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Isolation, Identification And Charactrization Of *Brucella* From Bovine Raw Milk Using New Biochemical And Biophysical Methods

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Abstract

Background: - Brucellosis is a zoonosis which still threatens public and animal health in many countries of the world. It infects variety of domestic animals and man causing incapacitating disease. To control and eradicate brucellosis is extremely important to diagnose it promptly and accurately. Accurate diagnostic and standard procedures are critical for the success of the brucellosis control and eradication. The diagnosis and differentiation between Brucella species and their different biovars has been based on serotyping, phage typing, and sensitivity to dyes, CO 2 requirement, H2S production, and metabolic properties. However, the variability of some phenotypic characteristics in different Brucella strains impairs the identification of species and biovars. Therefore, the addition and evaluation of other biochemical and biophysical tests considered essential for the detection and identification of *Brucella.*

AIM: - to evaluate the ability of sugars fermentation test indole test and growth under different PH concentrations to diagnose, identify and differentiate Brucella species.

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MATERIAL AND METHODS:-This is an experimental cross sectional study and it was carried out by direct isolation identification and characterization of brucella species from raw bovine milk.50 samples were collected,25 were positive to milk ring test five pure isolates out of them suspected brucella species were isolated. The isolation and identification were performed by using conventional standards methods these strains confirmed by using PCR technique. Sugar fermentation tests, Indole test, and growth under different PH concentrations tests used as additional new tests don't used conventionally for the identification of *Brucella* species.

Results:-in sugars fermentation test tow strains gave positive results for all four sugars (glucose, lactose, maltose, and sucrose), and positive Indole tests, the growth of tow strains was between 4 and10 PH concentration

Conclusions: - the new tests gave significant results which contributes with conventional tests to the differentiate between *Brucella* species.

Introduction: - . Although milk and dairy products are important components of a healthy diet, if consumed unpasteurized, they also can present a health hazard due to possible contamination with pathogenic bacteria. These bacteria can originate even from clinically healthy animals from which milk is derived or from environmental contamination occurring during collection and storage of milk. The decreased frequency of bovine carriage of certain zoonotic pathogens and improved milking hygiene have contributed considerably to decreased contamination of milk but have not, and cannot, fully eliminate the risk of milk borne disease. Bovine tuberculosis and tuberculosis are classic examples of zoonotic milk borne diseases. Brucellosis is a zoonosis which still threatens public and animal health in many countries of the world. It infects variety of domestic animals and man causing incapacitating disease. It is occurs worldwide and the most common mode of transmission is consumption of unpasteurized dairy products or meat of infected animals. It is also transmitted through direct skin contact and is occupational hazards for farmers, veterinaries, butchers and laboratory workers. Persons to person transmission are not epidemiologically significant. Aerosols contemning *Brucella* are considered to be highly infectious. The disease is caused by a group of bacteria known scientifically as the genus Brucella. This genus includes six major species namely *B. abortus, B. melitansis, B. suis, B. canis, B. ovis and B. neotomae.* Recently new species was discovered namely B. marine mammals. Each species of Brucella divided into some of biotypes like B. abortus include 6 biotypes, B. melitensis include 3 biotypes. B. suis include 5 biotypes. These biotypes can be recognized by biochemical test and the ability of growth in the presence of dyes agglutination with monospecific antisera for B. abortus (A) and B. melitensis (M) and DNA hybridization. [3] Brucellosis diagnostic tests fall into two categories:

Those that demonstrate the presence of organisms and those that detect an immune response to their antigens. , the identification of the different species is of great epidemiological importance. Brucellosis and its agents are included within the list of zoonosis that requires surveillance. Several biological samples can be used for monitoring and laboratory confirmation of the Brucella spp. Infection. The collected samples can be examined fresh or frozen and transported to the laboratory. Samples of milk, vaginal swabs, blood, aborted materials (aborted foetus, foetus membranes) and carcasses may be used. The differentiation between Brucella species and their different biovars has been based on serotyping, phage typing, and sensitivity to dyes, CO_2 requirement, H_2S production, and metabolic properties. However, the variability of some phenotypic characteristics in different Brucella strains impairs the identification of species and biovars. Therefore, the design of stable markers based on DNA is presently considered essential for the detection and identification of *Brucella*. Indirect diagnosis methods are widely routinely used in control and eradication and surveillance programs for ruminants. Immunological tests are required at each step of intervention against brucellosis: the evaluation of the prevalence, the assessment of the efficacy of control and elimination measures, and the confirmation of eradication of disease through surveillance. , the identification of the different species is of great epidemiological importance. Brucellosis and its agents are

included within the list of zoonosis that requires surveillance. This study to describe and discuss the merits of various direct methods applied in vitro (mainly in blood or milk) the recent literature on brucellosis diagnostic tests was reviewed. these tests are applied with different goals , such as national screening, confirmatory diagnosis ,certification, and international trade the validation of such diagnostic tests is still an issue ,particularly in wildlife.[9]the main objective of this study is to evaluate the ability of sugars fermentation test indole test and growth under different PH concentrations to identify and differentiate *Brucella* species.

MATERIALS AND METHODS:-

This is a cross section study conducted in Khartoum state (Farms of faculty of animal production university of Khartoum,50 samples of row milk from non-vaccinated (dairy bovine) ,Milk from individual animals were collected from non-vaccinated (dairy bovine) , after Stripping all the teats of the lactating animal after disinfection using sterile swabs containing 70% ethyl alcohol solution. Around 50 ml of the sample were collected using sterile 100-ml Falcon tubes. Falcon tubes were identified using unique barcode labels. For preservation and transportation to the Central veterinary research laboratories for analysis and storage samples were stored in ice bag. [9]

For safety consideration analysis of samples for biological threat agents is performed within a certified class 2 biological safety cabinet. The procedures described below function to rule out presumptively identified *Brucella* isolates. Laboratory coats and gloves were worn when processing specimens and performing tests .Safety glasses or shields are recommended.

All sample processing s and tests were carried out in Central veterinary research laboratories, Soba, Khartoum state Since September 20201 to November 2021

Milk samples were tested to Milk Ring Test [11],all of positives sample to Milk Ring Test processed for bacteriological examination .

Isolation and identification of suspected brucella species form positive MRT samples: -

1 Culture:-

•Under aseptic condition and in biological safety cabinet level 2 milk samples which were given a positive result to MRT cultured by taking a loop full of milk sample after well mixing and were inoculated on (OXOID GC AGAR BASE)

•culture media Code: - CM0367 Culture media (according to the central veterinary research laboratories protocol) within 10 hours of collection. Specimens were refrigerated at (4-10)0C until inoculation. GC (Gono Coccus) media .Plates were inoculated with sample material and incubated aerobically and in the presence of 5–10 % carbon dioxide at 37 °C. These plates examined 3–7 days post-inoculation for bacterial growth. Colonies having a characteristic of *Brucellae* were sub cultured for purification and identification. Identification of *Brucella* isolates done according to standard procedures. [8]

Identification and typing:-

Colony morphology and staining; Oxidase test Urease test; Catalase test Indole test, Agglutination with Monospecific antisera,

production of hydrogen sulphide, Growth on dye plates, and CO_2 requirements for growth.

. Molecular identification of the isolates using PCR technique:-

DNA extraction:-

1/tow colonies of fresh bacterial growth were used, the colonies were put in a test tube containing 1 ml of distilled water.

2/boiled for 10 minutes in a water bath.

3/centrifuged for 5 minutes at 1000 rpm.

4/five microliters of supernatant were used for PCR.

PCR procedures:-

Bruce-ladder PCR mix preparation (per one reaction, final volume of 2µl)

PCR buffer 10× 1× 2.5 µl

dNTPs (2 mM) 400 µM each one 5.0 µl

Mg 2+ (50 mM) 3.0 mM 1.5 µl

Bruce-ladder eight pair primer cocktail (12.5 µM) 6.25 pmol each one 7.6 µl

H_2O (PCR-grade) – 7.1 µl

DNA polymerase* 1.5 U 0.3 µl

*As this assay is a multiplex PCR with eight pairs of primers in the same tube reaction, best results are obtained when high quality DNA polymerase is used (for instance, Immolase DNA polymerase [Bioline], Titanium Taq DNA polymerase [Clontech], or PFU DNA polymerase [Biotools B&M Labs.]).NOTE: include always a negative control Without DNA and a positive control with B. suis DNA Add 1.0 µl of template DNA

Mixed gently by vortex and briefly centrifuged to collect all components to the bottom of the tube

Amplification by PCR

1/amplified using thermo cycler for 2 hours

2/the amplified DNA evaluated by agarose gel electrophoresis and subsequent eththidium bromide staining.

Detection of amplified product and interpretation of results

Analyse the PCR products (7 µl) by electrophoresis (120 V for 1 hour) in a 1.5% agarose gel in TBE buffer (89 mM Tris/HCl, 89 mM boric acid, 2.0 mM ethylene diamino tetra-acetic acid [EDTA], pH 8.0).

Use 1 kb plus DNA ladder as a molecular size marker. Visualise bands with UV light after staining with ethidium bromide.

Carbohydrate fermentation test:-

1/Inoculum working suspensions of *Brucella* in sterile distilled water will obtained by harvesting the growth on forty-eight hour.

2/The suspensions will used within twenty-four hours of the time of harvest, and their concentrations will adjusted, if necessary, to contain between 10 and 30 billion cells per 0.1 ml inoculum by using McFarland standard(1 MFU)

3/Basal fermentation medium:-

The different potential ingredients and the other variables which will examine, together with results obtained, are summarized below. Usually, only one or two strains of each species were employed for exploratory examinations of each variable Additional strains will include in the final examination of these factors.

The medium finally adopted consisted of:-

O Carbohydrate, 1 g

O Cresol red, 2 mg

o Agar, 200 mg per cent in 0.01 M Sorensen's phosphate, Eight-tenths ml of the basal medium will dispensed into a 12 or 13 mm tube and sterilized at 120 C for 15 min; 0.1 ml of filter sterilized, 10 per cent, carbohydrate solution and 0.1 ml of inoculum will then added.

4/ the inoculated fermentation tubes will incubate aerobically at 37 ^{0} C. Initially, readings were taken after 1, 2, 4, 6, and 10 days' incubation; however, upon progressive improvements in the basal medium a final reading could be made at the second day with strains of *Brucella. melitensis.* Occasionally more than 4 days' incubation was required when unduly small inoculum will used [9].

Growth under different pH concentrations:-

Bacterial strains were inoculated in nutrient broth medium having different pH value (2, 4,6,7,9 and 10). Inoculated Peptone tubes were incubated at 37˚C for 24 – 48hrs. And the growths were observed daily. PH was adjusted using NaOH and HCl. [2]

RESULTS:-

50 samples were collected and screened for *Brucella* species by Milk Ring Test 25 of them (50%) were positive.

All of these positive milk samples were examined by culture staining biochemical tests and agglutination to Monospecific antisera, five isolates gave suspected *Brucella* features in colonial morphology and staining pictures but not in biochemical tests these isolates confirmed by PCR technique, two brucella strains were isolated, identified, and confirmed as *Brucella.melitensis.*

	G.S	ZN.S	Oxidase.	Catalase	Urease	H_2S	CO_2	Indole
4	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
5	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
25	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
31	-ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve
45	-ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve

Table(1) biochemical tests

Agglutination with Monospecific antisera			Grow in dye media	
NO	A	M	1/50000 B.F	1/50000 THIONIN
4	+ve	-ve	-ve	+ve
5	+ve	-ve	-ve	-ve
25	+ve	+ve	-ve	+ve
31	+ve	+ve	+ve	-ve
4	+ve	+ve	+ve	-ve

TABLE (2) Agglutination with monospecific antisera

Carbohydrate fermentation					Growth in different PH conc.					
NO	G	L	S	M	2	4	6	7	8	10
4	+ve	+ve	+ve	+ve	-	-	+++	++	++	+
5	+ve	+ve	+ve	+ve	-	-	-	-	-	-
25	+ve	+ve	-ve	+ve	-	+	+++	++	++	-
31	+ve	+ve	+ve	+ve	-	+	++	++	++	+
45	+ve	+ve	+ve	+ve	-	+	++	+++	+++	+++

Table (3) Sugars fermentation test and growth in different PH conc.

DISCUSSION:-

in this study milk samples were collected from 50 dairy cow to detect antibodies against Brucella using Milk Ring Test overall, 25(50%) were positive by Milk Ring Test this ratio was higher from other on that was resulted from a study done by Najibullah Mohammed et.al Milk Ring Test for spot identification of Brucella abortus infection in single cow herds (2014) in which they found that 18.35% of milk samples were positive with Milk Ring Test [23]

In this study all isolates from row cow milk were B .melitensis in other side study done by Shahzad et al for Isolation and identification of bovine Brucella isolates from Pakistan by biochemical tests and PCR resulted that most isolates were isolated from milk the most isolates were from cattle all of them were Brucella abortus[17]

In this study B. melitensis was isolated from raw bovine milk by culture and confirmed by qPCR, and suggested the transmission of brucellosis between goats and cattle In other study carried out by Martin Wainaina et al for detection of Brucella species in raw milk from various livestock species raised under pastoral production systems in Isiolo and Marsabit Counties, northern Kenya which use qPCR for direct detection of Brucella species from milk without culture ,detected B.abortus in goats and suggested transmission of brucellosis between cattle and goats[18]

Our study used biochemical and metabolic activity of sugar tests by ordinary manual method used in labs. For identification of Brucella species and gave results did not correlate to with the classical biotypeing scheme, and this result agreed with a result of study done by Al Dahouk and his collages for differential phenotyping of Brucella species using a newly developed semi-automated metabolic system showed that Brucella melitensis biovars formed a homogenous group although the metabolic activity don't correlate with the classical biotypeing ,subgroup within the species could still be defined. [20]

In recent study conducted in Ethiopia by Muluken etal for isolation and identification of Brucella melitensis using bacteriological and molecular tools concluded that the microbiological and biochemical characteristics of the isolates indicated that the isolates were Brucella melitensis [19]

The tow *Brucella. Melitensis* isolates growth was normal to heavy at pH ranged from 4 – 10. This indicates the ability of *Brucella.melitensis* for growth in wide range of ph. and Acid tolerance may relate to virulence as has been discussed previously. These results are matched to results of previous study carried by Basil A Abbas.Ali

B. Talei. Isolation, Identification and bio typing of brucella species from milk At Basrah province.J.Vet.Res.Vol.9, No.1, 2010. [3]

In indole test tow strains gave positive result this result don't agree with other results in different study carried out by Basil A Abbas.Ali B. Talei. Isolation, Identification and bio typing of brucella species from milk At Basrah province.J.Vet.Res.Vol.9, No.1, 2010. [3]

Sugars fermentation test gave positive result for all sugars used (glucose, maltose, lactose, sucrose) expect one of these strains gave negative result for sucrose sugar. This result differ from other result in other study conducted by M.J .PICKETT and E. L. NELSON fermentation of carbohydrates speciation within the genus Brucella(1954) which gave positive fermentation result with glucose only[9]

These results variation explained by the variation and the epidemic consideration of the strains. , especially in this a typical strain of *Brucella melitensis*

CONCLUSION:-

This study concluded that out of 50 samples of raw cow milk were 25 positive by Milk Ring Test.

All of these 25 samples were cultured and 5 out of them were suspects to be Brucella species according to basic conventional tests for direct diagnosis of Brucella species.

These five strains were tested for indole test two of them gave positive result and the rest were negatives

These five strains tested for carbohydrates fermentation tests and gave positive result for all sugars used (glucose, maltose, lactose, and sucrose) expect one of these strains gave negative result for sucrose sugar.

In the test of growth under different PH concentrations one of the suspected isolate didn't grow at all, the rest four isolates don't grow in concentration 2, and the growth of them were in the concentration between 4 and 10 with the variation in the degree of growth. Two of these isolates were identified and confirmed by qPCR(qualitative Polymerase Chain Reaction) as *Brucella.melitensis* species .

RECOMMENDATIONS:-

1/ The study recommends is to develop a miniaturised semi-automated system for reliable identification of genus *Brucella*e and the differentiation of its species based on comprehensive metabolic activity testing.

2/These tests recommended to be included in the direct conventional test for brucella identification isolated from milk to a set or a kit similar to API 20, as well as biotex and micronut system plates and other semi-automated techniques. This will replace time consuming tube methods and reduce the risk of infection,

3. The study recommends further studies to understand the metabolic activity of the brucella species for different substrates like other sugars and amino acids.

5/recommend to do further studies to the rest of the isolates to identify the genus and species of them

6/recommend to do a another survey to detect the prevalence of B.melitensis as a typical species in a cattle in different areas

4/. Strict measures are needed to ensure the safety of milk sold to the general population

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ETHICAL CONSIDRATION:-

This study was approved by ethical committee of faculty of medical laboratory sciences _Alzaiem Al-Ezhari university, (Farms of faculty of animal production university of Khartoum), Central veterinary research laboratories, Soba, Khartoum state.

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