	96 well	Petri dish	Flow	Perfusion
1	FV2[d]	Baculo[c]	Rabbit	Freund's[a]	X	X	X	X
2	ID1-DBL4	Baculo[c]	Rabbit	Freund's[a]	X	X	X	X
3	ID1-ID2a	S2[b]	Rabbit	Freund's[a]	X	X	X	X
4	ID1-ID2a	S2[b]	Rabbit	Alhydrogel	X	X	X	X
5	ID1-ID2a	S2[b]	Mouse	IDRI LS-127	X	X	X	X
6	FV2[d]	Baculo[e]	Rat	Freund's[a]	X	X	Not done	X
7	ID1-ID2a	S2[b]	Rat	Alhydrogel	X	X	Not done	X
8	NTS-ID2a	E. coli	Rat	Alhydrogel	X	X	Not done	X
9	DBL3-4	Baculo[c]	Rat	Freund's[a]	X	X	Not done	X
10	DBL2-3	Baculo[c]	Rat	Freund's[a]	X	Not done	Not done	X

[a] Primary dose in Freund's complete adjuvant (Sigma-Aldrich) followed by booster injections in Freund's incomplete adjuvant.
[b] Expression using the pExpreS2-1 plasmid (Expres2ion) in *Droshophila* Schneider 2 cells (Expres2ion).
[c] Expression using the baculovirus vector pAcGP67-A (BD Bioscience) in High-Five insect cells (BD Biosciences).
[d] FV2: Full-length VAR2CSA.

coated with 20 μL of 2 μg/ml Decorin and incubated at 4 °C in a humidity chamber overnight. Each spot was blocked with 3% BSA at room temperature for 2 h. 5×10^6 IE/ml late-stage IE in PBS with 2% FBS were pre-incubated with immune-sera or control pre-immunization serum from each species at 1:10, 1:100, 1:1000 and 1:2000 dilution for 30 min at room temperature. 20 μL of the IE/serum suspension or IE control was added to each spot and allowed to settle for 21 min at room temperature. Background binding was evaluated on BSA coated spots. Each sample was tested in duplicate in four individual experiments. Unbound IEs were washed off with PBS with 2% FBS on a rotation table and bound IE were fixed with 1.5% Glutaraldehyde. Bound IE were visualized with a Nikon Eclipse TE2000-E and 3 images of each spot were taken using NIS-Elements F at 10× magnification. IE were counted in ImageJ and converted to IE/mm^2.

A binding ratio was obtained by dividing the median number bound IE in each sample by the median number bound IE on control spots with IE incubated with pre-immunization sera from the same species and at the same concentration.

The inter-assay variation of FCR3-CSA binding was determined using the mean binding on each day to calculate a CV.

The proportion of IE binding to a spot was estimated using the formula (IE/mm2∗spot area)/(added IE/spot).

2.4.3. Flow assay

The flow assay was performed mainly as previously described [32]. Channels ($0.04 \times 0.01 \times 2.8$ cm (w × h × l)) on biochips (Vena8Flouro+; Cellix).were coated with 2 μg/ml Decorin in PBS at 4 °C overnight and blocked with 2% BSA in PBS for one hour at room temperature. FCR3 VAR2CSA cultures were adjusted to 4–5% late stage trophozoites and 1% hct (corresponding to 1.1×10^8 - erythrocytes/ml). IE in RPMI were incubated with serum from immunized rodents in concentrations 1:10, 1:100 and 1:1000 for 30 min at room temperature. Pre-immunization serum at 1:10 from each species served as control. The samples were centrifuged at 800*g* for 12 s and the pellet was resuspended in RPMI with 2% serum.

Binding of IE was quantified by allowing IE to flow through channels at 0.5 dyne/cm^2 [33] for five minutes and IE adhering in the channel were counted in five fields at 40× magnification or in 10 fields at 100× magnification. For each test sera, three independent experiments were performed in triplicates.

A binding ratio was determined as the IE binding in the presence of test sera/IE binding in the presence of control sera.

The inter-assay variation of FCR3-CSA binding was determined using the mean IE binding in the channels on each day to calculate a CV.

The proportion of IE binding in the channel was estimated using the formula (IE/mm^2 ∗ channel surface area (mm^2)/(((Time (min) ∗ flow rate (μl/min)) + channel volume (μl)) ∗ IE/μl)). Time was defined as duration of the experiment (5 min) and an average of 2.5 min counting.

2.4.4. Placental perfusion assay

Ex vivo perfusion of a placental cotyledon was performed mainly as previously described [19]. Briefly, the maternal and fetal circulation of a cotyledon was re-established in a placenta donated immediately after delivery. Flushing both circulations with medium washed out maternal and fetal blood. The experiments were performed in 4–5 one-hour phases with IE or IE pre-incubated with immune-serum in the maternal circulation (1% haematocrit) separated by an open-loop wash phase to remove unbound IE and serum.

The proportion of IE in the maternal circulation was determined by flow cytometry as previously described [19,34].

The binding ratio was expressed as the median percentage bound IE in the presence of sera relative to the control phase without sera.

Inter-assay variation was calculated using the per cent bound IE at t = 60 in the control phase of each perfusion and expressed as a CV.

2.5. Statistical analysis

The inter-assay variation in parasite binding inhibition was analysed by Spearman rank correlation.

Graphs and statistical analysis were performed in R [35].

2.6. Ethical considerations

The perfusion study was approved by the ethical review board in Capital Region of Denmark (reference nr H-1-2012-103). Informed written consent was obtained from participating women. The animal study was approved by the Danish Animal Experiments Inspectorate (approval number: 2013-15-2934-00902/BES). Anaesthesia of mice and rats was initiated by inhalation of 5% Isoflurane and maintained by 2–3% Isoflurane before full bleed. Rabbits were anesthetized before full bleed with Ketamine (35 mg/kg) and Xylazine (5 mg/kg) and euthanized with pentobarbital (150 mg/kg). The methods were carried out in accordance with the approved guidelines for human subjects and experimental animals.

3. Results and discussion

The inhibitory capacity of sera was tested in four binding assays that are summarised in Table 2. Three *in vitro* assays test IE binding to decorin coated on plastic surfaces, while the *ex vivo* perfusion assay test binding to placental tissue. The 96-well assay is a standardized high throughput assay where several plates can be run simultaneously. Quantification of bound IE relies on the incorporation of tritium labelled hypoxanthine into the parasite nucleic acids, followed by liquid scintillation counting [13]. This requires equipment and facilities for radioactive material, which may limit the transferability of the assay. Alternatively, colorimetric methods may be used [36]. The petri dish assay requires a minimum of equipment and is easily transferred between laboratories [14]. Dilution series of two sera can be accommodated in one dish, and several dishes can be run simultaneously. Quantification relies on imaging in a microscope, with either manual or automatic counting with appropriate software. The flow assay requires a pump system and associated chips with channels. One sample can be tested in each channel, and dilution series of 2–3 sera can be tested in one day. The perfusion system requires a perfusion chamber, pumps, access to placental tissue and relatively large amounts of reagents. One condition can be tested in each one-hour phase and 4–7 phases conducted in one day.

3.1. Potential bias towards assessment of inhibition of sub-populations of IE

The variation in overall binding of VAR2CSA expressing IE is shown in Fig. 1 and Table 2. The 96-well assay had the least inter-assay variation (CV 8%). Due to the high capacity, experiments were performed on only three occasions, which may limit the variation. The largest inter-assay variation was seen in the flow assay, where the binding efficiency in control measurements differed with a factor of 14 between assays performed on different days. As shown in Fig. 1, the majority of the parasite cultures used in the flow assay resulted in a low and comparable binding, while

Table 2
Assay overview.

Assay	Throughput [a]	Binding substance	Condition for IE binding	Washing method	IE binding quantification	% IE binding in control (median & range)	CV (%)
96-well	High: 6 sera/plate. 10 plates/day	CSPG	Static	Resuspension	Liquid scintillation counting	27 (12–37)	8
Petri dish	Medium: 2 sera/plate. 4 plates/day	CSPG	Static	Flooding, rotation	Imaging in microscope, automatic counting	53 (11–95)	33
Flow	Low: one sera/channel. 2–3 channels/day	CSPG	Flow	Continuous flow	Imaging in microscope, manual counting	8.9 (3.5–48)	102
Placental perfusion	Low: one sera/phase. 4–7 phases/day	Placental tissue	Flow	Continuous flow	Flow cytometry estimation of IE in circulation	75 (53–92)	15

IE: infected erythrocyte, CV: coefficient of variation (standard deviation of the control binding day means/mean of the control binding day means), CSPG: chondroitin sulphate proteoglycan

[a] The number of sera that can be tested/day with the number of concentrations and replicates used in this study.

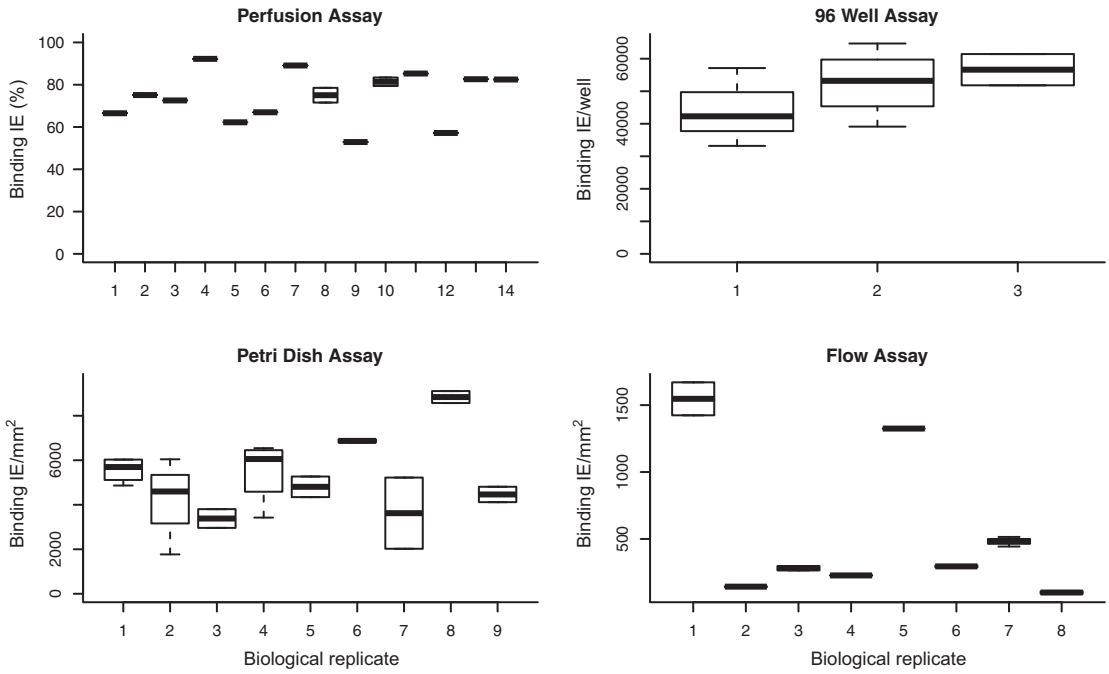

Fig. 1. Comparison of the inter- and intra-assay variation. The figure shows variation in binding of FCR3-CSA to decorin or placental tissue within and between days. The variation between phases/plates/dishes/channels is shown in one boxplot while the day-to-day variation is shown between boxplots. Placental perfusion assay, IE binding (%) after 60 min perfusion (n = 17 control phases performed in 14 perfusions). 96 well assay, mean IE/well binding on each plate (n = 9 plates performed on 3 days). Petri dish assay, mean IE/mm^2 binding in each petri dish (n = 24 petri dishes performed on 9 days). Flow assay, mean IE/mm^2 binding in each channel (n = 15 channels performed on 8 days).

two cultures resulted in markedly higher and comparable parasite binding. All cultures used had been cultured according to standard protocols. This assay is particularly sensitive to temporary obstructions of flow, which may increase over-all binding. However, in the case of obstruction, an experiment was terminated and excluded. Although we did not observe deviations during this study the high variation demonstrates the fragility of this assay. Also the petri dish assay had a relatively large inter-assay variation (CV 33%), as well as a large intra-assay variation (Fig. 1).

The highest proportion of added IE that bound in the assay was seen in the placental perfusion assay where 75% of the perfused IE disappeared from the circulation (Table 2). In the petri dish assay 53% of the added IE adhered to spots and in the 96-well assay 27% of the added IE adhered to the wells. In both the petri dish and 96-well assays, saturation and the wash step intensity may affect the number of bound IE, leading to an underestimation of the proportion of IE that can bind in the assay. The lowest proportion of binding was seen in the flow assay, where 8.9% of added IE adhered in the decorin-coated channel. The strikingly low proportion of binding in this assay is likely influenced by the flow dynamics in the channel and the lack of the three-dimensional villous tree, which architecture may increase parasite binding. However, the relatively low median binding in this assay may also suggest that the assay favours binding of a sub-population of high avidity binding IE.

In this study, the same concentration of the CSPG decorin (2 μg/ml) was used in the *in vitro* assays. In the flow assay, BeWo cells [16], human trophoblast cultures [17] and placental cryosections [18] have been used. While BeWo and human trophoblast cultures may present a more relevant receptor for IE binding, cell cultures lack the three-dimensional architecture of the villous tree, which likely contributes to adhesion.

In the flow assay a shear stress of 0.5 dyne/cm^2 was used. Recent studies suggest that the shear stress on the syncytiotrophoblast range between 0.5 ± 0.2 and 2.3 ± 1.1 dyne/cm^2, and that the binding of IE decrease with increasing shear stress [17]. In the perfusion assay the shear stress on the syncytiotropoblast is not known due to the complexity of the system. However, the flow rate in the maternal circulation resembles that of the placenta and the intervillous flow is mimicked as the perfusate percolates the villous tree [19].

Table 3
Serum concentration required to obtain 50% binding inhibition.

Serum	VAR2CSA construct and adjuvant	96-well assay	Petri dish assay	Flow assay	Perfusion assay[a]
1	FV2, Freund's	1:2000	1:2000	1:10	1:100[b]
5	ID1-ID2a, IDRI LS-127	1:2000	1:1000	1:10	1:100
6	DBL1-6, Freund's	1:2000	1:100	Not done	1:100
2	ID1-DBL4, Freund's	1:100	1:100	No inhibition	No inhibition
3	ID1-ID2a Freund's	1:100	1:100	No inhibition	No inhibition
7	ID1-ID2a, Alhydrogel	1:100	1:100	Not done	No inhibition
8	NTS-ID2a, Alhydrogel	1:100	1:10	Not done	No inhibition
4	ID1-ID2a, Alhydrogel	1:10	No inhibition	No inhibition	No inhibition
9	DBL3-DBL4, Freund's	No inhibition	No inhibition	Not done	No inhibition
10	DBL2-DBL3, Freund's	No inhibition	Not done	Not done	No inhibition

[a] Concentration in total volume perfusion medium (100 ml). Pre-incubation performed in 10 ml medium.
[b] A subset of the FV2 inhibition experiments has previously been published by Pehrson et al. [19].

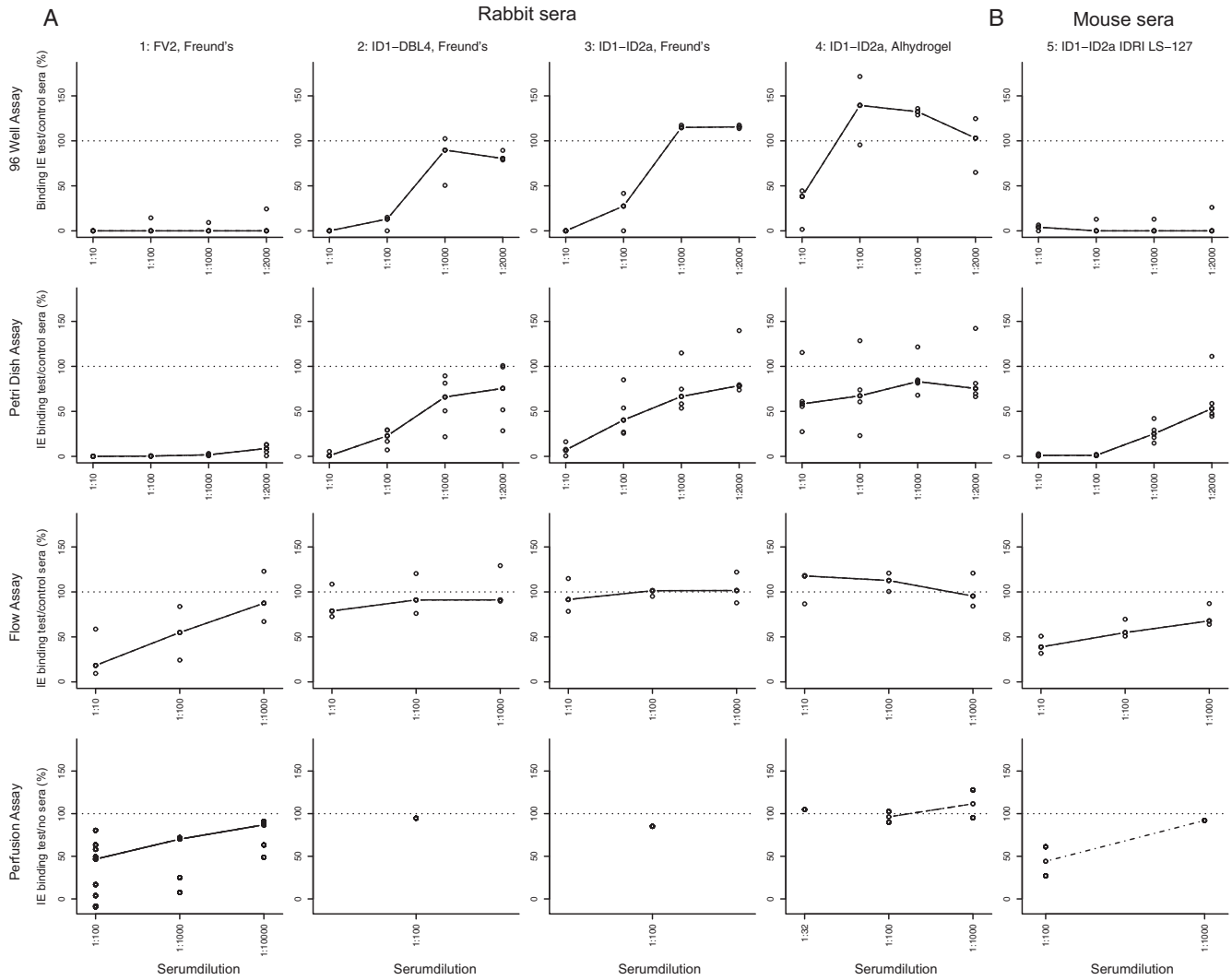

Fig. 2. Comparison of the assay sensitivity in measuring induction of binding inhibitory antibodies Binding inhibition by (A) rabbit sera, (B) mouse sera, and (C) rat sera. The y-axis shows the binding ratio of test sera/control sera. The x-axis shows the serum dilution. Individual experiments are shown as circles and the experiment median is shown as circles connected by a line. The dotted lines represent binding in the control samples incubated with pre-immunization sera. FV2: Full-length VAR2CSA.

3.2. Differential concentration of immune-serum needed for inhibition in various assays

The immune-sera used for comparing binding inhibition in the different assays are shown in Table 3 and the binding inhibitions are shown in Fig. 2A–C. In general, inhibition was achieved with low concentrations of immune-sera in the 96-well and petri dish assays, whereas high immune-sera concentrations were required to obtain inhibition in the flow assay and the placental perfusion assay. Several sera inhibited well at a low dilution, but failed to inhibit parasite binding at higher dilutions, demonstrating the importance to test serum in dilution series in the 96-well and petri dish assay to be able to compare the inhibitory activity of antibodies induced by homologous vaccines [7,18,26,27,37–41].

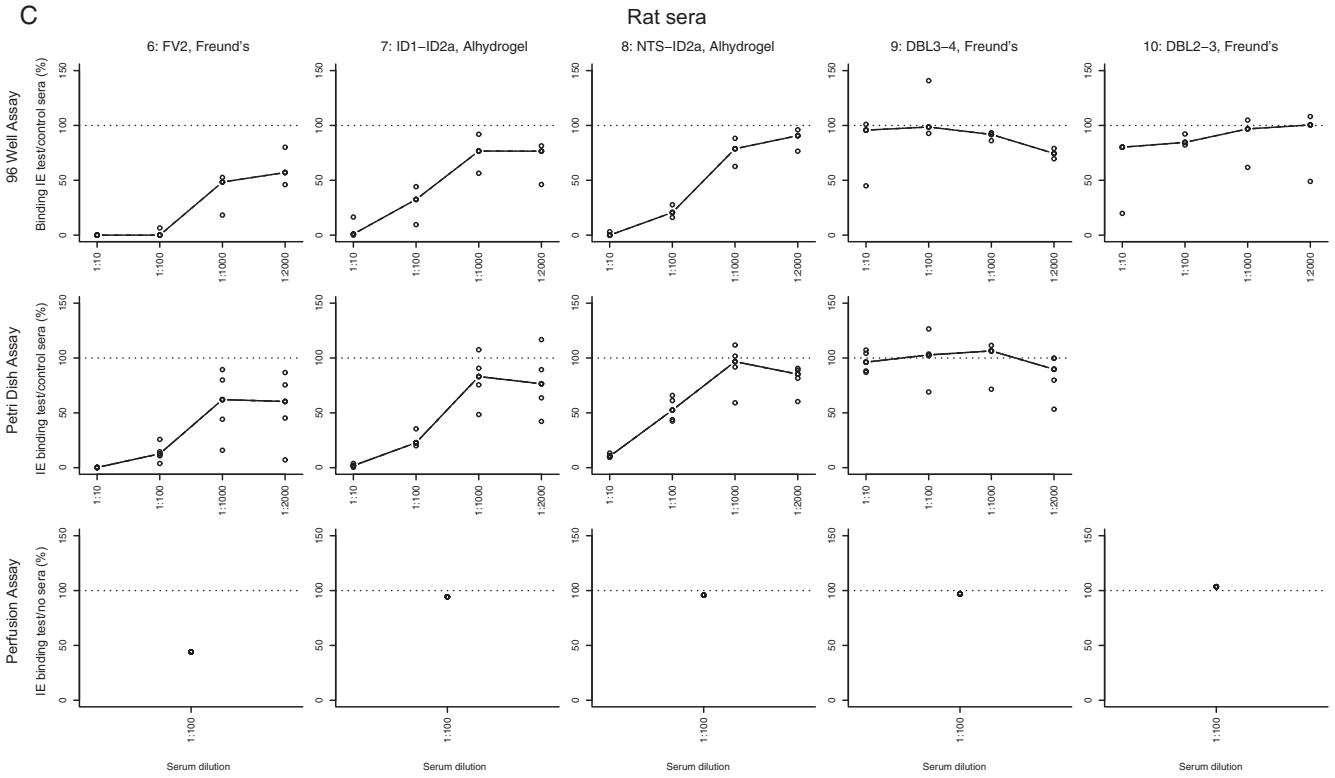

Fig. 2 (*continued*)

Only sera against the full-length VAR2CSA protein (from both rabbit and rat) and the minimal CSA binding region, ID1-ID2a, with a liposomal adjuvant, inhibited parasite binding in the flow and perfusion assays. There are several possible explanations why it would be more difficult to inhibit binding in the perfusion model. It is likely that the IEs have a higher affinity for the oncofetal CSA [42] in the placenta compared to bovine decorin, therefore higher antibody levels or affinity may be needed to block that binding. CSA is abundant in the placenta, and parasites bind both to the syncytiotrophoblast and in the intervillous space in the perfusion model as *in vivo* [19,43]. Therefore, IEs are likely to be surrounded by CSA, enabling a higher avidity binding in placental tissue. In addition, the blood flow through the intervillous space is slow [17], which may favour IE adhesion.

3.3. Does the overall binding level influence the measured binding inhibition level?

There was a positive correlation between IE binding in control measurements and serum binding inhibition at low serum concentrations in the petri dish assay (1:2000: rho = 0.58, $p < 0.0001$, 1:1000: rho = 0.43, $p = 0.005$, Fig. 3). Therefore, when comparing the inhibitory capacity of sera analysed in different petri dishes and on different days, it may be necessary to ensure similar binding between experiments. A similar, but not statistically significant effect was seen in the 96-well assay, however, the effect of IE control binding on measured inhibition was much lower and the assay therefore appears to be more robust to inter-assay variation. In the flow assay, there was a trend towards higher binding inhibition with higher CSA binding, and although only significant at one concentration (1:100: rho = 0.50, $p = 0.030$), it supports the hypothesis that the low number of bound IE observed in the majority of the flow experiments represents a subpopulation of strongly adherent IE. We did not observe a significant correlation between binding and inhibition in the perfusion assay, however the number of data points available is not sufficient to draw any conclusions.

4. Conclusions

Despite the fact that a highly standardised parasite line under selection to express VAR2CSA was used, all the CSA binding assays suffered from considerable inter- and intra-assay variation. This variation was a factor two-three in the perfusion and 96-well assays but considerably higher in the petri dish and flow assays.

The binding inhibition results highlight the importance of running dilution series of immune-serum and assuring comparable parasite binding between experiments. Choice of assay may depend on study objective. It is evident that much higher concentrations of immune-sera are needed to inhibit parasite binding to CSA in the placenta as compared to CSA immobilized on plastic. This is likely due to the fact that in the placenta the overall density of CSA surrounding the IE will be much higher as compared to a one dimensional petri dish or a tube. To differentiate constructs that can elicit inhibitory antibodies in pre-clinical development, assays with higher sensitivity for detecting binding inhibition, such as the 96-well and petri dish assays, may be an advantage. These assays, in particular the 96-well assay, also have a higher capacity. The perfusion model more closely represents *in vivo* conditions and may be more informative regarding the efficacy in humans. However, the model is resource demanding, which limits the number of samples that can be tested and the assay is not sensitive to detect small effects. The overall concordance in determining the inhibitory capacity of individual sera across the *in vitro* and *ex vivo* assays, demonstrates the possibility of using one of these assays in a surrogate endpoint after initial smaller vaccine efficacy trials [44]. Until such data are available, it will not be possible to determine the level of vaccine-specific antibody-mediated inhibition that corresponds to protection *in vivo*, as antibodies acquired

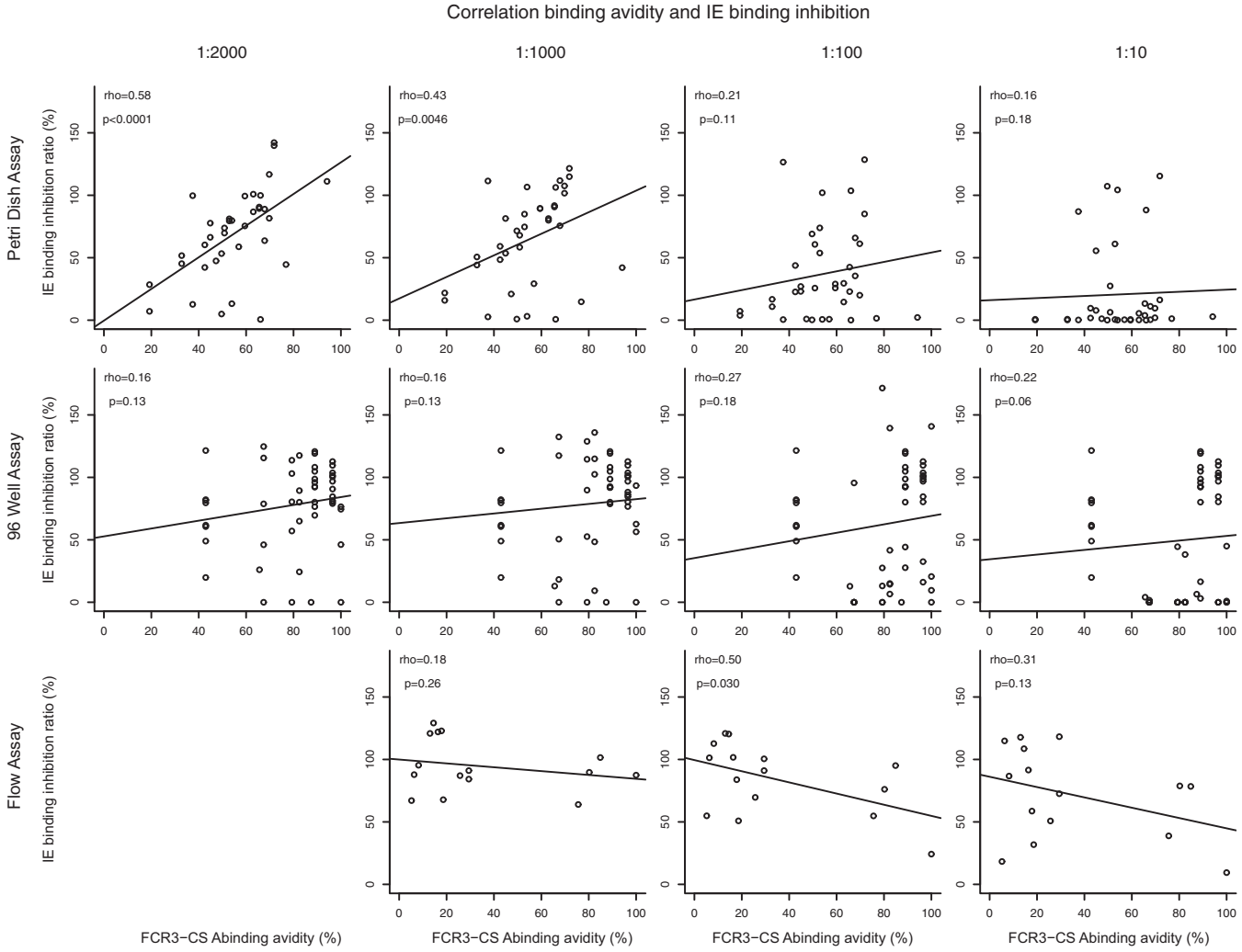

Fig. 3. Correlation between FCR3-CSA IE binding avidity and antibody mediated binding inhibition. The upper panel shows the petri dish assay, the middle panel shows the 96 well assay and the lower panel shows the flow assay. Binding inhibition was tested at three or four different concentrations. The x-axis shows the control IE binding (i.e. without VAR2CSA specific antibodies) in each assay relative to the maximum CSA binding observed. The y-axis shows the binding ratio of test sera/control sera (i.e. binding in the presence of VAR2CSA specific antibodies).

in natural infections are also generated against parts of VAR2CSA that are not included in the vaccine, thus restricting such analysis. However, we hypothesize that the placental perfusion system most likely reflects the capacity of inhibitory antibodies *in vivo*. Thus, if a 50% inhibitory capacity (IC50) at 1:100 serum dilution achieved in the perfusion system corresponds to *in vivo* protection; a surrogate endpoint would be an IC50 of 1:2000 dilution in the 96-well assay and 1:10 dilution in the flow assay based on the results obtained here. The relationship with inhibition in the petri dish assay appears less clear, however, petri dish assays are less complex and relatively easy to transfer between laboratories. In this study there appears to be a consistent relationship between inhibition in the 96-well assay and the perfusion assay, which, in combination with the high throughput of the 96-well assay, may favour its use in the clinical development of a var2csa-based vaccine.

The vaccine formulation containing the ID1-ID2a construct with a potent liposomal adjuvant [31], currently in phase I clinical trials, was inhibitory in all assays. Sera from mice immunised with this formulation effectively inhibited parasite adhesion in perfused placental tissue at a 100-fold dilution. This is an encouraging result, conferring hope that this formulation will also inhibit parasite accumulation *in vivo*.

Conflicts of interest

AS and TGT are owners of European patent WO2004067559 (Compounds useful in the diagnosis and treatment of pregnancy-associated malaria). The authors declare that they have no competing interests.

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Contributors

CP, MAN, LM and YA designed the study. CP, KKH, MS, WAJ MAN, AS, MR and YA performed the experiments, CP, KKH, MAN,

TGT, AS, YA analysed the data. CP drafted the manuscript. All authors revised the manuscript and approved the final version.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2016.12.028.

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