Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 1, 2021, Pages. 3300 - 3319

Received 15 December 2020; Accepted 05 January 2021.

Development of Rapid Immunological and Molecular tools for Diagnosis of Brucellosis Infection in Indian Population

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Type of Article –Study Protocol

Conflict of Interest -None

Funding: The study was supported by Central India Institute of Medical Sciences, Nagpur as an part of in-house funding project.

Abstract:

Background: In India, human brucellosis is common yet neglected due to lack of prompt diagnosis. To constitute an effective algorithm for diagnosis of brucellosis infection, development of rapid yet specific tests for screening of infection in Indian communities is needed.

Objective of the present study is to develop and evaluate rapid immunological (ELISA) and molecular tools (LAMP PCR) to improve existing diagnostic capacity for brucellosis infection.

Methodology:A prospective observational study will be conducted in patients attending the outpatient department (OPD) of Central India Institute of Medical Sciences (CIIMS) Nagpur, other hospitals. A total of 312 individuals suspected of brucellosis will be recruited inclusion criteria, additional risk factors, and clinical symptoms .The clinical diagnosis of human brucellosis will be carried out using standard gold standard microbiological, serology and

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 1, 2021, Pages. 3300 - 3319

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molecular tools. An in house ELISA assay based on Whole cell antigens of brucella isolates will be optimized and developed for immunological diagnosis of brucellosis in blood. In addition to ELISA, a point of care molecular assay (LAMP PCR) will be developed targeting species and virulence specific genes for rapid molecular diagnosis. Statistical analysis will be performed using MedCalc statistical software to determine sensitivity and specificity and concordance analysis for determination of diagnostic accuracy.

Conclusion: A rapid, specific, safe, efficiaousand affordable diagnosis using immunological (ELISA) and molecular assay (LAMP PCR) will be developed for brucellosis diagnosis to be used as alternative or in lieu to existing conventional diagnostic tools

KeyWords: Human Brucellosis, Enzyme Linked Immunosorbent Assay (ELISA), Polymerase Chain reaction (PCR), Loop Mediated Isothermal Amplification Assay (LAMP PCR)

INTRODUCTION:

Human Brucellosis has been a major neglected zoonotic disease of public health concern due to its prevalence in areas of high endemicity in India (1,2).It is one of the causes of pyrexia of unknown origin (PUO) in endemic areas (3**)**A wide incidence of Brucellosis is reported all over Indiaranging from 17-34% with an average prevalence rate of 9.5%. Clinical manifestations includeacute or sub-acute febrile illness. In 5-10% cases, existing infection may contribute with neurological complications which lead to Neurobrucellosis (4,5) Diagnosis of Brucellosis includes microbiological, culture and serological assays each having its own relative merits. Although available diagnostic tests are rapid and cost-efficient they have sensitivity and specificity constraints. Detection of brucella using serological methods is based on IgG and IgM assays as these consists of various cocktail of immunodominant proteins having its functional and regulatory response. These antigens further helps in the identification of the disease. Detection of *Brucella* specific antibodies in blood against such antigens using ELISA technique will yield a rapid yet affordable approach for diagnosis compared to commercial kits.

Similarly development of rapid molecular tools like Loop mediated isothermal amplication (LAMP) techniques for diagnosis of brucellosis can significantly improve the diagnostic scenario for brucellosis infection (6)LAMP is an isothermal nucleic acid amplification technique which can be characterized as rapid, robust and much more specific and sensitive with conventional methods and can be implemented in an low resource setting areas(7)Despite the conceptual complexity, the technique is simple to use and detection can be carried out in just one step. offered advantages limited studies are available in India which have utilized diagnostic utility of LAMP for brucellosis infection in humans (8)Similarly, no studies are available which demonstrates utility of rapid lateral flow assays for brucellosis.

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Experimental design: In the present study, cross sectional study will be carried out for a total of 312 participants attending the outpatients departmentof CIIMS with suspected confirmed and controls cases and will be recruited as per mentioned (Table 1).

Table 1: Inclusion and exclusion criteria for participant recruitment.

Inclusion criteria	**Confirmed brucellosis cases**	**Suspected brucellosis cases**
Demographic characteristics		
Age	**Above 18 years**	**Above 18 years**
Gender	**Males and females**	**Males and females**
Clinical symptoms: Fever, joint pain, joint swelling, back pain, chest pain, sweating, fatigue, neck stiffness, pyrexia of unknown origin (PUO)	Positive	Positive
Risk factors: Consumption of raw milk/meat/milk products(cheese, ghee) and contact with animals, history of abortion,	Positive	Positive
Conventional serological/Molecular and/ or Culture	Positive	Culture –ve/ positive by either serology/ molecular tools
Exclusion criteria		
Demographic characteristics		
Age	Above 18 years	Above 18 years
Tests for ruling out tropical illness: Leptospirosis, Scrub typhus IgM detection tests, Dengue NS1 detection test and Malaria Antigen test	Negative	Negative
Controls Criteria **Age**	Above 18 years	Above 18 years
Conventional serological/Molecular and/ or Culture	Negative	Negative
Clinical symptoms: Fever, joint pain, joint swelling, back pain, chest pain, sweating, fatigue, neck stiffness, pyrexia of unknown origin (PUO)	Negative	Negative

Objective 1: To develop rapid point of care molecular assays (LAMP PCR) for diagnosis using different genus of Brucella

Reagents required for Molecular biology Assay:

A) DNA Extraction Reagents:

1. 10x Phosphate buffer saline – 1x

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2. Sodium Dodecyl Sulphate (SDS) -10%
3. Sodium Chloride (NaCl)- 5M
4. CethylTrimethyl Ammonium Bromide (CTAB)-10%
5. Chloroform
6. Isoamyl alcohol
7. Sodium Acetate- 3M
8. Ethanol-70%
9. Proteinase K – 50 mg

Preparation of the Reagents:

i) 10x Phosphate buffer saline

Table 2: Components required for preparing 10x Phosphate buffer saline with different concentration.

Sr No	Components	Concentration
1	NaCl	80gms
2	KCl	2 gms
3	Disodium Hydrogen Phosphate (Na2HPO4)	14 gms
4	Sodium Dihydrogen Phosphate (NaH2PO4)	2 gms

Dissolve all ingredients in 800ml of distilled water and make up the volume till 1 litre of pH 7.4 Sterilize the prepared reagent by autoclaving.

ii) **10% Sodium Dodecyl Sulphate (SDS)** – Weigh 10g of SDS and dissolve it in 100 ml distilled water. Mix thoroughly

iii**) 5M Sodium Chloride (NaCl)** - 29.22 gms in 100 ml of distilled water. Mix thoroughly.

iv**) 10%CethylTrimethyl Ammonium Bromide (CTAB) -**Weigh 0.41 gm of NaCl and mix it in 8 ml distilled water. Now add part by part 1g of CTAB to theNaCl solution in order to avoid any kind of lumps during the preparation.

v) **Chloroform**- Add chloroform and isoamyl alcohol in the ratio of 24:1

vi) **3 MSodium Acetate**- 2.4gms of sodium acetate and in 10 ml distilled water. Mix thoroughly

vii) **70%Ethanol**- Take 70ml of absolute ethanol solution and mix in 30ml of distilled water.

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Instruments used:

Figure 1: List of instrument required for molecular extraction protocol.

Sr.No	Instrument Name	Reference Images
1	Water Bath (Make : REMI)	
2	Heating Block (Make : REMI)	
3	Cooling Centrifuge (Make : REMI C-24 BL/5-06)	
4	Vortex Mixture (Make: REMI)	

B) Gel electrophoresis:

Table 3: List of components required for electrophoresis with the concentration used for preparing gel.

Sr No	Reagents	Concentration
1	TAE Buffer	50x
2	Ethidium Bromide(EtBr)	10 mg/ml
3	Agarose	3%
4	Loading Dye	6x
5	Molecular Markers	1000bp

Preparation of the reagents:

1. **50X TAE Buffer** – Dissolve 48.8g of Tris and 3.722gms of EDTA to prepare 200 ml. Adjust pH and keep on stirrer. Add 11.42 ml of glacial acetic acid in the prepared solution .Mix thoroughly.

2. **Ethidium Bromide solution (Etbr)**–Weigh 10 mg of Etbr and mix it in 1 ml of distilled water.

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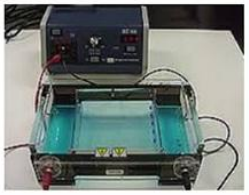
(**Caution**: While preparing EtBr wear precautionary safety measures as the reagent is carcinogenic)

3**. 3% Agarose gel**:Weigh 2 gms of agarose powder in 100 ml 1XTAE (for larger gels).Dissolve itfor 1-2 min in microwave. Add 12 µl of Etbr in the gel. Dissolve properly. Allow it to solidify for 20 mins.

Instruments used:

Fig 2: List of instrument required for gel electrophoresis analysis.

Sr No	Instrument Name	Reference Image
1	Electrophoresis Assembly	
2	Microwave	

Protocol:

DNA Extraction-

DNA will be extracted according to the Phenol-Chloroform extraction method.

1. Blood sample collected will be initially processed using FicollHistopaque protocoland further centrifuge at 2736 g

2. Discard the supernatant and process using 100 µl pellet. Now add 400 µl of 1 x PBS solution and 15µl SDS with 3 µl Proteinase K. Incubate at 55^0C for 1-1.30hr.

3. Add 100 µl NaCl and 80 µl CTAB in a heating block for 65^0C.Next, add equal amount of phenol and chloroform .Centrifuge at 16416g of high speed.

4. Separate the aqueous layer and add equal amount of Chloroform and centrifuge in a high speed of 16416 g

5. Again remove the aqueous layer andmix properly with 0.6 volumes of Isopropanol and 3M Sodium acetate.

6. Wash the supernatant with 70% ethanol, dried and dissolve it in 25 µl of 1x Tris –EDTA buffer solution

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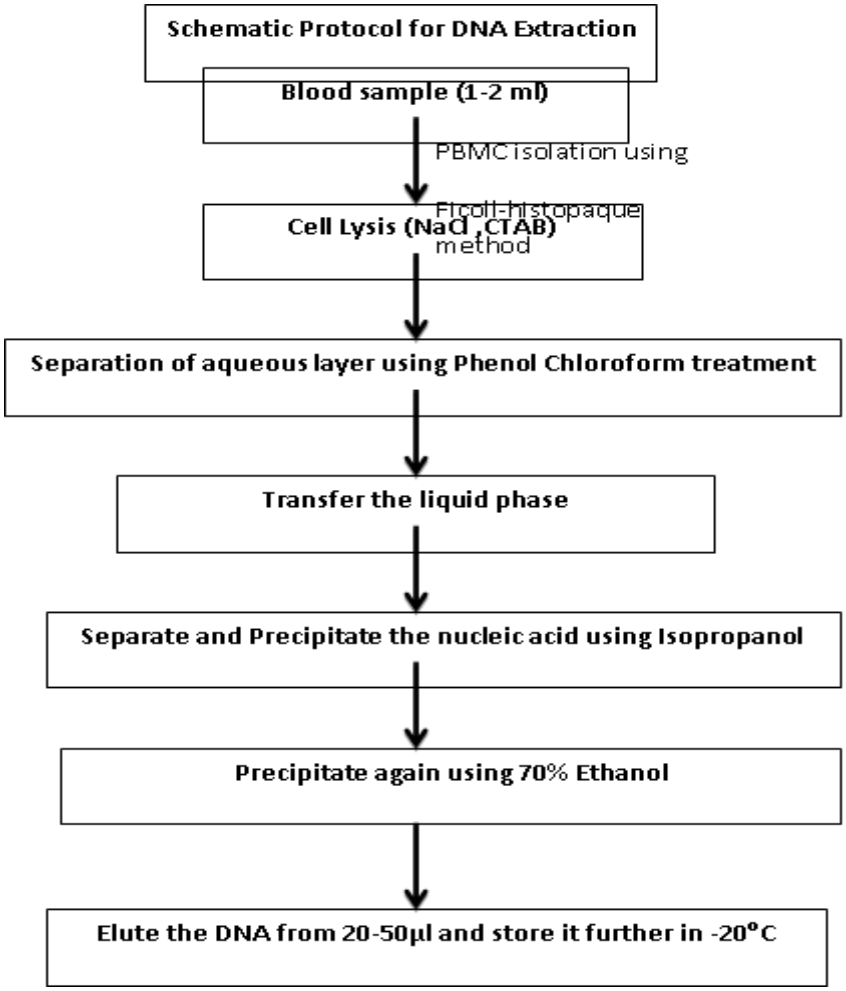

Fig 3: Schematic Protocol for DNA Extraction

B. Conventional PCR

PCR Master Mix:

PCR master mix will be prepared with a 50-µlreaction containing template, 1X PCR buffer, 100 nMprimer, 200 mMdeoxyribonucleoside triphosphate, and 1 U of Taq polymerase. Thereaction will be performed in a thermal cycler (ProFlex PCR system, Applied Biosystems). Amplification protocol consist of denaturation at 93°C for 5 min, 35 cycles of 90°C for 1 min annealing temperature 60°C for 30 s, and further extension at 72°C for 1 min, and 72°C for 7 min. The PCR analysis will be further done on a 2% agarose gel to determine the sizes of the amplified product(7)

C. LAMP PCR:

Table 4: List of targeted genes for diagnosis of Brucella using point of care assay

Sr No	Target Gene	Primer sequence
1	Bcsp31	All primers will be designed by using (Primer Explorer) and also using reference literature
2	Omp25	
3	Vir B	

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Designing of Primers for In- House LAMP assay development:

In the study, primers will be taken from the existing available literature and will be further standardized and evaluated. Further, using primer designing software in house primers will be designed.

Synthesis of Primers:

The selected primers from the literature will be further synthesized from the commercial company Eurofins.

Dissolving lyophilized primers and preparation of working stocks:

- Dissolving of primers will be done according to the manufacturer's instructions.
- Centrifuge the primer tube at 6840 g for 10 minsin a cooling centrifuge to help settle down any kind of particulates while transportation.
- Mix the appropriate amount of TE/water to make up the required concentration of 100µM. Prepare aliquots of the stock primer and store at -20°C for further use.

Table 5: Literature based Sequence of oligonucleotides for Bcsp31 gene and Vir B gene of brucella

Brucella gene target	Primer	Sequence	Reference
Bcsp31	F3	5'- GCTTTACGCAGTCAGACGT-3'	7
	B3	5'- GCTCATCCAGCGAAACGC- 3'	
	FIP	5'AGGCGCAAATCTTCCACCTTGCGCCTATTGGGCCTATAACGG- 3'	
	BIP	5'-GGCGACGTTTACCCGGAAATTCAGGTCTGCGACCGAT-3'	
	LF	5'- CCTTGCCATCATAAAGGCC-3'	
	LB	5'- CGTAAGGATGCAAACATCAA -3'	
VirB	Primer	Sequence	8
	F3	5'- ATACGCGCAACAGCTCAA-3'	
	B3	5'- TCGAATTGTGGAACAAGGCA- 3'	
	FIP	5- GCTGGTGGCACTATTGCGCAC3'	
	BIP	5'-GGTGATTTCGTGCGCACATGG -3'	
	LF	5'- TCACTTGCACCGTTCATTCC-3'	
	LB	5'- TTGACAACTAGGCGTTCCTG -3'	

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PCR Master Mix Protocol:

To prepare a 25µl reaction mixture add 40 pmol/L of FIP and BIP, 20 pmol /L of Loop Primers and 5 pmol/L of outer primer, 1.4mM dNTPs, 0.8mol/L betaine, 8mmol/L of MgSo4, 8 U of *Bst*DNA polymerase and template DNA (8,9,10)

Table 6: Components of Master Mix required for PCR amplification of LAMP PCR

Components	Stock Concentration
Nuclease free Water	-
Betaine	5M
MgSO4	100mM
Buffer	10x
dNTP's	2.5mM
Primer	40:20:05
Bst Polymerase	8000U/ml

PCR Amplification protocol: The amplification protocol includes three stages.The detailed description is included in (table 8)

Table 7: Amplification protocol for point of care assay

STAGES	STEPS	TIME
Denaturation, annealing and extension	Amplification of DNA at 63°C	35 minutes
Termination	Termination of amplification of DNA at 95°C	2 minutes

Analysis of LAMP product:

LAMP PCR tubes can be analyzed by visual detection LAMP products can be detected without electrophoresis using ambient light after the addition of DNA dyes to the reaction tube. Further if required, electrophoresis of LAMP products in a 3% agarose gel can help distinguish specific amplification from non-specific amplification.

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Objective 2: To develop rapid and affordable whole cell lysate & synthetic peptide based immunological assays (ELISA) for diagnosis of Brucellosis infection

Standardization and evaluation of ELISA based on Whole cell lysate of Brucella strains:
Materials required for preparing Brucella Culture:

1. Brucella Broth
2. Brucella Selective supplement
3. Horse Serum
4. Methanol
5. Glucose

Preparation of the reagents:

i**) Brucella Broth**: Weigh 14 gms of Brucella broth and dissolve it n 500 ml distilled water .Slightly warm the solution and autoclave it at 121^{0}C for 30 mins .Keep sure to maintain the pH at 7

ii) Brucella Selective Supplement: Add 10 ml of methanol and sterile distilled water in the proportion of 1:1 in the lyophilized tube. Incubate it for 10-15 mins at 37 ^{0}C

iii) **Horse serum:**Commercially available.

iv) **10% Glucose**: Weigh 10gms of Glucose and dissolve it into 100 ml of distilled water. Mix thoroughly.

2. Reagents required for the preparing WCL:

1. Brucella Culture
2. 10X PBS
3. Lysis Buffer- 100 ml solution
4. Acetone
5. Cut off columns
6. Sodium dodecyl sulphate

Preparation of the reagents:

i)10XPBS:

Table 8: Reagents required for preparing 10x Phosphate buffer saline with different concentration.

Sr No	Components	Concentration
1	NaCl	80gms
2	KCl	2 gms
3	Disodium Hydrogen Phosphate (Na2HPO4)	14 gms
4	Sodium Dihydrogen Phosphate (NaH2PO4)	2 gms

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ii) Lysis Buffer:

Table 9: Reagents required for preparing lysis buffer as per the mentioned concentration

Sr No	Reagents	Concentration
1	NaCl	1 gm
2	Triton X*	2 ml
3	SDS	2 gms
4	Tris	2gms
5	EDTA	0.5M
6	Urea	5 M
7	Thiourea	2M

Mix all the above reagents .Use 5 ml per 1 mg of pellet of WCL .Add 100 mg/ml lysozyme (50 µl) and protease inhibitor cocktail (100 µl) before just adding lysis buffer. Add DNase after sonication. (*: Triton X will be used for modifications in whole cell lysate protocol)

Instruments used:

Figure 3: Instruments required for preparation of whole cell lysate using brucella culture.

Sr No	Instrument Name	Reference Image
1	Incubator	
2	Cooling centrifuge	
3	Shaking Incubator	
4	Sonicator	
5	Protein Quantification Machine	

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Protocol:

In house Brucella Culture:

- To prepare 500 ml of brucella broth medium add 10 ml ofstarter culture attaining the OD at 600 as per culture preparation method and add consecutively brucella selective supplement, inactivated horse serum, 10% glucose and further incubate it for 7-8 days to obtain the maximum growth required for whole cell lysate (11)

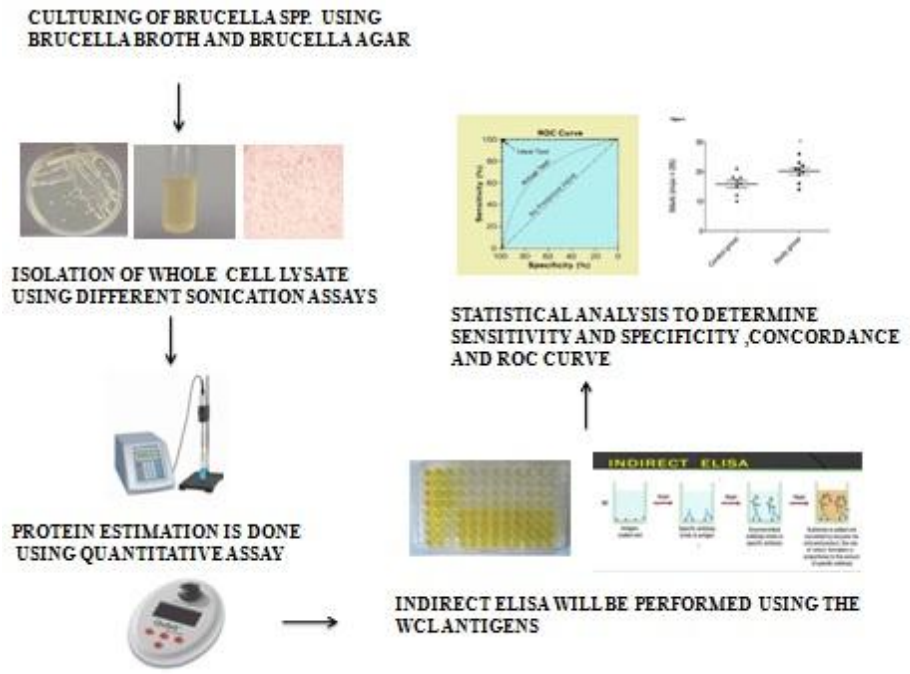

Fig 4: Schematic flow chart for preparation of whole cell lysate of Brucella for ELISA

Protocol for preparation of WCL of Brucella:

- After attaining the growth culture, collect the pellet and centrifuge it at 13680g
- Discard the cell debris in the form of supernatant and washoff the pellet thrice with chilled 1X PBS to remove the traces and centrifuge it at13680 g for 10 min.
- Treat with lysis buffer and sonicate it for 1 min.
- Centrifuge the pellet and pool the supernatant with ice-cold acetone and incubate for 2 hr at -20^0C.
- Treat the supernatant with acetone solution and centrifuge it at 13680g for 10 min to obtain the heavy amount of proteins.
- Dissolve the pellet in ice-cold 1X sterile PBS and concentrate it using cut off columns, and further quantify using Qubitfluorometer
- The pellet which is the proteins should be stored at 20 ^{0}C for further use (11)

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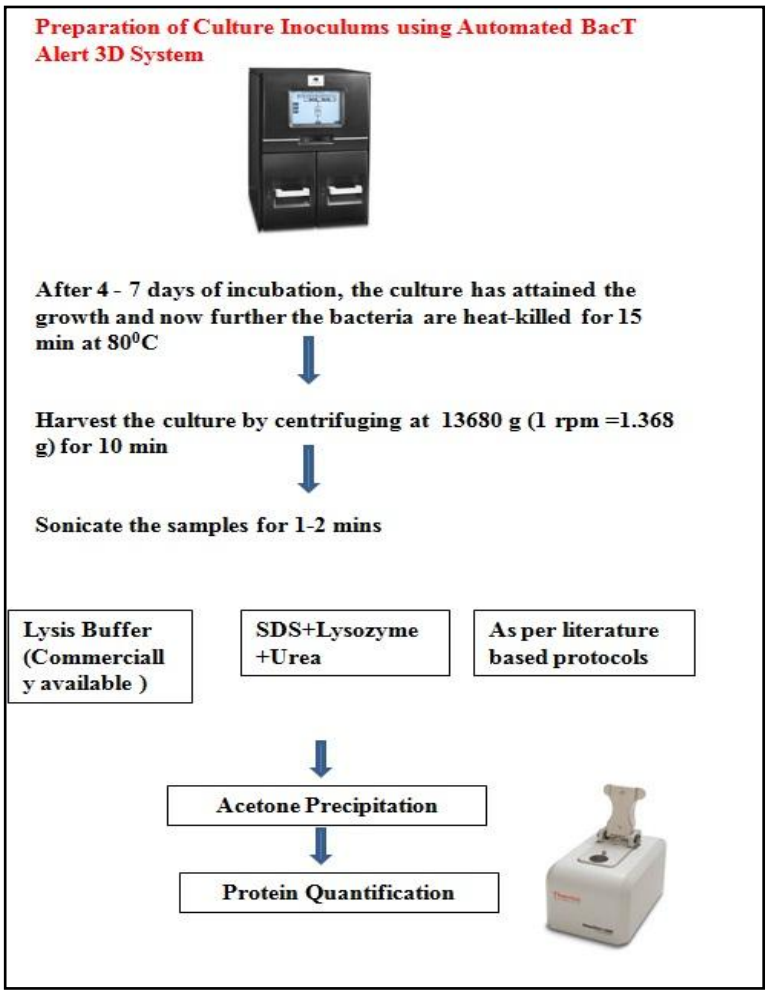
Fig 5. Schematic procedure of whole cell lysate using different protocols



Standardization of ELISA assays using whole cell lysate for diagnosis of Brucellosis:

The ELISA will be standardized using different concentration of whole cell lysates using checkers board titration method. The best concentration showing higher reactivity and lower cross reactivity in negatives and controls will be further used for evaluation and protocol development

Standardization of ELISA:

Ingredients for ELISA test:

1. 10XPhosphate Buffer saline (PBS)
2. Bovine Serum Albumin (BSA)- 0.5%
3. Goat Anti human IgM Horseradish peroxidase (HRP) conjugate
4. Goat Anti human IgG Horseradish peroxidase (HRP) conjugate
5. 3, 3, 5, 5'- Tetramethylbenzidine (TMB) substrate
6. Tween 20
7. Stop Solution-2N
8. Peptides

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Preparation of the reagents:

i) 10x Phosphate buffer saline

Table 10: Components required for preparing phosphate buffer saline using given concentration.

Sr No	Components	Concentration
1	NaCl	80gms
2	KCl	2 gms
3	Disodium Hydrogen Phosphate (Na2HPO4)	14 gms
4	Sodium Dihydrogen Phosphate (NaH2PO4)	2 gms

Dissolve all ingredients in 800ml of distilled water and make up the volume till 1 litre .The pH of the prepared should be 7.4 Sterilize the prepared reagent by autoclaving.

ii) **BSA:** Weigh 50mg and dissolve it in 10 ml 1x PBS .Mix thoroughly.

iii) 3, 3, 5, 5'- Tetramethylbenzidine (TMB) substrate: Commercially available store at 4^0C until further use.

v) Stop Solution: Measure 27.6 ml Sulphuric acid concentrated and dilute it to 500 ml distilled water. Mix it properly.

Instruments used:

Fig 6: Instruments required for performing ELISA assay.

Sr No	Instrument Name	Reference Image
1	Incubator	
2	Cyclo- Mixer for Vortexing	
3	ELISA plate reader	
4	pH Meter	
5	Laboratory Centrifuge	

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ELISA:

Peptide designing: Reference sequence of immunodominantbrucella antigens (OMP-31, OMP-25, Lipopolysaccharide, Type IV secretion protein) will be obtained from UniProtKB/Swiss-Prot protein data base and peptides will be synthesized using bioinformatics tool. The obtained sequences will be further analyzed using multiple sequence alignment NCBI BLAST for checking thenon redundant protein database sequences. According to the NCBI blast analysis further,antigenic sequences of the proteins will be selected. The antigenic peptides further will be designed using online software titled "Molecular Immunology Foundation-Bioinformatics software (MIF Bioinformatics software)" which predicts immunodominant epitopes from particular antigenic sequences. Immunogenic peptides will further be checked of suitability for antibody production using the Innovagen software. Final sets of peptides after scrutiny will be then synthesized through GenicBio lab, Shanghai, China. The obtained peptides will be stored at -20^{o}C until further use.

Table 11: List of targeted proteins used for designing peptides for indigenous in-house ELISA assay.

Sr No	Target Proteins	Peptide designing
1	Brucella melitensis type IV secretion protein	All peptides will be designed using software developed by "Molecular Immunology Foundation-Bioinformatics software (MIF-Bioinformatics software)"
2	Brucella melitensis outer membrane protein 25	
3	Brucella melitensis outer membrane protein 31	
4	Brucella melitensis lipopolysaccharide	

Protocol for In-House ELISA:

Antigen Coating: The wells of microtiter plates will be coated with 100 µl of brucellaantigen having different concentrations 5ng, 10ng, 20ng, 50ng, 100ng diluted in PBS and incubate further at 37°C for 3hrs. Later add 0.5% BSA in the antigen wells and incubate for 2 hrsto block the plate at 37°C.

Sample analysis: In the antigen coated plate, Add PBS and wash three times and further add 100 µl of serum sample (dilution in PBS) and incubate it for 35 min .Again wash the wells and incubate itwith secondary antibody and further incubate for 30 min at 37°C.

Repeat the above mentioned step again with PBS, and add 100 µl of TMB/ H2O2 substrate at room temperature for 2-3 minutes. Add up 100 µl of 2.5N H2SO4 and measurethe absorbance of each well at 450nm(12, 13, 14)

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Fig 7: Schematic Protocol for Antibody Coating

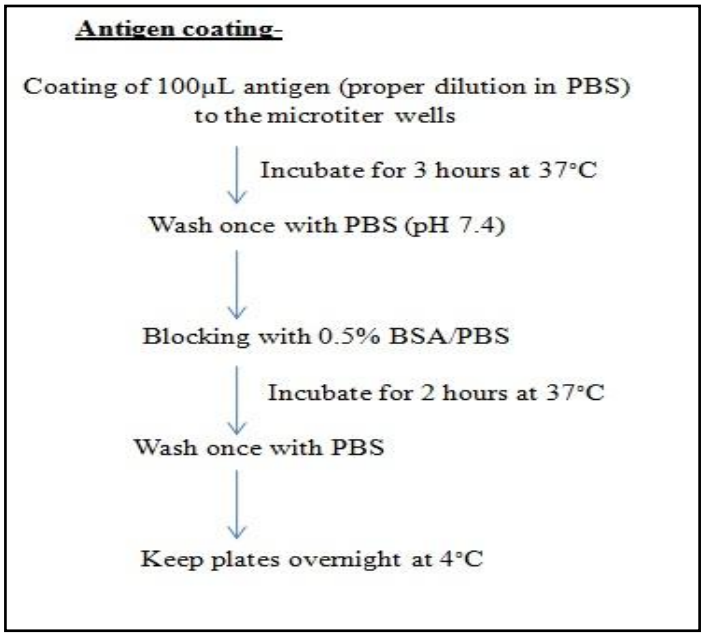
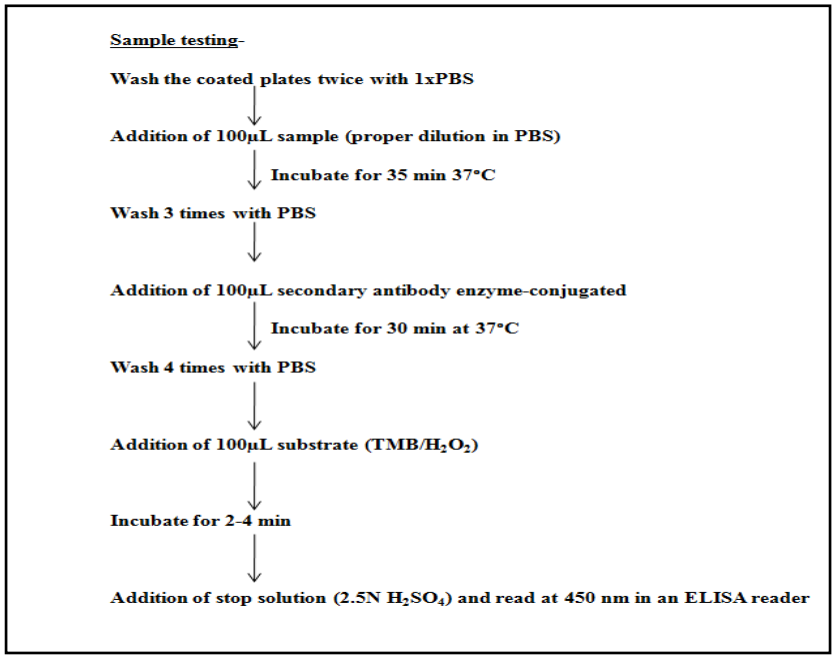


Fig 8: Schematic Protocol for Sample testing after antigen coating



Standardization and evaluation of peptide ELISA for diagnosis of Brucellosis:
Protocol for peptide ELISA will be standardized using Checkerboard titration methods using combinations of different concentration of peptides along with antibody dilutions. The best combination showing maximum reactivity in positive samples with minimum background in negative controls will be used further for final evaluations in suspected cases and confirmed cases.

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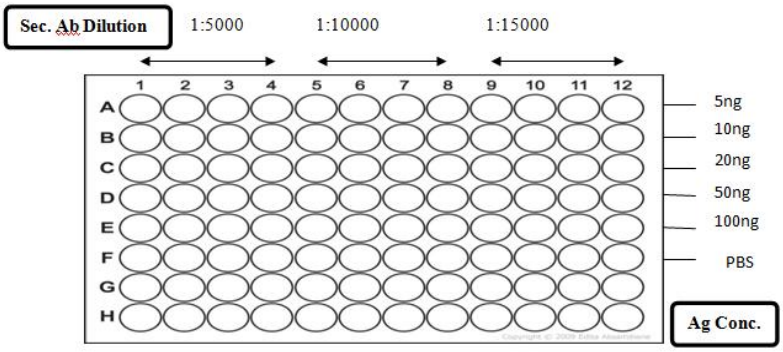
Figure 9: Various different concentrations and different dilutions of antigens (vertically) and secondary antibody (horizontally) used for standardization.



Result Interpretation: Measure the absorbance value of each well at 450 nm. Further using Medcalc statistical tool calculate the cut-off value by measuring the absorbance in the suspected and confirmed cases and calculate the sensitivity and specificity of the developed assay (12, 13, 14)

Conventional screening tests:

Rose Bengal test (RBT)

Place 20 µl of serum sample on a glass slide and an 20 µl of Rose Bengal Antigen .Mix it gently. Incubate for 2-3 mins at ambient temperature and further observe the agglutination (15, 16)

Standard Agglutination Test (SAT)

- 80µl of phenol saline to be added in 1st well of the first column of the U bottom plate.
- Add, 50µl of phenol saline in 2ndwell/ column and onwards lane. Add 20µl of serum in the 1st well of the first column and mix it uniformly.
- Transfer 50µl from the above uniform mixture from 1st well to 2nd& consecutively dilute the serum (1:2 dilutions) up to 11th well and discard in the next tube .
- Add 50µl of plain Brucella antigen in all the wells and incubate it overnight at 37ºC.

The plate will be observed from underneath for a mesh-like structure (agglutination) at the bottom of wells. The highest dilution of serum showing positive agglutination will be considered at a titer of the serum. The mixture of antigen and serum dilutions shall be incubated for 16–24 h at 37°C (17,18)

EXPECTED RESULTS:

The diagnostic confirmation of brucella includes various immunological and molecular assays which may lead to the limitation in detection the disease .In the study , in house ELISA will be developed using culture secretome of brucella to detect antigenic proteins and to develop low cost effective diagnosis for brucella .The results will be compared with commercial ELISA test kits andapproximately cut offs will be determined by sensitivity and specificity. Diagnostic accuracy will be developed using concordance analysis.Molecular

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 1, 2021, Pages. 3300 - 3319

Received 15 December 2020; Accepted 05 January 2021.

assay based on point of care test LAMP PCR will be developedusing different species of brucella to diagnose bed side patients for brucella in robust, cost effective, and more sensitive way. Comparison between groups (confirmed, suspected & controls) will be calculated using the Chi square test in MedCalc statistical software (version 10.1.2.0) and a difference with $p<0.05$ will be considered as significant. Odds ratios using the bivariate analysis will be analysed using SPSS software (version 22.0). Figures and graphs will be prepared using Graph pad prism 5 and sigma plot updated version. Concordance between individual tests will be calculated using kappa test in MedCalc software.

DISCUSSION:

Diagnosis of Brucellosis includes microbiological, culture and serological assays each having its own relative merits. Although available diagnostic tests are rapid and cost-efficient they have sensitivity and specificity constraints. Detection of brucella using serological methods is based on IgG and IgM assays as these consists of various cocktail of immunodominant proteins having its functional and regulatory response. These antigens further helps in the identification of the disease. Detection of *Brucella* specific antibodies in blood against such antigens using ELISA technique will yield a rapid yet affordable approach for diagnosis compared to commercial kits. Hardly any study on brucellosis particularly in relation to this technique is available. Few of the related studies were reviewed (19-22).

Troubleshooting:

Table 12: Represents trouble shooting with respect to PCR, ELISA and PCR

Sr. No.	Step	Problem	Possible reason	Possible solution
1	PCR	Carry over contamination	Contamination can be carried over from previous PCR due to aerosols, contaminating pipettes, surfaces, gloves and reagents.	Carryover contamination can be reduced by unidirectional workflow, Proper cleaning of pipettes, making frequent and small aliquots of reagents, aseptic cleaning techniques, proper separation of reagents stored together
2	PCR	Low purity	Purity of DNA affected	Use of purification kits, remove traces of contaminants like phenol, EDTA, Proteinase k with chemical or enzymatic DNA purification protocol, re-wash the DNA with 70% ethanol to remove salts and ions
3	ELISA	Uniform low readings	Incorrect wavelength, Insufficient development time, capture antibody did not bind to the plate	Check filter and readers, increase development time, dilute PBS without additional proteins, re-qualify the reagents of choice
4	ELISA	High Signal	Substrate solution mixed to early and turning colour uniformly	Make fresh buffers, substrate solution to be mixed and used immediately, wash plates properly, check dilutions, titrate if necessary

Conflict of Interest:

The authors declare no conflict of interest

Acknowledgement:

The work was supported by Central India Institute of Medical Sciences, Nagpur.

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