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An Overview of Introducing Various Laboratory Tests for Diagnosis of Human Brucellosis in the Republic of Macedonia

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Abstract

Aim. To present various laboratory tests introduced for diagnosis of human brucellosis in the Republic of Macedonia.

Methods. Various laboratory tests were implemented in our lab, as screening or confirmatory tests, some in studies some as a routine diagnostic test, such are: - Agglutinations tests: Rose Bengal test (RBT), Slide Agglutinations test, Standard tube test (SAT), Wright test, Antihuman globulin test (Coombs); - 2-Mercaptoethanol test (2 ME); - Complement fixation test (CFT); - Indirect enzyme immunoassay (ELISA); - Competitive enzyme immunoassay (cELISA); - Fluorescent polarization assay (FPA); - PCR-based assays from peripheral blood samples.

Results. Our comparative studies on a number of samples from patients at different stages of the disease showed: - Sensitivity of: culture 17,7%, RBT 96%, SAT 84%, Coombs 86%, 2-ME 46,5%, cELISA 98%, ELISA 98%, FPA 86 %, R.A.P.I.D PCR-56%, and -Specificity of: culture 100%, RBT 97%, SAT 100%, Coombs 100%, cELISA 98%, ELISA 100%, FPA 92%, and R.A.P.I.D PCR 100%.

Conclusion. ELISA is the best serology test for diagnosis of human brucellosis. FPA and cELISA are promising tests but need further studies on a larger number of human samples. PCR detection of *brucella* DNA enables the overcoming of problems and requirements of *brucella* isolation and identification.

Introduction

Diagnosis of human brucellosis is based on clinical features and laboratory tests (culture, serology testing and molecular techniques).

Culture as a "Gold standard" is primarily difficult, hazardous, time-consuming, lacks sensitivity to patients with chronic brucellosis, and requires highly skilled personnel and special level III bio safety cabinets. Bacteriological isolation and identification is not routinely implemented since no adequate laboratories and especially trained technicians exist in Macedonia.

The epizootic in animals and epidemic of human brucellosis in the Republic of Macedonia started in 1980 and still remain a serious veterinarian, medical and economic problem. From 1980 until the end of 2009, about 11,500 human cases were reported and underwent treatment for brucellosis. Diagnostic tests following WHO and reference laboratories recommendations were first introduced by Prof. D-r Borivoje Sokolovski *et al.* [1-4]. The

microbiology lab of the Institute of Preventive Medicine at the Military Hospital in Skopje was from the beginning of the epidemic a National reference lab and centre for education for the diagnosis of human brucellosis.

Diagnosis of human brucellosis is based on clinical features and laboratory tests (culture, serology testing and molecular techniques). The variable symptoms, subclinical and atypical infections, in both acute and chronic stages, make diagnosis of human brucellosis difficult.

With the development of different diagnostic tests most of them were subsequently implemented in our laboratory.

Culture as a "Gold standard" is primarily difficult, hazardous, time-consuming, lacks sensitivity to patients with chronic brucellosis, and requires highly skilled personnel and special level III bio safety cabinets. Bacteriological isolation and identification are not routinely implemented since no adequate laboratories exist in Macedonia. However, modern blood culturing and automatic identification and susceptibility testing systems, in a few laboratories, with all the risks, are used for isolation, identification and even antibiotic testing.

Materials and Methods

Materials

Blood samples were collected from patients from departments for infectious diseases of five hospitals in Macedonia and samples from field studies across the country. Diagnosis was based on epidemiological data, clinical findings and laboratory tests. A total 725 sera from 592 patients were tested with classical serology methods, 1.100 sera for ELISA, 330 blood samples for PCR and 90 for culture. Many of the patients were on treatment when the blood samples were collected. Control samples were obtained from 100 healthy, voluntary blood donors and 69 sera from healthy persons from endemic areas. Simultaneously, when taking samples for serology testing, 4 ml of blood in tubes with EDTA (anticoagulant) were collected for PCR.

Methods

Different tests were implemented in our lab, as screening or confirmatory tests, some in studies some as a routine diagnostic test, such are:

- Agglutinations tests:
  - Rose Bengal test (RBT), Slide Agglutinations test;
  - Standard tube test (SAT), Wright test;
  - Antihuman globulin test (Coombs);
  - 2-Mercaptoethanol test;
- Complement fixation test (CFT);
- Indirect enzyme immunoassay (ELISA);
- Competitive enzyme immunoassay (cELISA);
- Fluorescent polarization assay (FPA);
- PCR-based assays from peripheral blood samples.

Agglutinations tests:

a) Rose Bengal test (RBT), Slide Agglutinations test. RBT is highly sensitive, and is mostly used as a screening test. Antigen is performed from a concentrated suspension of *B. abortus* 99-Weybridge. The test is positive if agglutination appears within 4 min. False positive results appear due to cross-reaction with *Yersinia enterocolitica*. RBT (RhoneMerieux, bioMerieux) and BAB (Inep) tests were used.

b) Standard tube test (SAT), Wright test. A test for the detection of agglutinable IgM and IgG antibodies is performed as a standard tube test in serial of dilutions of the sera from 1/10 to 1/1280. To each tube 0.5 ml of 10% of *Brucella abortus* 99-Weybridge (Veterinary Institute, Zagreb) antigen is added, and incubated for 24 h at 37C^0. The SAT is positive if agglutination appears e" 1/160. SAT cannot distinguish IgM from IgG antibodies [5, 6]. Mostly positive in the acute stage of the disease , thetest is less sensitive in sthe ub-acute and chronic stage.

c) Antihuman globulin test (Coombs). A test for detecting non-aglutinable IgG and/or AgA antibodies. The test is positive if the agglutination appears in tubes with twofold or higher dilutions than the SAT test result [6]. Tests were performed according to standard procedure using rabbit anti-human globulins (anti IgG, IgM, IgA, Immunological Institute, Zagreb).

d) 2-Mercaptoethanol test (2-ME). The test is performed as a SAT [6]. The diluents (0. 85% NaCl contain 0.05 M of 2-ME/ Merck). 2-ME destroys disulfide bonds of IgM antibodies so only IgG remains as agglutinable antibodies (if present). The test helps to confirm the stage of the disease and response to the antibiotic treatment. As a routine test 2-ME was performed only in our microbiology lab, starting in 1996 [7, 8].

Complement fixation test (CFT)

The principle of the test is that IgG fixes the complement well while IgM does not. In early stages of the

disease when IgM only are present, CFT is usually negative. In acute brucellosis CFT titres appears in the second month and reach the maximum at the fourth month of the disease. CFT is usually positive in sub-acute and chronic brucellosis. High titres remain about 12 months. Sera were diluted from 1/4 to 1/64. The test is negative if 100% haemolysis appears in sera diluted 1/4.

Indirect enzyme immunoassay (ELISA)

Developed by Carlsson *et al.* in 1976, iy distinguishes different classes of antibodies (IgM, IgG, and IgA) important for the diagnosis of the stage of the disease [9].

ELISA is a method of quantitative and qualitative detection of sera antibodies (IgM, IgG, IgA).

ELISA micro plates with *Brucella* LPS antigen attached (Novum diagnostica, Vircell) and a Tecan classic ELISA reader were used in our study.

Competitive enzyme immunoassay (cELISA)

cELISA was developed to distinguish vaccinal from infective antibodies in animals [10]. The principle of the test is that vaccinal antibodies have a lower affinity due to shorter exposure to the antigen and due to immune elimination compared with field infection in which the antigen persisted, resulting in an increased affinity of antibodies [9]. The most commonly used format of cELISA utilizes SLPS from *B. abortus* as antigen, passively attached to a polystyrene matrix. Tests were performed with VLA, Weybridge, UK, test kits.

Fluorescent polarization assay (FPA)

FPA was developed as an accurate test that can be performed under field conditions outside the diagnostic laboratory, allowing rapid and accurate diagnosis of brucellosis in animals [9]. The basis of the test is that a molecule in solutions rotates randomly at a rate inversely proportional to its size. For diagnosis of brucellosis, a fluorescence polarization analyzer (FPM-1, Jolley) is used to obtain a background measurement of the fluorescence of diluted serum. An antigen consisting of an OPS fragment prepared from *B.abortus* strain 1119-3, approximately 22 k-Da in size, labelled with FITC (Fluorescein isothyocianate) is added and incubated for 2 min, followed by final reading. The result is presented in milipolarization units/mP [11, 12]. Tests were performed with test kits, FPA and instructions at the Animal Diseases Research Institute, Canadian Food Inspection Agency, Nepean, Ontario, Canada.

PCR-based assays from peripheral blood samples

Brucella has been isolated from human tissue samples: blood, urine, cerebrospinal fluid, which is suitable for analysis by PCR [13-16].

Brucella genome, consists of two circular chromosomes, has been completely sequenced for *B. melitensis, B.abortus* and *B. suis*. *B. melitensis* genome contains 3,294,931 base pairs (bp): chromosome I of 2,117,144 bp and chromosome II of 1,177,787 bp. *Brucella abortus* chromosome I contains 2,124,241 bp and chromosomes II is 1,162,204 bp.

Genes encoding DNA replication, protein synthesis, core metabolism, and cell-wall biosynthesis can be found on both chromosomes.

The R.A.P.I.D.™-PCR (Ruggedized Advanced Pathogen Identification Device) that we have used in our research is a 32 sample capacity, automated instrument. Monitoring the fluorescence from the double-stranded DNA dye (SYBR® Green) followed by differentiation of products by melting curves or from TaqMan® probes (6-FAM-oligo-TAMRA,) allows inexpensive quantification of a low initial template copy number. The R.A.P.I.D. system can complete a 40-cycle reaction in less than 20 minutes (6 to 30 min.). This research was performed at the Armed Forces Institute of Pathology, Washington DC, USA.

Primers in study:

IS711. *B. abortus:* Forward primer (Fa144) 5'CAT TGA AGT CTG GCG AGC A 3' [19]. Reverse primer (R 301) 5' TAT CGT CGT ATT GCG CTG C 3' [19]. *B. melitensis:* Forward primer (Fm 167) 5'AGC GTG ACG AAG CAC TGT CT 3' [20]. Reverse primer (R 301) 5'TAT CGT CGT ATT GCG CTG C 3' [19]. *B. suis:* Forward primer (Fs 194) 5' AGC GTG ACG AAG CAC TGT CT 3' [20]. Reverse primer (R 301) 5' TAT CGT CGT ATT GCG CTG C 3' [19].

BCSP31. Forward primer (F 622) 5' GCG TTG GGA GCG AGC TTT 3' [18]. Reverse primer (R 681) 5' GCC AGT GCC GAT ACG GAA 3' [18], and TagMan Probe (230) 6FAM-CGG TTG CAC AGG CCC CGA CA-TAMRA [20].

Culture

Culture as a "Gold standard" is primarily difficult, hazardous, time-consuming, lacks sensitivity to patients with chronic brucellosis, and requires highly-skilled per-

sonnel and special level III bio safety cabinets. Bacteriological isolation and identification is not routinely implemented since no adequate laboratories exist in Macedonia.

Results and Discussion

Agglutination tests. Agglutination tests were performed according to standard procedures.

The sensitivity of the Rose Bengal test (RBT) depends on the stage of the disease. Different results ranging between 100% Araj [17], 98.8% sensitivity Nikolovski (18), 86.6% Chernicheva [19] in acute brucellosis, 44.5% in chronic brucellosis and 16.2 % in healthy persons in an endemic area. The results in our study were similar, 96% sensitivity and 95.4% specificity in acute brucellosis, 59% in patients after treatment and 7% in health persons in an endemic area.

Standard tube test (SAT, Wright test) in acute brucellosis showed a sensitivity of 93. 8% by Nikolovski *et al.* [20], 87, 8% Namasito *et al.* [21].

In our study, sensitivity was 84% and specificity 100%. Cross reaction with *Yersinia* is not significant due very rare isolation in humans with diarrhoea, but never isolated in patients with brucellosis.

Antihuman globulin test (Coombs). Moreno *et al.* [22] found 97% sensitivity. In our study sensitivity was 86%, specificity 100% in patients with acute brucellosis, without any statistically significance towards SAT.

2-Mercaptoethanol test (2-ME). Comparing SAT and 2-ME test results it was possible to prove which antibodies were present in the sera (IgM, IgG or both). Gandara *et al.* [23] found 2-ME positivity in 63, 8%, that provides additional information on the stage of the disease. We found the 2-ME test positive in 53 (46.5%) of 114 SAT positive sera, which was statistically significant. Patients with positive 2-ME and SAT positive had only IgG in their sera, while patients with negative 2-ME and positive SAT had IgM antibodies (acute stage). Titres of 2-ME decrease faster than SAT titres (>18 months), meaning a good response to antibiotic treatment.

Complement fixation test (CFT). In our study CFT was positive in 42 % in patients with acute brucellosis and 39 % in patients after treatment. This test did not provide additional information on the stage of the disease or response to the antibiotic treatment, so we stopped using this test in diagnosis of brucellosis.

Indirect enzyme immunoassay (ELISA). In 1995, at the Veterinary Instute Skopje, for the first time in Macedonia and most probably in the Balkan area, ELISA testing was used in diagnosis of human brucellosis by Bosnjakovski and Taleski [16] with Dr Bomellie's test kits. At that time mostly in-house test kits were available.

Barbudhe *et al.* [25] in their ELISA study on 80 sera found a sensitivity of 89% and specificity of 77%, Colmenero *et al.* [26] in a prospective study of 50 patients with acute brucellosis found sensitivity of 90% for IgM and 68% for IgG. Araj *et al.* in 1986 published results of a study of 173 patients with a sensitivity and specificity of 98%, and two years later in a large study of 573 sera found specificity and sensitivity of 100% [27, 28].

Results were calculated towards Cut-off (CO) as follows: CO=MN + 0.250, where MN presents the average values of two negative controls. Results 10% over Cut-off were positive, results 10% lower than Cut-off were not.

Results showed the statistically significant higher sensitivity (98%) and specificity (100%) of ELISA against classical serology tests. Sensitivity to SAT, Coombs and ELISA was 82 %, 86% and 98% respectively and a specificity of 100% in all tests [16].

Positive and negative prognostic value for ELISA IgM was 98%, positive prognostic value for SAT was 82%, Coombs 89% and the negative prognostic value for SAT 84.7%, and 90% for Coombs.

Competitive enzyme immunoassay (cELISA). A number of papers have pointed to high sensitivity and specificity in the diagnosis of brucellosis in small ruminants, but no papers have been published on the diagnosis of human brucellosis. Lucero *et al.* have determined sensitivity of 98.3 and 98. 4% with cut-off values of 28 and 30%, respectively, for sera from 116 individuals found positive by classical tests. For the 51 culture positive patients, cELISA found 100% positive [29].

The results in our comparative study with classical serology tests and cELISA on 73 human sera are as follows: All negative in healthy voluntary blood donors (No=10), 31 positive in patients with acute brucellosis (No=31), six positive in previously treated (chronic disease) and 26 negative patients (recovered). These results showed statistically significant higher sensitivity and specificity (98%) in acute disease then the classical serological SAT test (84%), Coombs (86%) [30].

Fluorescent polarization assay (FPA). Sensitivity of FPA was 86% and specificity 92%.

Animals studies (bovines) by Nielsen and Gall, from 1990, (n=8669), 1994, 1996, 1998 and 1999 (n=14037), then Samartino *et al.* in 1999, (n=733), Dajer *et al.* in 1999, (n=590) showed: Sensitivity between 93.5% and 99% and Specificity from 97.2% to 100%.

The potential use of FPA in diagnosis of human brucellosis has been assessed by Lucero *et al.* Based on 340 sera from asymptomatic blood donors with no evidence of brucellosis, the specificity of FPA was found to be 97.9% using a cut-off value of 72 mP, and the sensitivity 96.1% with 76 sera from *brucella*-infected patients [31].

Our study of human samples (n=217), using cut-off 90 mP, showed: Sensitivity of 86% and Specificity of 92%.

FPA is a very promising tool in the diagnosis of human brucellosis in addition to diagnosis in animals. Further studies concerning sensitivity, specificity and the best cut-off values are needed.

PCR-based assays from peripheral blood samples. The first PCR-based assays, used for genus-specific identification of *Brucella*, amplified genes encoding: 43-kDa outer membrane protein of *B. abortus* (primers NP, amplicon size 635 bp); 31-kDa *B. abortus* protein (primers B4/B5, size 223 bp); omp-2/ membrane extern *B. abortus* protein (JPF/JPR, size 193 bp); *B. abortus* 16S rRNA (Ba148-167F/Ba928-948R primers, size 800 bp); *B. abortus* 16S rRNA (F4/R2 primers, size 905 bp).

The most frequently described PCR target for the diagnosis of human brucellosis is the *bcsp31* gene encoding a 31-kDa antigen conserved among *Brucella* spp.

An insertion sequence (IS*711*) element named *IS6501*, 836 bp in length, occurs 20-35 times in the *B. ovis* genome and 5-15 times in other *Brucella* species. Most *Brucella* species contain at least one copy of *IS711* at a unique chromosomal location. The multiplex assay consisting of one common primer anchored in the *IS711* and a species-specific primer that binds to the unique sequence allowed species identification determined by the size of the amplicon.

In 100 patients with acute brucellosis confirmed by ELISA, the PCR results were: 10 positive with IS711 primers and 56% with BCSP31 primers.

In 100 patients after treatment and persistent symptoms, from 23 ELISA positive, 2 were IS711 positive and 17 BCSP31 positive. PCR positive results confirmed the chronic stage or relapse of the disease.

In 100 patients after treatment, without symptoms, 2 were PCR (IgM and IgG ELISA positive), and needed further treatment.

In all 30 health persons (control group) PCR and ELISA were negative.

In our study sensitivity was 10% for IS711 and 56% for BCSP31, while specificity was 100% for each test. The positive prognostic value was 100 % for each test. The negative prognostic value of IS711 25% and BCSP31 40.5%, showed no very high confidence in negative results.

Culture. Published culture results vary. Moreno *et al.* found 70% positive blood cultures [22], Eissa *et al.* found 75% positive, [32], Shehabi *et al.* found 44.4% positive [33]. Modern authomatic haemoculturing systems allow higher positive culture results.

Cultures were performed in the Armed Forces Institute of Pathology, Washington DC, USA. Polimorphonuclear cells (PMC) obtained with VacutainerÒCPT™ (Cell preparation tube with Sodium Citrate) in BBL™SEPTI-CHEK™- liquid media were cultivated. All samples were incubated over four weeks with cultivating weekly on *Brucella* agar that were further incubated for one week at 37C^0, in 10% CO2 atmosphere. A total of 16 isolates (17.7%) were obtained from 90 cultivated samples. Using RAPID PCR, *brucella* DNA was confirmed in all isolates (culture specificity 100%). Determining the *brucella* DNA with RAPID PCR from cultures and blood cultures is faster, more economical than the standard methods of isolation and identification, and avoids the risk to employees of intra-laboratory infections. Only *Brucella melitensis* biotypes 2 and 3 were confirmed from isolates from Macedonia.

Conclusions

Diagnostic tests for human brucellosis were implemented following recommendations of WHO and world reference laboratories.

Bacteriological isolation and identification have not been routinely implemented yet, since no adequate laboratories and specially trained technicians exist in Macedonia.

Classical serologic tests are still the most frequently-used tests in the diagnosis of human brucellosis.

FPA and cELISA are promising tests but further studies with a larger number of human samples are

needed to determine the best cut-off.

The sensitivity and specificity of ELISA showed a statistically significant superiority in comparison with classical serological tests.

The possibility of routine use of ELISA and PCR enables the overcoming of the well-known problems in the diagnosis of human brucellosis and provides significant help in the treatment of patients and during epidemiological studies.

More effective PCR detection of *brucella* DNA requires sampling at the beginning of the disease (bacteraemia still present), and concentration techniques or larger volumes of blood for processing. PCR allows the overcoming of problems of bacterial isolation and identification.

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