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Short communication

A prototype of the direct agglutination test kit (DAT-Canis) for the serological diagnosis of canine visceral leishmaniasis

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A B S T R A C T

This report describes the stege I/II development of a new direct agglutination test (DAT) for the diagnosis of canine visceral leishmaniasis (CVL) using freeze-dried antigen produced Coomassie blue-stained *Leishmania* (*Leishmania*) *infantum* promastigotes. In stage I, 16 canine serum samples, collected from eight dogs carrying CVL and eight healthy dogs, were assessed with the DAT using 2-mercaptoethanol (2-ME), N-acetyl-cysteine (NAC), kaolin or NAC plus urea (NAC + U) to improve the assay conditions. Stage II assessed the diagnostic accuracy with 100 serum samples collected from dogs with symptomatic CVL and clinically healthy dogs, comparing the four different sample diluents. The CVL-DAT prototype kit showed equivalent performances when 2-ME, NAC or NAC + U were used: 97.1% sensitivity (CI: 83–99.8%), 97% specificity (CI: 88.5–99.5%) and a 97% diagnostic accuracy (CI: 90.8–99.2). With kaolin, a 94.1% sensitivity (CI: 79–99%), 97% specificity (CI: 88.5–99.5%) and 96% diagnostic accuracy were observed (CI: 89.5–98.7), with no statistically significant differences among the four reagents ($p = 1.0$). The NAC plus urea in sample diluent decreased non-specific agglutination, promoted a better defined sharp-edged blue spot and was thus chosen as a component for the new DAT prototype to diagnose canine VL, designated DAT-Canis.

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1. Introduction

Visceral leishmaniasis (VL) is an anthropozoonotic disease in Brazil caused by *Leishmania* (*Leishmania*) *infantum*, which is transmitted to the vertebrate host by the Phlebotominae sandfly species. Despite the Brazilian strategies for control of VL this disease still represents a serious public health problem due to its wide geographical distribution and the severity of its clinical manifestations, which can lead to death if not treated.

Several species of mammals have been found to be naturally infected with *Leishmania* spp., and dogs (*Canis familiaris*) are known to establish the peridomestic cycle of VL in urban, *peri*-urban and rural areas. In part, this fact is supported by the high rates of prevalence of VL in dogs (CVL) and the high skin parasitism in the infected dogs that favours infection of the vector, leading them to be considered as the largest reservoir hosts for *L. infantum* (Verçosa et al., 2012), and by the fact that canine VL cases precede or occur simultaneously with human VL cases (Prado et al., 2011).

For a CVL serological diagnosis, an enzyme-linked immunosorbent assay (EIE Leishmaniose visceral canina, Bio-Manguinhos-FIOCRUZ, Rio de Janeiro, RJ, BR) as a screening test and an indirect fluorescent antibodies test (IFI-Leishmaniose Visceral Canina, Bio-Manguinhos-FIOCRUZ, Rio de Janeiro, RJ, BR) as a confirmatory test were available to the Public Health Laboratories (LACENs) by the Brazilian Ministry of Health to assess canine seroprevalence until 2011. To interrupt the cycle of VL transmission, dogs with titers higher than 1:40 were euthanized (Ministério da Saúde 2006).

Since 2012, a rapid test (TR DPP® Leishmaniose Visceral Canina Biomanguinhos-FIOCRUZ, Rio de Janeiro, RJ, BR) has been used as a screening test, and the enzyme-linked immunosorbent assay (EIE-Leishmaniose Visceral Canina) has been made available to the Public Health Laboratories (LACENs) as a confirmatory test for the serological diagnosis of CVL (Ministério da Saúde 2011). This new protocol (combined DPP and ELISA) improved the diagnostic accuracy and has shown prevalence rates and incidence indexes greater than what was shown by the previous protocol (ELISA and IFAT) (Coura-Vital et al., 2014). However, the ELISA test (Biomanguinhos-FIOCRUZ) has variable sensitivity and specificity rates for CVL diagnosis, probably due to antigenic similarities among the *Leishmania* species. In areas where *Trypanosoma can-*

inum or *Trypanosoma cruzi* circulate, cross-reaction can occur with these serological tests, as some epitopes are shared with the species of the family *Trypanosomatidae* (Zanette et al., 2014). The low to moderate diagnostic accuracy of the ELISA test (Peixoto et al., 2015) makes the method inadequate for use as the confirmatory test, as recommended by the Health Ministry of the Brazil (Ministério da Saúde, 2011).

Alternatively, the direct agglutination test (DAT) can constitute a stool to select the *L. infantum* infected dogs, because it is a relatively simple test and very suitable test for CVL diagnosis even under harsh field conditions. Furthermore, the dog-DAT® based on freeze-dried *L. donovani* promastigote antigen (Royal Tropical Institute, Amsterdam, NL) applied in sera sample of dogs from the metropolitan region of Belo Horizonte, Minas Gerais state, Brazil, had better sensitivity (100 and 93%), specificity (91 and 95%) than IFAT (da Silva et al., 2006; Ferreira et al., 2007).

Considering the importance of a precise diagnosis of infected dogs for the control of VL, the aim of this study was to develop a prototype kit of the direct agglutination test to be used for CVL diagnosis.

2. Materials and methods

2.1. Serum samples

The samples used in this study were constituted of a part of a larger study and were collected from dogs of different breeds and ages living in different endemic neighbourhoods of the city of Belo Horizonte, Minas Gerais state, Brazil, from July to August 2013. Blood samples were collected by venipuncture and transferred to tubes without anticoagulant. Serum samples were obtained by centrifugation at 2.500 rpm for 15 min and stored at −70 °C until use. The samples were subjected to IFAT and ELISA tests (Bio-Manguinhos-FIOCRUZ) according to the manufacturer's instructions and the recommendations of the Brazilian Ministry of Health (Ministério da Saúde 2006). The seropositive dogs were sedated with xylazine, and anaesthetized intravenously with thionembutal before bone marrow aspiration. Bone marrow aspirates were inoculated into the biphasic medium Nicolle-Novy-McNeal (NNN)-LIT containing 10% bovine calf serum. The myelocultures were maintained at 25 °C and examined for viability and motility of flagellates once a week.

The stage I assays were performed using sixteen canine control samples, eight from dogs with CVL confirmed by clinical, myeloculture and serologic tests (IFAT and ELISA) and eight from healthy dogs confirmed by serologic tests (IFAT and ELISA).

The stage II was done to assess the performance of the prototype for CVL diagnosis. Based in an estimated sensitivity of 98%, confidence interval: 95% and alpha error: 5%, a minimal of 30 positive and negative samples were calculated (WOAH, Validation Guideline 3.6.1, 2014).

Thus, 34 serum samples collected from dogs naturally infected (CVL group), clinically classified as symptomatic through clinical examination, myeloculture and serological tests (IFAT and ELISA), and 66 serum samples from dogs (Control group), classified as healthy by clinical examination and negative results from serological tests (IFAT and ELISA), were used in stage II of this study.

All samples were aliquoted, blinded coded and tested with the DAT protocol using four different reagents in the sample diluent.

2.2. Development of the DAT for CVL diagnosis (stage I)

2.2.1. Antigen preparation

The antigen was prepared with *L. infantum* promastigotes, isolated from a patient seen at the Reference Centre of Leishmaniasis of the Centro de Pesquisas René Rachou/FIOCRUZ and deposited as MHOM/BR/2002/LPC-RPV in the *Leishmania* Collection of the Instituto Oswaldo Cruz-FIOCRUZ, according to Oliveira et al. (2013). Briefly, the antigen was prepared with *L. infantum* promastigotes that were recovered after four days of cultivation in biphasic medium NNN-LIT containing 20% heat-inactivated foetal bovine serum (FBS). Initially, the suspension was centrifuged for 10 min at 10 g at 25 °C and then incubated for 30 min in a biological oxygen demand incubator at 26 °C (FANEM, São Paulo, SP, Brazil) to separate dead from viable parasites. Next, the supernatant was carefully transferred into Falcon® tubes, washed three times with Locke's solution by centrifugation and then resuspended and incubated in Locke's solution containing 0.4% trypsin. After further washes, the pellet was resuspended and incubated with saline (0.9% NaCl) plus 1% sodium citrate (SCS) containing 2% formaldehyde (v/w). Next, the parasites were washed with SCS and stained with SCS containing 0.1% Coomassie blue R-250. After several washes with SCS until the supernatant became clear, the pellet was resuspended with stabilizing solution, and the concentration of promastigotes was adjusted to an absorbance of 0.500 in a spectrophotometer (λ = 590 nm). The total amount of antigen obtained was aliquoted into vials (5 ml/vial), frozen in a freezer at −70 °C and subjected to freeze-drying in a lyophilizer (Alpha 2–4 LDplus, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, GE) stabilized at 0.023 mbar and −55 °C for 24 h.

2.2.2. Standardization of the DAT for CVL diagnosis

To select the optimal conditions for the antigen in the DAT for CVL diagnosis, different stage I assays were performed with the DAT using 2-ME at 0.5, 0.1, 1.5 and 2.0 M, NAC at 0.001, 0.0025, 0.005 and 0.01 M and NAC at 0.005 M plus urea at 0.1, 0.25, 0.5, 1.0 and 2.0 M in the sample diluent to define the best concentration of each reagent. Kaolin was used at a 10% concentration, based on previous work (Oliveira et al., 2011). The optimal conditions to perform the DAT for CVL diagnosis are described as follows.

First, 5 ml of a rehydration solution (0.9% sodium chloride and 0.1% sodium azide) were added into the antigen vial, which was carefully homogenized. The sera were then diluted in samples diluent (0.9% sodium chloride and 0.1% sodium azide) containing 0.15 M 2-ME, and a two-fold dilution series was made from 1:100 to 1:102.400 in a V-shaped microtiter plate (Sarstedt Inc., Newton, NC, US). The microplate was incubated for one hour at room temperature, and 50 μl of the DAT antigen suspension was added to each well of the microplate containing 50 μl of diluted serum. After a minimum incubation of 18 h at room temperature, the end titer was read, as the dilution immediately before the well with a clear sharp-edged blue spot identical in size to the negative control reaction.

The same protocol was used to test the samples in the DAT using samples diluent containing 0.005 M N-acetyl-cysteine (DAT/NAC) or 0.005 M N-acetyl-cysteine plus 1 M urea (DAT/NAC + U).

To perform the DAT using kaolin in the sample diluent (DAT/kaolin), the sera were diluted 1:100 in samples diluent containing 10% kaolin, homogenized, incubated 15 min at room temperature and centrifuged by 15 min at 1.500 g. Then, 50 μl of the supernatant was used make a two-fold dilution series from 1:100 to 1:102.400 in a V-shaped microtiter plate (Sarstedt Inc., Newton, NC, USA), following the same procedure described above.

2.3. Diagnostic precision (repeatability and reproducibility)

To measure the repeatability, eight sera from the CVL group were retested by the DAT, using different reagents in the sample diluent, five times in the same day by the same researcher, maintaining the same conditions. The repeatability was measured by the variation coefficient from the titer results transformed in Log10.
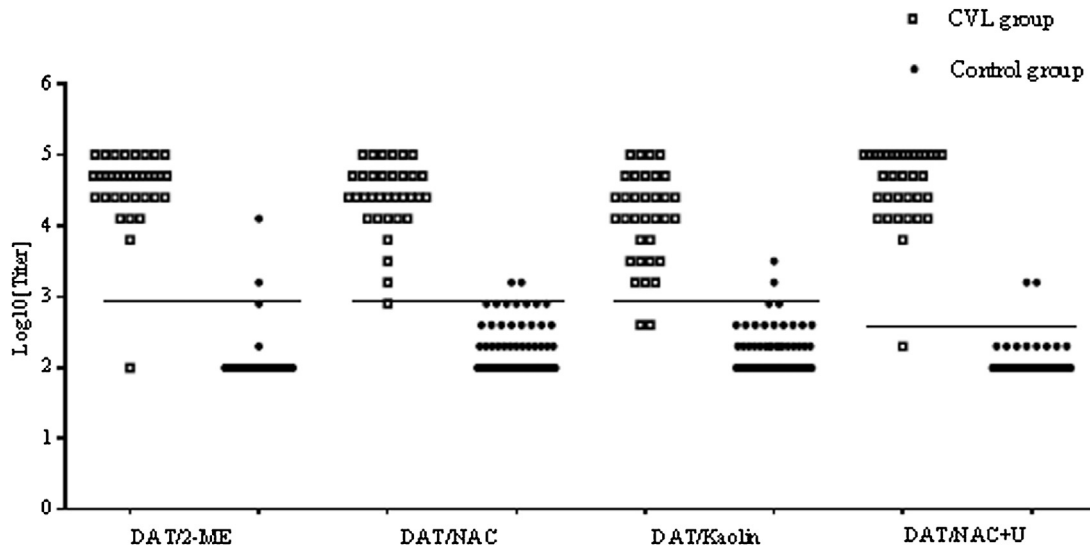
Fig. 1. Positivity of DAT using 2-ME (DAT/2-ME), NAC (DAT/NAC), Kaolin (DAT/Kaolin) and NAC plus Urea (DAT/NAC + U) in the sample diluent and applied in the CVL group (n = 34) and Control group (n = 66). The line represents the cut-off of the DAT using different reagents in the sample diluent.

The reproducibility of the DAT, performed with different reagents in the sample diluent, was measured through the weekly retesting of 15 sera from the CVL group by the same researcher. The titer results were transformed in Log10 and used for the variation coefficient calculation.

2.4. Performance of the DAT for CVL diagnosis (stage II)

To evaluate the diagnostic performance of the DAT, 100 canine serum samples were tested with four different reagents in the sample diluent following the optimal conditions defined in the stage I. At the end of testing, the data bank was accessed, and the samples were reclassified as 34 serum samples collected from dogs with CVL (CVL group) and 66 serum samples from health dogs (Control group). The sensitivity, specificity and diagnostic accuracy were measured using the obtained results.

2.5. Statistical analysis

The repeatability (intra-test precision) and reproducibility (inter-test precision) levels were measured by the variation coefficient (VC) of the titers transformed in Log10 as determined in the retesting of the positive control samples using the equation VC = (Standard deviation/Mean) x100.

The diagnostic parameters of the DAT were calculated with the following formulas: sensitivity = TP/(TP + FN) x100%; specificity = TN/(TN + FP) × 100%; accuracy = TP + TN/n × 100%, where TP represents true positive, TN true negative, FN false negative, FP false positive and n total samples. The x^2 test was employed for comparison of sensitivity, specificity and diagnostic accuracy rates, considering a significance level of 5% error probability.

All obtained DAT titer results, using different reagents in the sample diluent, were transformed to Log10 and plotted in a graph to compare the quantitative results. The variables of the CVL group were individually assessed with the W-test for normality and evaluated by the non-parametric Spearman's correlation, considering significance at the 0.01 level (two-tailed), using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, US).

2.6. Ethical considerations

This study was submitted and approved by the Ethics Committee for Research with Animals of the Oswaldo Cruz Foundation (CEUA/FIOCRUZ) under protocol number LW-76/12. All procedures involving experimental animals were conducted according to the guidelines of the Brazilian College for Experiments with Animals (Colégio Brasileiro de Experimentação Animal/COBEA).

3. Results and discussion

Variation coefficients (VC) ranging from 0 to 5.9%, 0 to 3.9%, 3.3 to 7.3% and 0 to 3.6% were obtained in the DAT repeatability analysis using 2-ME, NAC, kaolin and NAC plus urea in the sample diluent, respectively. The VCs of the reproducibility analysis ranged from 2.9 to 6.5%, 2.8 to 11.6%, 6.7 to 22% and 3.1 to 11.9% using 2-ME, NAC, kaolin and NAC plus Urea in the sample diluent. Such, the DAT developed for CVL diagnosis showed better repeatability when NAC plus urea was added in the sample diluent (CV <3.6%) and better reproducibility when using 2-ME in the sample diluent (CV <6.5).

In this study, only the dilution of the samples to determine the titer of the samples was considered. Thus, 1:800 (Log10 = 2.9) was defined as a better cut-off point for the DAT using 2-ME, NAC or kaolin in the sample diluent. In contrast, the better cut-off point for the DAT using NAC plus urea (DAT/NAC + U) was defined as 1:400 (Log10 = 2.6) (Fig. 1). Only one sample from the CVL group presented false-negative results for the DAT using 2-ME, NAC or NAC plus urea in the sample diluent. Two samples from the CVL group presented false-negative DAT results using kaolin in the sample diluent. From the Control group, two samples presented false-positive DAT results using any of the tested reagents in the sample diluent (Fig. 1).

Table 1 shows the sensitivity, specificity and diagnostic accuracy of the DAT used for 100 sera samples collected from CVL carrier dogs and healthy dogs. The DAT had a sensitivity ranging from 94.1 to 97.1% and a diagnostic accuracy from 96 to 97%, using 2-ME, NAC, kaolin or NAC plus urea in the sample diluent. The specificity was 97% for any of the reagents used in the sample diluent. In this way, the serologic parameters presented by the DAT using different reagents in the sample diluent were equivalent, except for DAT/kaolin, which presented two false negative results, decreasing the sensitivity to 94.1% and diagnostic accuracy for CVL diagnosis

Table 1

Sensitivity specificity and diagnostic accuracy rates of the DAT using different reagents in sample diluent applied in the CVL (n = 34) and control (n = 66) groups.

DAT	Sensitivity (n = 34) % (CI%)	Specificity (n = 66) % (CI%)	Diagnostic Accuracy (n = 100) % (CI%)
2-ME in the Sample diluent	(33) 97.1 (83.0–99.8)	(64) 97 (88.5–99.5)	(97) 97 (88.2–98.1)
NAC in the Sample diluent	(33) 97.1 (83.0–99.8)	(64) 97 (88.5–99.5)	(97) 97 (90.8–99.2)
Kaolin in the Sample diluent	(32) 94.1[a] (79.0–99.5)	(64) 97 (88.5–99.5)	(96) 96[a] (89.5–98.7)
NAC plus Urea in the Sample diluent	(33) 97.1 (83–99.8)	(64) 97 (88.5–99.5)	(97) 97 (88.2–98.1)

[a] Differences of sensibility and diagnostic accuracy rates from DAT/Kaolin and DAT with anyone reagents: $p = 1.0$.

to 96%, although without a significant difference ($p = 1.0$) (Table 1). These results are similar to the data reported in other studies performed with Dog-DAT® (Royal Tropical Institute, Amsterdam, NL) produced with *L. donovani* promastigotes. The new DAT prototype shown to be less sensitive but more specific than Dog-DAT®, that presented sensitivity of 100% and specificity 91% (da Silva et al., 2006). Other authors found sensibility of 93% and specificity of 95% for Dog-DAT®, respectively. Furthermore, only one sample from *Ehrlichia canis* infected dog presented cross reaction in the Dog-DAT® (Ferreira et al., 2007).

In this study, the two samples from the Control group that presented false-positive results by DAT/kaolin were negative by the DAT using other reagents in the sample diluent. In contrast, the two samples that showed false-positive results by the DAT using 2-ME or NAC, only one was positive by the DAT/NAC + U. This could be due to the mechanism of action of the reagents in inhibiting the non-specific interaction of the antibodies with the antigens present in the membrane of the parasites. The 2-ME and NAC break disulphide links in proteins, while kaolin absorbs proteins, such as the non-specific antibodies, macroglobulins and isoagglutinins that promote non-specific agglutination. Urea is chaotropic agent and inhibits weak interactions between epitopes and antibodies at the concentration used in this study (1 M). Experimentally, the use of NAC plus urea decreased non-specific agglutination and promoted a better defined sharp-edged blue spot. The log-transformed DAT titers using different reagents in the sample diluent and those from the 34 samples of the CVL group were correlated. Two samples showed the same results by the DAT using any of the reagents in the sample diluent, and there was frequent overlapping between the obtained results using 2-ME or NAC. The paired test showed positive correlations between DAT/2-ME and DATI/NAC (Spearman

coefficient of 0.67), DAT/2-ME and DAT/kaolin (Spearman coefficient of 0.74) and DAT/NAC and DAT/kaolin (Spearman coefficient of 0.73). In contrast, the correlation of results obtained by the DAT using 2-ME, NAC or kaolin versus DAT/NAC plus urea was low (Spearman coefficient >0.5) (Fig. 2). This may be due the presence of urea, but this fact did not decrease the titers of the positive samples. Surprisingly, frequent positive samples showing higher titers were observed with DAT/NAC + U. The use of 2-ME can injure the eyes, respiratory tract and nervous system, and thus its manipulation requires the use of a cabinet fume hood and personal protection equipment to protect the laboratory technician and prevent the release of toxic gas into the laboratory environment. In contrast, the DAT/kaolin requires an incubation of 15 min and centrifugation before the testing of the samples, which makes its use less practical compared with DAT using 2-ME, NAC or NAC plus urea in the sample diluent.

4. Conclusion

Thus, based on practicality and the results obtained, a prototype of the DAT kit using NAC plus urea in the sample diluent, was developed with *L. Infantum* promastigotes for canine VL diagnosis, here designated DAT-Canis. The prototype was composed of 10 freeze-dried antigen vials, one 10X concentrated rehydration solution vial, one 10X concentrated sample diluent vial and the instructions for use. The kit allows for 480 qualitative or 80 quantitative assays, including the positive and the reaction controls. It is recommended to perform two dilutions for the qualitative assay or a two-fold dilution series, starting from 1:100 to 1:102.400, for the quantitative assay. The production cost was estimated in US$35 per prototype, which is equal to the cost of DAT-LPC (Oliveira et al., 2013). The
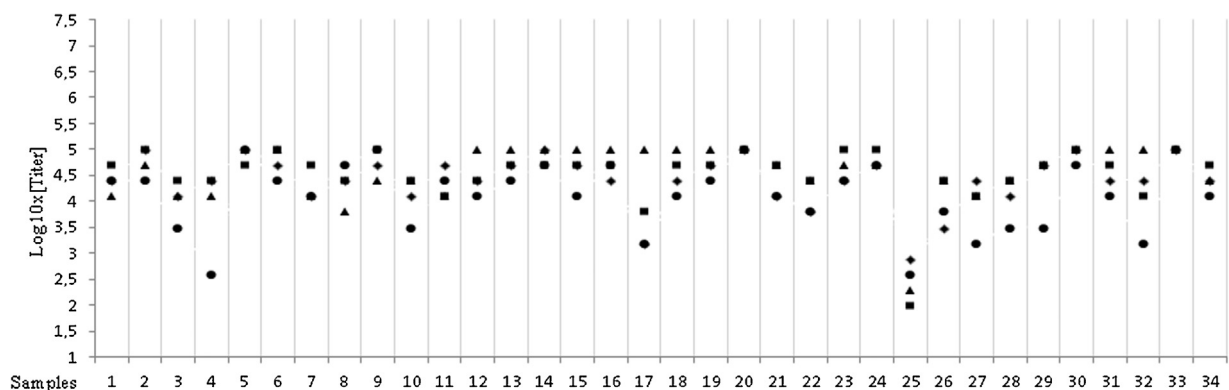

Fig. 2. Correlation analysis of Log10[titer] presented by 34 positive samples tested with DAT using 2-ME (♦), NAC (■), Kaolin (●) ou NAC + Ureia (▲). The majority of results is overlapping, as by example the sample 20 that presented exactly the same result by DAT-LPC using different reagent in the sample diluent. DAT-LPC/2-ME x DAT-LPC/NAC: Spearman coefficient = 0.67; DAT-LPC/2-ME × DAT-LPC/Kaolin: Spearman coefficient = 0.74; DAT-LPC/2-ME × DAT-LPC/NAC + U: Spearman coefficient = 0.42; DAT-LPC/NAC × DAT-LPC/Kaolin: Spearman coefficient = 0.73; DAT-LPC/NAC × DAT-LPC/NAC + U: Spearman coefficient = 0.50; DAT-LPC/Kaolin × DAT-LPC/NAC + U: Spearman coefficient = 0.37.

price of each sample quantitatively assayed is about US$0.44, considering only expenses of the reagents used in the production and execution of the test.

In this study, the test demonstrated good precision and diagnostic accuracy and may contribute to the improvement of the control of disease. However, the DAT was used only on samples from CVL and healthy dogs and not on samples from asymptomatic dogs, which was considered as the principal limitation of this study. Currently a large field trial to evaluate the usefulness of this test as a marker of asymptomatic infection is under way.

Acknowledgments

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