Rapid detection of human and canine visceral leishmaniasis: Assessment of a latex agglutination test based on the A2 antigen from amastigote forms of Leishmania infantum

Behnaz Akhoundi a, Mehdi Mohebali a,b,* Saeedeh Shojaee a, Mahmoud Jalali c, Bahram Kazemi d, Moigan Bandehpour d, Hossein Keshavarz a,b, Gholam Hossein Edrisian a,b, Mohammad Bagher Eslami e, Hossein Malekafzali f, Ameneh Kouchaki d

a Department of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran
b Center for Research of Endemic Parasites of Iran (CREPI), Tehran University of Medical Sciences, Tehran, Iran
c Department of Biochemistry, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran
d Center of Cellular and Molecular Research, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran
e Department of Pathobiology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran
f Department of Bistostatics and Epidemiology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran

highlights

- A2 antigen (42–100 kDa) was prepared from amastigote form of Leishmania infantum.
- A good degree of agreement was found between A2-LAT and DAT with human sera (0.914).
- A good degree of agreement was found between A2-LAT and DAT with dog sera (0.968).

abstract

The diagnosis of visceral leishmaniasis (VL) in humans and animal reservoir hosts is difficult, particularly in rural areas where the disease is endemic and laboratory facilities are limited. This study aimed to develop a latex agglutination test (LAT) for the rapid detection of anti-Leishmania antibodies against the A2 antigen derived from the amastigote form as well as those against crude antigens derived from the promastigote form of an Iranian strain of Leishmania (Leishmania) infantum. The A2 antigen (42–100 kDa) was prepared from the amastigote form of L. infantum, purified with electrophoresis and compared with the crude antigen from the promastigote form of L. infantum. Both antigens showed appropriate intensity reactions, were selected using dot blotting of positive and negative pooled sera and used to sensitize 0.9-μm latex beads. The tests were carried out on sera from 43 symptomatic, human patients with VL confirmed by parasitological examination and direct agglutination test (DAT), 30 healthy controls and 32 patients with other infections but without VL. Canine sera were collected from 63 domestic dogs with VL confirmed using parasitological examination and direct agglutination test and 31 healthy dogs from areas non-endemic for VL. Compared with the controls, human sera from DAT-confirmed patients yielded a sensitivity of 88.4% (95% CI, 82.1–94.5%) and specificity of 93.5% (95% CI, 87.0–99.7%) on A2-LAT (amastigote) when 1:3200 was used as the cut-off titre. A good degree of agreement was found between A2-LAT and DAT (0.914). LAT required 3–5 min to complete, versus the 12–18 h needed for DAT. Compared with...
1. Introduction

Visceral leishmaniasis (VL) is a life-threatening, systemic disease caused by an intracellular protozoan parasite belonging to the *Leishmania donovani* complex (WHO, 2010). Mediterranean VL is a potentially fatal protozoan infection that is endemic to some parts of Iran (Mohebali et al., 2005, 2006). VL caused by *Leishmania infantum* in humans and animal reservoirs has been reported from different parts of Iran (Mohebali et al., 2005). Almost 68.4% of human VL cases in Iran have been detected in the rural areas of the northwestern and southern regions, where health facilities are not well established and VL often coexists with brucellosis, tuberculosis and other infectious diseases (Mohebali et al., 2005, 2006). Parasitological, serological and molecular methods are used for the diagnosis of human VL (Zijlstra et al., 2001). Different conditions such as kind of *Leishmania* antigens, manufacture’s production, geographical location and clinical signs can be effect on the results of the tests (Zijlstra et al., 2001).

Rapid and early detection of human *L. infantum* infection by a fast, sensitive and non-invasive tool is highly necessary, as it will enable prompt treatment and therefore decrease the mortality rate of human VL. Of the currently available serological tests for the diagnosis of VL, the direct agglutination test (DAT) is simple, highly specific and sensitive, cost-effective and reliable (Mohebali et al., 2006; Harith et al., 1989). Nonetheless, the DAT has some limitations, particularly in the field; these include the requirement of a long incubation time (12–18 h) and serial dilutions of sera. Since VL occurs mainly in areas where health services are poorly developed, research has been focused on the development of a simple, cheap and accurate diagnostic test for VL. Few of the available serological methods for the diagnosis and seroepidemiological study of VL are suitable for field applications (Harith et al., 1986, 1989; Mohebali et al., 2005, 2006, 2011), which require relatively simple and rapid tests.

Attar et al. (2001), using rabbit anti-promastigote sera, demonstrated an antigen in the urine of both experimentally infected cotton rats and human VL patients by using a capture enzyme-linked immunosorbent assay (ELISA) and an easy-to-perform latex agglutination test (LAT) (Attar et al., 2001). The Kalon Biological Company (UK) designed a serological kit for diagnosis of VL based on the investigation by Attar et al. However, this kit is expensive to prepare pooled sera. Each pooled serum contained material from individual sera. A2-LAT of canine sera from DAT-confirmed cases yielded a sensitivity of 95.2% (95% CI 95.0–95.4%) and specificity of 100% (95% CI 100%) when 1:320 was used as the cut-off titre. A good degree of agreement was found between A2-LAT and DAT (0.968). Similarly, the sensitivity and specificity of Pro-LAT (promastigote) was calculated to be 88.4% and 91.9%, respectively for human sera and 96.8% and 90.3%, respectively for canine sera. No statistically significant differences were observed between A2-LAT and Pro-LAT for the detection of human and canine *L. infantum* infections. In conclusion, A2-LAT and Pro-LAT could be used in parallel to screen for *L. infantum* infections in humans and dogs in areas endemic for VL in Iran.

Peripheral blood samples (2 ml) were collected from suspected human and canine VL in various geographical locations of Iran (Mohebali et al., 2005, 2011). Thirty human sera (20 positive and 10 negative) and 30 dog sera (20 positive and 10 negative) used to prepare pooled sera. Each pooled serum contained material from 5 to 10 different sera collected from different geographical zones in Iran.

The human positive pooled sera were clinically, parasitologically (microscopically and culture) and serologically (DAT, indirect immunofluorescence assay [IFA], rK39) positive. No anti-*Leishmania* antibodies were detected in the control sera collected from healthy individuals who lived in non-endemic areas. The human positive pooled sera were divided into groups according to their DAT titres: high (antibody titre, $\geq 1:102,400$), intermediate (antibody titre, $1:12,800–1:51,200$), low (antibody titre, $1:400–1:6400$) and
negative (no anti-Leishmania antibodies detected). Similarly, positive pooled sera collected from dogs were divided into four groups: high (antibody titre, $\geq 1:20,480$), intermediate (antibody titre, $1:1280$–$1:5120$), low (antibody titre, $1:40$–$1:640$) and negative (no anti-Leishmania antibodies detected).

For evaluation of latex agglutination test (LAT) as rapid detection of anti-Leishmania antibodies against A2 antigen derived from the amastigote form of Iranian strain of Leishmania (Leishmania) infantum, 73 human serum samples (43 positive and 30 negative) and 32 serum samples from other human infectious diseases (VL negative) included on cutaneous leishmaniasis ($n = 13$), hydatidosis ($n = 5$), malaria ($n = 2$), leprosy ($n = 4$), toxoplasmosis ($n = 3$), hepatitis B ($n = 2$) and tuberculosis ($n = 3$) were used. Moreover, 94 canine serum samples (63 VL-positive and 31 VL-negative) were evaluated.

2.2. Parasitological examination

Parasitological examinations were performed on patients who were clinically suspected to have VL and had DAT titres $\geq 1:3200$ and on clinically positive domestic dogs with DAT titres $\geq 1:320$. Bone marrow smears were prepared from infected humans as well as domestic dogs, fixed with methanol, stained with 10% Giemsa and examined microscopically for the presence of amastigotes under high magnification (1000×). When necessary, culture was carried out in biphasic culture media (prepared from nutrient agar containing 10% whole rabbit blood overlaid with normal saline containing 100–200 U/ml penicillin G and 1 μg/ml streptomycin). The inoculated cultures were incubated at 21°C for up to 6 weeks and examined weekly for the presence of promastigotes.

2.3. Cell culture

In brief, promastigotes of the Iranian strain of L. infantum MCAN/IR/07/Moheb-gh. (GenBank accession No. FJ555210) were cultivated in RPMI1640 medium containing HEPES, l-glutamine, 20% fetal calf serum and penicillin/streptomycin at 25°C and 20% CO₂. After 1 day, the amastigote forms were examined under a microscope and subjected to acid phosphatase assay. Some procyclic promastigotes and 42–100 kDa (A2 fraction) from amastigotes were disrupted (three cycles of 12-s sonification with amplitude 90%) by sonicator (UP200H, Hielscher). Next, the suspension was centrifuged at 8000g for 1 h at 4°C, and aliquots of the resultant supernatant were analyzed using immunoblotting with one-dimensional sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) with pooled sera (Kumari et al., 2012).

2.4. Acid phosphatase assay

Acid phosphatase assay was performed as described by Doyle and Dwyer (Doyle and Dwyer, 1993). In brief, the culture supernatants of 80 × 10⁶ amastigotes and promastigotes were used for the assay. P-nitrophenyl phosphate was used as the substrate. Enzyme activities were reported as the number of nanomoles of substrate hydrolyzed to p-nitrophenol per minute at 30°C. Protein concentration was determined by the Bradford method, and the ratio of acid phosphatase to protein was determined for both stages. Next, the pellet of amastigote-containing culture media was used for soluble protein preparation.

2.5. Preparation of soluble proteins

In this step, 1 mM phenylmethylsulphonyl fluoride was added to the pellets. The cell suspension was then disrupted (three cycles of 12-s sonification with amplitude 90%) by sonicator (UP200H, Hielscher). Next, the suspension was centrifuged at 8000g for 1 h and aliquots of the resultant supernatant were analyzed using immunoblotting with one-dimensional sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) with pooled sera (Kumari et al., 2012).

2.6. SDS–PAGE and Immunoblot assay

SDS–PAGE was performed under denaturing and reducing conditions with a 15% acrylamide–3% bisacrylamide gel, as described by Laemmli (Laemmli, 1970). The gels were stained with Coomassie blue G250 or electroblotted. We used the protein ladder Fermentas SMO431, which contains six proteins ranging in size from 14.4 kDa to 116 kDa. The separated proteins were transferred from the gel to a nitrocellulose paper (50 mA, 16 h), blocked with 2.5% skim milk in phosphate-buffered saline (PBS, pH 7.4) and washed five times with PBS for 5 min. The proteins were detected using a human pooled sera (dilution 1:50) and horseradish peroxidase anti-human conjugate (Sigma A8792) with dilution 1:400. Enzyme activity was revealed using diaminobenzidine (Fluka 32741) as a chromogenic substrate. Color development was stopped by washing the membrane in distilled water. The weight of dark bands was determined, and Sartorius filtration tubes (10 kDa) were used (SM13239) to decrease the large number of promastigote fractions. The soluble proteins of the promastigotes were centrifuged at 2000g for 90 min and nonspecific bands were deleted.

2.7. Electroelution

After identification of the immunodominant antigens, SDS–PAGE was repeated in order to load large amounts of the proteins. Proteins with a molecular mass of 12–16 kDa and 29–36 kDa from promastigotes and 42–100 kDa (A2 fraction) from amastigotes were cut by reverse or negative dyeing (0.2 M imidazole and 2.7% ZnCl₂). These fractions were eluted by electrophoresis in running buffer (20 mM Tris-base, 150 mM glycerine, 0.01% SDS) using an Electro-Elutor (Biorad 422) at 10 mA for 3 h. The proteins were further visualized by SDS–PAGE. The purified fractions were quantified by the Bradford method.

2.8. Dot blot

The reaction intensity of the purified fractions and the first promastigote and amastigote lysates were investigated by the dot blot method. The process of dot blot was the same as that of immunoblot, except that each antigen (50 μg/ml) was directly spotted onto the nitrocellulose membrane, which was cross-linked at an intensity of 120 mJ, 1 min, using an ultraviolet light (UVitec). Negative pooled serum and a serum sample each from a patient with cutaneous leishmaniasis and one with a hydatid cyst were tested. Different human and canine pooled sera at 1:500 dilution were used as the primary antibody.

2.9. DAT antigen preparation and performance

DAT antigen was prepared in the protozoology unit of the School of Public Health at Tehran University of Medical Sciences. The principal phases of the procedure for preparing the DAT antigen were mass production of promastigotes of the Iranian strain of L. infantum (MCAN/IR/07/Moheb-gh., GenBank accession No. FJ555210) in RPMI1640 medium supplemented with 20% fetal bovine serum, trypsinization of the parasites, staining with Coomassie Brilliant blue and fixing with 1.2% formaldehyde (Harith et al., 1986,1989; Edrissian et al., 1996; Mohebali et al., 2005, 2011). Human sera were diluted from 1:10 to 1:102,400 with normal saline (0.9% NaCl) containing gelatin and 0.78% β-mercaptoethanol.
DAT and parasitological results were calculated as follows: Validity and agreement of A2-LAT and Pro.-LAT in comparison with positive; TP, true positive; FN, false negative; and FP, false positive.

2.10. LAT antigen preparation

The A2 and promastigote lysates proteins identified by the dot blot were candidates for LAT. Latex beads (0.9 μm; Sigma CLB9) were activated by mixing overnight at 4 °C with 2 mg/ml EDC (1-ethyl 3-dimethylamino propyl carbodiimide; S 4895907820, Merck) added to 0.1 M PBS (pH 7.6) to obtain a dilution of 1:10. The suspension was then centrifuged at 800g for 10 min at 4 °C and washed twice with the EDC-containing PBS. Finally, 900 μl PBS was added to the resultant sediment. The mixture of latex beads and the A2 fraction and promastigote lysate was mixed for 2 h at 40–50% shakes per min at room temperature for covalent coupling. At the end of this procedure, 0.01 g/ml bovine serum albumin was added to the beads. The latex beads sensitized with the two antigens were stored at 4 °C.

An aliquot of 5 μl serum in 0.1 M PBS (dilution 1:2; pH 7.6) was mixed with 5 μl of each latex antigen, and the solution was shaken at room temperature for 3–5 min.

2.11. Ethical approval

The trial was reviewed and approved by the ethics committees of Tehran University of Medical Sciences and the Centre of Diseases Control, Ministry of Health, Treatment and Medical Education, Iran in accordance based on Helsinki Declaration. All the samples were prepared after oral/written informed consent from each patient or his/her parent or dog’s owners. No personal information on the patients has been presented in this research.

2.12. Data analysis

The sensitivity and specificity of A2-LAT (A2 fraction) and Pro.-LAT (promastigote protein) were calculated as follows:

\[
\text{Sensitivity} = \frac{TP}{TP + FN} \times 100\% \\
\text{Specificity} = \frac{TN}{TN + FP} \times 100\% \text{ where } TN \text{ represents true negative; } TP, \text{ true positive; } FN, \text{ false negative; and } FP, \text{ false positive.}
\]

Validity and agreement of A2-LAT and Pro.-LAT in comparison with DAT and parasitological results were calculated as follows:

\[
\text{Validity} = \frac{(\text{Sensitivity} + \text{Specificity})}{2} \text{ and Agreement } = \frac{TP + TN}{Total}
\]

Statistical analyses were conducted using the SPSS software, version 13.5 (SPSS Inc., Chicago, IL, USA), with a probability (p) value of <0.05 considered as statistically significant. For evaluation of the reproducibility of LAT, 20 human sera and 20 canine sera were selected using a table of randomized numbers and subjected 2 times to LAT under the same conditions.

3. Results

3.1. Acid phosphatase assay

The ratio of acid phosphatase to protein derived from promastigote and amastigote forms of *L. infantum* were determined 0.5 and 0.2, respectively.

3.2. SDS–PAGE and immunoblot assay

After SDS–PAGE and immunoblotting of soluble proteins from the promastigotes and amastigotes of the Iranian strain of *L. infantum*, two antigen fractions of 12–16 and 29–36 kDa from the promastigotes and a 42–100 kDa A2 fraction from the amastigotes were consistently recognized (Fig. 1, Fig. 2).

3.3. Dot blot

The qualitative results of dot-blot of human pooled sera for the selection of purified fractions and the first promastigote and amastigote proteins for binding to latex beads are shown in Fig. 3. To obtain better results, the dot blot was repeated with low-positive and high-positive canine pooled sera with DAT (Fig. 4).

The A2 proteins and promastigote lysates yielded the most intense reaction with dot blot (Fig. 4).

3.4. LAT

The results of 199 human and canine sera evaluated with A2-LAT and Pro.-LAT are shown in Table 1. The sensitivity, specificity and agreement of the A2-LAT and Pro.-LAT for human and canine sera are shown in Table 2. Compared with the controls, human sera from DAT-confirmed patients yielded a sensitivity of 88.4% (95% CI, 82.1–94.5%) and specificity of 93.5% (95% CI, 87.0–99.7%) on A2-LAT when 1:3200 was used as the cut-off titre. A good degree of agreement was found between the A2-LAT and DAT (0.914). Furthermore, LAT required 3–5 to complete, versus the 12–18 h needed for DAT. Compared with the controls, A2-LAT of canine sera from DAT-confirmed cases yielded a sensitivity of 95.2% (95% CI, 95.0–95.4%) and specificity of 100% (95% CI, 100%) when 1:320 was used as the cut-off titre. A good degree of agreement was found between A2-LAT and DAT (0.968). Similarly, the sensitivity and specificity of Pro.-LAT was calculated to be 88.4% and 91.9%, respectively for human sera and 96.8% and 90.3%, respectively for canine sera.

Of the 43 DAT-positive sera, three samples with a DAT titre of 1:102,400, one with a titre of 1:51,200 and one with a titre of 1:12,800 tested negative on A2-LAT (5 false-negative results). Of
the 30 DAT-negative samples obtained from healthy controls, 2 tested positive on A2-LAT. In the case of the canine samples, A2-LAT yielded three false-negative results (two samples with a DAT titre of 1:20,480 and one with a titre of 1:320) and no false-positive results.

The reproducibility of the A2-LAT method was determined to be 100% by re-testing two times on 20 human sera and 20 canine sera of the positive and negative serum samples.

Pro.-LAT yielded five false-negative results and three false-positive results in the case of human sera, and two false-negative results (of a total of 63 canine samples; both had DAT titres of 1:20,480) and three false-positive results (of 31 DAT-negative samples) in the case of canine sera. One sample each from a leprosy patient and a tuberculosis patient tested positive on both LATs (false positive). No statistical correlation was found between A2-LAT and Pro.-LAT (p > 0.05). The reproducibility of the LATs was determined to be 98% after repeating the test three times on a few of the DAT-positive and DAT-negative human and canine sera.

4. Discussion

Early diagnosis, rapid tests and prompt treatment are essential to reduce the mortality and morbidity rates from VL in endemic areas (Desjeux, 2004). As the clinical manifestations of VL are non-specific, confirmatory tests are required for a definitive diagnosis (Chappuis et al., 2007). The tests currently in use, though highly specific, usually require sophisticated equipment and are expensive and time-consuming.

LAT is a simple, easy, inexpensive and rapid test for routine screening for VL in endemic areas where laboratory facilities are limited. Katex is a new modified LAT for the detection of leishmanial urinary antigen in VL patients; it was developed by Attar et al. (2001), who reported that it had 100% specificity and 68–100% sensitivity, using urine collected from confirmed VL cases and controls from Brazil, Yemen and Nepal. Other studies have reported different sensitivities and specificities for Katex, including (in order) 95.2% and 100% in Sudan, 67% and 99% in India, 57%, > 90% and 47.7%, 98.7% in two studies in Nepal, 73.9% and 82.4% in Ethiopia and 82.7% and 98.9% in Iran (Safi et al., 2003; Rijal et al., 2004; Mo-laei et al., 2005; Diro et al., 2007; Boelaelter et al., 2008). One of the main drawbacks of Katex is that the urine samples need to be boiled prior to testing in order to reduce the rate of false-positive results. Immunogenicity and protective efficacy of A2 antigen against different Leishmania species were studied by few investigators. In a study that was carried out on A2 antigen derived from L. donovani and L. infantum for immunization and protection against L. ama-
zonensis or L. chagasi infections, an appropriate protection was produced (Zanin et al., 2007).

Fernandes et al. (2008) used rA2 for immunization of dogs against canine visceral leishmaniasis and partial protection was achieved (Fernandes et al., 2008). Another study was done by the mentioned investigators in 2012, dogs, mice and nonhuman-pri-mate immunized with rA2 mixed with saponin, alum and IL-12. Their results shown vaccinated animals could produce significantly increased levels of total IgG and IgG2, but not IgG1 anti-A2 antibo-dies, IFN-α and low IL-10 levels were detected in vaccinated ani-mals before and after challenge, as compared to control animals (Fernandes et al., 2012).

Porrozzi et al. (2007) used an ELISA technique based on crude and recombinant leishmanial antigens (rA2, rK39, rK26) for serodi-agnosis of symptomatic and asymptomatic L. infantum visceral infections in dogs. Their results shown “antigens rK26 and rK39 provided very high sensitive ELISAs for the detection of symptomatic dogs (94% and 100%, respectively), followed by crude soluble antigen (89%) and rA2 (76%). Conversely, rA2 was more sensitive for asymptomatic dogs (89%) in comparison with rK39, rK26 (both 75%), and crude soluble antigen (59%). Some cross reactivity was identified in sera from dogs with other infections (L. braziliensis and Leptospira interrogans), but the rA2 protein provided more specificity (96%).” (Porrozzi et al., 2007).

In our study, fraction A2 antigen and LAT were replaced to re-combinant A2 and ELISA and used to detect L. infantum-infections in human and canine sera. Our results shown that A2-LAT had a lower rate of false positivity than Pro.-LAT for human and canine sera. However, the electroelution technique used for concentration of the samples and the recombinant A2 antigen are expensive and demand a high level of skill. Furthermore, while the culture of the promastigote forms of L. infantum is relatively easy to perform and produces large amounts of proteins in a short time, the culture of amastigotes of Leishmania species for the preparation of A2 pro-teins is difficult and time-consuming. In this study, the accuracy of A2-LAT and Pro.-LAT for the detection of VL in human and canine sera was identical; both antigens can be used in parallel to screen for VL in endemic areas in Iran.

The differentiation of Leishmania parasites from the promasti-gote to the amastigote stage requires acidic pH and high tempera-ture (35–37 °C). These conditions induce the synthesis of several antigens, and there have been several attempts to characterize the molecules that are specifically expressed by the amastigote stage (Zhang et al., 1996). Based on studies on LAT (Mohebali and Mohammadi, 1997; Safi et al., 2003; Rijal et al., 2004; Riera et al., 2004; Molaei et al., 2005; Diro et al., 2007; Boelaert et al., 2008; Ghati et al., 2009) different strains of L. donovani have been used for the preparation of sensitized latex beads (with antibody or antigen). In this study, however, an Iranian strain of L. infantum was used for bead sensitization and the comparative evaluation of the LAT and DAT for the detection of anti-L. infantum antibodies in human and canine sera.

In conclusion, the LAT can be used as a qualitative screening test for a large population of individuals and domestic dogs suspected to have VL in endemic areas. The results of LAT can be obtained within 3–5 min; further, retesting of LAT-negative samples with DAT is unnecessary. LAT-positive samples, however, should be sub-jected to a DAT for confirmation. Therefore, numerous serum sam-ples can be easily screened with a combination of these two tests within a short time. Further research to validate this test in larger populations in areas that are endemic and non-endemic for VL is recommended. The use of other concentrations and sizes of latex beads may be useful to reduce the rate of false-positive reactions.

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### Table 2

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<thead>
<tr>
<th>A2-LAT</th>
<th>Pro-LAT</th>
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<tbody>
<tr>
<td>Serum type</td>
<td>Sensitivity</td>
</tr>
<tr>
<td>Human</td>
<td>88.4</td>
</tr>
<tr>
<td>Dog</td>
<td>95.2</td>
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TP = true positive. TN = true negative.
FN = false negative. FP = false positive.

$^a$ Agreement = TP + TN/total.

$^b$ Validity=(Sensitivity + Specificity)/2.

References


