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ORIGINAL ARTICLE

AC-ELISA and RT-PCR assays for the diagnosis of triatoma virus (TrV) in triatomines (Hemiptera: Reduviidae) species

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Abstract Triatoma virus (TrV) is the only entomopathogenic virus found in triatomines. TrV replicates in cells of the midgut epithelium of triatomines, causing a high mortality rate and delayed development of the infected insect. In this work, we report an antigen-capture enzyme-linked immunosorbent assay (AC-ELISA) and a reverse transcription-polymerase chain reaction (RT-PCR) assay for detection of TrV infection. For antiserum production, rabbits and hens where inoculated with purified TrV. Antiserum reactivity was checked by immunodiffusion, and its specificity was confirmed by western blot and AC-ELISA. Totally 90 fecal samples from *T. infestans* were analysed. AC-ELISA and RT-PCR results correlated well with transmission electron microscopy (EM) observations, which are considered the gold standard, with Kappa values of 0.73 for AC-ELISA and 0.93 for RT-PCR when compared with EM. Applications and complementary uses of the two techniques reported in this work are discussed.

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Introduction

Triatoma virus (TrV) is a spherical non-enveloped virus found in *Triatoma infestans* (*T. infestans*) bugs [1, 2]. The viral genome is a positive-sense single-stranded RNA molecule of approximately 10,000 nucleotides [3]. TrV particles are about 30 nm in diameter, and the capsid is composed of four proteins, VP0, VP1, VP2, and VP3. In 2002, the International Committee on Taxonomy of Viruses classified TrV as a member of the family *Dicistroviridae*, genus *Cripavirus*, along with a small group of insect RNA viruses including cricket paralysis virus [4]. TrV replicates in cells of the intestinal epithelium of triatomines, causing high mortality, delayed development due to its effect on the molting process, and reduced fecundity [2, 5, 6]. The pathogenicity and the mode of transmission of TrV have been partially elucidated, indicating coprophagy as one of the main routes of infection in laboratory colonies [5, 7].

The biological, ecconomical and medical relevance of TrV is based on the fact that triatomines represent a very important link in the transmission of *Trypanosoma cruzi* (*T. cruzi*), the causative agent of Chagas' disease. This disease is an endemic zoonosis in Latin America, affecting 9.8 million people [8], and 15–30% of the patients have cardiac lesions or irreversible lesions in other organs [9]. To date, more than 60 species of triatomines have been found to be affected by predators, parasitoids, ecto- and endoparasites and pathogens, as well as other undetermined

symbiotic associations [10, 11]. TrV is the only viral pathogen that has been found in triatomines. It was proven to be infective in seven species that are vectors for *T. cruzi*: *T. infestans, T. pallidepennis, T. platensis, T. delpontei, T. rubrovaria, Rhodnius* (*R.*) *prolixus* [2], *T. patagonica* [12], *T. guasayana* (Rozas-Dennis et al., unpublished result), *T. dimidiata* (Zeledón 2007, personal communication; Guhl 2007, personal communication), and *T. maculata* (Guhl 2007, personal communication). Importantly, TrV can potentially be used as a biological control agent against domiciliated and non-domiciliated vectors of Chagas' disease, especially due to its vertical transmission and high pathogenicity [3, 5].

In this paper, we report the development of two specific, fast and sensitive assays for detection of TrV infection by antigen-capture enzyme-linked immunosorbent assay (AC-ELISA) and reverse transcription-polymerase chain reaction (RT-PCR). These two techniques will be useful to screen TrV infection in feces, and eventually in other insects. Furthermore, these techniques will allow the study of the natural prevalence and geographic distribution of TrV virus in populations of Triatominae in Argentina as well in other Latin American countries.

Materials and methods

Viral purification

Viral particles were purified from insects obtained from an experimentally TrV-infected colony (by feeding upon virus-contaminated substrata) of *T. infestans*, maintained at the Centro de Estudios Parasitológicos y de Vectores (CEPAVE), La Plata, Argentina. Totally 30 infected adult insects were dissected, and their midguts were homogenized in 10 ml of NMT buffer (0.01 M NaCl, 0.001 M $MgCl_2$ and 0.04 M Tris–HCl, pH 7.4). The homogenate was clarified and centrifuged at 140,000*g* for 3 h at 4°C to obtain TrV particles. The pellet was then resuspended in NMT buffer and layered on top of a continuous sucrose gradient (10–30%, w/v). After centrifugation at 64,000*g* for 3 h at 4°C, 2-ml fractions were obtained using a peristaltic pump. The selected fractions, measured at 260 nm, were diluted in NMT buffer and then centrifuged at 44,000*g* for 2 h at 4°C. This final pellet was resuspended in 1 ml TE buffer (Tris–EDTA) (1 mM EDTA, 10 mM Tris–HCl, pH 7.4). The virus was stored at −20°C and was later used for the production of hyperimmune antisera for the AC-ELISA assay and as a positive control in the RT-PCR assay. TrV-free *T. infestans*, maintained at the Centro de Estudios Parasitológicos y de Vectores (CEPAVE), La Plata, Argentina, were used as a negative control.

Antisera

The antibodies used in the AC-ELISA were prepared by inoculating two female rabbits and two hens with purified TrV. Rabbits were inoculated by subcutaneous injection of 0.5 ml of purified virus (37 μg) mixed with the same amount of Freund's complete adjuvant (Sigma-Aldrich Chemical Co., USA). A second dose was administered to the animals at day 15 after the first inoculation. The third dose was administered at day 45 (0.5 ml of virus mixed with 0.5 ml of Freund's incomplete adjuvant). The procedure for the production of anti-TrV in hens was the same as that for production in rabbits, except that the hen inoculations were performed by intramuscular injection of virus without adjuvant. Sera obtained from bleeding rabbits and hens were centrifuged at 2,000 rpm for 3 min; sera were recovered and stored at −20°C.

Immunodiffussion, SDS-PAGE and Western blot analysis

In order to confirm the presence and evaluate the concentration of specific TrV antibodies from inoculated rabbits and hens, double immunodiffussion assays in agar gels were performed in 1% agar in Tris 0.05 M and 8.5% NaCl, pH 8, for rabbits or 1% agar in 0.85% NaCl, pH 8, for hens. For Western blot analysis, TrV proteins were separated in 12.5% polyacrylamide gels using the discontinous system [13]. One gel was stained with Coomassie blue and then destained in an acid/methanol solution. The other was transferred to a nitrocellulose membrane (0.45 μm, Sigma-Aldrich Chemical Co., USA) by semi-dry electrotransfer (BioRad Laboratories, Canada) with transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol), and the membrane was blocked with 3% gelatin in TTBS (0.05% Tween-20 in TBS buffer, 20 mM Tris–HCl, pH 7.4, 0.5 M NaCl). The membranes were cut in strips and incubated with anti-TrV polyclonal antibodies diluted 1:40 in 1% gelatin TTBS. The strips were treated with anti-rabbit and anti-hen IgG peroxidase conjugates at a 1:1,000 dilution, and subsequently treated with a solution containing the chromogen DAB (3,3′diaminobenzidine, Sigma-Aldrich Chemical Co., USA).

Fecal samples

TrV-free *T. infestans* was used as a negative control, and *T. infestans* infected with TrV was used as a positive control. Two groups of 45 insects each, were maintained at the Centro de Estudios Parasitológicos y de Vectores (CEPAVE), La Plata, Argentina. Fecal samples were obtained by applying pressure to the abdominal region, and

these were resuspended in 200 µl PBS and kept in microtubes.

Standardization of the AC-ELISA

To determine the optimal reagent concentrations, several combinations and dilutions of each reagent (purified TrV, *T. infestans* fecal samples, rabbit antisera and hen antisera) were tested on polystyrene plates (checkboard titrations) [14]. Hen TrV antisera served as the capture antibodies and the rabbit TrV antisera were used as the detector antibodies for AC-ELISA assay. As a third antibody, commercial goat anti-rabbit IgG peroxidase conjugate was used (Sigma-Aldrich Chemical Co., USA). To determine the optimal dilution of the reagents, rabbit TrV antisera (1:10 to 1:10,240 dilutions) were evaluated against peroxidase-conjugated goat anti-rabbit (1:400 to 1:51,200 dilutions). Then, hen TrV antisera (1:10 to 1:10,240 dilutions) and purified TrV (1:50 to 1:6,400 dilutions) were titrated at the same manner. During this optimization process, different incubation periods (1–2 h) and concentrations of blocking reagent (2–5% nonfat dry milk in PBS-0.05% Tween 20) were tested. Purified TrV particles were used as a first step for standardization of the AC-ELISA test. Fecal samples of *T. infestans* were titrated from pure to 1:128 dilution. The best combination of the highest reagent dilution with the highest sensitivity and lowest background was selected for each reagent. Briefly, rows of 96-well microtiter plates were coated with 100 µl/well of a 1:300 dilution of hen hyperimmune TrV antisera in carbonate-bicarbonate buffer (0.05 M, pH 9.6) and incubated at 4°C overnight. After incubation and at each washing step, the plates were rinsed 4 times with PBS/0.05% Tween 20. Subsequently, the plates were blocked for 1 h at 37°C with 200 µl 5% nonfat dry milk in PBS to minimize nonspecific binding. Then, 100 µl of the fecal samples, diluted 1:4 in PBS/0.05% Tween 20, were placed in each well and incubated for 1.5 h at either room temperature or at 37°C. Rabbit TrV antisera diluted 1:400 in PBS/0.05% Tween 20 was applied for 1 h at 37°C. Next, 100 µl of a commercial goat anti-rabbit IgG peroxidase conjugate (Sigma-Aldrich Chemical Co., USA) diluted 1:3,000, was applied. Reactions were developed using 100 µl of the chromogen substrate (0.1 M citric acid, 0.2 M PO_4HNa_2, 10 µl H_2O_2, 30 vol) and 30 mg ABTS (2,2′ azino-bis-3 ethylbenzthiazoline-6-sulfonic acid, Sigma-Aldrich Chemical Co., USA) and read after 5, 10 and 15 min of incubation in a microplate reader (Titertek Multiskan, USA) with a 405-nm filter. Negative and positive control samples were included in each microplate. The data were analyzed as follows: normalized OD = raw OD of sample—raw OD of negative control/raw OD of positive control—raw OD of negative control. To determine a reliable cut-off, a total of 50 samples of TrV-free insects from the *T. infestans* colony were used to establish the normal range of variability during the AC-ELISA test.

RNA extraction

TrV RNA (vRNA) was extracted from purified virus and feces of *T. infestans*. Up to 0.3 mg of fecal samples resuspended in 50 µl PBS were homogenized in TRIZOL reagent (GIBCO-Invitrogen, USA), and vRNA was purified according to the manufacturer's instructions. RNA concentration was determined by measuring absorbance at 260 nm (A_{260}) in a spectrophotometer ($A_{260} = 1$ is equivalent to 40 µg/ml RNA). A ratio of $A_{260}/A_{280} = 2.0$ was obtained for pure vRNA.

RT-PCR

Pure vRNA (0.2 µg) was used as a template for RT-PCR. The first-round PCR was performed according to the OneStep RT-PCR protocol (QIAGEN, USA). It involved reverse transcription followed by one round of amplification. The following oligonucleotide primer pairs were used: TrVA- 5′ TCAAAACTAACTATCATTCTGG 3′ (nt 7427 to 7448 in TrV ORF2 sequence) and TrVB- 5′ TTCAGCCT-TATTCCCCCCC 3′ (nt 8240–8258), with an expected product of 832 bp. Primer sequences were selected from TrV-ORF2 regions that code for VP2 and VP3 capsid proteins, based on the GenBank AF 178440 sequence. Products were visualized on 1.2% agarose gels stained with ethidium bromide, and their sizes were determined by comparison against DNA markers (Promega, USA). To standarize the technique, purified TrV was used as a template, then fecal samples were used.

Electron microscopy

Negative-staining electron microscopy (EM) examinations were performed as described previously [15]. A total of 45 negative and 45 positive fecal samples were selected. Each sample was examined for approximately 10 min. This technique is considered the gold standard for evaluation of TrV infection.

Statistical analysis

AC-ELISA and RT-PCR assays were compared with EM to evaluate their specificity and sensitivity. Kappa values were calculated for the AC-ELISA and RT-PCR assays to measure their agreement with the EM results.

Results

Virus and antiserum characterization

Purification of virus particles from intestinal contents of infected insects results in a viral suspension of approximately 2.6 mg/ml protein. Four bands, corresponding to the four structural proteins of the TrV (VP0, VP1, VP2 and VP3), were detected by SDS-PAGE, with molecular weights of approximately 45, 39, 37 and 33 kDa, respectively, according to their electrophoretic mobility (Fig. 1a). The antisera of rabbits inoculated with TrV were positive against the TrV virus by immunodiffussion up to 1:128 dilution (Fig. 1b), The antisera of hens inoculated with TrV were positive against the TrV virus by immunodiffussion up to 1:16 dilution. The specificity of rabbit and hen anti-TrV polyclonal antibodies was confirmed by Western blot analysis (Fig. 1c).

AC-ELISA

The optimal dilutions of reagents in our AC-ELISA were 1:300 for the primary serum antibody TrV antisera obtained from inoculated hen, 1:4 for fecal samples, 1:400 for the secondary antibody TrV (rabbit antisera), and 1:3,000 for goat anti-rabbit peroxidase-conjugate. The reading time was optimized at 15 min. Optimal results for detection of TrV infection in fecal samples by AC-ELISA were obtained using 5% nonfat dry milk in PBS-0.05% Tween 20 as blocking solution. Up to 0.3 mg of fecal samples resuspended in 200 µl PBS showed a positive result at 1:64 dilution. The AC-ELISA values of fecal samples showed a normal distribution (data not shown). Assuming a 5% probability of misclassification (mean ± 2SD), the upper limit of an AC-ELISA value for the negative insect was 0.12. These fecal samples were used to compare the AC-ELISA test against EM and RT-PCR. Of a total of 90 fecal samples analyzed, 35 were positive (38.9%) and 55 were negative (61.1%) (Table 1).

RT-PCR

Fecal samples of TrV-infected *T. infestans* were analyzed by RT-PCR using the primer pair mentioned in "Materials and methods". The results are shown in Fig. 2. The expected 832-bp RT-PCR product was obtained from 0.2 µg of pure vRNA, and bands of the same size were found in the positive analyzed samples (Fig. 2). Out of a total of 90 fecal samples analyzed by RT-PCR, 46 were positive (51.1%) and 44 were negative (48.9%) (Table 1).

EM

The fecal samples analyzed with RT-PCR and AC-ELISA were also tested by EM. The viral particles detected in fecal samples by negative-staining EM are illustrated in Fig. 3. As expected from previous observations [1, 15], the average size of TrV particles was approximately 30 ± 2 nm.

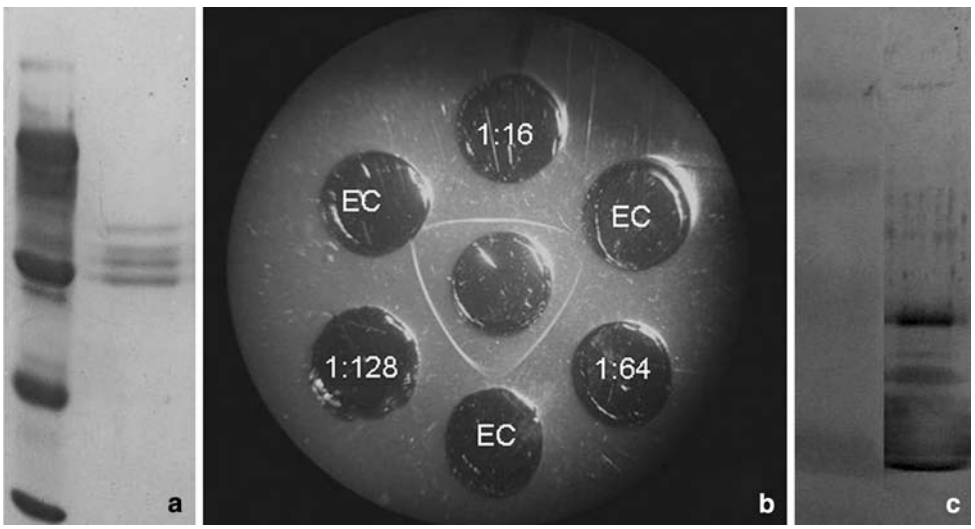

Fig. 1 **a** This picture shows the four bands corresponding to the structural proteins of the triatoma virus, VP0, VP1, VP2 and VP3, with molecular weights of 45, 39, 37 and 33 kD, respectively, detected by SDS-PAGE. **b** Different dilutions of rabbit TrV antisera (1:16, 1:64, 1:128) tested by immunodiffussion against purified virus, showing the positive immunoprecipitation lines. EC means empty cavity. **c** Western blot analysis confirming the specificity of the rabbit TrV polyclonal antibody shows the four (VP0, VP1, VP2 and VP3) structural proteins of triatoma virus

Table 1 Detection of TrV in fecal samples by EM, AC-ELISA and RT-PCR

	AC-ELISA		RT-PCR	
	Positive	Negative	Positive	Negative
EM positive	34 (37.8%)	11 (12.2%)	44 (48.9%)	1 (1.1%)
EM negative	1 (1.1%)	44 (48.9%)	2 (2.2%)	43 (47.8%)
Total	35 (38.9%)	55 (61.1%)	46 (51.1%)	44 (48.9%)

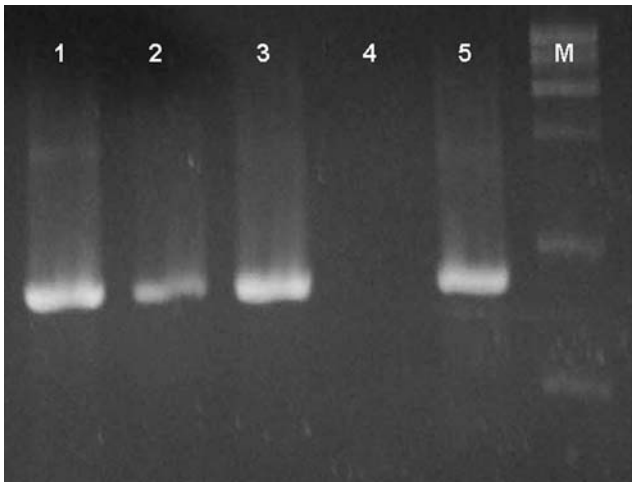

Fig. 2 Detection of triatoma virus (TrV) by RT-PCR in intestinal contents and fecal samples of *Triatoma infestans*. M: 1000-bp molecular ladder. *1–2* positive intestinal content samples. *3* positive fecal sample. *4* negative intestinal content sample. *5* purified TrV, used as a positive control

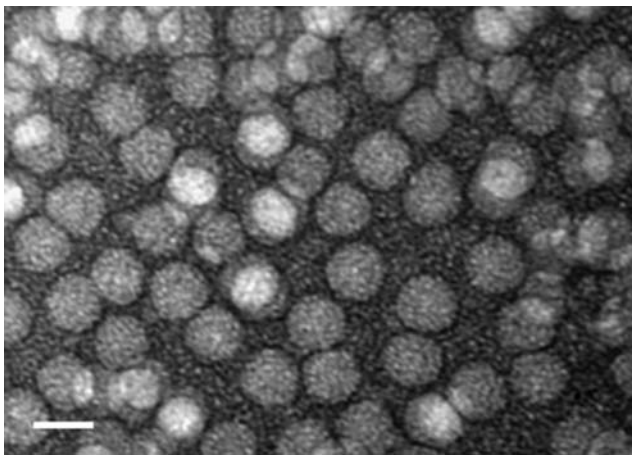

Fig. 3 Electron micrograph [negative staining, 3% (w/v) uranyl acetate] of viral particle preparations from fecal samples. *Bar* 30 nm

Statistical analysis

The concordance obtained after analysis of AC-ELISA and RT-PCR results against the EM test is shown in (Table 2). The AC-ELISA and RT-PCR assays showed different agreements with EM, with a Kappa value of 0.73 for AC-ELISA and 0.93 for RT-PCR. When compared with the EM gold standard, AC-ELISA and RT-PCR had 75.55 and 97.78% sensitivity and 97.77 and 95.55% specificity, respectively.

Table 2 Sensitivity, specificity and concordance values of AC-ELISA and RT-PCR with EM as the gold standard test for TrV detection

	Sensitivity %	Specificity %	Kappa value
ELISA versus EM	75.55	97.77	0.73
RT-PCR versus EM	97.78	95.55	0.93

Discussion

TrV is the only virus found in triatomines. Since TrV was discovered in *T. infestans* [1] and is geographically distributed in Argentina [16], its potential use as a biological control agent against the vectors of Chagas' disease could be considered a valid option. Nevertheless, the prevalence of TrV in triatomines in other Latin American Chagas' endemic areas is still unknown. The development of rapid, non-expensive and reliable diagnostic techniques to detect TrV infection could be a useful advantage in prevalence studies. Although EM is very reliable for virus particle detection [15], this technique is labour-intensive and time-consuming. In addition, EM may have a poor sensitivity at low virus titers, and it requires expensive equipment, making this technique inappropriate for screening a large number of samples.

RT-PCR and AC-ELISA have been used to detect a variety of RNA viruses, including picorna-like insect viruses [17–20]. The data reported here show that RT-PCR and AC-ELISA techniques are sensitive tests for the detection of TrV infections in fecal samples of triatomines.

In our experiments, RT-PCR was more specific and sensitive than AC-ELISA, with a Kappa value of 93% despite the fact that RT-PCR on insects are usually hampered by the presence of inhibitory components [21]. RT-PCR is more time-consuming and costly than AC-ELISA, but the latter assay has been tested in different virus infections [22–24] and seems to be more convenient for screening a large number of samples. We suggest that RT-PCR should be used for suspicious samples in which AC-ELISA has given negative or borderline results, in order to reduce the risk of false negatives.

In conclusion, the use of the assays reported in this study could be valuable in the analysis of TrV host range, prevalence and geographical distribution of the virus. They may also provide a valuable tool for evaluating the

potential use of TrV as a biological control agent against triatomines.

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