Evaluation of Rose Bengal Test in Comparison with PCR for Diagnosis of Human Brucellosis

Thesis

Submitted for the Partial Fulfillment of Master Degree
In Tropical medicine

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Introduction

Brucellosis is an important zoonotic disease, of nearly worldwide distribution ***(Mitka et al., 2007).*** Notably, in Mediterranean countries and the Middle East ***(Refai, 2002).*** As the most common zoonotic disease, it remains a significant public health concern ***(Pappas et al., 2006)***.

Brucella is a Gram negative coccobacilli pathogen that is distinguished from most other pathogens because it does not have "obvious virulence factors "like" capsules, fimbriae and flagella, therefore, it is non-motile ***(Moreno & Moriyon, 2002)***. Phenotypic characteristics, antigenic variation and prevalence of infection in different animal hosts have resulted in the initial recognition of six species: B. abortus (cattle), B. melitensis (goats and sheep), B. suis (swine), B. canis (dogs), B. ovis (rams) and B. neotomae (desert rats) ***(Bricker et al., 2000).*** Recently, other Brucella species were isolated from marine mammals. But species considered important agents of human disease are B. melitensis, B. abortus, B. suis and B. canis ***(CFSPH, 2009)***.

Brucellosis is also, an occupational hazard of laboratory scientists infected by the inhalation of aerosols in a microbiology laboratory setting ***(Groussaud et al., 2007).*** Most human disease is caused by B. abortus, B. suis, B. melitensis and B. canis ***(Cloeckaert et al., 2003).***It is most frequently associated with the consumption of unpasteurized dairy products or direct contact with infected animals or animal

products ***(Jevitt et al., 2005).*** Person-to-person spread of brucellosis is extremely rare. But it can be transmitted through breast feeding, transplacental route, sexual contact and tissue transplantation ***(CDC, 2007; WHO, 2009).*** The disease is characterized by acute and chronic infections in animals leading to abortion and infertility ***(Paulsen et al., 2002).***

In humans, brucellosis is a systemic, febrile illness and can be associated with chronic debilitating infection of major organ systems that may include bone, kidney, brain, epididymis, liver, ovary and gallbladder ***(Groussaud et al., 2007).***

The clinical symptoms of human brucellosis are nonspecific, and several other febrile diseases may be simulated. The infection is characterized by protean manifestations and prolonged recurrent febrile episodes. So, that the disease is referred to as "undulant fever" ***(Mitka et al., 2007).*** Although, many organ systems may be involved, brucellosis is rarely fatal ***(Mutnal et al., 2007).***

However, Because of the predilection to affect joints, the vague symptoms and chronic nature of the disease, symptoms can result in relatively long-term disability ***(Maloney, 2008; Al-Nassir et al., 2009).***

The Epidemiology and Surveillance Unit of the Egyptian Ministry of Health and Population has recorded a substantial increase in the number of patients with brucellosis in the recent past, from 24 cases in 1988 to 1429 in 1998 ***(ESUE, 2001).*** An earlier report (1992), described the distribution pattern of

human cases of brucellosis during the period 1982–1991: the infection rate was generally low except in 1987 and 1991, when there were marked increases in numbers of cases. This was clearly observed in Alexandria and Menofiya, and also in Giza and Damietta ***(Wassif, 1992)*.**

Due to its heterogeneous and poorly specific clinical symptom- matology, the diagnosis of brucellosis always requires laboratory confirmation; either by isolation of the pathogen or by demonstration of specific antibodies. Culture provides direct evidence of the presence of the pathogen and is the gold standard ***(Pappas et al., 2006)*.** But blood-culture sensitivity is often low, ranging from 50 to 90 % depending on the disease stage, Brucella species, culture medium, quantity of circulating bacteria and the blood-culture technique employed **(*Mantur et al., 2004*).**

Hence, serological tests play a major role in cases when the disease cannot be detected by blood culture. Serological tests include serum tube agglutination test (STAT), Rose Bengal test (RBT), complement fixation test(CFT) and indirect Coombs test, Previous studies found ELISA to be an effective method for diagnosis of brucellosis ***(Osoba et al., 2001)***. However, the interpretation of these tests is often difficult, particularly in patients with chronic brucellosis, in reinfections, relapses and in areas of endemicity where a high proportion of the population has antibodies against brucellosis ***(El Miedany & El Gaafary, 2003).***

The polymerase chain reaction (PCR) assays provide a rapid and highly sensitive method of differentiating the major Brucella groups that will be valuable for clinical & forensic applications. Also, it had excellent sensitivity, specificity and was able to detect all of the cases of acute disease ***(Foster & Richard, 2008).*** PCR is a very useful tool for the rapid diagnosis of acute brucellosis, a good marker for the post treatment follow-up and the early detection of relapses ***(Mitka et al., 2007).***

Febrile syndromes with no apparent focus are a cause of great concern in patients. They, therefore, require a fast and precise etiological diagnosis as Brucellosis is one of the causes of febrile syndromes. Therefore, the aim of the present study is to analyze the diagnostic yield of simple traditional serological tests in comparison with PCR for the diagnosis of acute human brucellosis.

Aim of the Work

The aim of the present study is:

1. To analyze the diagnostic yield of RBT and STAT in comparison with PCR for the diagnosis of human brucellosis.

2. To evaluate the sensitivity, specificity, accuracy, the cost and the time consuming of RBT and STAT in comparison with PCR for the diagnosis of human brucellosis.

Chapter (1)

Historical Aspects of Brucellosis

The significance of brucellosis has been recognized since antiquity, as a type of fever, characterized by fairly regular remission or intermission, it has been recognized along the Mediterranean littoral since the time of Hippocrates in 450 B.C ***(Mantur et al., 2007)***.

Much later, in the eighteenth century, the disease had a strong association with military medicine. In 1751, Cleghorn - a British army surgeon stationed on the Mediterranean island of Minorca, described cases of chronic relapsing febrile illness and cited Hippocrates's description ***(Purcell et al., 2007).***

Local names, often attached to the term "fever", the predominant manifestations of brucellosis, were designed by exogenous military garrisons that may have been more vulnerable than the indigenous population to infection from endemic brucellosis. Hence, "Mediterranean Fever" as well as the fevers of Malta, Constantinople, Naples, Gibraltar, Crete, Crimea, Levant, Syria and so forth ***(Rust et al., 2006; Mantur et al., 2007)***. Other synonyms were derived from its resemblance of malaria and typhoid e.g., typhomalarial fever and intermittent typhoid ***(Mantur et al., 2007)***.

In 1861, Marston - an assistant surgeon of the British Medical Department working in the Mediterranean, provided the first modern clinical description of the disease as he described his own infection and termed it "Mediterranean gastric remittent fever" ***(Rust et al., 2006; Purcell et al., 2007)***.

Dr. David Bruce - a Scottish physician researched Malta fever among British soldiers stationed on Malta in 1885. He described it as a disease clinically characterized by fever, profuse perspiration, splenomegaly, frequent relapses, rheumatoid or neuralgic pain, swelling of joint and orchitis ***(Gotuzzo & Carrillo, 2003)***. Then, in 1887, he isolated the pathogen from the spleen of British soldiers those died as a consequence of this disease. Bruce named it Micrococcus melitensis, derived from Melita (honey) the Roman name of the Isle of Malta ***(Bruce, 1887; Godfroid et al., 2005; Mantur et al., 2007)***.

Hughes - a colleague of Dr. Bruce, in a monograph in 1897, confirmed the Bruce finding and discussed clinical and pathological finding in 844 patients in more detail and suggested the name of "undulant fever" ***(Hughes, 1897; Rust et al., 2006; Purcell et al., 2007)***. Hughes' term "undulant fever" became the most widely accepted clinical designation until "brucellosis" obtained currency, although "Malta fever" has also shown some staying power as a designation ***(Rust et al., 2006)***. In the same year of Hughes' suggestion, Write and Smith detect antibodies against Micrococcus melitensis in human and

animal sera through agglutination test, which unraveled the zoonotic potential of the disease ***(Write & Smith, 1897; Nielsen, 2002; Mantur et al., 2007)***.

In 1904, owing to the high prevalence of "Mediterranean Fever" among the members of the British Army and Navy in Malta, the Royal Society of London, together with the Governor of Malta, established a commission with David Bruce as Chairman, to study the disease ***(Hall, 1989)***. In 1905, Dr Carruana-Secluna together with Dr Themistocles Zammit and other Maltese doctors working in this commission established the zoonotic principle of the role of animals in transmission of the disease to human. They demonstrated that more than half of Maltese goats were asymptomatically infected and that the organism could be transmitted to humans by the consumption of raw milk and milk products or by contact with infected goat urine ***(Gotuzzo & Carrillo, 2003; Rust et al., 2006)***. After one year, the British authorities depend on the result of the commission and prohibited the use of untreated goat milk this action produce an immediate decrease in deaths and infection in member of the British army in Malta ***(Hall, 1989; Nicoletti, 2002)***.

In cattle, the disease firstly appears as epidemic abortion in Latin America in 1864 and regarded as contagious abortion ***(Huddleson, 1943; Hall, 1989)***. In 1897, Bernard Bang, a Danish veterinarian from Copenhagen, identified an intracellular microorganism described as ‘Bacillus abortus’ as

the cause of abortion in cattle. The disease was named for him, 'Bang's Disease' in 1930 ***(Bang, 1897; Nicoletti, 2002; Mantur et al., 2007)***.

The third member of the group, which also bacillary in shape was recovered from aborted fetuses of swine by Traum in 1914 in united states of America(USA) and implicated as an agent of brucellosis in man by Huddleson in 1943 ***(Mantur et al., 2007)***.

In 1918, Alice Evans - an American bacteriologist proved that Micrococcus melitensis from goats and Bacillus abortus from cows could not be differentiated morphologically or by cultural and biochemical reactions but there were an antigenic differences which could be shown by agglutination absorption test. In the same year she suggested the possible pathogenicity of Bacillus abortus to man ***(Evans, 1918; Mantur et al., 2007)***.

In 1920, Evans confirmed that Micrococcus melitensis was a bacillus and not, as originally described a micrococcus. She also showed that Micrococcus melitensis, Bacillus abortus and isolates from pigs belonged to one genus ***(Hall, 1989; Mantur et al., 2007)***. Meyer and Shaw further confirmed Evans` observations and suggested the generic name Brucella in the honor of Dr. David Bruce ***(Mantur et al., 2007)***.

In 1943, Forrest Huddleson, a veterinary microbiologist, describe the organism of Brucella as aerobic, gram negative,

coccobacilli and did not require CO_2 for primary isolation ***(Huddleson, 1943; Hall, 1989)***.

In 1956, Buddle and Boyce discovered Brucella ovis, the cause of epididymitis in rams and then in 1957 Stonner and Lackman discover Brucella neotomae within desert wood rat in USA. In 1968, Carmichael described Brucella canis as an epidemic in beagle dogs ***(Pappas et al., 2005; Mantur et al., 2007)***. In 1994, Ewalt and Ross discover marine mammal Brucella strains ***(Ewalt et al., 1994; Ross et al., 1996)***.

Recently, new Brucella species was isolated from systemically infected common voles (Microtus arvalis) in 2000 in South Moravia, Czech Republic.The organism is characterized by rapid growth on standard media and high metabolic activity, which is atypical for Brucella. Their biochemical profile is more similar to that of Ochrobactrum spp., of which most species are typical soil bacteria ***(Hubalek et al., 2007)***. However, on the basis of RNA, isolates were allocated to the genus Brucella. Affiliation to Brucella was confirmed by DNA–DNA hybridization studies. This isolate was later described as a novel brucella species termed Brucella microti ***(Scholz et al., 2008; ICSP, 2008)***.

History of Brucella and the biological warfare:

Airborne transmission of brucellosis has been studied in the context of using Brucella species as a biologic weapon, researchers attempted to develop it into a biological weapon

beginning in 1942 and tested in field trials in 1944 and 1945 with animal targets. In 1954, it became the first agent weaponized by the old US offensive biological weapons program. By 1955, the United States was producing Brucella suis-filled cluster bombs for the US Air Force in Arkansas ***(Purcell et al., 2007; Maloney, 2008)***.

Development of Brucella as a weapon was halted in 1967, and President Nixon later banned development of all biological munitions on November 25, 1969. Although the munitions developed were never used in combat, studies conducted under the offensive program reinforced the concern that Brucella organisms might be used against US troops as a biological warfare agent ***(Maloney, 2008)***.

Chapter (2)

Bacteriology of Brucellosis

A- Causative organism:

Brucellae have been traditionally classified according to its differences in pathogenicity and preferred hosts into six named species: Brucella melitensis principally within sheep and goats, B. abortus in cows, B. suis in pigs, B. canis in dogs, B. ovis in sheep and B. neotomae in rodents ***(Mantur et al, 2007; CFSPH, 2009)***.Three biovars are recognized for B. melitensis (1-3), seven for B. abortus (1-6 and 9), and five for B.suis (1-5) ***(CFSPH, 2009).***

Formal names proposed for marine mammal isolates are; B. maris for all strains ***(Jahans et al., 1997)***, or B. pinnipediae for strains from pinnipeds and B. cetaceae for isolates from cetaceans ***(Cloeckaert et al., 2001)***. In the validation process, the etymology has been corrected and the validly published names become B. ceti sp. nov. (Cetaceans, as preferred hosts) and B. pinnipedialis sp. nov. (Pinnipeds as preferred hosts) ***(Foster et al., 2007; ICSP, 2008)***.

In humans, brucellosis can be caused by B. melitensis, B. abortus, B. suis biovars 1-4 and rarely, B. canis ***(Araj, 1999; CFSPH, 2009)***. One case of laboratory-acquired human infection and two cases of community acquired infection with a marine Brucella infection have been reported ***(Brew et al.,***

1999; Sohn et al., 2003). B. ovis, B. neotomae and B. suis biovar 5 have not been linked to human disease ***(CFSPH, 2009)*.**

Considering their high degree of DNA homology (> 90 % for all species**)**, Brucella have been proposed as a monospecific genus in which all types should be regarded as biovars of Brucella melitensis ***(Verger et al., 1985; Boschiroli et al., 2001; Cutler et al., 2005)***. This new classification includes Brucella melitensis 16M and 5 biovars: abortus, suis, canis, ovis and neotomae ***(Moreno et al., 2002)***. However, because of the differences in the animal reservoir and in the severity of the clinical disease associated with the different species this proposal has not yet met with complete agreement. So, the old classification of the genus is the classification used world-wide ***(EC, 2001; Mantur et al., 2007)***.

B- Morphology:

Brucellae are facultative intracellular coccobacilli (short rods) that measuring about 0.6 to 1.5 µm long by 0.5-0.7 µm wide. They are non-spore forming and lack capsules, native plasmid or flagella; therefore, they are non-motile ***(Corbel, 2002)***. They are Gram-negative and usually do not show bipolar staining. They are not truly acid-fast but resist decolouration by weak acids, thus stain red by the Stamp's modification of Ziehl-Neelsen (Z-N) method, which is sometimes used for the microscopic diagnosis of brucellosis from smears of solid or liquid specimens ***(EC, 2001)***.

C- Antigenic structure:

In all smooth strains the dominant surface antigen is lipopolysaccharide (S-LPS) O chain, which, depending on the three-dimensional structure forms A, M or C epitopes. Rough strains do not produce the O chain but have a common R epitope (R-LPS) ***(Corbel, 2002; Mantur et al., 2007)***. Numerous inner and outer membrane, cytoplasmic and periplasmic protein antigen have been characterized. Some are recognized by the immune system during the infection and are potentially useful in diagnostic tests ***(Goldbaum et al., 1993; Mantur et al., 2007)***.

D- Genome:

The complete sequencing of B.melitensis genome was achieved in 2002 ***(DelVecchio et al., 2002)***. The complete sequencing of B. abortus ***(Sanchez et al., 2001)*** and B. suis ***(Paulsen et al., 2002)*** has been accomplished as well. B. melitensis contains two circular replicons of 1.1 and 2.2 Mb, respectively, with a 57 percent GC content and no plasmids; 3197 open reading frames were sequenced, 2487 of which had an assigned function. B. abortus biovars 1 and 4 and B. suis biotype 1 are remarkably similar to B. melitensis. In contrast, B. suis biotypes 2 and 4 are composed of two replicons of 1.35 and 1.85 Mb, respectively, whereas B. suis biotype 3 is composed of a single circular replicon of 3.3 Mb ***(Pappas et al., 2005)***.