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Preparation and evaluation of a glycerol-preserved direct agglutination antigen for long-term preservation: a comparative study of the detection of anti-*Leishmania infantum* antibodies in human and dog

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ABSTRACT

Objective: To prepare and evaluate a glycerol-preserved antigen from an Iranian strain of *Leishmania infantum* (*L. infantum*) for use in glycerol-preserved direct agglutination tests (GP-DAT) as an alternative to freeze dried direct agglutination tests (FD-DAT) that use freeze-dried antigen. **Methods:** Glycerol-preserved DAT antigen was prepared and stored at different temperatures. We tested antigen stored at 4 ℃, 22-37 ℃ and 50 ℃ over a period of 365 days. Seven hundred twenty-nine serum samples were collected from different geographical zones of Iran from 2007-2009, and 80 of these samples were pooled to produce sera. Each pooled serum contained 10 sera. All positive and negative pooled sera were separately tested for anti-*L. infantum* antibodies with GP-DAT, FD-DAT and formaldehyde-fixed direct agglutination test (FF-DAT) antigens; tests were performed on both human and dog sera over a period of 12 months. **Results:** There was strong agreement between the results obtained using GP-DAT and FD-DAT antigens stored at 22-37 ℃ for 12 months for both human (100%) and dog (100%) pooled sera. The direct agglutination test results were highly reproducible (weighted kappa: GP=0.833, FD=0.979 and FF=0.917). **Conclusions:** Because GP-DAT antigen is highly stable over a range of temperatures and is easy to transport in the field, this type of antigen may be particularly useful in areas with endemic visceral leishmaniasis.

1. Introduction

Visceral leishmaniasis (VL) is the most severe form of leishmaniasis and has a fatality rate as high as 100% within two years if left untreated. The mortality rate for the disease can be reduced with early diagnosis and appropriate treatment[1]. Epidemiological studies have shown that Mediterranean VL caused by *L. infantum* occurs in different parts of Iran, and domestic dogs are the principal source of the infection[2].

The direct agglutination test (DAT) is an easily performed, highly sensitive, specific, reliable and cost-effective technique for the diagnosis and seroepidemiological study of VL in humans and dogs across different geographical regions[3-9].

However, use of the DAT has been limited because of its susceptibility to fluctuating temperature conditions and requirement for cold chain facilities, which may not be present in regions with endemic VL. A freeze-dried DAT antigen has been evaluated in the field and found to be adequately stable for long periods of time at temperatures up to 45 ℃, but preparation of this antigen requires

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sophisticated equipment, a continuous electrical supply and a high level of laboratory expertise[10].

In the present study, we report, for the first time, a glycerol-preserred (GP)-DAT antigen derived from an Iranian strain of *L. infantum*. The antigen was stored at various temperatures, and its ability to detect anti-*L. infantum* antibodies in cases of Iranian VL and canine visceral leishmaniasis (CVL) was compared with that of freeze dried (FD)-DAT and formaldehyde-fixed(FF)-DAT antigens.

2. Materials and methods

2.1. Study design

In this study, we evaluated the use of glycerol for the long-term preservation of DAT antigen. GP-DAT antigen was stored at 4 ℃, 22-37 ℃ or 50 ℃ immediately after preparation and subsequently used to detect the presence of anti-*Leishmania* antibodies in positive and negative pooled human and dog sera collected from different parts of Iran. Over a period of 365 days, the GP-DAT antigen was evaluated 12 times at monthly intervals. The results for GP-DAT and FD-DAT antigens kept at ambient temperatures (22-37 ℃) and FF-DAT antigen stored at 4 ℃ were presented. To determine the specificity of GP-DAT antigen for detecting anti-*Leishmania* antibodies, forty-one sera from patients with infectious diseases other than VL and 10 healthy control sera were used.

2.2. Subjects

The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the Tehran University of Medical Sciences in Tehran, Iran. Bone marrow and 2-3 mL of venous blood were collected from humans suspected to have VL and from domestic dogs in areas with endemic VL in the northwest and southern parts of Iran.

Sera from forty-one individuals with cutaneous leishmaniasis (n=12), hydatidosis (n=5), malaria (n=4), leprosy (n=4), toxoplasmosis (n=3), hepatitis B (n=3), tuberculosis (n=5), fascioliasis (n=3), HIV^+ (n=1) or borreliosis (n=1) were used to determine the specificity of GP-DAT antigen for detecting anti-*Leishmania* antibodies compared with FD-DAT and FF-DAT antigens.

Eighty qualified sera (40 human sera and 40 dog sera), out of 729 total serum samples collected from different geographical zones in Iran from 2007-2009, were used to make pooled sera. Each pooled serum contained 10 sera. All sera were separated from the blood samples and kept frozen until they were sent to the laboratory.

All pooled sera were tested in duplicate using GP-DAT, FF-DAT and FD-DAT antigens at the School of Public Health at the Tehran University of Medical Sciences.

2.3. Preparation of DAT antigens-FF-DAT antigen -GP-DAT antigen

The *L. infantum* antigens were prepared in the leishmaniasis laboratory at the School of Public Health at the Tehran University of Medical Sciences.

The principal steps for making DAT antigen were the mass production of promastigotes of the Iranian strain of *L. infantum* [MCAN/IR/07/Moheb-gh. (GenBank accession no. FJ555210)] in RPMI 1640 medium (Biosera, South America) containing 10% fetal calf serum (Biosera, South America), trypsinization of the parasites, staining with coomassie brilliant blue R-250 (Sigma, USA) and fixing with 1.2% formaldehyde[2,3].

Antigen was prepared under the same conditions as standard FF-DAT antigen, but after centrifugation of FF-DAT, the pellet was mixed with 1.2% (v/v) formalin citrate saline and preserved with 75% glycerol (Merck Lot No. 217K17776791) at 5×10^8 promastigotes/mL[11]. The GP-DAT antigen was divided into 0.5 ml aliquots; these vials were then stored at the ambient laboratory temperature (22-37 ℃), 4 ℃ and 50 ℃ for up to 12 months.

2.4. FD-DAT antigen

Twenty 5 mL vials of FD antigen (KIT Royal tropical Institute, Lot No. 0904, Netherland) were kindly provided by the Eastern Mediterranean Health Regional-World Health Organization (EMRO/WHO) as a gift and delivered to us by the office of the World Health Organization in Iran (Dr. Manenti). The FD antigens were stored at ambient temperatures (22-37 ℃) in the leishmaniasis laboratory at the School of Public Health, Tehran University of Medical Sciences in Tehran, Iran.

2.5. DAT performance

Monthly tests were performed between September 2009 and August 2010. To reconstitute the FD antigen, 5 mL of normal saline (0.9% NaCl) was added to each vial two days before testing. Glycerol-preserved antigen was reconstituted by adding 9 volumes of 1.2% (v/v) formalin citrate saline before testing.

The pooled human sera were diluted over a range of 1:100 to 1:102 400 with normal saline (0.9% NaCl) containing 0.2% gelatin and 0.78% β-mercaptoethanol (2-ME) (Sigma Lot No. 45H0508). The pooled canine sera were diluted from 1:10 to 1:204 80 with the same diluents, but 1.56% 2-ME were

added to the V-shape microtiter plates. The dog sera plates were incubated for 1 h at 37 ℃.

After adding the respective antigens, the microplates were manually shaken for one minute. Following 24 h incubation under ambient temperatures and moist conditions, the results were assessed[12,13]. The highest dilution showing agglutination was considered to be the titer. The positive controls were clinically, parasitologically (by microscopy and culture) and serologically (DAT, IFA and rK39) positive. No anti-*Leishmania* antibodies were detected in sera collected from healthy control subjects who lived in areas without endemic VL.

Glycerol-preserved DAT antigen was compared with FD-DAT and FF-DAT antigens. The results were read by at least two observers, and the titers were expressed as reciprocals of the highest dilution showing definite agglutination.

2.6. Data analysis

Statistical analyses were conducted using SPSS software version 13.5 (SPSS Inc., Chicago, IL, USA), with a probability (*P*) value of less than 0.05 considered to be statistically significant. The degree of agreement was determined by calculating the total number of positive samples with both antigens plus the total number of negative samples with both antigens divided by the total number of samples, using a 95% confidence interval.

3. Results

Glycerol was found to be an effective long-term preservative. Regarding storage temperature, DAT titers obtained with antigen preserved in 75% (v/v) glycerol against positive (n=6) and negative (n=2) pooled sera were compared to titers from FD-DAT and FF-DAT antigens.

All 6 positive pooled sera prepared from 30 confirmed VL patients and 30 infected domestic dogs with different anti-*Leishmania* antibody titers showed positive reactions to both FD-DAT and GP-DAT antigens stored at 4 ℃ and with GP-DAT antigens stored at 22-37 ℃ for a period of 12 months; however, 75% of FF-DAT antigens showed positive reactions to both human and dog positive pooled sera. Interestingly, 25.0% and 33.3% of confirmed human and dog pooled sera were detected with GP-DAT antigen stored at 50 ℃ for the first month after preparation. The glycerol-preserved antigen stored at 4 ℃ and 22-37 ℃ showed higher titers than the FF-DAT antigen, although storage of GP-DAT antigen at 22-37 ℃ did not produce autoagglutination. The results show that the FF-DAT antigen detected anti-*L. infantum* antibodies in human and dog sera for 6 months after preparation while the GP-DAT antigens stored at 4 ℃ and 22-37 ℃ were stable for at least 12 months.

With the exception of GP-DAT antigen stored at 50 ℃, DAT results were highly reproducible at the time of survey (weighted kappa: GP= 0.833, FD= 0.979 and FF= 0.917).

No anti-*L. infantum* antibodies were detected in sera collected from 41 patients with infectious diseases other than VL or from 10 healthy controls by GP-DAT, FD-DAT or FF-DAT antigens.

4. Discussion

Direct agglutination tests were introduced three decades ago using an antigen suspension composed of promastigote forms of *Leishmania donovani* for the detection of VL, but this technique required that the antigen be stored and transported in a cold chain[3]. The FD-DAT antigen was introduced to solve this problem; however, the major drawbacks of freeze-dried antigen are the requirement of sophisticated equipment, a continuous electrical supply and a high level of laboratory expertise[10,14].

A simple and economical method for making DAT antigen from *L. donovani* to detect anti-*Leishmania* antibodies was successfully introduced by Harith *et al* in Sudan for Kala-azar sufferers[15] and for PKDL in India[14]. Here, a GP-DAT antigen was prepared from an Iranian strain of *L. infantum* and stored at different temperatures for the purpose of detecting anti-*L. infantum* antibodies in positive and negative pooled sera collected from Iranian VL cases, patients with other infectious diseases, healthy controls and canine VL cases.

In order to decrease the influence of genetic variations in human and canine hosts, which is the most critical confounding factor in the study of this disease in diverse geographical regions[8,16-22], the positive pooled sera were collected from different areas with endemic VL and negative pooled sera were collected from areas without endemic VL.

It seems that long-term maintenance of GP-DAT antigen stored at 22-37 ℃ and 4 ℃ compared to FF-DAT antigen is related to the potentially protective effects of glycerol on the intact promastigote *L. infantum* that is used for DAT antigen preparation.

The GP-DAT antigen stored at 50 ℃ was of unacceptable quality; this is likely due to high temperatures having destroyed the antigen, which lead to decreased test sensitivity during the initial stages of the study.

In conclusion, GP-DAT antigen stored at 22-37 ℃ is highly stable at fluctuating laboratory temperatures, easy to transport and can be used for the serodiagnosis of human and canine VL without the requirement for specialized equipment for transportation or maintenance of a cold chain.

Conflict of interest statement

We declare that we have no conflict of interest.

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