Immunodiagnosis of *Trypanosoma cruzi* (Chagas' Disease) Infection in Naturally Infected Dogs

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*This study reports on the standardization of an enzyme-linked immunosorbent assay (ELISA) for detecting specific antibodies anti-*Trypanosoma cruzi *in naturally infected dogs. Sera from 182 mongrel dogs of all ages residing in four rural villages in Santiago del Estero, Argentina, were collected in November 1994 and preserved in buffered neutral glycerin. All sera were tested by indirect hemagglutination test (IHAT), indirect immunofluorescence test (IFAT), and ELISA using the flagellar fraction of* T. cruzi *as antigen. Dog sera from an area without vectorial transmission were used to calculate ELISA specificity and cut-off value. Eighty-six percent of sera had concordant results for all tests. All sera reactive for IHAT and IFAT were also reactive for ELISA, except in one case. Sera tested by ELISA when diluted 1:200 allowed a clearer division between non-reactive and reactive sera than when 1:100 with greater agreement among serologic techniques. The specificity of ELISA was 96.2%. Among 34 adult dogs with a positive xenodiagnosis, sensitivity was 94% both for ELISA and IFAT. ELISA is the first choice for screening purposes and one of the pair of techniques recommended for diagnostic studies in dog populations.*

Key words: *Trypanosoma cruzi* - serology - chronic infection - dogs - surveillance

Serological tests used to detect specific antibodies anti-*Trypanosoma cruzi* in humans allow to confirm a chagasic infection when two or more serological assays are positive (Cerisola 1969). Methodological standardization to achieve this aim has been emphasized in a collaborative work among reference laboratories from Argentina, Brazil and United States (Camargo et al. 1986). Serologic assays are required for the diagnosis of patients, identify uninfected blood donors, in serologic surveillance after application of insecticides (Chuit et al. 1989), and to determine population serologic profiles (Segura et al. 1985). Parameters to evaluate the goodness of a serologic method include sensitivity, specificity, predictive values, the standardization of methods and reagents, and reproducibility, simplicity and cost of the assay (Cura et al. 1992).

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Many mammal species constitute important reservoirs of *T. cruzi*, especially dogs (Minter 1976). Dogs are a frequent source of blood meals for domestic triatomines (Gürtler et al. 1997), and in the Argentine *chaco*, a risk factor in the domestic transmission of the parasite (Gürtler et al. 1991), and a natural sentinel of *T. cruzi* transmission (Castañera et al. 1995). Therefore, a reliable test to identify infected dogs, that can be semi-automated for high capacity testing of blood samples, may be of help in serologic surveillance. For them, serologic techniques employed in patients must be adapted and standardized. Unfortunately, serological reactions applied to human sera are usually employed in domestic and laboratory animals without verifying the validity of test results. Determination of the appropriate cut-off titer for enzyme-linked immunosorbent assay (ELISA), as applied to dog sera, is currently lacking. In other studies (e.g. García Vázquez et al. 1995), serodiagnosis for *T. cruzi* infection in dogs was made using ELISA as the only serologic test. Regarding serum dilutions for ELISA, Barr et al. (1991) chose 1/64 for dogs, while others employed 1/100 for dogs, 1/40 for mice and 1/10 for monkeys (Mirta Moreno personal communication).

In a previous study (Lauricella et al. 1993), sera from 482 mongrel dogs were used to estimate sensitivity, specificity and the minimal diagnostic

titer for complement fixation, direct agglutination, indirect hemagglutination (IHAT), and indirect immunofluorescence (IFAT) tests. The purpose of the present study was to standardize an ELISA for detecting anti-*T. cruzi* antibodies in dogs residing in an endemic area for Chagas' disease in northwest Argentina. We also report on the correlation between serological and parasitological results and between ELISA and IFAT or IHAT.

MATERIALS AND METHODS

Study area - The survey was carried out in four rural villages: Amamá, Mercedes, Trinidad and Villa Matilde, Departamento Moreno, Province of Santiago del Estero, Argentina. The villages were situated within 9 km of each other in semiarid, hardwood thorny forest habitat at 27°12'S, 63°02'W, 140 km distant from the capital city. It is a highly endemic area for *T. cruzi*; the prevalence of seropositivity for *T. cruzi* reached 34% in humans and 65% in dogs from Amamá in 1992 (Gürtler et al. 1996). In October 1992, Amamá and the other villages were sprayed with deltamethrin (Cecere et al. 1996).

Sera collection - All 96 houses were visited and 182 (77%) of 237 mongrel dogs of all ages were bled by venipuncture in November 1994. Fifty five dogs were not bled by one of the following reasons: owners did not permit bleeding (12); absence of dogs during two visits to the house (16); dog behavior dangerous for handlers or animals that escaped to the forest (15), and unhealthy animals or insufficient blood samples (12). A rapid assessment of each individual's clinical state was carried out.

At the field site, samples were allowed to clot, kept at 4ºC overnight and serum separated into two aliquots; one was diluted 1:1 in buffered neutral glycerin and stored at room temperature until testing and the other was frozen for future evaluations. Addition of glycerin allows preservation of anti-*T. cruzi* antibodies in human sera for long periods (Pérez et al. 1989); its usefulness for dog sera has already been demonstrated (Lauricella 1991).

Serology - All serological tests were carried out at the Instituto Nacional de Parasitología "Dr. Mario Fatala Chabén" in Buenos Aires without regard to the identity or previous test result of each individual dog. IFAT was carried out as described by Alvarez et al. (1968). A formaldehyde solution containing *T. cruzi* epimastigotes (Tulahuén strain) was used as antigen. A phosphate buffer (PB) pH 7.2 was used to dilute the antigen until reaching 15-20 parasites per microscopic field at 400 X. We used a sheep anti-dog IgG (H&L chains) conjugated with fluorescein isotiocyanate (The Binding Site Limited, Birmingham, England) diluted 1:500 in PB with 1:22.000-Evans' Blue. Dog sera doubling dilutions from 1:16 to 1:64 in PB, were placed on the slides, incubated at 37°C during 30 min, and rinsed three times with PB. Slides were then incubated with the conjugate for 30 min at 37ºC, rinsed three times with PB, covered with buffered neutral glycerin and cover glasses, and microscopically examined for fluorescence. Reactive sera from xenodiagnosis-positive dogs having high or low antibody titers for *T. cruzi* and one non-reactive dog serum from an area without vectorial transmission of *T. cruzi* (Buenos Aires City) were used as controls. For IHAT, a commercially available kit was used (Polychaco S.A.I.C, Buenos Aires, Argentina), and sera titrated in double dilutions to 1:128. Minimal IFAT and IHAT diagnostic titers of seropositivity for *T. cruzi* were 1:16 for both assays (Lauricella et al. 1993).

ELISA was carried out in 96-well microtiter polystyrene plates coated with an homogenate of the flagellar fraction of *T. cruzi* (Segura et al. 1977). To determine the optimal concentration, the antigen was diluted from 4 to 100 mg/ml in phosphate buffer saline (PBS) pH 7.2. The plateau was observed at 20 mg/ml; this concentration was used thereafter. Fifty ml of antigen were added to each well and left overnight at 4°C. The plates were washed three times with 120 ml/well of PBS containing 0.01% Tween 20 (PBS/Tween); vacant plastic sites were blocked with 100 mg/well PBS-3% skimmed milk (Molico, Nestlé) for 1 hr at 37°C and washed with PBS/Tween as before.

Each serum sample was diluted to 1:100 (E100) and 1:200 (E200) in PBS-1% skimmed milk and tested in duplicate. Fifty ml of each serum sample were added to each well and incubated for 1 hr at 37°C. The plates were washed three times with PBS/Tween and then incubated with 50 ml/well peroxidase-conjugated rabbit anti-dog IgG (H&L chains; Biosys, France) diluted to 1:1,200 in PBS-1% skimmed milk for 1 hr at 37°C. Seven dilutions of peroxidase-conjugated anti-dog IgG, ranging from 1:300 to 1:3,300, were done to determine the best titer under our laboratory conditions; from these, the 1:1,200 dilution was selected and used thereafter. After washing three times with PBS/Tween, 50 ml of a substrate solution of 0.04% *o*-phenylenediamine-dihydrochloride (Sigma Chemical Co., Saint Louis, Mis., USA) in 0.01% (v/v) H_2O_2 in citrate buffer (pH 5.0) were added to each well. Plates were maintained in the dark at room temperature.

When the substrate dilutions started to show a yellowish color, usually within 8-15 min, the reaction was stopped by adding 50 ml/well of 2.5N ClH. No difference was found when 1N H_2SO_4 was used. Within 5-15 min after adding ClH, the absorbance was analyzed on a digital spectrophotom-

eter reader MR700 (Dynatech Lab. Inc., Buenos Aires, Argentina) at 490 nm. The mean absorbance of each pair of duplicate sera was calculated; if the difference between both values was more than 30%, the sample was retested. Five dog sera reactive for *T. cruzi* from xenodiagnosis-positive animals and five non-reactive dog sera from Buenos Aires, all with concordant IFAT and IHAT results, were used as controls. Each plate had two positive and one negative control sera.

To calculate the specificity of ELISA and the cut-off value, sera from 53 dogs from Buenos Aires were tested by IFAT, IHAT and ELISA as before. Sera were provided by the Instituto de Zoonosis "Dr Luis Pasteur" and came from owned pure-bred and mongrel dogs of all ages. The cut-off value was estimated in two ways: (1) the mean absorbance plus three times the standard deviation (SD) of the results obtained with negative dog sera from Buenos Aires; and (2) the absorbance of the negative control mean (n=5) plus 0.13 times the positive control mean (n=5) (Pan et al. 1992). In addition, we estimated the absorbance that gave a sharper division between non-reactive and reactive sera in the study dog population (i.e., giving the lowest number of discordant samples among serologic techniques). Twenty dog sera from Buenos Aires and 20 xenodiagnosis-positive reactive dog sera were tested by ELISA three times to evaluate reproducibility.

Xenodiagnosis - Thirty-four dogs that had a positive xenodiagnosis for *T. cruzi* in late 1992 (Gürtler et al. 1996) were used to determine ELISA sensitivity. Each dog had been identified by its name, age, sex and number of family house. In those xenodiagnoses, 20 laboratory-reared uninfected third instar nymphs of *Triatoma infestans* were fed on each dog during 25 min. Fecal droplets of each individual bug were obtained by abdominal pressure, mixed with physiological saline solution, covered with 22 x 22 mm^2 cover glass and microscopically examined for *T. cruzi* infection at 400 X approximately 30 and 60 days after feeding.

RESULTS

Using 53 dog sera from Buenos Aires, the mean absorbance of ELISA E200 was 0.03 (SD=0.05). Thus, the cut-off value calculated as mean plus 3 SD was 0.18. No statistically significant differences between E100 and E200 mean absorbances were observed. Using the second procedure to calculate the cut-off, the mean absorbance of negative control sera was 0.08; the mean absorbance of positive control sera was 0.70. Thus, the cut-off value was 0.17. All 53 dog sera from Buenos Aires were non-reactive for IFAT and IHAT, but two of them had borderline absorbances of 0.18 by ELISA E200. Therefore, the specificity of IFAT and IHAT was 100% while that of ELISA E200 was 96.2%.

Fig. 1 shows the frequency distribution of absorbances for ELISA E100 and E200. More diluted sera (1:200) allowed a clearer division between reactive and non-reactive sera; no serum fell within the absorbance interval 0.14-0.23. Using E100, eight of nine sera with an absorbance in the range 0.14-0.23 (i.e., within 20% of the calculated cut-off values 0.17 and 0.18) were non-reactive by IFAT and IHAT. Using E100, an absorbance value of 0.20 provided the lowest number of discordant results among techniques.

Of the 182 sera tested, 57 (31%) and 74 (41%) sera were reactive for *T. cruzi* by IHAT and IFAT, respectively (Table I). Seventy-eight (43%) sera were reactive for E100 (data not shown) and 73 (40%) were reactive for E200. Seven sera showed discordant results between E100 and E200; five sera reactive for E100 were negative for E200 and the other techniques. Two sera had positive or negative concordant results among E100, IFAT and IHAT. Therefore, we selected E200 as the best serum dilution and used it in the following analyses.

Comparison among ELISA E200, IFAT and IHAT shows that 156 (86%) sera had concordant results among the three techniques (Table I). Of the remaining 26 sera with discordant results, 14 were negative only for IHAT, four were positive only for ELISA and five were positive only for IFAT; the other three sera showed various combinations of results. Though 54 sera were reactive for the three techniques, 70 sera were reactive by at least two techniques. All sera reactive for IHAT and IFAT were also reactive for ELISA E200, except in one case.

TABLE I

Comparative results among ELISA (E200), indirect immunofluorescence (IFAT) and indirect hemagglutination (IHAT) tests against *Trypanosoma cruzi* in 182 dogs; Amamá and close villages, November 1994

		IFAT		
ELISA	IHAT	Positive	Negative	Total
Positive	Positive	54[a]	1	55
	Negative	14	4	18
	Total	68	5	73[c]
Negative	Positive	1	1	2
	Negative	5	102[b]	107
	Total	6	103	109
Total			108	182

a: positive for three techniques; *b*: negative for three techniques; *c*: ELISA reactive sera.

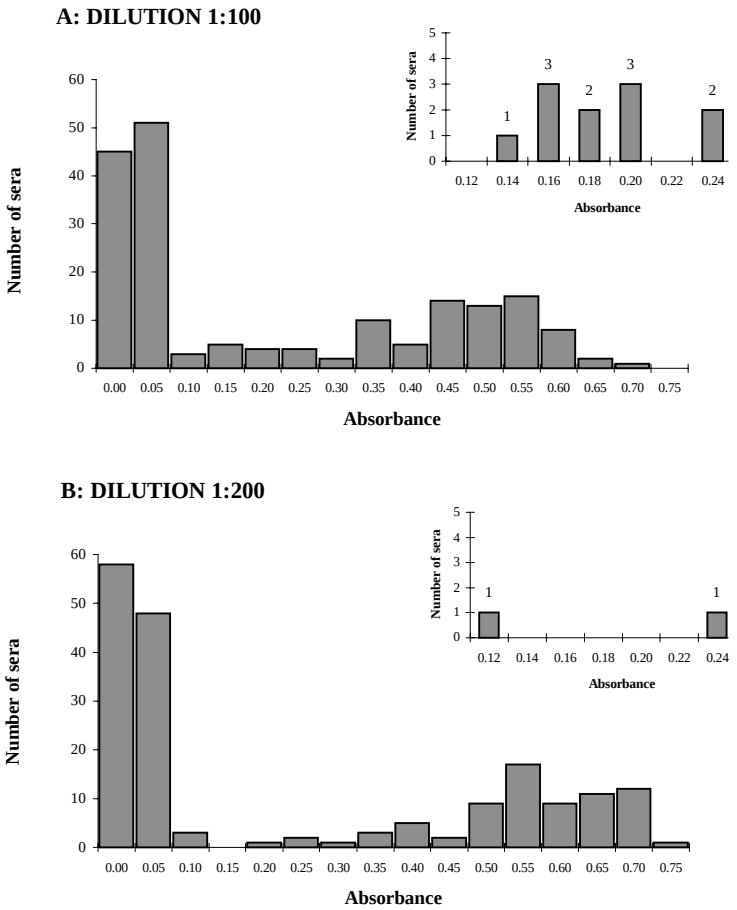

Fig. 1: frequency distribution of ELISA absorbance (lower class limits shown) for 182 dog sera from Amamá and neighboring villages, November 1994. A: 1:100 serum dilutions (E100); B: 1:200 serum dilutions (E200). Embedded figures show the distribution of sera close to the cut-off values; numbers on each bar are numbers of sera.

Fig. 2 shows the joint frequency distribution of titers for ELISA E200 and IFAT. Among 12 sera with discordant results, the test that was reactive showed significant (not borderline) titers. Using IFAT as the reference for ELISA E200, co-positivity was 0.92 (67/73) and co-negativity was 0.94 (103/109).

All sera from the 34 dogs with a positive xenodiagnosis showed seroreactivity for *T. cruzi* by ELISA E200 except in two cases (Table II); one of them was also not reactive for IFAT and IHAT. Twenty-two sera were reactive for the three assays. Sensitivity estimates were calculated as the proportion of the xenodiagnosis-positive dogs tested that were reactive for a given technique. The sensitivity of ELISA and IFAT was 94% (32/34), whereas IHAT sensitivity was 68% (23/34).

Fig. 3 shows the mean (±SD) absorbance of 20 dog sera from Buenos Aires and 20 xenodiagnosis-positive reactive dog sera tested by ELISA E200

TABLE II

Concordance among ELISA (E200), indirect immunofluorescence (IFAT) and indirect hemagglutination (IHAT) tests in sera from 34 dogs with a positive xenodiagnosis; Amamá and close villages, November 1994

		IFAT		
ELISA	IHAT	Positive	Negative	Total
Positive	Positive	22[a]	1	23[b]
	Negative	9	0	9
	Total	31	1	32[c]
Negative	Positive	0	0	0
	Negative	1	1[e]	2
	Total	1	1	2
Total		32[d]	2	34

a: positive for three techniques; *b*: IHA sensitivity = 68% (23/34); *c*: ELISA sensitivity = 94% (32/34); *d*: IFAT sensitivity = 94% (32/34); *e*: negative for three techniques.

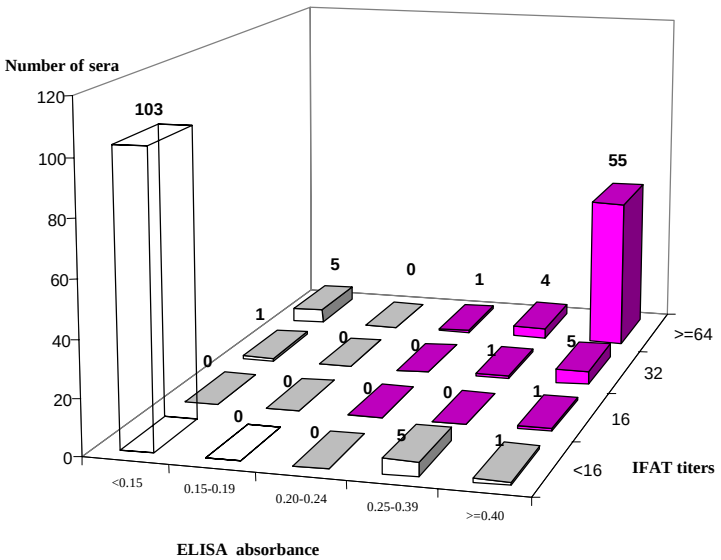

Fig. 2: joint frequency distribution of ELISA (E200) absorbances and titers for indirect immunofluoresce test (IFAT).

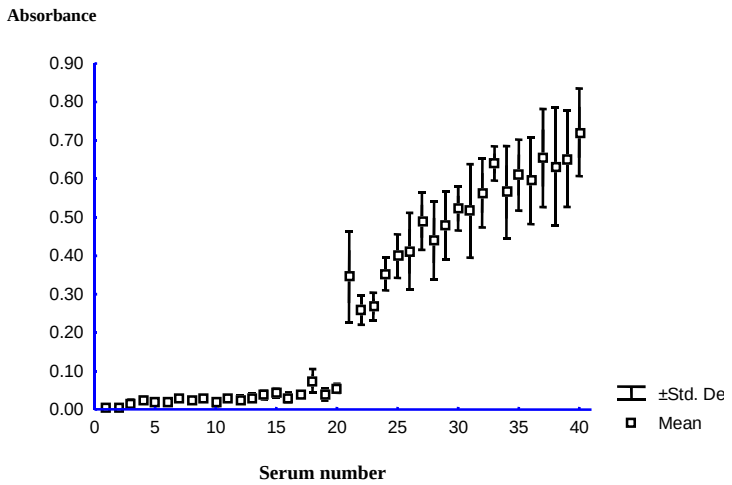

Fig. 3: mean absorbance and standard deviation (SD) for 20 dog sera from an area without vectorial transmission and 20 xenodiagnosis-positive dog sera tested by ELISA (E200) three times.

three times. The overall coefficient of variation of absorbance values was 16% for xenodiagnosis-positive reactive dog sera and 36% for negative control sera. In spite of increasing variability among readings with increasing absorbance values, no reactive dog sera fell below the cut-off value.

DISCUSSION

Our study shows that ELISA was as sensitive as IFAT, which in previous studies was shown as the most sensitive technique among those assayed (Lauricella et al. 1993). However, both ELISA and IFAT revealed sera reactive for *T. cruzi* that the other technique failed to detect.

The serum dilution 1:200 (E200) allowed a clearer division between positive and negative sera than 1:100 (see Fig. 1); five of seven ELISA E200-negative sera were negatively concordant with the other serological techniques. Considering the weak health status of dog populations from these impoverished rural areas, it is likely that low titers of unspecific antibodies due to other infectious agents were eliminated by using more diluted sera.

The cut-off value for ELISA E200 varied little from 0.17 to 0.18 by two procedures; the frequency distribution of absorbances in Fig. 1 showed no data within 20% of cut-off values. However, two

(3.8%) dogs from Buenos Aires tested positive by ELISA E200 when those cut-off values were used. When E100 was used, an absorbance of 0.20 provided the lowest number of discordant samples among techniques; the interval from 0.14 to 0.23 included eight of nine sera non-reactive for IFAT and IHAT, most of which would have been considered positive with a cut-off of 0.17 or 0.18. Taking a more conservative approach, a cut-off value of 0.20 for both serum dilutions would provide both a sensitive and specific serodiagnosis for *T. cruzi*.

Of 34 sera from xenodiagnosis-positive dogs, ELISA E200 missed two samples, one of which tested positive by IFAT. Both dogs had a positive xenodiagnosis 1-2 years before, and one of them also tested positive by ELISA and IHAT. Therefore, both dogs were not in the acute, serologically unresponsive, phase of infection by November 1994. Moreover, one of the ELISA-negative, xenodiagnosis-positive dogs tested positive by ELISA and IFAT in 1996 (unpublished results). Mislabeling of sera or a wrong identification of the animal were unlikely due to tight control procedures. These two dogs might have been either severely undernourished, which is not uncommon in the study area, immunosupressed or both. Regarding malnutrition, mice on a protein-deficient diet dropped their specific *T. cruzi* immune response (Carlomagno et al. 1996). Human beings infected with *T. cruzi* or *Toxoplasma gondii* and subjected to organ transplants were seronegative as a consequence of immunosuppresive treatment (Vázquez et al. 1993, Derouin et al. 1993).

Leishmania sp. and *T. cruzi* produce serologic cross-reactions that can be differentiated by immunoblotting (Chiaramonte et al. 1996). However, very few human cases of *Leishmania* sp. were notified in Departamento Moreno (Santiago del Estero) since 1992, and all of them occurred far from our study area (Yadón 1996). In addition, dogs from the study villages did not show apparent signs of *Leishmania* infection, such as desquamation, alopezia, erythema, ulceration and depigmentation (Opitz 1996). Therefore, the likelihood of "false positive" serologic reactions due to *Leishmania* sp. in the study dogs seems remote. Using sera from dogs residing in Buenos Aires, we showed ELISA E200 to be 96.2% specific. However, we did not test sera from dogs showing specific diseases.

Greater uniformity in binding antigens to plates is achieved with less heterogeneous antigen preparations (Venkatesan & Wakelin 1993). In our study, we used the flagellar fraction of *T. cruzi*. However, in a preliminary comparison between the flagellar fraction and a total homogenate of *T. cruzi* as applied to ten positive and ten negative control sera, we obtained comparable results with both antigens using ELISA E200 (unpublished).

The present sensitivity estimate for IFAT agreed with a previous one (Lauricella et al. 1993), in spite of using different commerically available conjugates. For IHAT, however, differences between a previous (84%) and the present sensitivity estimate (68%), separated by almost ten years, are not uncommon when different batches of commercially available kits are used. In any case, our xenodiagnosis-positive set of sera was limited in number. Therefore, sensitivity estimates may be markedly affected by even one or two anomalous cases.

In human immunodiagnosis of Chagas' disease, ELISA has a high level of reactivity (small amount of antigens and antibodies required for diagnosis) while IFAT and IHAT have an intermediate level (Cura et al. 1992). As stated by Hoff et al. (1985), the most important practical advantages of ELISA are that reagents are relatively inexpensive and that the test can be read objectively and quantitatively using an ELISA plate reader. This makes ELISA specially apt for mass testing of serum samples. At present, ELISA is the first choice for screening anti-*T. cruzi* antibodies in dog populations for serologic surveillance after insecticide sprays and apparent elimination of triatomine bugs, and is a recommended technique for individual serodiagnosis.

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