CHAPTER - III

MATERIALS AND METHODS

The Serodiagnostic study of animal handlers for Brucellosis was conducted in the Department of Microbiology, School of Health Sciences, Kannur University, over a period of two years. 445 Serum samples were collected from slaughterhouse workers, Veterinary practitioners and animal breeders. The whole study population was grouped based on the risk of occupational exposure to Zoonotic infections. The individuals participated in the study was informed about the importance of the study and explained the details of test going to be carried out to analyse on their samples. A questionnaire was collected from each individual involved in the study. A consent letter also has been collected from all the participants.

3.1 SELECTION OF STUDY MATERIALS

Serum samples from occupationally exposed individuals, who are frequently and repeatedly exposed to animals as part of their profession were the materials used for this study. Three groups of populations were selected based on the risk of exposure to animals. The first group was labeled as Group I which included Slaughter house workers, Group II consisted of individuals working in veterinary and associated fields and Group III consisted of cattle, Buffalo and Goat breeders. The entire three groups were having members from both genders. The study population was selected by random sampling method.
3.2 STUDY MATERIALS (Table No. 1)

Serum samples collected from 445 Animal and non animal handlers were grouped into the following

3.2.1 Group I (210 serum samples)

3.2.1.1 Group I A - consisted of 110 serum samples collected from Slaughter house workers

3.2.1.2 Group I B - formed the control group which consisted of 100 serum samples collected from factory workers who were supposed to be less exposed to animals

3.2.2 Group II (165 serum samples)

3.2.2.1 Group II A - consisted of 83 serum samples collected from Veterinary doctors

3.2.2.2 Group II B - consisted of 47 serum samples collected from livestock inspectors and attenders

3.2.2.3 Group II C - formed the control group which consisted of 35 serum samples collected from relatively healthy individuals working in schools and offices

3.2.3 Group III (70 serum samples)

3.2.3.1 Group III A - consisted of 25 serum samples collected from farmers having cow at their house

3.2.3.2 Group III B - consisted of 18 serum samples collected from farmers having goat at their house
<table>
<thead>
<tr>
<th>Group</th>
<th>Description</th>
<th>Number of Participants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I A</td>
<td>Slaughter house workers</td>
<td>110</td>
</tr>
<tr>
<td>I B</td>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>Group II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II A</td>
<td>Veterinary Doctors</td>
<td>83</td>
</tr>
<tr>
<td>II B</td>
<td>Live stock Inspectors/Attenders</td>
<td>47</td>
</tr>
<tr>
<td>II C</td>
<td>Control</td>
<td>35</td>
</tr>
<tr>
<td>Group III</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III A</td>
<td>Farmers having cow</td>
<td>25</td>
</tr>
<tr>
<td>III B</td>
<td>Farmers having goat</td>
<td>18</td>
</tr>
<tr>
<td>III C</td>
<td>Farmers having buffalo</td>
<td>12</td>
</tr>
<tr>
<td>III D</td>
<td>Control</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>445</td>
</tr>
</tbody>
</table>
3.2.3.3 **Group III C** – consisted of 12 serum samples collected from farmers having buffalo at their house

3.2.3.4 **Group III D** – formed the control group which consisted of 15 serum samples collected from persons having neither cow, goat and buffalo at their house.

3.3 **STUDY METHODS**

3.3.1 **Gathering information from study population**

   A prior patient consent form (Annexure – I) was obtained from each person of the study population. Information were collected from each person on a proforma (Annexure – II), A detailed history from each person of the study population were taken, which included their name, age, occupation, nature of work in their respective profession, History of consuming raw milk, History of fever (nature and duration) and complaints of joint pain, presence of lymph adenopathy and previous history of orchitis were also noted.

   The type of exposure was then graded as follows:

   Grade 1: Exposure by consuming milk, meat and their products.

   Grade 2: Exposure by direct or close contact with cow/buffalo/goat.

3.3.2 **Collection of Blood**

   Venous blood (4ml) was collected, allowed to clot and centrifuged for 3 minutes at 1000 r.p.m. Serum was pipetted out and utilised for the test. A total of 445 serum samples were
collected, among which 295 were from animal handlers and 150 sera were from the non-animal handlers which formed the control group.

3.3.3 Screening of serum for Brucella antibody

All the serum samples were tested for Brucella antibody by Rose Bengal Plate Test, Standard Tube Agglutination Test (STAT) and IgG and IgM ELISA methods. All the 445 serum samples were processed by the standard reagents and Kits supplied by authorised manufactures. The tests performed were according to the protocol supplied by the Indian Veterinary Research Institute, Izatnagar, UP.

3.3.3.1 Rose Bengal Plate Test - RBPT (Plate No. 1)

The test was performed by the guidelines given by Indian Veterinary Research Institute, Izatnagar, UP. It is a suspension of pure smooth culture of Brucella strain 99 phenolised and stained with 1% Rose Bengal Dye. It is buffered at Ph 3.65 using lactic acid buffer.

Suppliers: Indian Veterinary Research Institute, Izatnagar, UP.

Kit contents: Brucella coloured antigen, White tile, Mixing sera, Positive and Negative Controls

Principle:

Brucella antibody if present in the patient serum will specifically bind with the antigen present in coloured reagent and will make visible agglutination reaction which can be read by the naked eye.
**Method:**

All the reagents and serum samples were brought at room temperature before starting the test and the antigen bottle was shaken thoroughly before use. One drop of antigen (0.03ml) was placed on each square of the plate. With a 0.1 ml pipette, one drop of serum was placed by the side of the antigen drop. With a spreader the antigen and serum drop were thoroughly mixed and spread to an area about 2.5 cm diameter. The plate was manually rotated for 4 minutes. With each set of test sera, a known positive and negative control sera were also included (Morgan *et al.*, 1978)

The results were read after 4 minutes of mixing the reagent. The formation of clear clumps was considered as positive test while the absence of clumps was considered as negative reaction.

Interpretation: The test was examined for agglutination in bright light. Any degree of agglutination was taken as positive and no agglutination was taken as negative.

**3.3.3.2 Standard Tube Agglutination Test - STAT**

The test was performed by the guidelines given by Indian Veterinary Research Institute, Bareli, UP. STAT was performed by the procedure described by Alton *et al.* (1975).

**Principle:**

Plain antigen of *Brucella abortus* S99 was used for the test. It is a pure smooth suspension of *Brucella abortus* strain 99 in
phenol saline, standardised against anti Brucella abortus serum (1000 IU/ml) to give 50% agglutination with 1: 500 dilution of serum. The blocking effect of the sera can be removed by prior heating of the serum at 55°C for 30 minutes or by using 4% saline as the diluent for the test. The most reliable method for obviating the blocking effect is by detecting the antibodies using the coombs test.

**Suppliers:** Indian Veterinary Research Institute, Bareli, UP.

**Kit contents:** Brucella suspension Plain antigen of Brucella abortus S99 was used for the test.

**Method:**

Doubling dilution of test serum, starting from 1:5, was done with 0.5% phenol saline in 10 sugar tubes. Antigen bottle was shaken well and brought to room temperature. Equal amount of (0.5ml) Brucella abortus antigen was added to each tube. The content of each tube were mixed by rolling in between the palms. The dilution ranged from 1:10 in the first tube, to 1:5120 in the tenth tube. After mixing, the test tubes were incubated at 37°C for 24 hrs. The results were compared with the antigen control tube showing agglutination. With each set of test sera, a known positive and negative were also included.

A titre of 80 IU per ml and above after overnight incubation at 37°C was taken as positive. All the test tubes, starting from 1:20 to 1: 2560 were thoroughly checked for agglutination.

The tubes were taken out of the water bath and kept on the bench at room temperature for an hour, for observation. All the tubes were examined against light and tubes of test series were compared with antigen control tubes for degree of opacity of the
supernatant fluid. The result of the test was recorded as shown in table

**Interpretation:**

The highest dilution of test serum tube comparable with tube III of antigen control tube was taken as the end titre of the serum. The result was expressed in international units per ml of serum by doubling the serum titre showing 50% agglutination. 80 IU/ml or above was considered significant, 40 IU/ml as doubtful and less than 40 IU/ml as negative.

**3.3.3.3 Indirect Enzyme Linked Immunosorbent Assay - I-ELISA**

(Plate No. 2)

**Introduction:**

Indirect Immunoenzyme assay was used to detect Brucella IgG and IgM antibodies from human serum. The method was based upon the binding of antibodies in the test samples with the antigen absorbed on the polystyrene surface.

**Suppliers:** The kits were supplied by VirCell microbiologists was used for Indirect Elisa with serum samples of study population.

**Kit Contents:**

- 96 Microtitre plate coated with Brucella antigen.
- Wash buffer 20X concentrate
- Sample diluting buffer
• Positive and Negative controls
• Monoclonal IgG, IgM Peroxidase conjugate
• TMB Substrate
Plate No. 1:
Rose Bengal Plate Test

Plate No. 2:
I - ELISA
• Stop solution.

**Principle:**

The assay requires the use of an antibody labelled with a tag such as peroxidase or phosphatase and an enzymatic substrate that produce a visible signal, indicating the presence of antigen. Colourimetric, chemiluminescent or fluorescent system may provide qualitative or quantitative result, where signal levels are measured in a spectrophotometer, luminometer or flurometer. In a properly optimised assay, the intensity of the signal generated is proportional to the amount of antigen present.

**Method:**

The samples were diluted with the diluents provided with the kit. The diluents are a blue coloured phosphate buffer containing protein stabilizers and Proclaim. For detecting Brucella IgG and IgM Antibody screening separate antigen coated wells and procedures were used.

**3.3.3.3.1 Brucella IgG ELISA**

After bringing the kits and reagents to room temperature, 5μl of sample and 75μl of the serum diluents to each of the test wells. Negative and positive controls were also put up in each set of the test. After shaking the wells for 2 min. the plates were incubated at 37°C for 45 minutes. After the initial set of incubation the plates were washed for removing unbound compounds.100μl of IgG conjugate was added immediately after the first set of washing and incubated for 30 min. at 37°C. After the second set of washing the wells were treated with substrate, Tetra Methyl Benzidine (TMB)
and the colour developed after 15 minutes of incubation in Dark was measured using an ELISA reader.

The colour development is terminated by the addition of a stop solution, which changes the colour from blue to yellow. The resulting dye is measured spectrophotometrically at the wavelength of 450 nm. The concentration of the IgG antibodies is directly proportional to the intensity of the colour.

3.3.3.3.2 Brucella IgM ELISA

After bringing the kits and reagents to room temperature, 25µl of IgG sorbent to each of the required wells except in control. 5µl of sample and 75µl of the serum diluents to each of the test wells. Negative and positive controls were also put up in each set of the test. After shaking the wells for 2 min, the plates were incubated at 37°C for 45 minutes. After the initial set of incubation the plates were washed for removing unbound compounds. 100µl of IgM conjugate was added immediately after the first set of washing and incubated for 30 min. at 37° C. After the second set of washing the wells were treated with substrate, Tetra Methyl Benzidine (TMB) and the colour developed after 15 minutes of incubation in Dark was measured using an ELISA reader.

The colour development is terminated by the addition of a stop solution, which changes the colour from blue to yellow. The resulting dye is measured spectrophotometrically at the wavelength of 450 nm. The concentration of the IgM antibodies is directly proportional to the intensity of the colour.
Observation:

The Substrate will develop colour in the presence of enzyme. Microtitre wells with colour indicate the presence of antigen antibody reaction. The reaction was stopped by adding 1% $\text{H}_2\text{SO}_4$ before putting the wells in the Elisa reader. The intensity of colour development varies with the amount of antigen antibody in the test serum.

Calculation

Antibody Index = (Sample O.D./Cut off serum mean O.D.) x 10

<table>
<thead>
<tr>
<th>Antibody Index</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 9.0</td>
<td>Negative</td>
</tr>
<tr>
<td>9 - 11</td>
<td>Equivocal</td>
</tr>
<tr>
<td>&gt; 11.0</td>
<td>Positive</td>
</tr>
</tbody>
</table>

Interpretation:

In the acute stage of the disease there is an initial production of IgM antibodies, followed closely by production of IgG antibodies. IgG-class antibodies may decline after treatment; however, high levels of circulating IgG-class antibodies may be found without any active disease. Chronic brucellosis shows a predominance of IgG-class antibodies with little or no detectable IgM.
3.4 CALCULATION OF SENSITIVITY AND SPECIFICITY

3.4.1 Sensitivity of the test

The sensitivity of a clinical test refers to the ability of the test to correctly identify those patients with the disease.

If \( a = \) True positive, \( b = \) False positive, \( c = \) False negative & \( d = \) True negative

\[
\text{Sensitivity} = \left[ \frac{a}{a+c} \right] \times 100
\]

3.4.2 Specificity of the test

The specificity of a clinical test refers to the ability of the test to correctly identify those patients without the disease.

\[
\text{Specificity} = \left[ \frac{d}{b+d} \right] \times 100
\]
ANNEXURE - I

PATIENT CONSENT FORM

Study Title: Serodiagnostic study of animal handlers for Brucellosis

This is a medical research study conducted by Deepthy B. J., Research scholar, Department of Microbiology, School of Health Sciences, Palayad, Kannur. You are being asked to take part in this study because you are in the selected group of study.

In this study, researchers are collecting blood samples to study the diseases related to your profession. If you agree to be in this study we will collect the blood by putting a needle into a vein onto your arm. One small tube of blood will be taken this will take about 5 minutes.

We will do our best to protect the information we have collected from you. Information which identifies you will be kept secure and restricted. If information from this research is published or presented at scientific meetings your names and identification will not be used. All those details will be destroyed when this research is complete.

If you wish to be in this study please sign below.

Date Participant’s signature of consent

Date Person obtaining consent
ANNEXURE - II

INFORMATION COLLECTED FROM THE STUDY POPULATION

General information

Name:
Age:
Sex:
Weight:
Address:
Occupation:

Specific Information:

History of Fever:
Nature of fever:
Joints pain:
Night sweat:
Lymph node enlargement:
History of Orchitis:
History of Abortion:
Habit of drinking raw milk: