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ORIGINAL PAPER

Changes of RAPD profile of *Trypanosoma cruzi* II with *Canova* and Benznidazole

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Chagas disease, caused by the protozoan *Trypanosoma cruzi*, involves immunomediated processes. *Canova* (*CA*) is a homeopathic treatment indicated in the diseases in which the immune system is depressed. This study evaluated the Random Amplification of Polymorphic DNA (RAPD) profile of *T. cruzi* under the influence of *CA* and Benznidazole (BZ). Mice infected with the genetic lineage of *T. cruzi* II (Y strain) were divided into 4 groups:
Infected animals treated with saline solution (control group); treated with *CA*; treated with *BZ*; treated with *CA* and BZ combined.
Treatment was given at the 5th–25th days of infection (D5–25). The parasites were isolated by haemoculture in Liver Infusion Tryptose (LIT) medium: at D5 (before treatment), D13, 15 and 25 (during treatment) and D55 and 295 (after treatment). DNA was extracted from the mass of parasites. RAPD was done with the primers λgt11-F, M13F-40 and L15996, the amplified products were eletrophoresed through a 4% polyacrylamide gel. Data were analyzed by the coefficient of similarity using the DNA-POP program.
163 markers were identified, 5 of them monomorphic. *CA* did not act against the parasites when used alone. The RAPD profiles of parasites treated with BZ and *CA* + BZ were different from those in the control group and in the group treated with *CA*. The actions of the *CA* and BZ were different and the action of BZ was different from the action of *CA* + BZ. These data suggest that *CA* may interact with BZ. The differences in the RAPD profile of the Y strain of *T. cruzi* produced by BZ, *CA* + BZ and the natural course of the infection suggest selection/suppression of populations. *Homeopathy* (2008) **97**, 59–64.

Keywords: *Trypanosoma cruzi*; *T. cruzi* II; RAPD; *Canova* medication; Benznidazole; Homeopathy

Introduction

Chagas disease is caused by *Trypanosoma cruzi*. There are an estimated 16 million infected people in South Americat[1] 3.5 million of them in Brazil.[2] The disease includes immunomediated processes and the presence of the parasite is important in maintenance and clinical evolution of the disease, particularly the cardiomyopathy justifying aetiological treatment.[3] Recent studies support an autoimmune pathogenesis for chronic Chagas' myocarditis. However, the characteristics of the inflammatory infiltrate in human myocarditis include a wide variety of cells, many of them not involved in autoimmunity.[4] It has been demonstrated that residual antigens of the parasite, present during the chronic phase of infection, are sufficient to maintain the immune response and consequently damage to the host tissue.[5] In Brazil, only Benznidazole (BZ) is licensed to treat this disease. This drug can

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cause serious adverse effects and is of low efficacy in the chronic phase of Chagas disease.[6,7] As most of the infected population is in this phase, the search for an alternative treatment has been the purpose of many studies.[8,9]

Homeopathy is one of the most used complementary or alternative medicine methods in the world.[10] *Canova* (*CA*) is a medicine developed according to homeopathic techniques of Hahnemann. The final product contains *Aconitum* (Ranunculaceae) 11dH, *Bryonia* (Curcubitaceae) 19dH, *Thuja* (Cupresaceae) 19dH, *Arsenicum album* (arsenic trioxide) and *Lachesis* (Viperidae) 18dH and less than 1% alcohol all in distilled water. *CA* is already in use by patients in Brazil, it appears to have very few adverse effects but until now only a few pre-clinical studies have been published. It is claimed to enhance the individual's own immunity, promoting an immunological response against several pathologic states. It is indicated in diseases in which the immune system is depressed.[11–13] *CA* is said to induce natural defenses and has been referred to as a modifier of the biological response.[14] It interferes in the production of cytokines, interleukin (IL)-2, interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α). It produces, *in vitro*, changes in the morphology of macrophages altering the distribution of integrins, actin filaments and crystallizable fragment of antibody (FC) receptors.[14] There are few controlled experiments on homeopathy for immunological conditions, there are insufficient data to reach a firm conclusions.[15]

Research done by our group[9] has demonstrated that *CA* modifies the course of experimental *T. cruzi* infection. It changes the pattern of the tissue tropism of the Y strain of *T. cruzi* which is related to the genetic subdivision *T. cruzi* II,[16] increasing significantly the parasitism in the skeletal muscle and mainly in cardiac muscle. The doubt that emerges from this study is whether this change is related to genetic changes of the strain submitted to the action of *CA* or not. To deal with this question, the Random Amplification of Polymorphic DNA (RAPD) technique was used. RAPD may identify polymorphisms that are detected as fragments of DNA, amplified by polymerase chain reaction (PCR), that are present in one but not in another sample.[17,18] We used this technique to study the action of the *CA* and Benznidazole in experimental murine infection.

Material and methods

Parasites and inocula

A *T. cruzi* Y strain[19,20] belonging to the genetic subgroup *T. cruzi* II was used.[16] The inoculum was of 10,000 trypomastigotes per animal by single intraperitoneal injection.

Animals

Forty male Swiss mice aged 4 weeks, were divided into experimental groups of 10 animals each:

Group 1: control: infected animals treated with saline solution,
Group 2: infected animals treated with BZ,
Group 3: infected animals treated with *CA*,
Group 4: infected animals treated with *CA* + BZ.

Parasites were obtained at D5 (before treatment), D13, 15 and 25 (during treatment) and D55 and 295 (after treatment). Blood was collected by venepuncture at the orbital plexus using 3 animals at each evaluated time point, the parasites were cultured in haemoculture in LIT medium. The animals were anesthetised for venepuncture and at D295 were euthanased by ketamine chloridrate (50 mg/kg) and xylazine (10 mg/kg) by single intraperitoneal injection.

Canova (*CA*)

CA is produced in drops, inhalant and intravenous forms, only by authorized pharmacies and laboratories. Canova is standardized and authorized by competent agencies for medical application. It is currently registered as a magistral formula, according to Law Nº 5991/73. This commercial medicament follows Hahnemann's homeopathic techniques. *CA* is a colorless and odorless solution. These experiments were performed with commercial Canova purchased from Canova do Brasil, batch numbers 2401221 and 2409181.

Benznidazole

N-Benzil-2-nitro-1-imidazolacetamide produced by the pharmaceutical company Roche under commercial name of Rochagan was used. To dissolve Benznidazole water and gum arabic were used.

Treatment

The treatment was begun on the 5th day after infection. The *CA* was dispensed in a dose of 0.2 mL/day for each animal, with a syringe into the oral cavity, for 20 consecutive days. BZ suspension was administered in a dose of 100 mg/kg of animal per day in 0.2 mL orally, for 20 consecutive days. The control group was treated with saline solution in a dose of 0.2 mL/day for each animal, orally for 20 consecutive days.

Genetic characterization

Method of obtaining a cellular mass of parasites: after 20 days of culture at 28°C, the erythrocytes were eliminated and the cultures were maintained until approximately 1×10^9 parasites. The material obtained from the 3 animals used for each evaluated time per treatment, was pooled.

DNA extraction and dosage

The DNA was extracted and dosed from the cell mass of each sample as described by Macedo.[21]

Determination of the RAPD profiles

The total DNA was in a concentration of 1 ng/mL and amplified by the RAPD technique. Three primers were used: λgt11-F (5'GACTCCTGGA GCCCG3'), M13F-40 (5'GTTTTCCCAGTCACGAC3') and L15996 (5'CTCCAC CATTAGCACCCAAAGC3') in accordance to Gomes *et al*.[22] After amplification, 10 μL of the products were eletrophoresed in polyacrilamide gel (4.5%), silver stained and

photographed. We used a marker of molecular weight of 1 kb plus DNA ladder (Gibco BRL).

Analysis

The presence or absence of bands was analyzed. The coefficient of similarity (CS) was calculated by using the DNA-POP program. The phenogram was generated from the coefficients of dissimilarity (CD) (CD = 1 − CS) based on the matrix of genetic distance obtained through the method UPGMA (Unweighted Pair Group Method with Arithmetic Averages), data obtained from the average of the 3 markers.

Ethics

This work was approved by the Experimental Animal Research Ethics Committee of Universidade Estadual de Maringá (UEM).

Results

Figure 1 shows the electrophoresis gels of the 3 primers used (λgt11-F, M13F-40 and L15996). The genetic profiles of parasites obtained from the different treatments with the 3 primers resulted in a good amplification. Due to the variation in the intensity and low number of large bands above 1650 bp, only bands between 200 and 1650 bp were considered in the analysis. In this interval 163 markers were identified, 158 of them polymorphic (93.6%) and 5 monomorphic (5.4%). For the primer λgt11-F, 3 monomorphic markers were observed in the region of 300 bp, between 300 and 400 bp and at 200 bp (Figure 1a). For the primer L15996, 2 monomorphic markers were observed, 1 close to 100 bp and the other at approximately 200 bp (Figure 1b). These 5 monomorphic markers were present at all evaluated time points. For the primer M13F-40 no marker was observed (Figure 1c).

Figure 2 shows the matrix for the averages of the coefficient of similarities for the 3 primers. The CS obtained from the profile of RAPD of parasites obtained from animals before the treatment, the control group treated with saline and the group treated with the *CA* until the D13 day were greater than 80% (Figure 2a). The similarity between the control group and group treated with *CA* on D15 day of infection was 80% (Figure 2b). The CS for the control group on D13 and D15 was 53% (Figure 2c). The CS for RAPD profile for the group of parasites taken from animals before treatment and in the control group on D15 day was 52% (Figure 2d). The CS obtained from animals before treatment and the BZ-treated group on the D13, 15, 25, 55 and 295 was 61%, 53%, 67%, 61% and 47%, respectively (Figure 2e). The similarity between parasites obtained from group treated with *CA* and *CA* + BZ was 53% and 58% for D13 and 15 day of infection, respectively (Figure 2f). The CS between the parasites obtained before treatment and group treated with *CA* + BZ was 55%, 44%, 49% and 63% on D13, 15, 25, 55 and 295 day of infection, respectively (Figure 2g). The similarity between the parasites got from group treated with BZ and *CA* + BZ was 45%, 65%, 66% and 74% on D13, 15, 25, 55 and 295 days of infection, respectively (Figure 2h).

The phenogram obtained by UPGMA with 3 markers for the different groups is shown in Figure 3. This phenogram reflects the finding that parasites obtained on D13 in the control group and the group treated with *CA* were genetically different from the parasites treated with BZ and *CA* + BZ.

Discussion

This study evaluated the effect of *CA* and BZ on the evolution of the RAPD profile of *T. cruzi* in experimentally infected mice. The results showed differences in the RAPD profiles for the 3 primers used. The region considered in the analysis was between 200 and 1650 bp. Although D'Ávila *et al*.,[23] used the same primers, they considered the region 250–3000 bp as the basis for analysis. These authors had as the purpose of correlating the profile of RAPD with the clinical aspects of Chagas disease, analyzing several strains of *T. cruzi*. In this study, the analysis was done in a shorter time frame than that of D'ávila *et al*.[23] Because we used a single strain the differences were smaller.

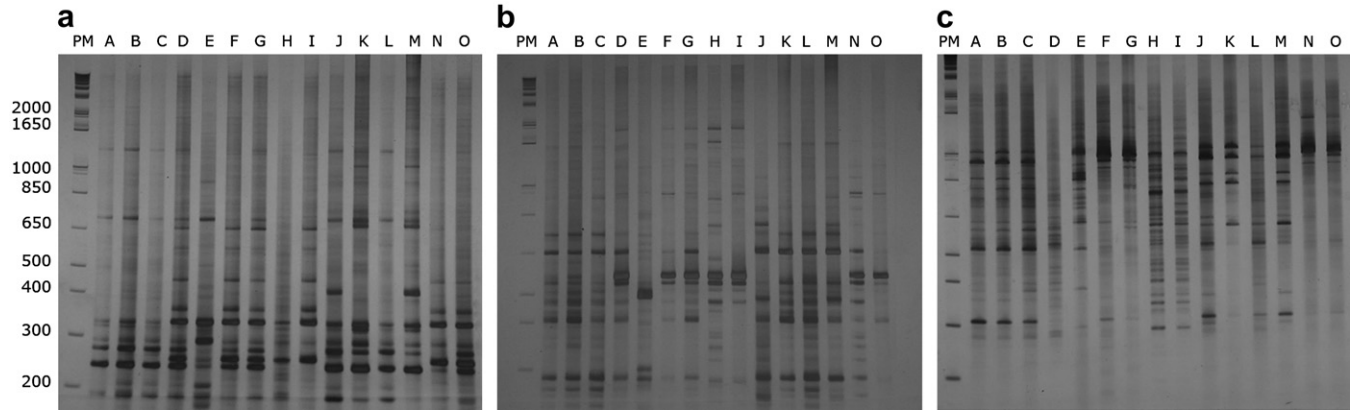

Figure 1 a–c: Polyacrylamide gel showing RAPD profiles of Y strain *T. cruzi* obtained from mice, treated with saline, Benznidazole and *Canova* (*CA*). Primers used: λgt11, L15996 and M13F-40. Left scale molecular weight size markers of some DNA fragments of 1 kb plus ladder scale (Gibco BRL). A: D5 (before treatment); B: D13 saline control; C: D13 treated with *CA*; D: D13 treated with BZ; E: D13 treated with *CA* + BZ; F: D15 control; G: D15 *CA*; H: D15 BZ; I: D15 *CA* + BZ; J: D25 BZ; K: D25 *CA* + BZ; L: D55 BZ; M: D55 *CA* + BZ; N: D295 BZ; and O: D295 *CA* + BZ.

	13º C	13º CM	13º BZ	13º CM+BZ	15º C	15º CM	15º BZ	15º CM+BZ	25º BZ	25º CM+BZ	55º BZ	55º CM+BZ	295º BZ	295º CM+BZ
BT	0.83*a*	0.85*a*	0.61*e*	0.55*g*	0.52*d*	0.53	0.53*e*	0.44*g*	0.67*e*	0.49*g*	0.61*e*	0.63*g*	0.47*e*	0.48*g*
13ºC		0.86*a*	0.59	0.56	0.53*c*	0.53	0.58	0.46	0.62	0.51	0.68	0.62	0.45	0.45
13ºCM			0.60	0.53*f*	0.53	0.56	0.56	0.47	0.65	0.51	0.67	0.63	0.49	0.52
13ºBZ				0.45*f.h*	0.63	0.57	0.60	0.60	0.58	0.33	0.53	0.55	0.46	0.52
13ºCM+BZ					0.54	0.55	0.57	0.48	0.46	0.43	0.50	0.49	0.42	0.48
15ºC						0.80*b*	0.58	0.63	0.48	0.46	0.49	0.58	0.55	0.65
15ºCM							0.56	0.58*f*	0.49	0.47	0.50	0.55	0.54	0.61
15ºBZ								0.65*h*	0.51	0.46	0.56	0.53	0.55	0.53
15ºCM+BZ									0.34	0.34	0.49	0.48	0.52	0.54
25ºBZ										0.66*h*	0.64	0.74	0.53	0.56
25ºCM+BZ											0.67	0.74	0.50	0.56
55ºBZ												0.66*h*	0.49	0.54
55ºCM+BZ													0.61	0.64
295ºCM+BZ														0.74*h*

Figure 2 Matrix of CS obtained by visual and computer analysis of the profiles of RAPD from Y strain of *T. cruzi* isolated from mice treated with saline control (C), *Canova* (*CA*), Benznidazole (BZ) and *CA* + BZ. The numbers represent the day of infection in which the parasites were obtained (BT = before treatment). (a) CS from the group treated with saline solution and treated with CA until D13, (b) CS between control group and CA D15. (c) CS between control group on D13 and 15, (d) CS between before treatment and control group D15, (e) CS between before treatment and BZ at D13, 15, 25, 55 and 295, (f) CS between-group treatment with *CA* and *CA* + BZ at D13 and 15, (g) CS between BZ and *CA* + BZ at D295, (h) CS between BZ and *CA* + BZ at D13 15, 25, 55 and 295.

The 5 monomorphic markers identified in the region 200–1650 bp, because they are present at all evaluated time points, may be considered as genetic markers of the Y strain. These markers did not change during in the experiment, which leads us to believe that there were no genetic changes in the studied parasites.[24]

The parasites obtained from animals treated with *CA* showed high degree of similarity with control in terms of RAPD profile before treatment, the profiles of the control and group treated with *CA* evolved in the same way at D13 and 15 (during treatment). These data show that the change in the tropism observed by Pupulin *et al*.[9] were not caused by genetic change of *T. cruzi* due to the effect of *CA*, it may be related to other mechanisms. According to our results there are evidences that immune mechanisms are involved in the change of tropism above mentioned. These evidences may be confirmed by preliminary data from Pupulin *et al*.[25] showing changes in the levels of TNF-α in mice infected with *T. cruzi* and treated with *CA*.

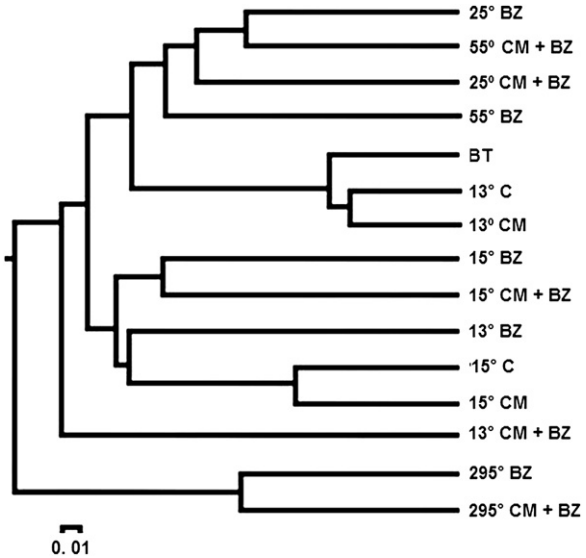

Figure 3 Phenogram obtained by UPGMA for 163 markers obtained by primers: λgt11, L15996 and M13F-40, at different days of infection and different treatments. C – saline control; BZ – infected animals treated with BZ; CA – infected animals treated with *CA*; *CA* + BZ – infected animals treated with *CA* + BZ.

The RAPD profiles of parasites under the action of BZ were different from those for the control group and the *CA* treated group. Similarities between parasites from animals treated with *CA* + BZ compared with BZ (45% and 65% at D13 and D15, respectively), or *CA* (53% and 58% at D13 and D15, respectively), were lower than those for the control group or the group treated with *CA* alone. This implies that the actions of *CA* and BZ are different and the action of BZ is different from the action of *CA* + BZ together. On comparing the RAPD profiles from groups treated with *CA* + BZ and BZ with the profile of parasites before treatment, the coefficients of similarity obtained are low and the association of complex (*CA* + BZ) presents, at most time points, less similarity to the untreated control group than BZ alone.

These data suggest that the action of *CA* interacts with that of BZ. Although this interaction was most marked at D13 (during treatment, similarity 45%), it was continued until D295 day of infection, decreasing progressively to 74%. BZ and *CA* modulate the immune system.[26,14] Romanha *et al*.[27] suggest that the activation of the immune system by the parasite and endogenous IFN-γ, has an essential role in the effects of BZ against the infection by *T. cruzi*. On the other hand, it has been shown that *CA* stimulates macrophage activity increasing adhesion, spreading and phagocytic activity of these cells, which have a significant function in the defense mechanisms of the host against *T. cruzi*.[14,28,20]

In experimental infection with *Leishmania amazonensis*, *CA* has an immunomodulatory effect, reducing the progress of the infection and limiting its dissemination. *In vitro CA* reduces the level of infection, inducing the production of

NO by macrophages.[11] In *in vivo* infection, when administered orally or subcutaneously in mice, *CA* reduces infection by *L. amazonensis* resulting in smaller lesions and fewer parasites in lymph nodes and spleen. Other work suggests that *CA* changes the oxidative metabolism of macrophages[14,29] and interferes in the production of TNF-α, in *in vitro* and *in vivo* infections.[25]

The changes in the RAPD profiles showed not only the action of BZ or the association *CA* + BZ but also spontaneous change during the course of the infection, since the saline-treated control group also showed changes without the disappearance the monomorphic markers. The results are based on pooled samples of material obtained from 3 animals at each evaluated time point. For each time point just 1 pooled sample was used in the polyacrilamide gel. These results are compatible with selection/suppression of sub-populations in the Y strain of *T. cruzi*. It is known that this strain is polyclonal consisting of several sub-populations. These sub-populations may be selected or suppressed depending on host pressure against parasites. Murta *et al*.[26] obtained clones of the Y strain resistant to BZ with RAPD profiles different from the parental strain.

In this study, the low similarity observed between the RAPD profiles of the parasites from animals treated with BZ and *CA* + BZ compared to the pretreatment profile produced is sufficient evidence to believe that some of these sub-populations were suppressed at least until D295, the last time point we evaluated. We tested a single pooled sample from each group at each evaluated time point, so it was not possible to detect within-group variation, which might be greater than the between-group variation observed. The variation related to the genetic changes of the strain may due to the mice's immune response, which can show individual variations, modifying the course of infection, and cause changes of the RAPD profiles, by selecting or suppressing sub-populations of the parasite.

Conclusion

This is the first published study to evaluate the mechanism of action of a homeopathic medicine on the course of experimental *T. cruzi* infection using a molecular technique. The inclusion of an allopathic medicine as a 'positive control' enriched the result. The markers identified may be useful in the future analysis of results. The changes in the profiles of RAPD of the Y strain of *T. cruzi* may be due to BZ, the association of *CA* + BZ as well as the course of infection itself. Although changes in the profile of RAPD with *CA* were not observed, the association of *CA* + BZ promoted changes in this profile, suggesting that the *CA* interacts with BZ: the 2 treatments together produced results different from the isolated action of BZ. The possibility that the variation could be attributed to the mice's own immune response should not be ignored. The change in the RAPD profile during course of infection itself is compatible with immune selection/suppression of different sub-population of the strain.

Repetition of similar experiments with a larger number of more discriminatory primers, and the use of clones of the parasite instead strains may produce an interesting model to evaluate the mechanism of action of CA and other treatments for *T. cruzi*.

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