

## Kinetic characterization and gene expression of adenosine deaminase in intact trophozoites of *Trichomonas vaginalis*

Marina Weizenmann<sup>1</sup>, Amanda Piccoli Frasson<sup>1</sup>, Muriel Primon de Barros<sup>1</sup>, Patrícia de Brum Vieira<sup>1</sup>, Denis Broock Rosemberg<sup>2</sup>, Geraldo Attilio De Carli<sup>3</sup>, Maurício Reis Bogó<sup>4</sup>, Carla Denise Bonan<sup>5</sup> & Tiana Tasca<sup>1</sup>

<sup>1</sup>Laboratório de Pesquisa em Parasitologia, Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil; <sup>2</sup>Departamento de Bioquímica, ICBS, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil; <sup>3</sup>Instituto de Geriatria e Gerontologia, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, RS, Brazil; <sup>4</sup>Laboratório de Biologia Genômica e Molecular, Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, RS, Brazil; and <sup>5</sup>Laboratório de Neuroquímica e Psicofarmacologia, Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, RS, Brazil

**Correspondence:** Tiana Tasca, Laboratório de Pesquisa em Parasitologia, Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Av. Ipiranga 2752, 90610-000, Porto Alegre, RS, Brazil. Tel.: +55 51 3308 5325; fax: +55 51 3308 5437; e-mail: tiana.tasca@ufrgs.br

Received 19 February 2011; accepted 11 March 2011.

Final version published online 26 April 2011.

DOI:10.1111/j.1574-6968.2011.02283.x

Editor: Albert Descoteaux

### Keywords

*Trichomonas vaginalis*; adenosine deaminase; intact trophozoites; adenosine; inosine.

### Abstract

*Trichomonas vaginalis* is a parasite that resides in the human urogenital tract and causes trichomonosis, the most prevalent nonviral sexually transmitted disease. Nucleoside triphosphate diphosphohydrolase (NTPDase), which hydrolyzes extracellular di- and triphosphate nucleotides, and ecto-5'-nucleotidase, which hydrolyzes AMP, have been characterized in *T. vaginalis*. The aim of this study was to characterize the adenosine deaminase (ADA) activity in intact trophozoites of *T. vaginalis*. A strong inhibition in adenosine deamination was observed in the presence of calcium and magnesium, which was prevented by EDTA. The apparent  $K_M$  value for adenosine was  $1.13 \pm 0.07$  mM. The calculated  $V_{max}$  was  $2.61 \pm 0.054$  nmol  $\text{NH}_3 \text{ min}^{-1} \text{ mg}^{-1}$  protein. Adenosine deamination was inhibited in the presence of erythro-9-(2-hydroxy-3-nonyl)adenine. Semi-quantitative reverse transcriptase-PCR experiments were performed and both ADA-related genes *ada(125)* and *ada(231)* mRNA were expressed, although *ada(231)* in higher quantity when compared with the *ada(125): $\alpha$ -tubulin* ratio. Furthermore, a phylogenetic analysis showed that the *T. vaginalis* sequences formed a clade with *Entamoeba histolytica* and *Dictyostelium discoideum* sequences, and it strongly suggests homologous functions in the *T. vaginalis* genome. The presence of ADA activity in *T. vaginalis* may be important to modulate the adenosine/inosine levels during infection and, consequently, to maintain the anti-inflammatory properties through different nucleoside-signalling mechanisms.

### Introduction

*Trichomonas vaginalis* is a protozoan parasite that causes trichomonosis, the most prevalent nonviral sexually transmitted disease worldwide (WHO, 2001). In women, the infection is clinically characterized by vaginitis and cervicitis (Petrin *et al.*, 1998; Lecker & Alderete, 2000). The pathogen has been associated with serious health consequences including adverse pregnancy outcomes (Klebanoff *et al.*, 2001), infertility (Grodstein *et al.*, 1993), predisposition to cervical cancer (Viikki *et al.*, 2000) and pelvic inflammatory

disease (Cherpes *et al.*, 2006), and it is a cofactor in HIV transmission and acquisition (Sorvillo *et al.*, 2001; Van Der Pol *et al.*, 2008).

At the infection sites, tissue stress or injury takes place and intracellular ATP can be released into the extracellular environment. Extracellular nucleotides such as ATP play a role as danger-associated molecular patterns (DAMPs) or 'alarmins' by acting as signalling molecules that contribute to inflammation and immune responses (Hanley *et al.*, 2004; Bours *et al.*, 2006). The crucial factors in purinergic signalling are the stimulation of nucleotide release, their

metabolism by enzymes acting in an extracellular manner and the presence of receptors that selectively bind the resulting products and mediate signal transduction (Gounaris & Selkirk, 2005). The purinergic signalling involves specific purinergic type 1 (P1) and type 2 (P2) receptors and is important in both neuronal and non-neuronal processes, including the modulation of inflammation and specific immune responses (Robson *et al.*, 2006; Sansom *et al.*, 2008).

The ecto-nucleoside triphosphate diphosphohydrolase family (ecto-NTPDases) is constituted by eight members (NTPDase1–8) that hydrolyze nucleoside di- and triphosphates to the monophosphate form. Nucleoside monophosphates may then be catalyzed to nucleosides such as adenosine by the action of ecto-5'-nucleotidase. Purine salvage and the regulation of blood clotting, inflammatory processes and immune reactions are among the major roles played by these enzymes to date (Sansom *et al.*, 2008; Burnstock & Verkhratsky, 2009). The adenosinergic signalling can be controlled by adenosine uptake via bidirectional transporters, followed by intracellular phosphorylation to AMP by adenosine kinase or deamination to inosine by adenosine deaminase (ADA; EC 3.5.4.4). ADA participates in the purine metabolism, where it degrades either adenosine or 2'-deoxyadenosine, producing inosine or 2'-deoxyinosine, respectively (Franco *et al.*, 1997). A phylogenetic study demonstrated the existence of different ADA-related members, which include ADA1, ADA2 and a similar deduced amino acid sequence named adenosine deaminase like (ADAL) (Maier *et al.*, 2005). Despite its intracellular location, ADA1 may occur on cell surface, anchored to two proteins, CD26 and A<sub>1</sub> receptors, acting as an ecto-ADA cleaving extracellular adenosine (Franco *et al.*, 1997). ADA has been described in mammalian cells and tissues, blood-feeding insects, mollusks and parasites, *Plasmodium lophurae*, *Trichinella spiralis*, *Fasciola gigantica* and *Hyalomma dromedarii* (Franco *et al.*, 1997; Gounaris, 2002; Mohamed, 2006; Ali, 2008).

The characterization and expression of S-adenosylhomocysteinase were described in *T. vaginalis*, which catalyzes the reversible hydrolysis of S-adenosylhomocysteine to homocysteine and adenosine (Minotto *et al.*, 1998). Those authors have previously reported the absence or the poor activity of ADA. It is important to mention that *T. vaginalis* is dependent on salvage pathways to generate *de novo* nucleotides (Heyworth *et al.*, 1982, 1984). Munagala & Wang (2003) demonstrated that adenosine is the primary precursor of the entire pool of purine nucleotides in *T. vaginalis*, and activities of ADA, IMP dehydrogenase and GMP synthetase were identified in trichomonads, suggesting a metabolic pathway able to convert adenine to GMP via adenosine. Our group has investigated the purinergic system in *T. vaginalis* throughout the extracellular nucleotide hydrolysis, and NTPDase and ecto-5'-nucleotidase activities

were described (Matos *et al.*, 2001; Tasca *et al.*, 2003, 2005). Considering that (1) extracellular nucleotides and nucleosides, such as adenosine and inosine, act as DAMPs playing a role in cell signalling that contribute to inflammation and immune responses (Bours *et al.*, 2006; Sansom *et al.*, 2008), and (2) the ectonucleotidase pathway has been characterized in *T. vaginalis*, the aim of this study was to characterize ADA activity, an enzyme involved in nucleoside metabolism, and to evaluate the relative mRNA expression of ADA-related genes in this mucosal parasite.

## Materials and methods

### Parasite culture and preparation of parasite suspensions

*Trichomonas vaginalis* clinical isolate TV-VP60 (Michel *et al.*, 2006) was used throughout this enzyme characterization study. The other five isolates were TV-30236 (from the American Type Culture Collection, ATCC) and the clinical isolates TV-LACM1, TV-LACM2, TV-LACH1 and TV-LACH2 from our Clinical Laboratory surveys (Universidade Federal do Rio Grande do Sul, Brazil). Trichomonads were cultured axenically *in vitro* and maintained in trypticase–yeast extract–maltose (TYM) medium (Diamond, 1957), pH 6.0, supplemented with 10% (v/v) inactivated bovine serum at 37 °C. Organisms from the logarithmic phase were evaluated before and after assays based on motility and viability using trypan blue (0.2%) exclusion. The parasites were then harvested by centrifugation and washed three times with phosphate-buffered saline (PBS) added with 2.0 mM EDTA and 2.0 mM EGTA. The final pellet was resuspended and used for the subsequent assays. *Trichomonas vaginalis* lysates were obtained in liquid nitrogen, at 0.1 mg<sup>-1</sup> protein<sup>-1</sup> mL<sup>-1</sup>, in the presence of 1.0 mM protease inhibitor cocktail.

### ADA assay

An aliquot from the parasite suspension was added to the reaction mixture containing 50 mM sodium phosphate buffer (pH 7.5) to maintain the protein concentration (50–150 µg mL<sup>-1</sup>) in the final volume of 200 µL. The samples were then preincubated for 10 min at 37 °C. The reaction was initiated with the addition of the substrate adenosine (3.0 mM) and stopped, after a determined time (10–40 min), by adding the samples on 500 µL of phenol-nitroprusside reagent (50.4 mg of phenol and 0.4 mg of sodium nitroprusside mL<sup>-1</sup>). Controls with the addition of the enzyme preparation after the termination of reaction were used to correct nonenzymatic deamination of the substrate. The reaction mixtures were mixed with 500 µL of alkaline-hypochlorite reagent (sodium hypochlorite to 0.125% available chlorine, in 0.6 M NaOH). Samples were

incubated at 37 °C for 15 min. The colorimetric assay was carried out at 635 nm (Giusti, 1974) to measure the ammonia produced by the enzymatic reaction and the ADA activity was expressed as  $\text{nmol NH}_3 \text{ min}^{-1} \text{ mg}^{-1}$  protein. In all assays, at least three different experiments were performed in triplicate. The protein quantification was performed in triplicate for the parasite suspensions (Bradford, 1976) using bovine serum albumin as a standard.

### Characterization of ADA activity

After the standardization of incubation time and the protein concentration in order to maintain the linearity of the enzymatic reaction, assays to determine the optimum pH were performed using 50 mM sodium phosphate buffer (mixture: 0.2 M disodium phosphate and 0.2 M sodium phosphate, pH 6.5–7.5) and sodium carbonate bicarbonate buffer (mixture: 0.2 M sodium carbonate and 0.2 M sodium hydrogen carbonate, pH 8.5). The apparent  $K_M$  and  $V_{\max}$  values for adenosine deamination were determined from Eadie–Hofstee plots using substrate concentrations from 0.40 to 3.0 mM. The substrates 2'-deoxyadenosine, guanosine and 2'-deoxyguanosine (all in 3.0 mM) were also assayed for ADA activity. The effect of the divalent cations  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  at 2.5 and 5.0 mM was observed by assaying in parallel a control without the cations and a control with cations and EDTA at the same concentrations. ADA activity was measured in the presence of erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA), a potent inhibitor of the ADA 1 isoenzyme, in increasing concentrations (5.0–25  $\mu\text{M}$ ). In order to determine as to how long the EHNA inhibition effect lasts, a 20-min incubation with the inhibitor was performed and the EHNA-treated trophozoites were incubated in culture medium (TYM). After different times (1, 6 and 24 h), the ADA activity was tested. The trichomonad-culture supernatants from EHNA-treated trichomonads were also collected to test in the *T. vaginalis*–neutrophils interaction assay.

### Analysis of gene expression by reverse transcriptase (RT)-PCR

Trophozoites were centrifuged and washed three times with PBS buffer (pH 7.2) for total RNA extraction using TRIzol reagent (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's instructions. The purity of the RNA was spectrophotometrically quantified by calculating the ratio between absorbance values at 260 and 280 nm. Afterwards, cDNA species were synthesized using the SuperScript<sup>TM</sup> III First-Strand Synthesis SuperMix (Invitrogen) following the supplier's instructions from 2.0  $\mu\text{g}$  of total RNA. PCR reactions were performed in a volume of 20  $\mu\text{L}$  using 0.1  $\mu\text{M}$  of specific primers for ADA, 2.5 mM  $\text{MgCl}_2$  and 0.5 U Taq Platinum (Invitrogen) in the supplied

reaction buffer. The sequences of  $\alpha$ -tubulin primers were in accordance with previously described data (Kucknoor *et al.*, 2005) and the PCR conditions were as reported in previous studies (Giordani *et al.*, 2010; Rückert *et al.*, 2010), using 0.5 M betain. All assays were carried out using 1.0  $\mu\text{L}$  of cDNA template. The conditions for all PCR were as follows: initial 1-min denaturation step at 94 °C, 1-min annealing step (*ada 125* and *ada 231*) at 57 °C, 1-min extension step at 72 °C for 35 cycles and 10 min of a postextension cycle at 72 °C. Negative controls were included for each set of PCR. PCR products were separated on a 1.0% agarose gel with GelRed 10 $\times$  (Invitrogen) and visualized with UV light. Band intensities were analyzed by densitometry using the freeware IMAGEJ 1.37 for Windows. The alpha-tubulin gene was used for normalization and all PCR products were run in a single gel. The results are representative of three different experiments.

### Identification of ADA-related *T. vaginalis* amino acid sequences

The identification of ADA-related *T. vaginalis* amino acid sequences was performed using the well-known ADA1, ADAL and ADA2 from humans (NP\_000013, NP\_001012987 and CAG30303), mouse (NP\_031424 and AAH52048), frog (Q6GP70, AAH97573 and AAX10952), chicken (NP\_001006290, NP\_001025718 and AAX10953) and fish (AAH76532, NP\_001028916, AAL40922 and XP\_687719) as queries to perform a BASIC LOCAL ALIGNMENT SEARCH (BLASTP function) via the GenBank database. ADA-related sequences from *Leishmania major* (XP\_843322), *Plasmodium falciparum* (XP\_001347573), *T. spiralis* (AAT39739), *Trypanosoma brucei* (XP\_823299), *Entamoeba histolytica* (XP\_655082), *Dictyostelium discoideum* (XP\_637270) and *Escherichia coli* (AAA16408) were also retrieved from GenBank and included in the phylogenetic analysis. The ADA protein sequences obtained were aligned with CLUSTALX program (Thompson *et al.*, 1997) and a phylogenetic tree was constructed with MEGA 4.0 program (Tamura *et al.*, 2007) using the statistical neighbor-joining method (Saitou & Nei, 1987) with proportional (*p*) distance.

### Isolation of human neutrophils

Human neutrophils were isolated as described previously (Boyum, 1968), with some modifications. Briefly, venous blood of healthy volunteers was collected on a heparin anticoagulant solution, centrifuged (250 g, 10 min, 24 °C) and the resulting platelet-rich plasma was discarded. Leukocytes were obtained following erythrocyte sedimentation in 2% Dextran T-500 and centrifuged (525 g, 20 min, 24 °C) through a layering on Histopaque 1077 (Sigma, St. Louis, MO). The neutrophil-enriched pellet was subjected to a 15-s hypotonic lysis to remove the remaining erythrocytes and

centrifuged (1000 g, 5 min, 24 °C). The purified neutrophils were resuspended in RPMI 1640 supplemented with 10% fetal bovine serum and 10 mM HEPES for the next experiments. The purity of neutrophils was confirmed morphologically (> 95%) and examined using flow cytometry (FACSCalibur, BD Bioscience, Franklin Lakes, NJ). The phenotypic analysis as performed by CELL QUEST BD and PAINT A GATE PRO BD softwares, after staining with fluorescein isothiocyanate (FITC)-conjugated anti-CD45, anti-CD15, anti-CD8 antibodies and phycoerythrin-conjugated anti-CD14, anti-CD22, anti-CD3 and anti-CD4 antibodies (BD Bioscience).

### Culture condition of neutrophils and *T. vaginalis*

Neutrophils ( $2.0 \times 10^5$ ) were co-cultured with live and lysed *T. vaginalis* ( $1.0 \times 10^4$ ), 1 h EHNA-treated and nontreated trophozoites, as well as trichomonad-culture supernatants from EHNA-treated trichomonads. All conditions were performed on a 96-well microplate, for 24 h, in the presence or not of  $100 \text{ ng mL}^{-1}$  lipopolysaccharide (used as a positive control),  $100 \mu\text{M}$  adenosine and  $100 \mu\text{M}$  inosine. After incubation, the cell-free culture supernatants were collected and subjected to quantification of nitrite immediately. The results are representative of at least three independent experiments.

### Measurement of nitrite production

The concentration of NO in culture supernatants was determined as nitrite using Griess reagent (Sigma) in accordance with the manufacturer's instructions.

### Statistical analysis

Data were expressed by mean  $\pm$  SD and analyzed by one-way ANOVA, followed by Tukey multiple-range test or Student's *t*-test, considering  $P < 0.05$  as significant. The analyses were performed using the SPSS software.

## Results

### Adenosine deamination as a function of time and protein concentration

The adenosine deamination in trophozoites of *T. vaginalis* was evaluated as a function of time and protein concentration in order to determine the correct assay conditions. The deamination promoted by ADA activity was linear up to 40 min (Supporting Information, Fig. S1a) and in the range of  $50\text{--}150 \mu\text{g protein mL}^{-1}$  (Fig. S1b). Therefore, we chose to use  $100 \mu\text{g protein mL}^{-1}$  from cultures in further enzyme assays. The viability of the trophozoites was not affected by any of the conditions used in the assays. When trophozoite

suspensions were incubated with their respective times and protein contents without the substrate adenosine, there was no significant production of  $\text{NH}_3$ . Therefore, the involvement of other  $\text{NH}_3$  sources was negligible in the assay condition tested.

### Influence of pH on ADA activity

To evaluate the influence of pH on ADA activity, the enzyme assays were carried out in a pH range of 6.5–8.5. The buffers used were sodium phosphate (used in a pH range from 6.5 to 7.5) and sodium carbonate bicarbonate buffer (assayed for pH 8.5). The results showed that the optimum pH for ADA was 7.5 (Fig. 1a); therefore, this value was chosen for the subsequent experiments.

### Effect of divalent cations on ADA activity

In order to investigate a possible effect of divalent cations on ADA activity,  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  were used. Both cations were able to decrease (approximately 50%) the ADA activity at the lower tested concentration (2.5 mM). When tested at a higher concentration (5.0 mM),  $\text{Mg}^{2+}$  inhibited 80% of ADA activity and  $\text{Ca}^{2+}$  completely abolished the activity. This effect is specifically caused by cations because it was prevented by the addition of EDTA (Fig. 1b).

### Kinetic parameters of *T. vaginalis* ADA

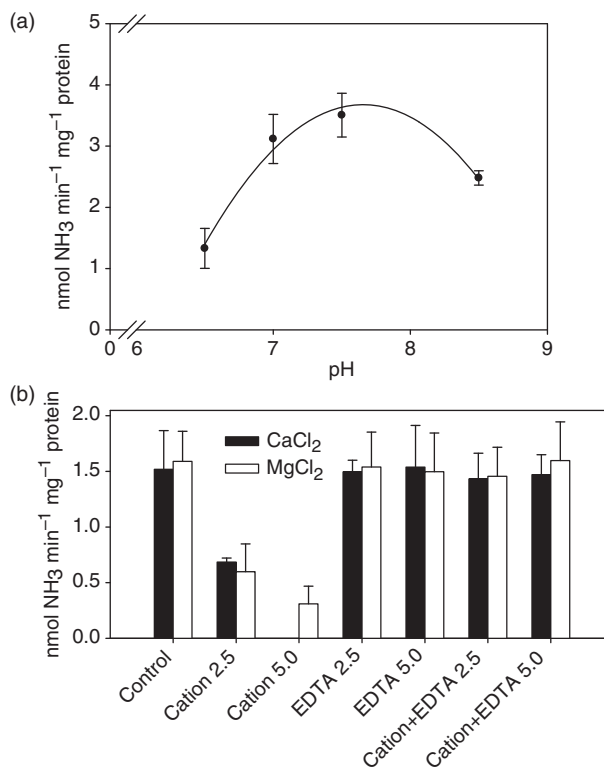
The adenosine deamination was determined at adenosine concentrations ranging from 0.4 to 3.0 mM (Fig. 2). The apparent Michaelis–Menten constant ( $K_M$  app) and maximum velocity ( $V_{\text{max}}$  app) were estimated from a Eadie–Hofstee plot (inset, Fig. 2). The apparent  $K_M$  was  $1.13 \pm 0.07 \text{ mM}$  (mean  $\pm$  SD,  $n = 4$ ), whereas the calculated  $V_{\text{max}}$  was  $2.61 \pm 0.054 \text{ nmol NH}_3 \text{ min}^{-1} \text{ mg}^{-1} \text{ protein}$  (mean  $\pm$  SD,  $n = 4$ ).

### Different nucleoside specificity

The relative substrate specificity of *T. vaginalis* ADA was determined (Table S1). Adenosine and 2-deoxyadenosine were substrates for ADA, presenting the activities  $1.9 \pm 0.6$  and  $2.9 \pm 0.5 \text{ nmol NH}_3 \text{ min}^{-1} \text{ mg}^{-1} \text{ protein}$ , respectively. Guanosine and 2-deoxyguanosine were not deaminated.

### Inhibition of ADA activity by EHNA

We measured the adenosine deamination in *T. vaginalis* in the presence and in the absence of EHNA, a potent inhibitor of ADA1 activity (Iwaki-Egawa & Watanabe, 2002; Sharoyan *et al.*, 2006; Rosemberg *et al.*, 2008). The incubation time of 20 min for EHNA inhibition was used because this was the optimal time for all enzyme assays, ensuring the linearity of the reaction. After the EHNA treatment, trichomonads were



**Fig. 1.** Influence of pH (a) and of divalent cations (b) on ADA activity in intact trophozoites of *Trichomonas vaginalis*. Data are means  $\pm$  SD of three different experiments (parasite suspensions) performed in triplicate.

metabolically active because they were inoculated in TYM medium for the subsequent experiments including the ADA assay and interaction with human neutrophils. Moreover, the parasites presented motility and cellular integrity checked using trypan blue dye exclusion after EHNA incubation at all concentrations. The inhibition was tested using several concentrations of EHNA (5, 10, 15, 20 and 25  $\mu$ M) and the results revealed a strong and dose-dependent inhibition, reaching the complete abolishment of activity at the highest concentration of EHNA (Fig. 3). Furthermore, the EHNA inhibition was long lasting, because no activity could be detected after passage in culture medium 1 and 6 h after the EHNA treatment (Table 1). The low ADA activity detected after 24 h ( $0.27 \pm 0.05$  nmol NH<sub>3</sub> min<sup>-1</sup> mg<sup>-1</sup> protein) was probably due to new trophozoites grown after the incubation in the culture medium.

### Interaction *T. vaginalis*-neutrophils

We have evaluated the interaction of EHNA-treated *T. vaginalis* on NO production by human neutrophils stimulated with *T. vaginalis*. Figure 4 shows that neutrophils alone produced low levels of NO ( $1.98 \pm 0.35$   $\mu$ M); however, when stimulated with lipopolysaccharide (positive control), the

concentration increased 35 times ( $70.26 \pm 14.69$   $\mu$ M). When the trichomonad-culture supernatants from EHNA-treated trichomonads and the *T. vaginalis* lysate were incubated with neutrophils, both conditions inhibited the NO production. On the other hand, and expectedly, the co-culture with intact *T. vaginalis* trophozoites produced a high amount of NO. However, when incubated in the presence of 1 h EHNA-treated parasites, the NO production effect was reverted. The same effect was observed with adenosine and inosine.

### Phylogenetic analysis and gene expression pattern of ADA in *T. vaginalis*

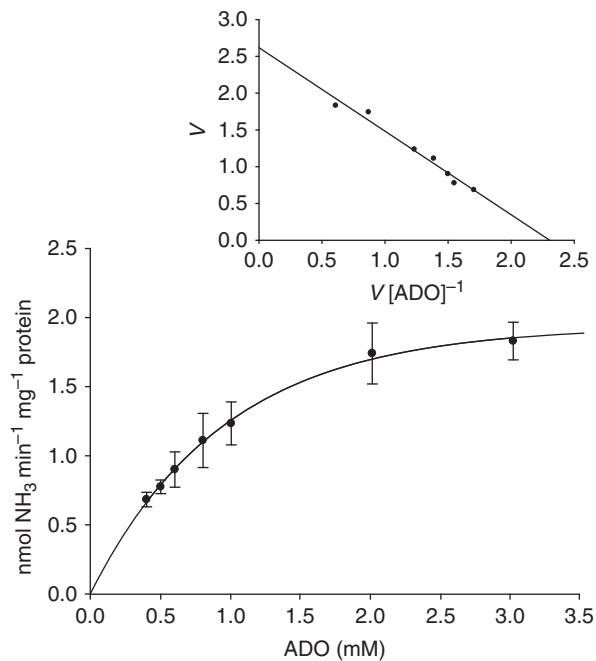
In order to identify the ADA-related sequences on *T. vaginalis* genome, we performed a phylogenetic analysis. NCBI BLAST searches of GenBank yielded two complete *T. vaginalis* ADA-related sequences (XP\_001317231 and XP\_001325125). Semi-quantitative RT-PCR experiments were performed and the relative abundance of ADA-related genes *ada(125)* and *ada(231)* mRNA vs.  $\alpha$ -tubulin was determined by densitometry. As shown in Fig. 5a and b, both genes were expressed, although *ada(231)* in higher quantity when compared with the *ada(125)*: $\alpha$ -tubulin ratio. The phylogenetic tree was constructed using the neighbor-joining method and proportional (*p*) distance (Fig. 5c). Four well-resolved terminal clades supported by high bootstrap values were identified, confirming the presence of two ADA orthologues for *T. vaginalis*. The first clade grouped consistently ADA1 vertebrate sequences and ADA-related sequence from *T. spiralis*. The second clade was formed by *E. histolytica*, *D. discoideum* and *T. vaginalis* sequences. The third clade grouped the ADAL sequences, whereas the fourth clade was formed by ADA2 sequences. *Plasmodium falciparum* and *L. major* ADA-related sequences were placed independently between the four clades mentioned. *Trypanosoma brucei* and *E. coli* were the most divergent sequences. The tree topology strongly suggests homologous functions on the *T. vaginalis* genome.

### ADA activity in different *T. vaginalis* isolates

In order to screen freshly isolated clinical isolates besides TV-VP60, we have determined ADA activity in five other *T. vaginalis* isolates. One isolate, TV-30236, is from ATCC and long term grown; the isolates TV-LACM1 and TV-LACM2 are fresh clinical isolates, obtained from female patients, while TV-LACH1 and TV-LACH2 are fresh clinical isolates from male patients. All isolates presented ADA activity, although we could not establish a relationship between isolate source and activity (Table S2).

### Discussion

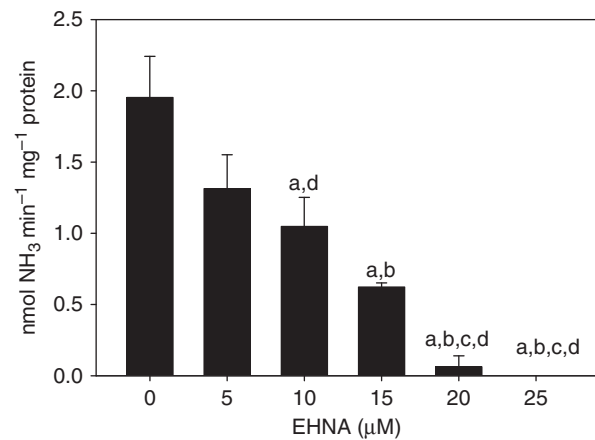
Herein, we described the biochemical properties of an ADA activity and two ADA-related sequences present on intact



**Fig. 2.** Dependence of ADA activity on adenosine concentrations in intact trophozoites of *Trichomonas vaginalis*. Data represent means  $\pm$  SD of four different experiments (parasite suspensions) performed in triplicate. Inset: Eadie–Hofstee plot for adenosine.  $V$ , velocity of ADA activity, expressed in  $\text{nmol NH}_3 \text{ min}^{-1} \text{ mg}^{-1} \text{ protein}$ ;  $V [\text{ADO}]^{-1}$ , velocity/adenosine concentration.

trophozoites of *T. vaginalis*. Cellular integrity was assessed, before and after the reactions, and the viability of the trophozoites was not affected by any of the conditions used in the assays. The influence of pH on the adenosine deamination in *T. vaginalis* was verified and the results demonstrated that the optimal pH for ADA activity reached at 7.5. It is known that vaginal pH in noninfected women is approximately 4.3, but can vary from below 4 to pH 7.5 during the menstrual cycle (Stevens-Simon *et al.*, 1994). In agreement, previous studies demonstrated that the optimal pH values for ADA activities from the camel tick, *H. dromedarii*, and from the trematode *F. gigantica* were also 7.5 (Mohamed, 2006; Ali, 2008).

Cation exposures (2.5 mM) were able to decrease the adenosine deamination in *T. vaginalis* in approximately 50%. Higher concentration of calcium (5.0 mM) completely abolished the enzyme activity and the presence of EDTA, a chelating agent, restored ADA activity. Previous data showed that zinc and other divalent cations are able to interact with other amino acid residues and induce an inhibition of the enzyme activity (Cooper *et al.*, 1997; Mohamed, 2006; Rosemberg *et al.*, 2008). Because zinc is toxic to *T. vaginalis*, we could not perform the experiments on the influence of this metal in ADA activity in intact trophozoites (Langley *et al.*, 1987; Houang *et al.*, 1997).



**Fig. 3.** Inhibition of ADA activity in intact trophozoites of *Trichomonas vaginalis* by EHNA. Data represent means  $\pm$  SD of three different experiments (parasite suspensions) performed in triplicate. Statistical analysis by one-way ANOVA. Statistically significant: <sup>a</sup>when compared with the control; <sup>b</sup>when compared with 5 mM; <sup>c</sup>when compared with 10 mM; <sup>d</sup>when compared with 15 mM ( $P < 0.05$ ).

**Table 1.** EHNA inhibition of *Trichomonas vaginalis* (TV-VP60) ADA activity

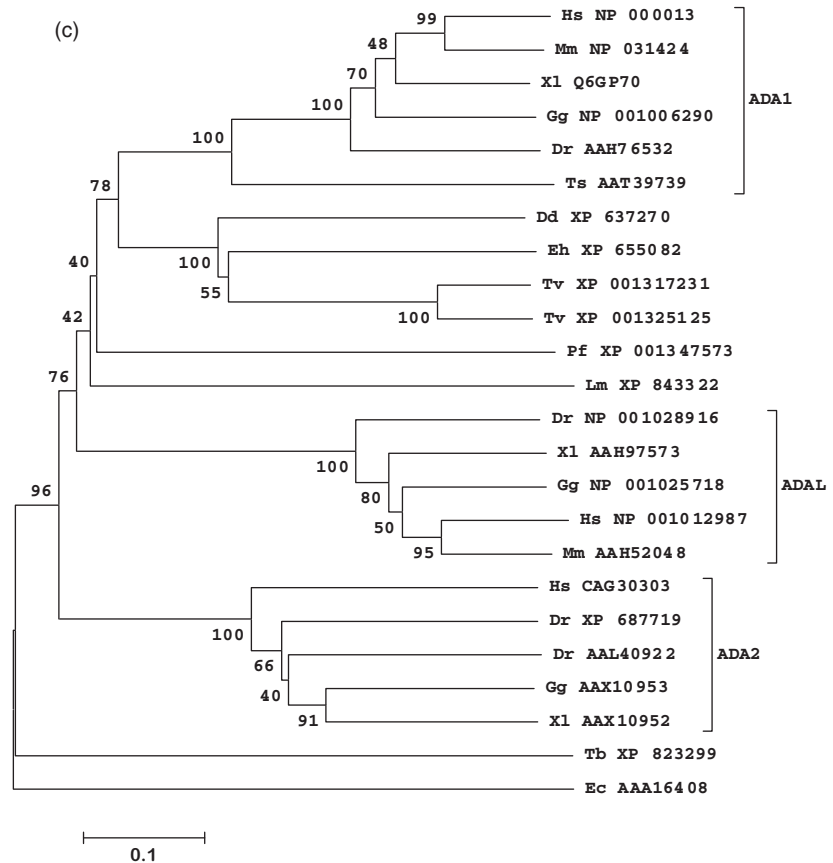
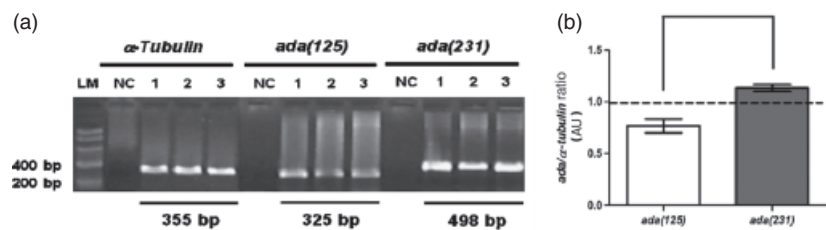
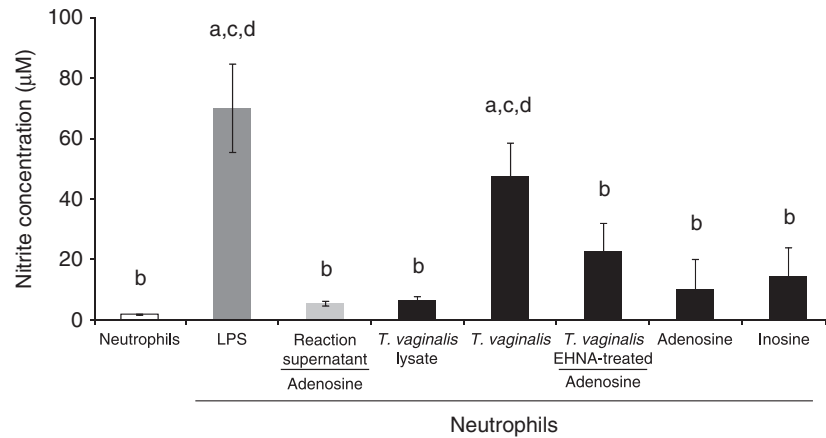
Condition	ADA activity ( $\text{nmol NH}_3 \text{ min}^{-1} \text{ mg}^{-1} \text{ protein}$ )
No EHNA	$1.80 \pm 0.05$
EHNA 20 min	ND
EHNA 1 h	ND
EHNA 6 h	ND
EHNA 24 h	$0.27 \pm 0.05$

ND, not detectable under the assay conditions used.

Additional studies are necessary to explain the relevance of the inhibition of ADA activity by calcium and magnesium in *T. vaginalis* physiology, because magnesium is the most abundant divalent cation in living cells, with a total cellular concentration between 14 and 20 mM (Schmitz *et al.*, 2007).

The substrate curve demonstrated that the apparent  $K_M$  for adenosine was around  $1.13 \pm 0.07$  mM and the estimated  $V_{\text{max}}$  for adenosine deamination was  $2.61 \pm 0.054$   $\text{NH}_3 \text{ min}^{-1} \text{ mg}^{-1} \text{ protein}$  in *T. vaginalis*. The kinetic data obtained in this study are in accordance with other studies related to ADA activity, although there are some variations of  $K_M$  among different ADA members. The  $K_M$  value of *H. dromedarii* ADA2 was estimated to 0.5 mM adenosine (Mohamed, 2006), which is relatively close to several ADAs from different sources, such as rat brain (0.45 mM) (Centelles *et al.*, 1988), bovine brain (0.4 mM) (Lupidi *et al.*, 1992), human (0.46 mM) and chicken liver (0.33 mM) (Iwaki-Egawa & Watanabe, 2002). However, lower  $K_M$  values were reported for ADA activity from mice intestine (0.023 mM) (Singh & Sharma, 2000) and from the sand fly *Lutzomyia longipalpis* (0.01 mM) (Charlab *et al.*, 2000).

**Fig. 4.** Effects of lipopolysaccharide (positive control), live and lysed *Trichomonas vaginalis*, *T. vaginalis* EHNA-treated and nontreated, as well as the supernatants of the EHNA reaction, adenosine and inosine (100  $\mu$ M), on NO production by neutrophils. Scale bars represent the mean  $\pm$  SD of at least three independent experiments. Data were analyzed by ANOVA, followed by Tukey test ( $P < 0.05$ ). Letters represent differences when compared with: <sup>a</sup>neutrophils alone (control); <sup>b</sup>neutrophils stimulated with nontreated intact *T. vaginalis*; <sup>c</sup>adenosine; <sup>d</sup>inosine.



**Fig. 5.** (a) Expression pattern of ADA-related genes *ada(125)* and *ada(231)* mRNA in *Trichomonas vaginalis* trophozoites. The amplifications resulted in a single product. (b) The results were expressed as OD of the ADA-related genes vs.  $\alpha$ -tubulin expression (mean  $\pm$  SE) of four independent replicate RT-PCR experiments. (c) Phylogenetic analysis of ADA-related family members. The deduced amino acid sequences were aligned with CLUSTALX program and the phylogenetic tree was constructed using the neighbor-joining method, proportional ( $p$ ) distance with MEGA 4.0 program. The phylogenetic tree grouped consistently. Tv, *T. vaginalis*; Hs, *Homo sapiens*; Mm, *Mus musculus*; Gg, *Gallus gallus*; Xl, *Xenopus laevis*; Dr, *Danio rerio*; Dd, *Dictyostelium discoideum*; Eh, *Entamoeba histolytica*; Lm, *Leishmania major*; Pf, *Plasmodium falciparum*; Ts, *Trichinella spiralis*; Tb, *Trypanosoma brucei*; ADA1, ADAL and ADA2 orthologous sequences.

Additional data on biochemical characterization revealed the strong preference of the *T. vaginalis* enzyme for 2'-deoxyadenosine as a substrate, even higher than that for adenosine. It was already demonstrated that the preference for both adenine nucleosides may be varied and adenosine and 2'-deoxyadenosine are the classical substrates for ADA (Iwaki-Egawa & Watanabe, 2002; Iwaki-Egawa *et al.*, 2004). In order to verify whether adenosine deamination in *T. vaginalis* may be altered in the presence of a classical inhibitor of ADA1, intact trophozoites were incubated in the presence and in the absence of EHNA. ADA activity from trichomonads was strongly inhibited by increasing concentrations of EHNA, reaching complete inhibition at the highest inhibitor concentration. Furthermore, the ADA inhibition by EHNA was shown to be long lasting; even after the inhibition experiment and the cultivation in TYM medium, the activity could not be detected after 6 h. After 24 h, a very low ADA activity was found, probably due to newly grown trophozoites. Importantly, EHNA-treated *T. vaginalis* reverted the NO production by neutrophils found in nontreated parasites, indicating the involvement of ADA in the immunomodulatory role of purinergic signalling.

Finally, to demonstrate the presence of ADA in *T. vaginalis* at the molecular level, the results revealed that two ADA-related gene sequences were expressed in trophozoites. In addition, the phylogenetic analysis showed four well-resolved terminal clades, confirming the presence of two ADA orthologues for *T. vaginalis* in the second clade with other protozoa species, *E. histolytica* and *D. discoideum* sequences.

*Trichomonas vaginalis* ADA could be involved in the inflammatory process generated during the infection. Neutrophils are the predominant inflammatory cells found in the vaginal discharge of patients with *T. vaginalis* infection (Demirezen *et al.*, 2000), and their recruitment is known to be mediated via interleukin-8 (IL-8) (Ryu *et al.*, 2004). Extracellular ATP stimulates IL-8 release and, conversely, adenosine inhibits IL-8 secretion (Bouma *et al.*, 1996; Kukulski *et al.*, 2009). Our contribution differs from that of Munagala & Wang (2003), who identified the presence of ADA activity in *T. vaginalis* lysates, in the parasites' cytoplasm. The present study was performed using intact trophozoites, indicating the presence of extracellular ADA activity. During the infection, it is conceivable that *T. vaginalis* requires the uptake of adenosine, the primary precursor of all purine nucleotides. Consequently, decreased amounts or the lack of adenosine as an anti-inflammatory agent could result in acute symptoms due to raised inflammation. To overcome this adverse situation, the parasite has ADA activity to degrade adenosine to inosine, which also presents anti-inflammatory effects (Haskó *et al.*, 2004). In addition, both endothelial cells and neutrophils have been consistently reported to release high levels of adenosine at sites of metabolic distress, inflammation and infection. The

concentrations of extracellular adenosine are below 1.0  $\mu\text{M}$  in unstressed tissues, whereas adenosine levels in inflamed or ischemic tissues can be as high as 100  $\mu\text{M}$  (Haskó & Cronstein, 2004). Therefore, in the microenvironment of trichomonad infection, ADA would modulate the adenosine:inosine ratio and the maintenance of related immunological properties through different nucleoside signalling mechanisms at immune cells. Further studies are necessary to better understand the physiological significance of this enzyme in *T. vaginalis* and the association with the mechanisms involved in specific host–parasite interactions.

## Acknowledgements

M.W. is a recipient of a fellowship from BIC/PROPESQ/UFRGS; P.d.B.V. and D.B.R. from CAPES; and A.P.F., from CNPq. This study received financial support from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil, T.T., grant #477348/2008-4).

## References

- Ali EMM (2008) *Fasciola gigantica*: purification and characterization of adenosine deaminase. *Exp Parasitol* **119**: 285–290.
- Bouma MG, Van den Wildenberg FA & Buurman WA (1996) Adenosine inhibits cytokine release and expression of adhesion molecules by activated human endothelial cells. *Am J Physiol Cell Ph* **270**: C522–C529.
- Bours MJL, Swennen ELR, Di Virgilio F, Cronstein BN & Dagnelie PC (2006) Adenosine 5'-triphosphate and adenosine as endogenous signaling molecules in immunity and inflammation. *Pharmacol Ther* **112**: 358–404.
- Boyum A (1968) Isolation of mononuclear cells and granulocytes from human blood. Isolation of mononuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1g. *Scand J Clin Lab Inv Suppl* **97**: 77–89.
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal Biochem* **72**: 248–254.
- Burnstock G & Verkhatsky A (2009) Evolutionary origins of the purinergic signalling system. *Acta Physiol* **195**: 415–447.
- Centelles JJ, Franco R & Bozal J (1988) Purification and partial characterization of brain adenosine deaminase: inhibition by purine compounds and by drugs. *J Neurosci Res* **19**: 258–267.
- Charlab R, Rowton ED & Ribeiro JMC (2000) The salivary adenosine deaminase from the sand fly *Lutzomyia longipalpis*. *Exp Parasitol* **95**: 45–53.
- Cherpes TL, Wiesenfeld HC, Melan MA, Kant JA, Cosentino LA, Meyn LA & Hillier SL (2006) The associations between pelvic inflammatory disease, *Trichomonas vaginalis* infection, and positive *Herpes Simplex* virus type 2 serology. *Sex Transm Dis* **33**: 747–752.



- Cooper BF, Sideraki V, Wilson DK, Dominguez DY, Clark SW, Quioco FA & Rudolph FB (1997) The role of divalent cations in structure and function of murine adenosine deaminase. *Protein Sci* **6**: 1031–1037.
- Demirezen S, Safi Z & Beksaç S (2000) The interaction of *Trichomonas vaginalis* with epithelial cells, polymorphonuclear leucocytes and erythrocytes on vaginal smears: light microscopic observation. *Cytopathology* **11**: 326–332.
- Diamond LS (1957) The establishment of various *Trichomonas* of animals and man in axenic cultures. *J Parasitol* **43**: 488–490.
- Franco R, Casadó V, Ciruela F, Saura C, Mallol J, Canela EI & Lluís C (1997) Cell surface adenosine deaminase: much more than an ectoenzyme. *Prog Neurobiol* **52**: 283–294.
- Giordani RB, Weizenmann M, Rosemberg DB, De Carli GA, Bogo MR, Zuanazzi JAS & Tasca T (2010) *Trichomonas vaginalis* nucleoside triphosphate diphosphohydrolase and ecto-5'-nucleotidase activities are inhibited by lycorine and candimime. *Parasitol Int* **59**: 226–231.
- Giusti G (1974) Adenosine deaminase. *Methods of Enzymatic Analysis* (Bergmeyer HU, ed), pp. 1092–1099. Academic Press, New York.
- Gounaris K (2002) Nucleotidase cascades are catalyzed by secreted proteins of the parasitic nematode *Trichinella spiralis*. *Infect Immun* **70**: 4917–4924.
- Gounaris K & Selkirk ME (2005) Parasite nucleotide-metabolizing enzymes and host purinergic signalling. *Trends Parasitol* **21**: 17–21.
- Grodstein F, Goldman MB & Cramer DW (1993) Relation of tubal infertility to history of sexually transmitted diseases. *Am J Epidemiol* **137**: 577–584.
- Hanley PJ, Musset B, Renigunta V, Limberg SH, Dalpke AH, Sus R, Heeg KM, Preisig-Muller R & Daut J (2004) Extracellular ATP induces oscillations of intracellular  $Ca^{2+}$  and membrane potential and promotes transcription of IL-6 in macrophages. *P Natl Acad Sci USA* **101**: 9479–9484.
- Haskó G & Cronstein BN (2004) Adenosine: an endogenous regulator of innate immunity. *Trends Immunol* **25**: 33–39.
- Haskó G, Sitkovsky MV & Szabó C (2004) Immunomodulatory and neuroprotective effects of inosine. *Trends Pharmacol Sci* **25**: 152–157.
- Heyworth PG, Gutteridge WE & Ginger CD (1982) Purine metabolism in *Trichomonas vaginalis*. *FEBS Lett* **141**: 106–110.
- Heyworth PG, Gutteridge WE & Ginger CD (1984) Pyrimidine metabolism in *Trichomonas vaginalis*. *FEBS Lett* **176**: 55–60.
- Houang ET, Ahmet Z & Lawrence AG (1997) Successful treatment of four patients with recalcitrant vaginal trichomoniasis with a combination of zinc sulfate douche and metronidazole therapy. *Sex Transm Dis* **24**: 116–119.
- Iwaki-Egawa S & Watanabe Y (2002) Characterization and purification of adenosine deaminase 1 from human and chicken liver. *Comp Biochem Phys B* **133**: 173–182.
- Iwaki-Egawa S, Namiki C & Watanabe Y (2004) Adenosine deaminase 2 from chicken liver: purification, characterization, and N-terminal amino acid sequence. *Comp Biochem Phys B* **137**: 247–254.
- Klebanoff MA, Carey JC, Hauth JC *et al.* (2001) Failure of Metronidazole to prevent preterm delivery among pregnant women with asymptomatic *Trichomonas vaginalis* infection. *New Engl J Med* **345**: 487–493.
- Kucknoor AS, Mundodi V & Alderete JF (2005) Adherence to human vaginal epithelial cells signals for increased expression of *Trichomonas vaginalis* genes. *Infect Immun* **73**: 6472–6478.
- Kukulski F, Ben Yebdri F, Lecka J, Kauffenstein G, Lévesque SA, Martín-Satué M & Sévigny J (2009) Extracellular ATP and P2 receptors are required for IL-8 to induce neutrophil migration. *Cytokine* **46**: 166–170.
- Langley JG, Goldsmid JM & Davies N (1987) Venereal trichomoniasis: role of men. *Genitourin Med* **63**: 264–267.
- Lehker MW & Alderete JF (2000) Biology of trichomonosis. *Curr Opin Infect Dis* **13**: 37–45.
- Lupidi G, Falasca M, Marmocchi F, Venardi G, Cristalli G & Riva F (1992) Adenosine deaminase from bovine brain: purification and partial characterization. *Biochem Int* **26**: 1053–1063.
- Maier S, Galellis J & McDermid H (2005) Phylogenetic analysis reveals a novel protein family closely related to adenosine deaminase. *J Mol Evol* **61**: 776–794.
- Matos JAA, Borges FP, Tasca T, Bogo MR, De Carli GA, Da Graça Fauth M, Dias RD & Bonan CD (2001) Characterization of an ATP diphosphohydrolase (Apyrase, EC 3.6.1.5) activity in *Trichomonas vaginalis*. *Int J Parasitol* **31**: 770–775.
- Michel R, Borges F, Wiltuschnig R, Neves F, Ribeiro J, Vieiro R, Vieira PB, Bohns G, Tasca T & De Carli GA (2006) Prevalência da tricomonose em mulheres residentes na Vila dos Papeleiros em Porto Alegre, RS. *RBAC* **38**: 127–130.
- Minotto L, Ko G-A, Edwards MR & Bagnara AS (1998) *Trichomonas vaginalis*: expression and characterisation of recombinant S-adenosylhomocysteinase. *Exp Parasitol* **90**: 175–180.
- Mohamed T (2006) Adenosine deaminase from camel tick *Hyalomma dromedarii*: purification and characterization. *Exp Appl Acarol* **40**: 101–111.
- Munagala NR & Wang CC (2003) Adenosine is the primary precursor of all purine nucleotides in *Trichomonas vaginalis*. *Mol Biochem Parasit* **127**: 143–149.
- Petrin D, Delgaty K, Bhatt R & Garber G (1998) Clinical and microbiological aspects of *Trichomonas vaginalis*. *Clin Microbiol Rev* **11**: 300–317.
- Robson SC, Sévigny J & Zimmermann H (2006) The E-NTPDase family of ectonucleotidases: structure function relationships and pathophysiological significance. *Purinergic Signal* **2**: 409–430.
- Rosemberg DB, Rico EP, Senger MR, Dias RD, Bogo MR, Bonan CD & Souza DO (2008) Kinetic characterization of adenosine deaminase activity in zebrafish (*Danio rerio*) brain. *Comp Biochem Phys B* **151**: 96–101.
- Rückert C, Stuepp CS, Gottardi B *et al.* (2010) *Trichomonas vaginalis*: dehydroepiandrosterone sulfate and 17[beta]-estradiol alter NTPDase activity and gene expression. *Exp Parasitol* **125**: 187–195.
- Ryu J-S, Kang J-H, Jung S-Y, Shin M-H, Kim J-M, Park H & Min D-Y (2004) Production of interleukin-8 by human neutrophils

- stimulated with *Trichomonas vaginalis*. *Infect Immun* **72**: 1326–1332.
- Saitou N & Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**: 406–425.
- Sansom FM, Robson SC & Hartland EL (2008) Possible effects of microbial ecto-nucleoside triphosphate diphosphohydrolases on host–pathogen interactions. *Microbiol Mol Biol R* **72**: 765–781.
- Schmitz C, Deason F & Perraud A-L (2007) Molecular components of vertebrate  $Mg^{2+}$ -homeostasis regulation. *Magnes Res* **20**: 6–18.
- Sharoyan S, Antonyan A, Mardanyan S, Lupidi G & Cristalli G (2006) Influence of dipeptidyl peptidase IV on enzymatic properties of adenosine deaminase. *Acta Biochim Pol* **53**: 539–546.
- Singh L & Sharma R (2000) Purification and characterization of intestinal adenosine deaminase from mice. *Mol Cell Biochem* **204**: 127–134.
- Sorvillo F, Smith L, Kerndt P & Ash L (2001) *Trichomonas vaginalis*, HIV, and Africans. *Emerging Infect Dis* **7**: 927–932.
- Stevens-Simon C, Jamison J, McGregor JA & Douglas JM (1994) Racial variation in vaginal pH among healthy sexually active adolescents. *Sex Transm Dis* **21**: 168–172.
- Tamura K, Dudley J, Nei M & Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**: 1596–1599.
- Tasca T, Bonan CD, De Carli GA, Battastini AM & Sarkis JJ (2003) Characterization of an ecto-5'-nucleotidase (EC 3.1.3.5) activity from intact cells of *Trichomonas vaginalis*. *Exp Parasitol* **105**: 167–173.
- Tasca T, Bonan CD, De Carli GA, Sarkis JJ & Alderete JF (2005) Heterogeneity in extracellular nucleotide hydrolysis among clinical isolates of *Trichomonas vaginalis*. *Parasitology* **131**: 71–78.
- Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F & Higgins DG (1997) The CLUSTAL\_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**: 4876–4882.
- Van Der Pol B, Kwok C, Pierre-Louis B et al. (2008) *Trichomonas vaginalis* Infection and Human Immunodeficiency Virus Acquisition in African Women. *J Infect Dis* **197**: 548–554.
- Viikki M, Pukkala E, Nieminen P & Hakama M (2000) Gynaecological infections as risk determinants of subsequent cervical neoplasia. *Acta Oncol* **39**: 71–75.
- World Health Organization (2001) Global prevalence and incidence of selected curable sexually transmitted infections. Overview and Estimates, WHO/HIV\_AIDS/2001.02 and WHO/CDS/CSR/EDC/2001.10. WHO, Geneva.

## Supporting information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Time (a) and protein (b) concentration curves to ensure linearity on ADA activity in intact trophozoites *Trichomonas vaginalis*.

**Table S1.** Substrate specificity of ADA from *Trichomonas vaginalis*.

**Table S2.** ADA-specific activities from different *Trichomonas vaginalis* isolates.

Please note: Wiley-Blackwell is not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.