UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
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Preservação da viabilidade de *Trichomonas vaginalis* em urina visando o diagnóstico laboratorial pelo exame direto a fresco

Mariana da Silva Dias

Porto Alegre, dezembro de 2015.

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Orientadora: Profa. Dr. Tiana Tasca
Coorientadora: Doutoranda Camila Braz Menezes

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"Desaprender para aprender. Deletar para escrever em cima. Houve um tempo em que eu pensava que, para isso, seria preciso nascer de novo, mas hoje sei que dá pra renascer várias vezes nesta mesma vida. Basta desaprender o receio de mudar"

Autora: Martha Medeiros

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A minha família, onde recarrego minhas energias.

Aos meus amigos, pelos bons momentos vividos.

E a todos que contribuíram direta e indiretamente para realização deste sonho.

Resumo

O método mais empregado no diagnóstico da tricomoníase é o exame direto a fresco de secreções vaginais e uretrais. Este método apresenta limitações importantes, visto que deve ser realizado imediatamente após a coleta pois é baseado na observação microscópica da morfologia do parasito. Considerando que essa técnica é a mais utilizada na rotina laboratorial, este estudo comparou diferentes soluções preservadoras amplamente utilizadas em laboratórios clínicos em diferentes temperaturas de armazenamento com o objetivo de melhorar a viabilidade dos trofozoítos para o diagnóstico de *T. vaginalis* em urina. Foi realizado um *screening* com seis soluções (salina glicosada pH 5,0 e pH 6,0; PBS pH 5,0 e pH 6,0; PBS + glicose pH 5,0 e pH 6,0), e a partir de análise estatística a solução salina glicosada pH 6,0 foi selecionada para realização dos testes em urina. A solução que melhor preservou os trofozoítos foi a salina glicosada pH 6,0 que manteve 61% de viabilidade em 6 horas de experimento. Esses dados demonstram a importância da correta preservação da amostra de urina para a realização do diagnóstico acurado da tricomoníase e sugerem a ampla utilização da solução salina glicosada pH 6,0 na rotina laboratorial clínica.

Palavras-chave: *Trichomonas vaginalis*, urina, solução preservadora, viabilidade, exame qualitativo de urina.

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Preservation of *Trichomonas vaginalis* viability in urine for laboratorial diagnosis by the wet mount examination

Mariana da Silva Dias, Camila Braz Menezes, Tiana Tasca*

Laboratório de Pesquisa em Parasitologia, Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Avenida Ipiranga, 2752, 90610-000, Porto Alegre, RS, Brazil.

*Corresponding author. Email: tiana.tasca@ufrgs.br

Abstract

The most common method used in diagnosis of trichomoniasis is the wet mount examination of vaginal and urethral secretions. This method has important limitations. Considering that it is the most widely used technique in the laboratorial routine and aiming to improve the sensitivity of the technique for diagnosis of *T. vaginalis* in urine, this study compared different preserving solutions commonly used in clinical laboratories at different storage temperatures. A screening was conducted with six solutions (glucose saline pH 5.0 and 6.0, PBS pH 5.0 and 6.0, PBS + glucose pH 5.0 and 6.0) and based on statistical analysis the best solution was selected to perform the tests in urine. The solution with better preservervation of the trophozoites, glucose saline pH 6.0 solution presented 61% of viability within 6 hours of experiment. These data demonstrate the importance of urine samples preservation for the accurate trichomoniasis diagnosis and suggest the use of glucose saline pH 6.0 solution in the clinical laboratorial routine.

Keywords: *Trichomonas vaginalis*, urine, preserving solution, viability, urine qualitative exam.

Introduction

Trichomonas vaginalis is a flagellated protozoan that causes trichomoniasis, the most common non-viral sexually transmitted disease (STD) worldwide [1] with an incidence of 276 million cases per year [2]. The impact of trichomoniasis on human health is significant since the infection has been associated with pregnancy complications [3], infertility [4], cervical and prostate[5] cancers [6] and most relevant is the association between the infection and increased HIV transmission rates [7]. Nitroimidazoles drug family, mainly represented by metronidazole, has been used for trichomoniasis treatment [8]. Although rare, this drug can cause hypersensitivity when used in large doses or for long periods and it is increasing the number of *T. vaginalis* clinical isolates which exhibit metronidazole resistance [9]. Considering the high percentage of asymptomatic cases reaching 85% in women [10] and 77% in men [11] and that the infection is not notifiable, the prompt and correct diagnosis of the infection is crucial, in order to manage appropriate treatment.

Until this year, the gold standard for the diagnosis of trichomoniasis was the culture method, now substituted by the molecular techniques, as stated by the CDC guidelines [8]. The most common method used in trichomoniasis diagnosis is the wet mount examination of vaginal and urethral secretions by observing the morphology and motility of the parasite flagella. This method although simple, fast and easy to perform, has important limitations, since it requires continued viability of parasites and *T. vaginalis* trophozoites does not survive outside the urogenital tract [12]. In this sense, fast processing, proper preservation of samples and microscopic examination at appropriate time can preserve the integrity and motility of trophozoites and improve diagnostic sensitivity. An advantage of the wet mount method is high specificity, as false-positive results are minimal. However, it can be misunderstood; clusters of white

blood cells may be confused with *T. vaginalis*, as they are similar in size and form. When compared with molecular tests, wet mount examination has low sensitivity, since it is necessary at least 10,000 trichomonas/ mL for detection by microscopy, while using molecular approaches less than 1 trichomonas/ mL is required for parasitic DNA detection [13].

Considering the vast application of wet mount examination in the laboratorial routine and aiming to improve the sensitivity of this technique for diagnosis of *T. vaginalis* in urine, this study compared different preserving solutions widely used in clinical laboratories in different storage temperatures in order to evaluate the best combination on samples preservation for the identification of trophozoites.

Materials and Methods

In this study, *T. vaginalis* ATCC 30236 isolate was used and six preservative solutions: glucose saline pH 5.0 and 6.0, PBS pH 5.0 and 6.0, PBS + glucose pH 5.0 and 6.0 were tested at room temperature (21°C) or 37°C. The day before the experiment, the trophozoites were adjusted to a density of $1.0x10^6$ trophozoites/ mL and incubated in trypticase-yeast extract- maltose (TYM) medium pH 6.0 for 24h [14]. After the incubation period, the parasites werewashed three times with the preservative solutions to be tested or TYM medium (control group) and then were resuspended in the same condition. Once the suspension was prepared, parasites were evaluated and counted in hemocytometer with trypan blue dye exclusion during a period of 8 hours. Throughout the incubation time the samples were incubated at both temperatures 21°C or 37°C. The tests were carried out in three independent experiments in triplicate and the media of viability of the tests was compared to the control group (trophozoites maintained in TYM to 37°C).

Based on the trophozoites viability observed after the incubation period with the six solutions, the next experiments were conducted to evaluate if the solution that showed the best performance in *T. vaginalis* trophozoite maintenance were able to keep the morphology and motility of the parasite in clinical specimen, urine. To choose the best solution we performed a statistical analysis (ANOVA). For this assay, four urine samples donated from a healthy volunteer were used. The urine was collected in a clean bottle and was placed in falcon tubes (approximately 5 mL per tube) and then different trichomonads densities, $1.0x10^6$, $1.0x10^5$, $1.0x10^4$ or $1.0x10^3$ trophozoites/mL were inoculated in the urine. Following, urine samples were centrifuged, the supernatant was discarded and the pellet containing trophozoites was resuspended in 1.0 mL of the preservative solution or TYM (control group). After that, samples were incubated for 6 hours at room temperature (21°C) or 37°C. Then, trophozoites were evaluated and counted in hemocytometer. The tests were carried out in three independent experiments in triplicate.

Finally, in order to investigate a possible interference of the preservative solution on the urinalysis routine test, this experiment evaluated if chemical and microscopic parameters were preserved in urine samples using glucose saline pH 6.0 as preservative solution. The urines included in this test presented or not some typical alterations. The chemical parameters were evaluated with commercial strips. Urine elements as cylinders, crystals, white blood cells, red blood cells, yeast, bacteria and epithelial cells were evaluated by microscopy. To conduct these tests, urines were firstly evaluated for chemical and microscopic parameters and after that were centrifuged in falcon tubes. The supernatant was discarded and the pellet was resuspended in 1 mL of saline glucose solution pH 6.0. The suspensions were incubated for 6 hours at room

temperature (21°C). At times 0 and 6 hours, the microscopic elements were evaluated and photographed in order to observe morphological parameters.

The experiments were performed at least in three independent assays, all in triplicate. The Statistical Package for the Social Sciences (SPSS) software version 18 was used to perform the statistical analysis. Data were expressed by mean ± standard deviation (S.D.) and analyzed by one-way analysis of variance (ANOVA) followed by Tukey multiple range test, considering $p < 0.05$ as significant.

Results

In this study, six solutions were tested in order to evaluate the potential of these solutions in the preservation of *T. vaginalis* trophozoites in urine samples, aiming to increase the sensitivity of the wet mount method at clinical laboratorial routine.

At first, we conducted a screening with the six solutions at room temperature (21°C) or 37°C. The results obtained for the two tested temperatures (21°C and 37°C) were evaluated during 8 hours of incubation and at the end of this period, in all preserving solutions, the trophozoites presented viability between 20 and 50% (Fig. 1 and Fig. 2).

Based on the results of this preliminary assay and taking into account the compatibility with the laboratorial routine, we selected 6 hours of incubation period for the next experiments. When the urine samples were maintained at room temperature (21°C) (Fig. 1) the number of trophozoites was the same in all solutions compared to control (times 0 and 2 hours). Considering 4 hours of incubation, only the glucose saline pH 5.0 solution induced a significant reduction in the parasites number compared to control ($p < 0.05$). Following 6 and 8 hours of experiment, all solutions produced significant decrease of parasites viability ($p < 0.05$). In 6 hours incubation, it can be

observed that trophozoites exhibited viability over 50%, at room temperature (21°C) with glucose saline pH 6.0, PBS pH 5.0 and PBS + glucose pH 5.0 presenting 61%, 53% and 51% of viability, respectively. It is clearly noted that glucose saline pH 6.0 was the solution with the best potential on the preservation of trophozoites viability (Fig. 1). The solution that presented the worst performance at this temperature in 6 hours of incubation period was glucose saline pH 5.0 demonstrating 39% of trophozoites viability.

In order to compare the two temperatures, the same evaluation was carried out at 37ºC. Results exhibited a similar profile to those at 21ºC: the trophozoites number was the same in all solutions compared to control at time 0 hour. Considering the time of 2 hours of incubation, the PBS pH 6.0 solution caused a significant reduction on parasite viability comparing to control ($p < 0.05$). Following 4, 6 and 8 hours of experiment, all solutions produced significant decrease of parasites viability when compared to control ($p < 0.05$). Figure 2 shows that, within 6 hours of experiment, the solutions presented more than 50% of parasite viability: glucose saline pH 6.0 and glucose saline pH 5.0 presenting 52% and 56% of viable trophozoites, respectively. It is possible to note that PBS + glucose pH 6.0 maintained only 30% of trophozoites viability, demonstrating that this solution was not appropriate to follow the viability studies in urine samples.

Based on these preliminary results we conducted the next experiments aiming to evaluate if the glucose saline pH 6.0 solution would be capable of preserving the trophozoites in urine since this biological sample is widely used in clinical laboratories in comparison with vaginal and urethral secretion. For this evaluation, the densities of $1.0x10^6$ and $1.0x10^5$ trophozoites/ mL were tested. In Figure 3 it can be observed that a good conservation of trophozoites in the urine was promoted by this solution with the best scores obtained when the highest density of trophozoites was applied. Although the

densities of $1.0x10^4$ and $1.0x10^3$ trophozoites/ mL would be more consistent with the amount of trophozoites found in urine samples of infected patients, in this work we were not able to detect trophozoites at these densities. This limitation could be attributed to the low sensitivity of the wet mount method.

Subsequently, the qualitative examination of urine performance was tested to rule out possible interferences of glucose saline pH 6.0 solution in urinalysis. No interference could be observed when the solution was employed to preserve urine samples. All chemical parameters were evaluated with commercial strips and urine elements such as cylinders, crystals, white blood cells, red blood cells, yeast, bacteria and epithelial cells were maintained the same at time 0 hour and after 6 hours of incubation in glucose saline pH 6.0 (data not shown). Therefore, this preserving solution did not interfere with the performance of the urinalysis test.

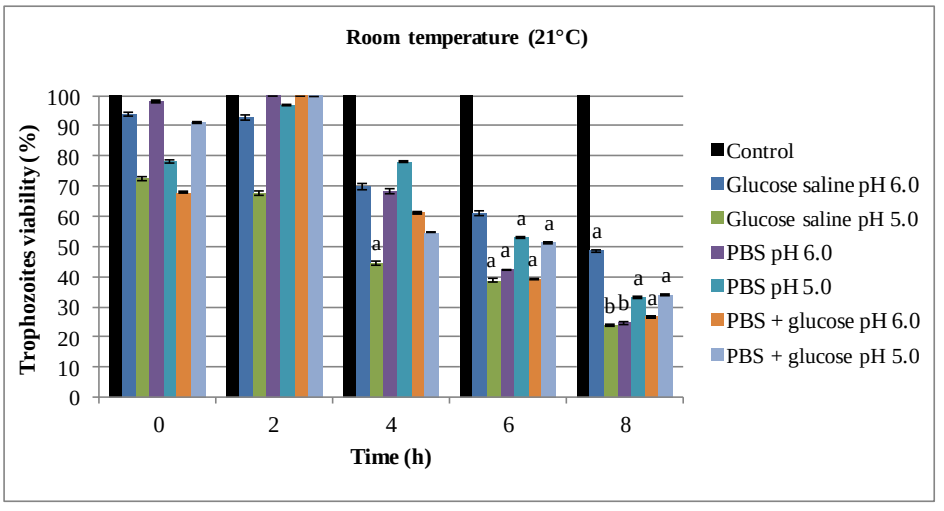

Figure 1: *T. vaginalis* viability in six different preserving solutions incubated at room temperature, 21ºC. Counting of parasites number was performed in relation to control (trophozoites in TYM medium). Data represent means ± standard deviation for at least

three experiments and were statistical analyzed by ANOVA ($p < 0.05$): [a] when compared with control, [b] when compared with control and glucose saline pH 6.0 solution.

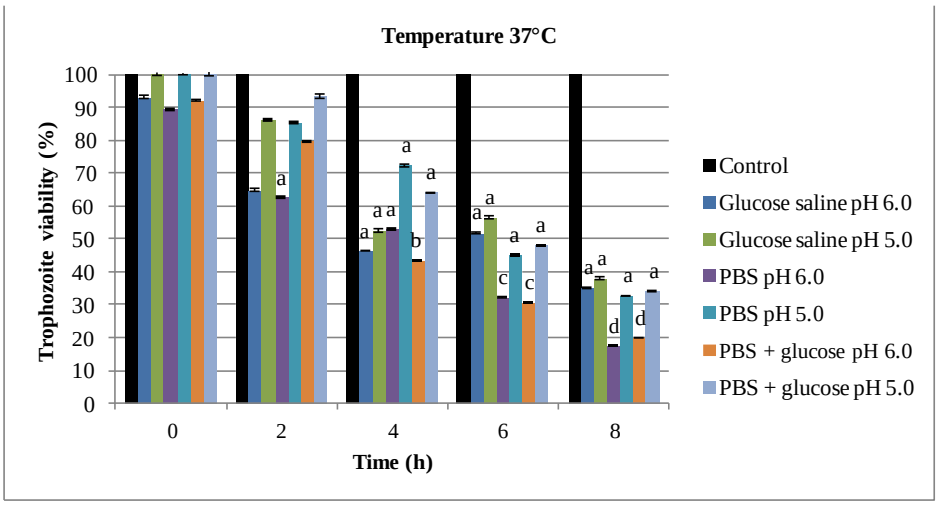

Figure 2: *T. vaginalis* viability in six different preserving solutions incubated at 37ºC. Counting of parasites number was performed in relation to control (trophozoites in TYM medium). Data represent means ± standard deviation for at least three experiments and were statistical analyzed by ANOVA ($p < 0.05$): [a] when compared with control, [b] when compared with control and glucose saline pH 6.0 solution, [c] when compared with control, glucose saline pH 6.0 and pH 5.0 solutions, [d] when compared with control, glucose saline pH 6.0 and pH 5.0, PBS pH 5.0 and PBS + glucose pH 5.0 solutions.

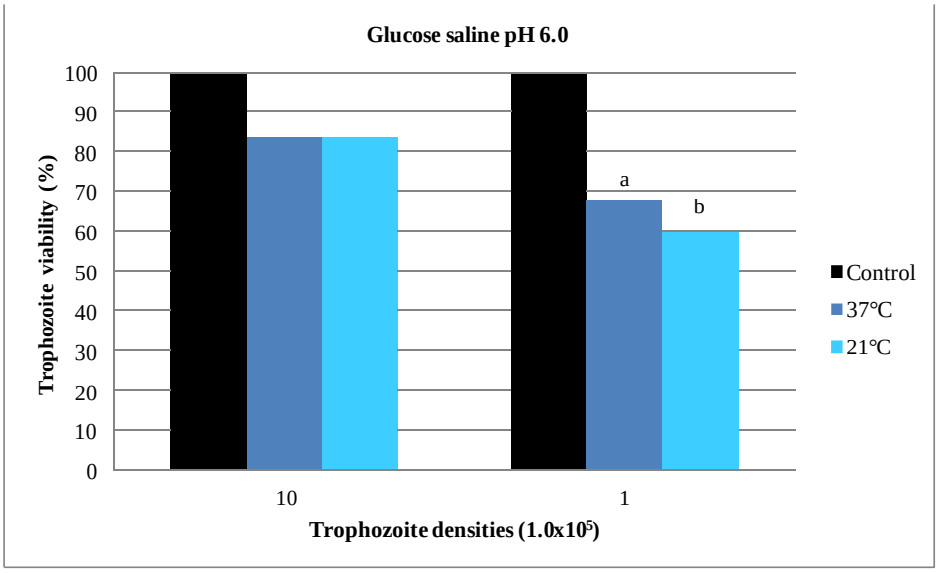

Figure 3: *T. vaginalis* viability in urine samples maintained in glucose saline pH 6.0 solution incubated at room temperature (21ºC) and 37ºC. Counting of parasites number was performed in relation to control (trophozoites in TYM medium). Data represent means ± standard deviation for at least three experiments and were statistical analyzed by ANOVA ($p < 0.05$): [a] when compared with control, [b] when compared with density 10 x 10^5 trophozoites/ mL.

Discussion

The most widely employed diagnosis of *T. vaginalis* infection in the laboratory routine is the wet mount examination of genital secretions, a simple, fast and easy method. Despite all these advantages, the technique presents low sensitivity (51% - 65%) and samples must be promptly processed and evaluated otherwise the parasites lose motility and viability, turning impossible the microscopic identification [8]. In addition to this limitation, more than 80% of patients infected are asymptomatic which

can delay the correct detection of the parasite [10, 11]. Considering these aspects, in this work we proposed a simple and low-cost method of preservation of the parasite in urine samples, since the qualitative and quantitative examination of urine is one of the most conducted in the laboratorial routine. Our intention was to demonstrate that along the time that the sample is stored and manipulated in the laboratory and by employing the correct preservative solution the trophozoites will be still alive and maintain the classical characteristics of motility and shape. This simple step of preservation will facilitate the tricho moniasis diagnosis in the urine and may increase the number of cases of parasite detection.

Initially, we carried out a screening, in order to evaluate the maximum period that trophozoites would maintain the viability in the tested solutions. It was clear that 8 hours period was too long and did not maintain the parasite viability. On the other hand, 6 hours of experiment showed promising results. From this assessment, we could elect the glucose saline pH 6.0 solution to continue our study. Sumadhya et al (2011), have demonstrated a better performance on the diagnostic method for *T. vaginalis* using solution glucose saline as preservation media for the samples [15]. These results are in complete agreement with our data, which demonstrated that the glucose saline pH 6.0 solution was the most promising for the trophozoite preservation, since it maintained the organism viability and major morphological aspects within 6 hours. Considering the parasite density of 1.0×10^6 trophozoites/mL, samples were very similar to the controls and maintained parasite viability as well as the trophozoites have remained piriforms, showing normal motility. In the density of 1.0×10^5 trophozoites/ mL it was still possible to easily identify the organsims; however with densities of 1.0×10^4 and 1.0×10^3 trophozoites/ mL, which are closer to the density found in the urine of infected patients, the visualization of the parasites was quite compromised since counting in

hemocytometer is not a highly sensitive method. Despite this limitation, a few trophozoites that occasionally were found could also be easily identified because they were highly motile. These data suggest that glucose saline solution is a suitable solution preserver for *T. vaginalis* trophozoites in the urine and could be easily applied in the routine diagnosis. Further studies with more sensitive counting methods will validate the use of glucose saline pH 6.0 solution considering low parasite densities.

One concern was the possible interference of preserving solutions in the urinalysis test. To rule out this issue, we tested the solution chosen based on the initial screening, glucose saline pH 6.0 solution, in several urine samples presenting different urinalysis results. Data demonstrated that the preserving solution did not cause any effect in the urine samples, as the same features found at 0 hour were maintained after 6 hours of incubation. This relevant finding contributes to the application of the glucose saline pH 6.0 solution in the preservation step during the examination procedure for direct trichomoniasis diagnosis.

In addition, we conducted a brief review on the literature regarding the preserver solution employed for trichomoniasis diagnosis (Table 1). For that, the following keywords were used in the search: "trichomoniasis and wet mount". The survey was done on the US National Library of Medicine (PubMed) for the period of 2005 to November 2015 in English. Most studies use saline solutions and vaginal secretion as clinical specimen (Table 1). Although this is the main biological fluid to detect *T. vaginalis*, the improvement in diagnosis methods such as nucleic acid amplification tests (NAATs) have detected the pathogen in both vaginal secretion and urine [7]. Taking into account the time of exposure in the preserver solution, with exception of the study by Stoner et al. (2013) [16], all reports that considered this information, maintained the parasites for very short times, 10 to 15 minutes. Our results revealed adequate preservation

(more than 50%) of *T. vaginalis* trophozoites in glucose saline pH 6.0 solution for up to 6 hours, an adequate time that is often taken to examine urine samples in the laboratorial routine.

Table 1: Brief review on the literature regarding the preserver solution employed for trichomoniasis diagnosis by wet mount microscopy method.

Solution for preservation	Specimen	Time	Reference
0.5 mL, 0.9% saline	Vaginal secretion	15 min	(Narayankhedkar et al., 2015) [17]
0.5 mL, 0.9% saline	Vaginal secretion	NR	(Ton Nu et al., 2015) [18]
0.85% sSaline	Vaginal secretion	NR	(Khatoon et al., 2015) [19]
0.85% sSaline	Vaginal secretion	NR	(Khatoon et al., 2014) [20]
0.2 mL, 5% glucose saline	Vaginal fluid	10 min	(Saleh et al., 2014) [21]
0.5 mL, Ringer solution	Vaginal secretion	NR	(Matini et al., 2014) [22]
0.2 mL, 5% glucose saline	Vaginal secretion	10 min	(Fule et al., 2012) [23]
0.5 mL, 0.9% saline	Vaginal secretion	NR	(Patil et al., 2012) [24]
0.5 mL saline	Vaginal secretion	NR	(Huppert et al., 2010) [25]
0.5 mL saline	Vaginal secretion	24 hours	(Stoner et al., 2013) [16]

NR: Not reported.

Herein we were able to maintain viable *T. vaginalis* trophozoites for the microscopic diagnosis for up to 6 hours after its processing with glucose saline pH 6.0 solution. Moreover, the solution did not cause any interference in the qualitative examination of urine. Our data demonstrate the importance of urine samples preservation for the accurate trichomoniasis diagnosis and suggest the use of glucose saline pH 6.0 solution in the clinical laboratorial routine.

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ANEXO:

INTERNATIONAL JOURNAL OF STD & AIDS
The Royal Society of Medicine Journals

1. Article types

When assessing the length of your contribution, allow 250 words for each table, figure or group of eight references, since all of these will contribute to its total length.

Editorials/Reviews

Suggestions for review articles are welcomed by the Editors and the subject matter should be outlined to the Editors, if possible before writing the article. Maximum length: 3500 words, with up to five tables or illustrations.

Original Research Articles

These are expected to contribute to the advancement of knowledge in the field of STD and AIDS. Maximum length: 3000 words, with up to five tables or illustrations.

Case Reports

These should be prepared in a narrative style and comprise a summary; a short introduction stating the reasons for reporting the case; the case report including history, investigations and treatment; and a discussion referring to the relevant literature. Maximum length 800 words with 1–2 tables or illustrations.

Audit Reports

These should be prepared in a narrative style and comprise a short summary, introduction, methods, results and discussion. Maximum length 800 words with 1–2 tables or illustrations.

Letters to the Editor

These should relate to articles published recently in the journal. Letters are usually less than 500 words.

2. Editorial policies

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All papers will be reviewed by independent referees, and authors may be requested to amend their contribution. The final decision about acceptance or rejection remains with the Editors. Papers on which the Editors are co-authors are handled by another member of the Editorial Board and are usually sent to at least two independent referees.

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As part of the submission process you will be asked to provide the names of peers who could be called upon to review your manuscript. Recommended reviewers should be experts in their fields and should be able to provide an objective assessment of the manuscript. Please be aware of any conflicts of interest when recommending reviewers. Examples of conflicts of interest include (but are not limited to) the below:

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Please note that the Editors are not obliged to invite any recommended/opposed reviewers to assess your manuscript.

2.2 Authorship

Papers should only be submitted for consideration once consent is given by all contributing authors. Those submitting papers should carefully check that all those whose work contributed to the paper are acknowledged as contributing authors.

The list of authors should include all those who can legitimately claim authorship. This is all those who:

1. Made a substantial contribution to the concept and design, acquisition of data or analysis and interpretation of data,
2. Drafted the article or revised it critically for important intellectual content,
3. Approved the version to be published.

Authors should meet the conditions of all of the points above. Each author should have participated sufficiently in the work to take public responsibility for appropriate portions of the content.
When a large, multicentre group has conducted the work, the group should identify the individuals who accept direct responsibility for the manuscript. These individuals should fully meet the criteria for authorship.
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