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TRABAJO DE INVESTIGACION

HEMOLYTIC ASPECTS OF STRAINS AND CLONES OF *Trichomonas vaginalis*

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ABSTRACT

*The in vitro hemolytic activity of live strains and clones of trophozoites of **Trichomonas vaginalis** was investigated. The isolates and clones were tested against human erythrocytes of groups A, B, AB, and O, and against erythrocytes of six adult animals of different species (rabbit, rat, chicken, horse, bovine, and sheep). Results showed that each of the isolates and clones tested lysed all human blood groups, as well as rabbit, rat, chicken, horse, bovine and sheep erythrocytes. No hemolysin released by the parasites could be identified. Hemolysis did not occur with trichomonads culture supernatants, and either with sonicated extracts of **T. vaginalis**, and nor with previously killed organisms.*

***Key-words:* Trichomonas vaginalis**, *hemolysis, hemolytic activity, erythrocytes isolates, clones.*

INTRODUCTION

Trichomonas vaginalis is a trichomonadid protozoan living in the human urogenital vaginal tract. The flagellated protozoan parasite is a common cause of the infection of the female tract. Trichomonosis is recognized as a major sexually transmitted disease, and its clinical presentation ranges from a totally asymptomatic infection to a severe vaginitis. The clinical features of trichomonosis in women are therefore limited to the urogenital tract.[1] In men infection is mostly asymptomatic, but in some cases it can lead to a mild urethritis which usually resolves spontaneously within 2 weeks.[2] Using an *in vitro* method, the

hemolytic activity of different species of Trichomonadida, such as *T. vaginalis,*[3-10] *T. gallinae,*[11] *T. foetus,*[5, 6, 12] and *T. suis*[5] has been studied. In this study we provide evidence that no hemolysin was released by isolates and clones of *T. vaginalis,* and hemolysis did not occur with trichomonads cultures supernatants, as well with sonicated extracts of parasite.

MATERIALS AND METHODS

Organisms: The *T. vaginalis* strains (VG, GB, Boa, Pc, Y, Na and Ba) used in this in this study were isolates from women with symptomatic vaginitis attending the Venereal Disease Department of the Charles Nicolle Hospital (Rouen, France), and the others (strains Jt and Jd) were obtained from Dr. Fernando Costa e Silva Filho (Universidade Federal do Rio de Janeiro, Brazil). *T. vaginalis* strains 30236 (sensitive to metronida-zole) and 30238 (metronidazole resistant) from the American Type Culture Collection (ATCC) were kindly provided by Dr. Marlene Benchimol (CBB-UENF, Campos, RJ, Brazil). All cells were cultured axenically *in vitro* in trypticase-yeast extract-maltose, Diamond medium[13] (TYM), without agar, pH 6.0, supplemented with 10% heat inactivated cold horse serum, in air, at 37° C. Isolates were subcultured every 48 h in TYM medium. The strains were kept alive in liquid nitrogen (-196° C) with 5% of dimethyl sulfoxide (DMSO). The trichomonads in the logarithmic phase of growth and subcultured every 48 h exhibited more than 95% mobility and normal morphology. The protozoa were counted with a hemocytometer and adjusted to a concentration of 1 x 10^6 living organisms per ml in TYM medium.

Isolation of clones: The solid medium for the isolation of clones (RC1, RC2, SC1, SC2, SC4, SC5, SC7) of *T. vaginalis* was a modification of Diamond TYM medium. Agar culture medium was prepared with 20 ml of the TYM warm, serum-free medium, containing 1.6% (w/v) of agar (Difco) and poured into a sterile 100 mm plastic petri dish. Each plate was then placed into airtight box containing a 5 g piece of solid CO_2 and left for at least 15 min to harden and absorb the CO_2. For the overlay 10 ml of the TYM containing 0.8% (w/v) of agar was melt and hold in a water bath at 40°C. It was quickly added 1 ml heat inactivated horse serum. The medium was cooled to 37°C, inoculate with 0.5 ml of *T. vaginalis* suspension (adjusted at 1 x 10^4 trophozoites per ml), and immediately poured over the base layer. The dishes were set in the CO_2.chamber to harden and absorb gas. As soon as the plates were hardened, they were transferred to a Anaerobac anaerobic jar (Probac) in which a 2 to 3 g piece of solid CO_2 had been allowed to evaporate. Three hydrogen-generating envelopes (Anaerobac) were placed inside the jar. The jar was then quickly sealed and incubated at 37°C for 3 to 5 days[14]. The clones were stored in liquid nitrogen (-196°C) with 5% of dimethyl sulfoxide (DMSO).

Cell lysates: Parasites lysates were obtained by sonication. Cells harvested in late exponential phase were washed three times in PBS pH 7.2 by centrifugation at 1.800 rpm. Washed cells were resuspended at a density of 1 x 10^6 per ml in PBS pH 7.2, then subjected to five cycles of sonication for 10 seg at 50 watts in ice bath. Samples were then centrifuged and supernatants filtred through a 0.22 mm filter membrane (Milipore) in order to perform the hemolysis assays.[2, 6]

Erythrocytes: Fresh human blood was obtained at the City Emergency Hospital (HPS) blood center, and also from volunteer donors. The blood was taken in an equal volume of Alsever's solution (dextrose 20.5 g, sodium citrate 8 g, citric acid 0.55 g, sodium chloride 4.2 g, distilled water to 1 liter), and from six different adults animals species:(rabbit, rat, chicken, horse, bovine, and sheep). All the erythrocytes were harvested, and washed three times by centrifugation (250 x g for 10 min) in equal volume of TYM medium. The supernatant was

discarded. Each experiment was perform using fresh erythrocytes from all human blood groups and adults animals. Whole human blood samples were previously examined, and determined to be hepatitis B antigen (HBsAg) negative, and human immunodeficient virus (HIV-antibody) negative. The erythrocytes were stored at 4° C.

Hemolysis assay: The trophozoites were harvested from a 24 h culture (viability >95%) in TYM medium, in air, at 37° C and washed three times in TYM medium by centrifugation (750 x g for 20 min). A volume of 50 ml of washed fresh undiluted erythrocytes was mixed with 2.5 ml of Hank`s balanced salt solution (HBSS) containing a total of 1 x 10^6 trophozoites of *T. vaginalis*[9] originated from a 24 h culture in TYM medium. After 18 h of incubation in air, at 37° C, without shaking, the mixture was centrifugated (250 x g for 10 min). Absorbance of the supernatants, and controls were measured at 540 nm with spectrometer,[4] and was compared with a standard curve obtained by osmotic lysis of the erythrocytes of each species. Control tubes were included in all assays and the spontaneous hemolysis was also controlled. The results were expressed as percentage of total hemolysis (100%). The mean and the standard error of the hemolytic activity of trichomonads with the different erythrocytes were calculated after performing the assay at least 12 times, and each sample was done in triplicate.

Statistical analysis*:* it was performed using the Student's t-test.

RESULTS

Results show that each of eleven isolates (Table 1 and 2), and six clones (Table 3 and 4) were capable of lysing all human blood groups A, B, AB, and O, as well as rabbit, rat, chicken, horse, bovine and sheep erythrocytes. The degree of hemolysis varied greatly from one isolate or clone to another, and varies according to donors origin of erythrocytes. Hemolytic activity was maintained after a serial transfer in axenic culture for six months. We found that *T. vaginalis* isolates tested had a hemolytic activity ranging between 49% and 94% with human erythrocytes (Table 1), and ranging between 41% and 56% with animal erythrocytes (Table 2). Experiments with *T. vaginalis* clones presented a hemolysis from 18% to 95% with human erythrocytes (Table 3), and from 32% to 93% with animal erythrocytes (Table 4). *T. vaginalis* isolated at the end of hemolysis assays were alive and were successfully cultured in TYM medium. The supernatants of hemolysis assays, tested in the presence of different species of erythrocytes did not induce any hemolytic activity. No hemolysin released by the parasites could be identified. Hemolysis did not occur with trichomo-nads culture supernatants from 18, 24 and 48 h kept at 37°C. Hemolytic activity was not observed with the hemolysis supernatant from 18 h, and neither with sonicated extracts of trichomonads, and nor with previously killed organisms. No enterotoxin was ever evident, and no hemolytic activity was observed with culture supernatants (details not shown).

Table 1. Hemolytic activity of eleven isolates of *Trichomonas vaginalis* on human erythrocytes groups

Isolates	No. of assays	Percentages of hemolysis* Human erythrocytes groups A	B	AB	O
VG	12	68 ± 0.2	52 ± 0.5	60 ± 0.6	67 ± 0.3
GB	12	92 ± 0.2	75 ± 0.2	76 ± 0.2	96 ± 0.2
Boa	12	76 ± 0.3	57 ± 0.3	76 ± 0.2	66 ± 0.7
Pc	12	70 ± 1.0	52 ± 1.1	65 ± 1.0	66 ± 0.6
Na	12	64 ± 0.4	49 ± 0.3	55 ± 0.3	79 ± 0.4
Y	12	89 ± 0.4	68 ± 0.3	58 ± 0.3	90 ± 0.4
Ba	12	70 ± 0.3	66 ± 0.3	67 ± 0.3	71 ± 0.5
Jt	12	88 ± 4.2	82 ± 4.2	88 ± 1.6	82 ± 2.6
Jd	12	88 ± 0.3	83 ± 0.4	88 ± 0.4	84 ± 0.3
30236 30238	12	75 ± 0.4	91 ± 0.2	53 ± 0.3	87 ± 0.3
	12	88 ± 2.9	95 ± 2.2	91 ± 3.0	94 ± 2.6

*Mean values ± one standard error of triplicate samples.

Table 2. Hemolytic activity of four isolates of *Trichomonas vaginalis* on six adult animals erythrocytes

Isolates	No. of assays	Percentages of hemolysis* Animal erythrocytes rabbit	rat	chicken	horse	bovine	sheep
Jt	12	88 ± 3.0	84 ± 2.3	85 ± 2.7	83 ± 4.2	69 ± 4.1	93 ± 1.2
Jd	12	88 ± 0.3	88 ± 0.3	70 ± 0.4	69 ± 1.0	89 ± 0.5	94 ± 0.3
30236	12	73 ± 0.5	67 ± 0.3	96 ± 0.2	83 ± 0.5	68 ± 0.4	66 ± 0.4
30238	12	77 ± 0.2	54 ± 0.2	68 ± 0.3	84 ± 0.2	59 ± 0.4	41 ± 0.2

*Mean values ± one standard error of triplicate samples.

Table 3. Hemolytic activity of seven clones of *Trichomonas vaginalis* on human erythrocytes

Clones	No. of assays	Percentages of hemolysis* Animal erythrocytes A	B	AB	O
RC1	12	73 ± 0.2	56 ± 0.2	59 ± 0.1	79 ± 0.2
RC2	12	65 ± 0.2	59 ± 0.3	56 ± 0.2	72 ± 0.3
SC1	12	82 ± 1.7	91 ± 5.3	74 ± 1.4	74 ± 6.3
SC2	12	95 ± 2.1	92 ± 0.4	84 ± 2.1	53 ± 2.7
SC4	12	77 ± 3.1	92 ± 1.4	48 ± 1.3	64 ± 2.3
SC5	12	84 ± 5.5	86 ± 1.8	58 ± 0.7	70 ± 4.3
SC7	12	56 ± 2.3	18 ± 0.8	20 ± 3.7	18 ± 1.4

*Mean values ± one standard error of triplicate samples.

Table 4. Hemolytic activity of six clones of *Trichomonas vaginalis* on six adult animals erythrocytes

Percentages of hemolysis* Animal erythrocytes

Isolates	No. of assays	rabbit	rat	chicken	horse	bovine	sheep
SC1 SC2	12	83 ± 3.6	86 ± 1.0	60 ± 0.9	74 ± 1.0	19 ± 1.6	38 ± 0.3
SC3 SC4	12	91 ± 1.5	67 ± 2.0	60 ± 3.0	55 ± 0.7	91 ± 1.7	44 ± 1.6
SC5 SC7	12	56 ± 2.2	61 ± 0.4	63 ± 0.7	91 ± 1.8	64 ± 0.6	43 ± 0.4
	12	53 ± 2.2	56 ± 0.0	52 ± 1.5	84 ± 0.0	67 ± 7.8	57 ± 1.0
	12	93 ± 2.8	67 ± 1.5	81 ± 5.7	72 ± 2.7	51 ± 7.1	32 ± 5.6
	12	68 ± 3.4	58 ± 0.0	58 ± 2.2	89 ± 0.0	51 ± 2.2	42 ± 0.9

*Mean values ± one standard error of triplicate samples.

DISCUSSION

The similarities of hemolytic activity observed in the results of the hemolysis assays performed with different isolates and clones of *T. vaginalis*, suggest that no changes in the hemolytic capacity had occurred. In this study we provide evidence that no hemolysin was released by the isolates and clones of parasite, and hemolysis did not occur with trichomonads cultures supernatants, as well with sonicated extracts of *T. vaginalis.*

A hemolytic activity has been demonstrated in several protozoan parasites such as *Trypanosoma congolense,*[15] and *T.brucei,*[16] *E. histolytica,*[17] *T. vaginalis,*[3-10] *T. gallinae,*[11] *T. foetus,*[5, 6, 12] and *T. suis.*[5] For *T. vaginalis*, Fiori *et al.*[2] showed that hemolysis was a contact and temperature dependent phenomenon and hypothetized that cytopathic effects could be related to pore-forming in the membrane of red blood cells. However, the contact of *T. vaginalis* and red blood cells is not a prerequisite for hemolysis which could also be due to a pH dependent lytic protein of more than 30 kDa released by the parasite under triggerig conditions.[18] López-Revilla and Said-Fernández[17] reported, that hemolysis depends on the suscep-tibility of red cell membranes to destabilization. Nevertheless, differences in suscebility to a given hemolysin exist in different individuals of the same animal species.[19]

Probably many mechanisms determine the pathogenic potential and hemolytic activity of the trichomonads trophozoites. It is essential that modern molecular characterization studies be conducted in conjunction with biological studies to determine the significance of hemolytic activity of *T. vaginalis*.

In our experiments, a hemolytic activity of isolates and clones of *T. vaginalis* have been clearly demonstrated. However, a relationship between hemolysis and cell pathogenicity is not clearly established, and probably the hemolytic activity does not follow the same mechanism in this parasite.

RESUMEN

Se investigó la actividad hemolítica de 11 cepas y 6 clones vivos de *Trichomonas vaginalis.* Las cepas y los clones fueron subsecuentemente estudiados frente a los eritrocitos de 7 especies animales adultas. Cada una das 11 cepas y 6 clones lisó los eritrocitos de todos los grupos sangüineos humanos, lo mismo que los de conejo, rata, pollo, caballo, bovino y ovino. No se detectó la liberación de hemolisina por el parásito (cepas y clones). Los resultados sugieren que la acividad hemolítica no es debida a la liberación de hemolisina por la *T. vaginalis* o por un producto de su metabolismo.

RESUMO

A atividade hemolítica de 11 cepas e 6 clones de *T. vaginalis* foi estudado *in vitro*. As cepas e os clones foram incubados com eritrócitos humanos do sistema ABO e com eritrócitos de seis animais adultos (coelho, rato, galinha, cavalo, bovino e ovino). Os resultados mostraram que todas as cepas e os clones de *T. vaginalis* hemolisaram as hemácias humanas e dos animais. Nenhuma hemolisina libertada pelo parasito pôde ser identificada. Os dados obtidos sugerem que a atividade hemolítica não é devida a libertação de uma hemolisina pelo *T. vaginalis* ou por um produto de seu metabolismo. A hemólise das hemácias não ocorreu com o sobrenadante das culturas do tricomonas, com também com os extratos sonicados e com os organismos previamente mortos.

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