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Validity of LAMP and molecular detection of human brucellosis among febrile negative malaria patients in Northern Kordofan State, Sudan

Abstract

Background: Brucellosis is a serious problem for man in contact with livestock and the diagnosis of this disease still depends on Cultivation and Isolation methods which can consume long time for diagnosis. Nowadays the invention of Loop-mediated isothermal amplification (LAMP) and other molecular technique can play role in rapid diagnosis and saving time with highly sensitive and specific results.

Aim: To determine the validity of new LAMP primer design by Mast group comparing to nested PCR technique. The old valid LAMP primer designed according to the published sequence of omp25 (GenBank accession No. gi 769744) to help in diagnosis of brucellosis among malaria negative febrile patients in Northern kordofan.

Methods: Seventy five blood samples were collected from febrile malaria negative patients of different ages from North Kordofan State, and examined by nested PCR and LAMP methods.

Results: The results showed that old LAMP primer came positive for 40 and negative for 35 of the patient's samples in other hand the new LAMP primer came positive for 65 patient's samples and negative for only 10 samples.

Conclusion: The sensitivity and specificity of new LAMP primer design by (Mast, Reinfeld, Germany) for detection of Brucella spp. was tested and validity of these primer show much better results when compared with nested PCR and another LAMP primer used. KAPPA value calculated to show the degree of agreement compare to the new primer results

Keywords: brucellosis, LAMP, nested PCR, negative malaria patients, Sudan

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Introduction

In 1904 brucellosis was reported in Sudan as disease that can infect both of human and cattle. *B. abortus* firstly isolated from farms in Khartoum, while *B. Melitensis* was reported in El Gezira from goat's milk among British residents. The disease was reported in different Sudan areas.[1] Brucellosis is livestock disease. Human infection occurs during contact with animals through infected materials like afterbirth or ingestion of animal products and inhalation of air borne agents. Dairy products like milk and cheese made from raw milk is important source of infection. Brucellosis detected livestock workers. Human to human transmission less reported.

Pasteurization of milk and Vaccination of cattle is very important mechanisms for prevention and control of disease in enzootic areas with high prevalence rates.[2] The prevalence of brucellosis in Khartoum suburban communities according to ages of the 14 brucella positive patients, 3(21.4%) aged 1-20 years and this comprised 0.8% of the total cases (362) examined. Eight (57%) aged 21-40 years, and this comprised 2.2% of the total cases examined. While two (14.3%) aged 41-60 years and this comprised 0.6% of the total cases; whereas one (7.1%) was from the group aged 60 and over, which comprised 0.3% of the total individuals surveyed. With reference to age, the main affected group aged between 20 and 40 years (P=0.003). The majority of positive cases (92.9%) were males, this comprised 3.6% of the total cases (362) examined. The risk of infections for those using milk and milk products is three times higher (78.6%) than for those who did not (21.4%) (P=0.004).[3]

The invention of the loop-mediated isothermal amplification LAMP has given relatively new DNA amplification technique. The LAMP amplifies DNA with high sensitivity depending on an enzyme with strand displacement activity under isothermal conditions. This technology uses four to six specially designed primers recognizing six to eight regions of the target DNA sequence, these primers making the LAMP reaction highly specific.[4,5] In last 10 years, LAMP has been used in laboratory setting to detect different pathogens and tumours.[6]

LAMP technology was developed by Notomi et al.[4] The reaction takes place in a single tube containing buffer, target DNA, DNA polymerase and primers. The tube is incubated at constant temperature at 64°C in water bath or heat blocks that providing constant temperature. The amplified product can be detected by naked eye as a white precipitate or a yellow-green color solution after addition of SYBR green to the reaction tube.[5]

Material and methodology

Study design

This is an analytic study carried out in Kordofan state's hospitals during the period between January 2017 up to June 2017.

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Clinical samples

Blood samples from 75 patients with febrile fever in contact with animal and/or consume raw milk product were included in this study. Blood samples were collected in EDTA and centrifuged at 5000rpm for 5minutes to obtain the serum. Then serum was immediately stored at -20°C until used for Rose Bengal serological test. Informed written consent was obtained regarding data and blood samples collection from each patient. Only patients who agreed to participate were enrolled in this study.

The DNA was extracted from blood samples and stored at -20 to be used for nested PCR and LAMP PCR.

Molecular methods

DNA extraction: DNA extracted was done by saturated sodium chloride method according to Reham et al.[7] modified from Miller et al.[8] By re-suspend the blood sample in red cell lysis buffer, the supernatant was discarded after centrifugation of the mixture then the white cell lysis buffer was added and incubated for 1hour at 65°C with 10%SDS and 20µl of protein's K.[8] 6M NaCl added before cold chloroform was added and centrifuged for 6minute.

The supernatant was healing by absolute ethanol and centrifuged again for 5minutes. This time the supernatant was discharged and the pellet was washed using 70% ethanol precipitate a pure DNA, and stored at -20°C until used.

Nested PCR: Multiplex nested RT-PCR was performed by processing the extracted DNA with four primers for the nested PCR which were synthesized according to Agricultural Industry Criteria of the People's Republic of China (NY/T 1467e2007) that amplifies a wanted region over two rounds of PCR in a volume of 25µl in the first round, 5µl of DNA mixed with 2µl of each primers outer primers; forward Bp1 and reverse Bp2. The sequences of these primers are shown in (Table 1). Reactions were performed in thermo-cycling conditions using PCR machine Techno (Japan) as follow: programmed for 35 cycles as following: (94°C, 49°C, and 72°C, for 1min, 60s and 10 min respectively) The second round of was done with 3µl of product from the first round as template with 2µl of each primers Inner primers; forward Bp3 And reverse Bp4 Programmed for 20 cycles as following: (94°C, 51°C, and 72°C, for 30s, 60s and 60s respectively followed by final extension of 6min at 72°C.) The PCR products from the second round was be electrophoresed on 1% agarose gel containing ethidium bromide and visualized above the UV light eliminator and the target amplicon was 419bp.[9]

Table 1 Nested PCR primers used in this study

Name of gene	Sequence	Size of band	Target gene
Bp1	CGT GCC GCA ATT ACC CTC		
Bp2	CCG TCA GCT TGG CTT CGA		bcsp31
Bp3	GAT GCT GCC CGC CCG ATA A	419 bp	
Bp4	GCA CCG AGC GAG CCT TGA AA		

LAMP PCR

In this study 2 LAMP primers design were used to detect *Brucella spp*. using isothermal amplification (LAMP) in constant temperature.

LAMP primers mix

The first primer mix of the old valid LAMP was designed according to the published sequence of omp25 (GenBank accession No. gi 769744).[4] These primers were F3, B3, FIP, and BIP. The sequences of these primers are shown in (Table 2).

Table 2 valid LAMP Primers used in this study

Name of gene	Sequence	Target gene
F3	G73ACGCCATCCAGGAACAG	
B3	G289CATCACCTTCAACACCGTAT	omp25
FIP	G171CCATAGCCAAGGTAAAGACCGG149C107GGTTGAAGTAGCTCCCCA	
BIP	C189AGCACCGTTGGCAGCATCA208T265CTGGTCCTGCTGGAAGTT	

The second new LAMP primer also design to detect the same *Brucella spp*. Using set of six LAMP primers to detect different *Brucella spp*. The primer mix was prepared of LPF, LPB, F3, B3, BIP and FIB primers.

Primer mix was heated for 15min in 95°C then exposed to shock freezing for 2-4min to prevent primers self binding.

LAMP condition

Reaction mix prepared using V6.28lamp pellets (Mast, Reinfeld, Germany), each pellet sufficient to run 12 reactions in 10µl volume. The mixture prepared of (24µl of 0.5M tris buffer, 67.2µl of distal water, and 4.8µl of primers) this mixture was divided into 12 reaction tubes (8µl per tube).

After that 2µl of template DNA was added to the mixture. These Reactions were performed by real time PCR analyzer at 63°C for 60min in isothermal conditions using real-time PCR machine Rotor-Gene Q presented by Qiagen company. The result read in FAM/SybrGreen channel. The second new LAMP primer was also treated using same procedure as the first valid LAMP primer.

Results

The results of nested PCR and LAMP PCR for two primers used for detection of human Brucellosis in serum from malaria negative febrile patients in northern kordofan State are shown in Table 3. The cross tabulation between results of PCR and each LAMP primers shown in Table 4 & Table 5. Table 6 shows the cross tabulation between the two LAMP primers results.

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Table 3 Frequency of PCR, valid LAMP, and new LAMP primer in febrile negative malaria patients in Northern kordofan State

Test	Positive	Negative	Total
PCR	55(73.3%)	20(26.7%)	75(100%)
Valid LAMP primer	40 (53.3%)	35(46.7%)	75(100%)
New LAMP primer	65(86.7%)	10(13.3%)	75(100%)

Table 4 Cross tabulation of PCR and New LAMP Primer results

		LAMP		Total
		Negative	Postive	
PCR	Negative	4	16	20
	Postive	6	49	55
Total		10	65	75

Kappa value=0.10

Table 6 Cross tabulation of New LAMP Primer and Valid LAMP Primer results

		Valid_LAMP_PRI		Total
		Negative	Positive	
NEW_LAMP_PRI	negative	6	4	10
	positive	29	36	65
Total		35	40	75

Kappa value=0.075

Discussion

Sudan is the largest Arab and African country, it is surrounded by nine countries and it is divided into 26 states. It is possesses a great livestock population about 138million animals,[10] playing pivotal roles in the economy and providing livelihoods for many people. Livestock in different parts of the world is threatened by brucellosis. Brucellosis is a contagious disease of animals which is transmitted to man. The disease is widely spread in many parts of the world particularly the Mediterranean and the Middle-Eastern countries.[11] On 2010, Adam Ahmed Adam Mustafa and Hassan Sidahmed Hassan study the Human Brucellosis in Khartoum State. Their findings revealed that eighty nine (89%) of the febrile patients had brucellosis using the Standard Tube Agglutination Test technique (STAT). The average age of brucellosis patient was 43.9 years. Sixty three (70.8%) of the brucellosis patients were males, and 26 (29.2%) were females. Fifty four (60.7%) of them had significant titres to Brucella melitensis while 23 (25.8%) patients had significant titers to *Brucella abortus*. Twelve (13.5%) patients had significant titres to both *Brucella melitensis* and *Brucella abortus*. They conclude that Brucellosis can be misdiagnosed as malaria or typhoid fever. Animal contact was found to be a significant risk fact.[12]

Our findings also came high as Adam Ahmed Adam Mustafa and Hassan Sidahmed Hassan but this results came using molecular methods which show that seventy one (94.6%) out of 75 samples toke from febrile negative malaria patient who had been consume dairy product, who had contact with livestock febrile negative malaria patient who had been consume dairy product or had contact with livestock were positive for *Brucella spp*. extracted DNA when they diagnosed by two molecular methods (nested PCR & LAMP) comparing to detection of serum antibody titers that they had used in their study. Twenty two of these seventy one positive samples (30%) were positive by only one of these methods.

Our result came agree with another study published by Laila F Nimri for diagnosis of Brucella established by PCR.[13] One hundred and twenty (72.7%) out of the 165 samples were positive by PCR. These patients had symptoms for more than one month to several years and were all positive by serology; recent infections of less than one month were negative by PCR. The DNA bands that appeared in the acute cases were brighter than those of the chronic cases. The PCR was negative on conclusion of the treatment for 25 of the 45 negative patients. The other twenty negative patients were suspected cases.[13]

That was very close to PCR result in our study that fifty five (73.3) samples of the 75 samples were positive.

Wei Ling Yu and Klaus Nielsen present a review of most of the currently used polymerase chain reaction (PCR)-based methods for identification of Brucella bacteria in biological samples on August 2010. They found that the gold standard for diagnosis of Brucellosis remains isolation of *Brucella spp* bacteria from samples. While PCR-based methods that identify nucleic acid fragments from the bacteria are more useful and practical. This is especially true for PCR tests targeting new species of *Brucella spp* from marine mammals. The sensitivity and specificity of most PCR-based methods are not well established and their real value for use with clinical samples and hence diagnosis has not been validated.[14] After one year of that Al-Ajlan HH1, Ibrahim AS and Al-Salamah AA suggest that serum and blood analysis by conventional and real time PCR is a convenient and safe method for rapid and accurate diagnosis of brucellosis.[15]

The results of the present study showed that (73.3%) was positive by PCR while (53.3%) was positive using a valid LAMP primer according to the published sequence of omp25 and the percentage of a new LAMP primer design by (Mast, Reinfeld, Germany), that show that molecular technique can be more sensitive than serological methods in detection of *Brucella spp*. disagree with Wei Ling Yu and Klaus Nielsen finding and these may be due to the specific primer targeting only specific regions for detecting of some strains, disregarding other strains. That's make the molecular technique more useful for identification more than detection.

A novel loop-mediated isothermal amplification (LAMP) assay was established by Guo-Zhen et al.[16] In Molecular and Cellular Probes on 2011 to detect Brucella species DNA in milk and blood samples of animals and humans. This LAMP assay based on the sequence of highly repetitive omp25 gene was able to detect 9 fg/ml *Brucella spp*. DNA with high sensitivity, which was 10 times higher than the nested PCR. The LAMP was evaluated for its specificity using 19 strains of six Brucella species and 28 related non-Brucella micro-organism strains as controls. The target 19 Brucella strains were all amplified, and no cross-reaction was found with all the non-Brucella micro-organism strains. Both nested PCR and LAMP assays were then used to detect *Brucella spp*. The LAMP assay should be a potential tool with high convenience, rapidity, sensitivity and specificity for the diagnosis of Brucellosis.[16]

We used an already valid LAMP primer in this study for detection of *Brucella spp*. trying to compare with a new LAMP primer design by mast group using PCR technique as a golden method. The old primer results showed low sensitivity and specificity for detection of brucellosis from malaria negative febrile patients than PCR. The lamp results came positive for 7(9.3%) samples out of 75 which found negative by nested PCR while nested PCR was positive for 22(29.3%) LAMP negative samples, KAPPA value for old LAMP primer vs PCR was 0.2 indicating slight agreement, while the new primer results show higher sensitivity and specificity than old one. It's results came positive for 16(21.3%) samples out of 75 which found negative by nested PCR while nested PCR was only positive for 6(8%) LAMP negative samples, KAPPA value for new primer vs PCR was 0.10 which also indicating slight agreement. But when we try to evaluate the agreement between these two primers we found that new LAMP gave 29(38.6%) samples positive and was negative by the old primer, while only 4(5.3%) samples positive by the old primer was negative by the new one. And there is no agreement between them using KAPPA value=0.075.[7]

Conclusion

Our study show very good validity for the new LAMP primer for *Brucella spp*. design by Mast GROUP than the old primer. Our finding show higher sensitivity and specificity for new LAMP than PCR in diagnosis of brucellosis. This study reveals the need for further investigations to compare LAMP with Cultivation and Isolation methods to evaluate the validity of LAMP to be used as gold stander method in future for diagnosis of Brucellosis. Accentual finding that LAMP can be considered the next gold stander molecular method for detection of DNA in samples.

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Conflict of interest

Authors declare that there is no conflict of interest.

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