A CAPILLARY-TUBE AGGLUTINATION TEST FOR MALARIA

by

H. F. Kortmann, Jan Leijjveld, J. P. J. Ross and K. F. Löhr

1. INTRODUCTION

A number of serological techniques have been utilized for the assessment of circulating antibodies in subjects with past or present malarial infections, and their application to the study of malaria has been reviewed by Voller & Bruce-Chwatt (1968). Most of the older tests such as precipitation, agglutination and complement fixation have not proved completely satisfactory and, of the newer techniques, the indirect haemagglutination test has yet to be thoroughly established. Immunofluorescence alone has developed as a practicable diagnostic aid. Many of these serodiagnostic methods, however, are of limited value by being too complicated or requiring sophisticated laboratory equipment and skilled technical supervision for efficient operation.

The Capillary-tube Agglutination (CA) Test avoids many of these difficulties and has been successfully employed to detect antibodies of several animal protozoan parasites. Ristic (1962) first described a CA test for the diagnosis of bovine anaplasmosis, using as antigen particulate suspensions of infected blood disrupted by ultrasound. The stability of these antigens which can be easily transported and stored for several months at 4°C, has greatly facilitated the scope of the test. Although capillary-tubes were used by Kreier et al. (1965) in an agglutination test for Plasmodium gallinaceum, his antigen consisted of whole parasites and suffered therefore many of the disadvantages of the older tests. It was Ristic’s basic method of antigen preparation, however, which has been subsequently modified to detect antibodies to Babesia bigemina (Löhr & Ross, 1969), Trypanosoma brucei sub-group and Trypanosoma rhodesiense (Ross, in press), Theileria parva and Theileria mutans (Ross & Löhr, in preparation).

The present report assesses the feasibility of further adapting ultrasonic techniques to establish a CA test for Plasmodium falciparum.

2. MATERIALS AND METHODS

Preparation of antigen. Schizonts and trophozoites infected red blood cells of P. falciparum were harvested in blood from heavily infected human placentae at the Maternity Clinic of the Magila Mission Hospital, situated in an area of North East Tanzania highly endemic for malaria. Extraction was commenced immediately after placental delivery by opening the maternal surface with cuttings about 20 mm deep and gently squeezing and washing the blood into phosphate buffered saline (PBS) at pH 7.2. The suspension was made up to 250 ml, centrifuged at 1000 g for 15 minutes and the supernatant discarded.

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The residue was then resuspended in 250 ml of PBS and the washing process repeated three times. A brownish top layer between 10 and 15 ml comprising 20 to 70 per cent. infected erythrocytes was pipetted from the final residue. Each collection was then stored at -70°C in a pool of material.

When required, aliquots of 50 ml were thawed and disintegrated by ultrasonic oscillation for five minutes (MSE 20 Kcs, 100 W, using 3/8 in (9.5 mm) probe at maximum amplitude). Operating temperatures were kept low by circulating chilled water through the treatment chamber. The resulting suspension was then centrifuged at 108 000 g for 30 minutes, maintaining a temperature of 4°C. The resulting pellet showed two distinct layers. The larger bottom layer was coloured dark brown and mostly composed of haemoglobin pigment. Only the upper reddish brown layer was retained and homogenized in a tissue grinder. The homogenate was then made up to 50 ml with PBS and recentrifuged at 108 000 g. This washing process was repeated until the supernatant was clear and there was no further evidence of a dark brown lower layer. The final pellet was now homogenized in three to five times its own volume of veronal buffered saline at pH 7.2 and centrifuged at 1000 g for 30 seconds. The supernatant which was the antigen was preserved by adding 40 per cent. formaldehyde to a concentration of 0.02 per cent., dispensed into 1 ml vials and stored at 4°C until required.

Disintegration of infected material released into suspension large amounts of haemoglobin pigment granules. Their removal was essential since they agglutinated spontaneously and obscured specific reactions. Separation was difficult however and, although it was possible to remove most of the pigment by differential centrifugation, there was concurrently a considerable loss of antigenic material and a disappointing harvest of effective antigen. For this reason, a satisfactory antigen was produced only from highly infected material in which at least 40 per cent. of the erythrocytes were parasitized. Material with lower parasitaemias produced poor quality antigens which failed to agglutinate many of the positive control sera. From a total of 40 malaria infected placentae, five batches of antigen were prepared of which only two, derived from the most heavily parasitized material, were satisfactory. As observed with bovine CA antigen preparation, the exposure time to ultrasound was highly critical. Under-treatment resulted in antigens which reacted non-specifically, giving false positive reactions while over-treatment progressively destroyed antigenicity (Löhr & Ross, 1969; Ross, 1971). Of the two satisfactory batches of antigens, one was later inadvertently destroyed in this manner. The remaining batch, consisting of 3 ml of antigen, was produced from the pooled extracts of three infected placentae. This was sufficient, however, to perform about 750 tests and adequate for the evaluation described below.

**Test procedure.** The test is carried out in capillary-tubes 90 mm long, having a bore of 0.5 mm. Antigen is drawn into the tube by capillary attraction to a length of about 10 mm and followed by test serum, or its dilution, until full. The sera had previously been inactivated in a water bath at 56°C for 30 minutes.

Mixing is thorough, provided care is taken to ensure that air bubbles do not develop at the interface. The tubes are then supported vertically in plasticine until read. In strong reactions, agglutination commences almost immediately, while moderate ones take an hour or more to appear. Both these reactions can be seen by the naked eye against a dark background with a top light source. Weak, doubtful and negative reactions are best confirmed by viewing by means of a hand lens or a stereo-microscope after 24 hours. If readings are to be delayed, tubes should be sealed with colloid to prevent evaporation.

**Test evaluation.** The CA test was validated by testing the prepared antigens against 55 undiluted negative control sera. These were obtained from normal individuals, 43 of whom had never visited a tropical country and 12 were recent arrivals in Africa from Europe with no intervening history of malaria.

For positive controls, serum samples were obtained from 70 local residents who attended the Magila Mission Hospital and could be presumed, on epidemiological grounds, to be regularly exposed to infections with *Plasmodium falciparum*. From the 70 local residents a group of
28 women had previously participated in a follow-up study. At the moment of examination, 14 of them had received a weekly dose of 300 mg chloroquine base for a period of five to 21 months (average 12 months). The remainder had received a placebo over a similar period. Monthly blood films from each woman were examined for malaria parasites. No parasites were seen in the examinations of the protected women, whereas patent parasitaemias were confirmed, on at least one occasion, in each of the non-protected women.

Two-fold serial dilutions were prepared from these sera and CA tested against the prepared antigens. The results were then compared with those from a parallel series of tests carried out by the Indirect Fluorescent Antibody (IFA) Test (Voller, 1964; Matola et al., 1971), using a homologous antigen.

Immunoglobulin (IgG and IgM) concentrations in the sera of the 28 women known from the follow-up study were also determined with the immunodiffusion technique of Mancini (1965), using commercially available immuno-plates and standards (Hyland Laboratories Inc., Los Angeles (United States of America)).

All sera were also tested for cross-reactions with CA antigens made in a similar way from the bovine parasites Anaplasma marginale and Babesia bigemina. Finally a few standard sera from cows recovering from experimental infections with Anaplasma marginale, Babesia bigemina and Theileria parva were tested with Plasmodium falciparum CA antigen.

Possible relationships between CA titres, IFA titres and immunoglobulin concentrations were then subjected to statistical analysis.

3. RESULTS

Application of the test. The antigen was unaffected when CA was tested against the 55 undiluted negative control sera derived from people with no history of malaria. On the other hand, distinct clear agglutination of the antigen occurred (see photograph) against the 70 positive control sera. Group comparisons of CA and IFA titres of the 70 sera from local residents and of immunoglobulin concentrations of the 28 sera from the follow-up groups are given in Table 1. CA titres are also compared individually with IFA titres in Figure 1, IgG levels in Figure 2 and IgM concentrations in Figure 3.

A not very strict, though highly significant, correlation exists between the titres obtained by the CA and IFA technique (Spearman: \( r = 0.46; \) p two-sided \( \approx 0.0005 \)). Between CA titres and IgG concentrations no significant correlation was present (\( r = 0.30; \) p two-sided \( \approx 0.13 \)). The correlation between CA titres and IgM concentrations, though also not very strict, can be considered as significant (\( r = 0.40; \) p two-sided \( \approx 0.05 \)).

A comparison of the results of the 14 sera of protected women with those of the 14 non-protected women revealed that the protected group had significantly lower CA (Wilcoxon two sample test, p two-sided \( \approx 0.0005 \)) and IFA (p two-sided \( \approx 0.05 \)) titres than the non-protected group. IgM concentrations were also significantly lower (p two-sided \( \approx 0.02 \)) in the protected group, but there was no significant difference in IgG concentrations of both groups.

Human sera which reacted negatively with plasmodial antigen were also non-reacting with CA antigens of Anaplasma marginale and Babesia bigemina. Amongst the 70 sera which reacted positively with plasmodial antigen, there were a few which also gave positive reactions with the CA antigen of A. marginale, but only when undiluted. Of these same 70 sera, 55 gave agglutination with the B. bigemina CA antigen when undiluted, while two sera still agglutinated with this antigen in a dilution of 1:2.5 and one of 1:5.

Some sera of animals recovering from experimental infections with Babesia bigemina, Anaplasma marginale and Theileria parva showed positive reactions with Plasmodium falciparum CA antigens, but only when undiluted.
DISCUSSION

The preparation of efficient CA antigens from blood protozoans requires, ideally, a prolific source of highly infected material from which parasites can be easily and economically separated, preferably in pure suspension. Concentration of schizonts and trophozoites in the placentae of pregnant women infected with *P. falciparum* is a well known phenomenon. As an antigen source, however, placentae have some disadvantages since heavy infections are uncommon and cannot be predicted from examination of the blood smears from the mother. Moreover, there is a considerable variation in the volume of blood which can be extracted from one placenta and also in the number of lymphocytes and macrophages in the extract which have to be removed, usually at the cost of considerable loss of antigenic material. In future CA antigens from other primate malaria parasites such as *Plasmodium knowlesi*, *P. fieldi* and *P. cynomolgi* spp. might be used as alternatives to *P. falciparum* and their cross-reactivity with CA antibodies to human malaria is worth investigation.

The positive reactions obtained with all sera from a hyperendemic malaria area and the negative reactions with all sera from individuals with no history of malaria suggest that the CA test for malaria has a good basic specificity. The correlation between CA titres and the titres obtained with the specific accepted IFA technique supports this suggestion.

The correlation between CA titres and IgM concentrations can also be considered as significant. One serum from a hospitalized patient having an abnormally high IgM concentration of non-specific origin was tested, however, it failed to agglutinate the malaria CA antigen. Hence, further testing of this antigen with other non-malarious sera of increased IgM concentrations is required. Only then could it be assessed whether capillary agglutination in malaria depends solely on increased IgM levels, as was the case in the Melano-floculation Test of Henry (Trensz & Raab, 1965; Voller, 1966) or would actually indicate the presence of antiplasmodial antibodies.

The observed cross-reactions of undiluted human malarial sera with CA antigens from bovine intra-erythrocytic parasites, and vice versa, might be explained by the occasional occurrence of heterophile antibodies between phylogenetically different families.

The CA test for malaria might be of value as an indicator of the efficacy of malaria control campaigns. It may also find useful application experimentally in the laboratory.

<table>
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<th>Titre-class</th>
<th>CA test No. of sera</th>
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<td></td>
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<tr>
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<td>70</td>
<td>Total 28</td>
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TABLE 1. DISTRIBUTION OF TITRES, OBTAINED BY CA AND IFA TECHNIQUES OF 70 SERA FROM INDIVIDUALS FROM A HYPERENDEMIC MALARIA AREA. ALSO OF IgG AND IgM CONCENTRATIONS OF 28 OF THEM.
ACKNOWLEDGEMENTS

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SUMMARY

A Capillary-tube Agglutination (CA) Test is described for the detection and titration of circulating antibodies to human malaria. Antigens consisted of particulate suspensions of Plasmodium falciparum infected blood from human placentae, disintegrated by ultrasound and washed free of soluble fractions and pigment by differential centrifugation. Control sera from individuals with and without histories of malaria were tested. Antigens were not agglutinated by the 55 negative control sera but were agglutinated by all 70 sera from individuals resident in a hyperendemic malaria area, in dilutions ranging from 1:1 to 1:2560. There was a not very strict, though highly significant correlation between these titres and titres obtained by the Indirect Fluorescent Antibody Test for malaria.

A group of 14 women, from the same hyperendemic area, were subjected to weekly chloroquine prophylaxis over an average period of one year, and serologically compared with an untreated but otherwise comparable group. The treated group had significantly lower CA and IFP titres than the untreated group. IgM concentrations were also significantly lower in the treated group, but there was no significant difference in the IgG concentrations of both groups. It was also shown that the CA serum titres of these 28 treated and untreated women were significantly correlated to their IFP titres and IgM concentrations but not to their IgG levels.

RESUME

Les auteurs décrivent un test d'agglutination en tube capillaire permettant de détecter et de titrer les anticorps antipaludéens circulants. On a obtenu les antigènes à Plasmodium falciparum à partir du sang de placentes de parturientes provenant d'une région à forte endémicité palustre du nord-est de la Tanzanie. Les globules rouges parasités par des schizontes ou des trophozoïtes ont été désintégrés au moyen d'ultrasons, après plusieurs lavages et élimination de la fraction soluble et des pigments par centrifugation différentielle.

On recueille d'abord le sang et on le lave dans une solution de phosphate à pH 7,2. On en prépare une suspension de 250 ml que l'on centrifuge à 1000 g pendant 15 minutes. On rejette le liquide et conserve le culot sur lequel on répète trois fois l'opération de lavage au tampon de phosphate, et l'on pipette enfin 10 à 15 ml du résidu final lequel contient entre 20 et 70 % des globules rouges infectés. On conserve au réfrigérateur à -70°C. En fonction des besoins, on retire par échantillons de 50 ml qui sont portés au gel et désintégrés aux ultrasons pendant 5 minutes (MSE 20 Kcs., 100 W) en opérant à la température de l'eau glaçée. La suspension est ensuite centrifugée 30 minutes à 108 000 g à 4°C. La couche inférieure du culot, contenant surtout l'hématozoine, est abandonnée. On récupère la couche supérieure qui est rendue homogène par passage dans un moulin à tissus et qui est soumise à
plusieurs lavages; on porte son volume à 50 ml avec du tampon au phosphate puis l'on centrifuge à 108 000 g. On répète l'opération jusqu'à ce que le liquide surnageant soit clair et qu'il n'y ait plus de dépôt brunâtre. Le culot final est mis en suspension homogène dans 3 à 5 fois son volume de véronal sodium à pH 7,2 et centrifugé à 1000 g pendant 30 secondes. On retire la couche supérieure qui contient l'antigène et qui est préservée par addition de 40 % de son volume d'aldéhyde jusqu'à concentration de 0,02 % puis on la met en ampoules de 1 ml et on la conserve à 4°C.

Le test est effectué dans des tubes capillaires de 90 mm de long sur 0,5 mm de diamètre intérieur. On aspire l'antigène sur environ 10 ml de longueur dans le tube capillaire et l'on complète la longueur du tube capillaire avec le sérum à titrer ou le sérum dilué. Les sérum auront été inactivés au préalable par mise en bain-marie à 56°C pendant 20 minutes. On agite soigneusement en évitant toute bulle d'air à l'interface et l'on dispose les tubes verticalement pour la lecture. Quand la réaction est fortement positive, l'agglutination commence presque immédiatement; quand elle est modérée, elle peut prendre une heure ou davantage. Les réactions sont visibles à l'œil nu sur fond noir, sous lumière apicale.

Des sérum témoins provenant de sujets ayant ou non souffert de paludisme ont également été testés. Cinquante-cinq sérum témoins négatifs n'ont pas provoqué l'agglutination des antigènes alors que les sérum de 70 sujets résidant dans une zone d'hyperendémie palustre ont provoqué l'agglutination à des dilutions allant de 1:1 à 1:2560. On n'a pas observé de corrélation étroite entre ces titres et les titres obtenus par la technique d'immunofluorescence indirecte; toutefois leurs valeurs sont hautement significatives.

Un groupe de 14 femmes, provenant de la même zone hyperendémique, a été traité chaque semaine à la chloroquine à titre prophylactique pendant environ une année. Les titres obtenus avec le test d'agglutination en tube capillaire et avec la technique d'immunofluorescence ont été sensiblement inférieurs aux titres correspondants d'un groupe semblable mais non traité. Les concentrations en IgM ont été également très inférieures dans le groupe traité, mais on n'a observé aucune différence significative de la concentration en IgG entre les deux groupes. On a également démontré que les titres de sérum obtenus avec le test d'agglutination en tube capillaire de ces 28 femmes respectivement traitées et non traitées sont dans un rapport statistiquement significatif avec les titres fournis par la technique d'immunofluorescence et avec les concentrations en IgM mais non avec les concentrations en IgG.
REFERENCES


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Voller, A. (1964) Bull. Wld Hlth Org., 30, 343


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Capillaries 1-3 are positive; 4-6 are negative. In the negative tubes a varying concentration of antigen is suspended. Some sedimentation can be seen, but no agglutination. The amount of antigen also varies in the positive tubes. In tube 1, excess of antigen remained suspended while in tube 3 the same serum agglutinated practically all the antigen.
FIG. 1. CORRELATION BETWEEN CA TITRES AND IPA TITRES OF 70 SERA FROM INDIVIDUALS LIVING IN A HYPERENDMIC MALARIA AREA.
FIG. 2. CORRELATION BETWEEN CA TITRES AND IgG AND IgM CONCENTRATIONS OF SERA FROM 28 WOMEN PARTICIPATING IN A FOLLOW-UP STUDY. OF THESE, 14 HAD RECEIVED MALARIA PROPHYLAXIS FOR AN AVERAGE PERIOD OF ONE YEAR. THE 14 OTHERS REMAINED NON-PROTECTED, AND REVEALED MALARIA PARASITAEMIAS DURING THAT YEAR ON AT LEAST ONE OCCASION.
FIG. 3. CORRELATION BETWEEN CA TITRES AND IgG AND IgM CONCENTRATIONS OF SERA FROM 28 WOMEN PARTICIPATING IN A FOLLOW-UP STUDY. OF THESE, 14 HAD RECEIVED MALARIA PROPHYLAXIS FOR AN AVERAGE PERIOD OF ONE YEAR. THE 14 OTHERS REMAINED NON-PROTECTED, AND REVEALED MALARIA PARASITAEMIAS DURING THAT YEAR ON AT LEAST ONE OCCASION.