**3. MATERIALS AND METHODS**

**3.1. Area and Study Population**

The study was conducted on commercial dairy cows at Chittagong region which is the south-east part of Bangladesh. The type of animals kept under commercial farming system were all cross of local with different exotic breeds (Friesian mostly). Among the population, 250 cows were selected from different farms randomly. The study was conducted only on the lactating dairy cows.

**3.2. Study design and farm selection**

The study was undertaken between February to July, 2011. The Dept. of Livestock Services of Chittagong maintains the register of commercial dairy farms at Chittagong. From that register 7 farms having a total of 250 cows were selected by simple random sampling method using the Excel software (Microsoft Inc., 2007). Four of them are situated within the Chittagong city and rests three are located at village areas. The study design is schematically shown in the Fig. 01.

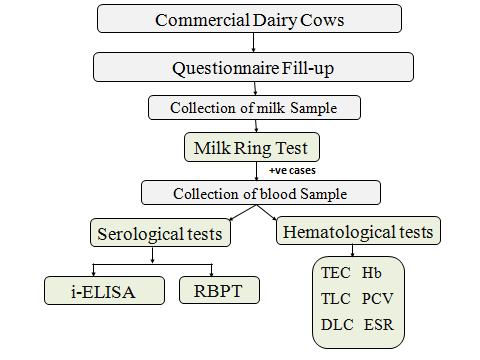


Fig 01: Schematic diagram of experimental design.

**3.3. Questionnaire design and data collection**

Information about each herd and the animals kept was collected by means of a structured questionnaire, which was completed at all the selected herds on a single visit. The questionnaire was designed to comprise mostly closed ended (categorical) questions to ease data processing, minimize variation, and improve precision of responses (Thrusfield, 2005). The questionnaire was filled up by repeated questioning to the farmers and also farm manager and attendant, taking records from register book by the author. Important herd and animal level data includes cattle location, total number of animals, breed, history of abortion and other reproductive disorders. A complete form of questionnaire is given in the Annex-I.

**3.4. Laboratory Preparation**

All items of glassware including screw capped test tubes, pipettes, cryotube vial, vacutainer, ependrof tube soaked in a household dishwashing detergent solution (‘Trix’ Recket and Colman Bangladesh Ltd.) for overnight, contaminated glassware were disinfected in 2% sodium hypochlorite solution prior to cleaning. The glassware were then cleaned by brushing, washed thoroughly by running tap water, rinsed four times in distilled water and finally sterilized by autoclaving at 1210C for 15 min under 15 lbs pressure per sq. inch. Autoclaved items were dried in a hot air oven at 500C. Disposable plastic like micropipette tips were sterilized by autoclaving. All the glassware was kept in oven at 500C for future use.

Other appliances used are test tube stand, cryotube vial rack, incubator, refrigerator, autoclave machine, hot air oven, centrifuge machines, centrifuge tubes, disposable 10 ml plastic syringe etc.

**3.5. Sample Collection and Handling**

In this study two different types of samples were collected namely milk and then blood.

**3.5.1. Milk sampling:** About 5ml of milk was collected from each cow into sterile screw capped test tube. In farm, milk was collected on the spot when it was milking. Then the udder especially teats were disinfected using medicated towel. Milk was collected from 4 teats of a cow and then labeled the ID on wall of the tube and stored in the ice box. Milk samples were screened by Milk Ring Test within 6 hours.

**3.5.2. Blood sampling:** The cows that are positive to Milk Ring Test were subjected to blood collection for separation of sera and hematological tests. About 10 ml of blood was collected from jugular vein using disposable sterile syringe (12 ml) after disinfection of the jugular furrow using Tr. Iodine. Immediately after that about 5ml of blood was transferred to EDTA (Di-sodium salt of ethylene diamine-tetraacetic) vial for hematological analysis and the rest 5ml was poured into another vacutainer for serological tests. The vacutainer kept inclined position for about 30 minutes to allow clotting and maintained at approximately +4°C in refrigerator until they were processed. In the laboratory, sera were separated by centrifugation at 2500 rpm (503g) for 15 min. and stored in 1.5 ml eppendrof tubes at -20°C until laboratory tests were performed. Hematological analysis was done within 6 hours of collection.

**3.6. Immunological Tests**

Milk Ring Test was done as a screening test. The cows that showed positive reaction to MRT were subjected to sera collection and indirect ELISA and Rose Bengal Plate tests were done to make a final diagnosis.

**3.6.1. Milk Ring Test (MRT):** The MRT was done in the Laboratory of Department of Microbiology, Faculty of Veterinary Medicine, Chittagong Veterinary and Animal Sciences University. After proper and uniform mixing using voltex, 1 ml of undiluted milk sample was taken from screw capped test tube into cryotube vial and 50 µl of colored brucellar antigen was added to it. Careful and uniform mixing was made using micropipette and placed in the incubator for 1 hour at +37°C (±2°C), then 18-20 hours between +2°C to +8°C. An individual test sample was interpreted as positive if the ring of cream equally or more colored than the underlying milk and negative if ring of cream less colored than the underlying milk (Fig. 02) as described by the reagent giving company INSTITUT POURQUIER-326 Rue de la Galera – 34090 Montpellier – France.

**3.6.2. Indirect-Enzyme Linked Immunosorbent Assay (i-ELISA):** The test is done in the Laboratory of Department of Medicine, Faculty of Veterinary Science, Bangladesh Agricultural University. The assay was performed according to the protocol and suppliance provided by the ELISA kit manufacturer company (Svanova Biotech AB, art. No. 10-2700-10, SE-751 83 Uppsala, Sweden). The sensitivity and specificity of this test is 97.4% and 85.8% respectively (Limet, 1988).

**Preparation of PBS- Tween Buffer for i-ELISA:** As working buffer 20x concentrate PBS- Tween Solution (PBST) was diluted into 1/20 in distilled water (DW). For this test 25 ml PBST solution was added to 475 ml DW to make it 500ml and was mixed thoroughly.

**Reconstitution of Anti-Bovine I­­­g­­G Conjugate for i-ELISA:** According to the procedure Liophilized HRP Conjugate was reconstituted with 11.5 ml PBS-Tween Buffer. Buffer was added carefully to the bottle. Then the solution was leaved for one minute and mixed thoroughly. According to the recommendation of the solution was prepared immediately before use.

**Test Procedure:** All reagents supplied by the manufacturer company were equilibrated to room temperature (18 to 25°C) before use. 100 µl of Sample Dilution Buffer was added to each well of polystyrene microtitre plate precoated with *Brucella abortus* antigen that would be used for serum samples and serum controls. The sample positions were recorded on a work sheet. After that 4 µl of undiluted Negative Control Serum (Reagent A) and 4 µl of undiluted Positive Control Serum (Reagent B) were added respectively to selected wells (well A1 and A2 for reagent A and well A3 and A4 for reagent B). Four (4) µl of serum sample was added to each well (other than A1, A2, A3 and A4). For confirmation both test and control sera were used in duplicates. After uniform mixing the plate was sealed and incubated at 37°C (98.6°F) for l hour. Then the plate was rinsed 3 times with PBS-Tween Buffer and filled up the wells at each rinse, emptied the plate and tapped hard to remove all remains of fluid. Then 100 µl of anti-bovine HRP Conjugate was added to each well and incubated at 37°C (98.6°F) for 1 hour. Again rinsed the plate according to the previous way. Then 100 µl Substrate Solution was added to each well and incubated for 10 minutes at room temperature (18 to 25°C). The reaction was stopped by adding 50 µl of Stop Solution to each well and mixed thoroughly. The Stop Solution was added in the same order as the Substrate Solution was added. The optical density (OD) of the controls and samples was measured at 490 and 620 nm wave length in a microplate photometer (BioTek). The OD was measured within 15 minu­tes after the addition of Stop Solution to prevent fluctuation in OD values. The raw OD was calculated by subtracting the OD values of 620 nm from 490 nm. After that ELISA units was calculated using Microsoft Excel worksheet 2007.

**Interpretations:** Interpretation of samples is shown in Table 01. (recommended by the manufacturer).

Table 01. Interpretation of indirect (antibody capture) ELISA.

|  |  |
| --- | --- |
| **Grade** | **ELISA unit limits** |
| Negative | < 2 |
| Weak positive | 2 to <7.5 |
| Positive | 7.5 to <30 |
| Strong positive | > 30 |

**3.6.3. Rose Bengal Plate Test (RBPT):** This test was also done in the Laboratory of Department of Medicine, Faculty of Veterinary Science, Bangladesh Agricultural University. All sera samples were tested using RBPT antigen (VLA Weybridge, UK). The test procedure recommended by Alton *et al.,* (1988) was followed. Briefly, 30*μ*l of RBPT antigen and 30*μ*l of the test serum were placed alongside on the RBPT plate, and then mixed thoroughly. The plate was shaken for 4min and the degree of agglutination reactions was recorded. The sample was classified positive if any agglutination was observed and negative if no agglutination (Fig. 03). The sensitivity and specificity of this test is 67.7% and 98.7% respectively (Limet, 1988).

An animal was considered to be positive if it tested positive on all three tests: the MRT, i-ELISA and RBPT. Results of each tests is shown in the Annex-II.

**3.7. Hematological tests**

The hematological tests like hemoglobin (Hb), packed cell volume (PCV), erythrocyte sedimentation rate (ESR), total erythrocyte count (TEC), total leucocyte count (TLC) and differential leucocyte count (DLC) were done in the Physiology Laboratory, Department of Physiology, Pharmacology and Biochemistry, Faculty of Veterinary Medicine, Chittagong Veterinary and Animal Sciences University.

**3.7.1. Hemoglobin test:** The haemoglobin was measured by Colorimetric Procedures. The test procedure described by Campbell (1995) was followed. N/10 HCl up to 2 mark was taken into an empty graduated tube. The blood was sucked up to the 20 μl mark of the measuring pipette and added it to the graduated tube Containing 0.1 N HCl. Thoroughly mixed it with blood and kept it aside undisturbed for 7-10 minutes. After 9-10 minutes, about 95% of the final brown colour was attained. The resulting brown fluid was then diluted with distilled water until it matched with the brown glass standard. The value of haemoglobin was read from the scale by noting the height of the column of the diluted acid haematin. The values were always represented in gm%.

**3.7.2. Packed cell volume (PCV) or Haematocrit value:** It was done by Wintrobe Method as approved by Howlett *et al* (2002). A Wintrobe tube (having a uniform 3mm bore and calibrated by a 10 cm scale with millimetre divisions) was taken. The scale on the left side was read from top to bottom for ESR while scale on the right in the reverse order for PCV. Anticoagulant added blood was taken in a syringe to which a needle was already fixed with a polyethylene tubing of a 2mm diameter. The tip of the polyethylene tubing having a long narrow end was inserted to the bottom of the haematocrit tube (Wintrobe tube) and the blood was forced out by pressure on the syringe. The pipette was slowly withdrawn simultaneously. The Wintrobe tube was filled up to the mark 0, i.e. 1 ml of blood. The tubes were placed in the centrifuge machine and centrifuged at the speed of 3000 rpm for 30 minutes. After thirty minutes, the packed cell volume values were read from the bottom to top. The packed cell volume was read in percentage.

**3.7.3. Erythrocyte sedimentation rate (ESR):** The value was also determined by Wintrobe Method as described by Campbell (1995). According to the method, **t**he blood was taken in a tube containing an anti-coagulant. Then a clear dry Wintrobe tube was taken and filled the tube up to the 10 cm mark withthe help of a syringe and needle with polyethylene catheter sufficient to reach tothe bottom of Wintrobe tube. The blood present above the mark was adjusted with the help of cotton. The tube was placed in the stand and kept it undisturbed. The fall of the corpuscles was noted at intervals of 10, 20, 30, 60 minutes.The erythrocyte sedimentation rate was always represented as mm/time interval.

**3.7.4. Total erythrocyte count (TEC):**  The method was followed according to Campbell (1995). Blood was drawn by suction into a Thoma red cell pipette up to 0.5 mark. The blood sticking on the outer surface of the pipette was wiped off. In case the blood has slightly passed 0.5 mark, it was adjusted by touching the tip of the pipette against cotton. The diluent (Hayem’s Solution) was then drawn up to 101 mark. While drawing the diluent, the pipette was then rotated between the finger and thumb in order to mix the blood thoroughly with the diluent. The pipette was clasped horizontally and mixed for half a minute for uniform mixing, the pipette was clasped loosely in one hand and rotate the attached rubber tube with thumb and index finger of the other hand. Rotation in one direction was avoided. Mixing was repeated each time before expelling a drop for examination. The Neubauer’s chamber was kept on a smooth surface. The cover glass was then placed on the counting chamber. Several drops of fluid were expelled from the pipette and discarded. This was done to expel the Hayem’s fluid in the capillary which was not mixed up with the blood. A small drop of mixed fluid was then placed between the cover glass and the ruled platform. The fluid was completely covered the chamber and none was run over the sides. The blood was allowed to settle for few minutes and examine for the uniform distribution of the red corpuscles in the chamber under low power. Then placed under the high power objective of microscope and the erythrocytes were counted in the central small squares. The number of cells in the four corner groups of 16 squares and one central one were recorded. Cells were counted in L (inverted or upright) form and omitting the cells lying on or outside the opposite line.



Fig 04:Counting Area in Neubauer's Chamber

**Calculations:**

Total Erythrocyte count = N x 10,000

N = Total count of RBC in 5 squares

The dilution was 1 to 200 (blood drawn to the 0.5 mark), then the total of cells found in

the 5 groups of 16 squares was multiplied by 10,000 in order to give the number of cells per

cu mm of blood. If the dilution was 1 to 100, then total number of cells wsa multiplied by 5,000.

The length of the side arm of smallest square is =0.05 mm

The area of the smallest squares is = 0.0025 sq. mm. i.e. (0.05 x 0.05 = 0.0025)

The depth = 0.1 mm

Total volume = 0.1 x 0.0025 = 0.00025 cumm

Since 80 such squares are counted the area covered is =0.00025x80=0.02 cumm

or the number of cells counted per cumm is = 1/0.02 or 50 as the dilution was 1 to 200.

The multiplication factor is = 50 x 200 = 10,000

**3.7.5. Total leucocyte count (TLC):** The blood was drawn by sucking into a thoma white cell pipette containing white bead to the mark 0.5 as described by Campbell (1995). Wiped off the excessive blood sticking to the outer side of the pipette. The blood in excess than the mark 0.5 was drawn out by applying against the cotton and drawn the Turk’s fluid into diluting pipette upto mark 11. The pipette was kept horizontal on the palm of the hand and mixed thoroughly by rotation with the other hand. Mixing with Turk's fluid caused haemolysis of RBC (Acetic acid) and stained WBC (Gentian violet). After mixing for 1 minute expelled several drops of fluid from the stem of the pipette containing only the Turk’s fluid. A small drop of mixed fluid was then placed between cover slip and the ruled chamber. Allow it to settle for some time and examine under 10X power of the microscope in four corner group of sixteen squares. The counting was made in L form or up and down omitting the cells lying on the other lines. This was done to remove the error of recounting.

**Calculation:**

Total Leukocyte count (T.L.C) = X × 50

X = Total number of cells counted in all four squares

50 = Multiplication factor

Multiplication factor = 20/ (1x 1 x 0.1 x 4) = 50 where

20 = dilution

1x1 = area of one large square

0.1 = depth between the cover slip and counting chamber

4 = No. of large squares in which counting was done

**3.7.6. Differential leukocyte count (DLC):** DLC was done by Slide Technique. A drop of blood was placed on the surface of the slide near one end. The slide was then held between two fingers and steadied with the little finger of the left hand. The blood may be spread by means of another glass slide known as spreader which was held in right hand. The spreader was held just to the left of the drop of blood and then it was pulled back to edge of the drop. The blood was spread out behind the spreader which was then pushed to the left. The movement must be quick and steady. The smear will be thin or thick according to the movement of the spreader i.e. slow or rapid and depending on the angle at which the spreader slide is held. The angle should be about 30 degree. A good smear should be smooth, homogeneous and without serrations, have even edges and should occupy approximately the middle third of the slide. The blood was allowed to dry on the glass in the open air.

Then fresh smear was placed in racks. The smear was stained and covered the smear with stain (Wright's Stain) and kept it for one minute. Fixation was completed during this phase. The stain was diluted with distilled water approximately using the same volume of water as that of stain. A greenish metallic scum comes up and the margin shows a reddish tinge. The stain was washed after 3 or 4 minute until the film was yellowish or pink and examined under oil immersion lens. Different leukocytes were counted and record with the help of mechanical cell counter and find out the percentage.

Fig 05: Differentiate leukocyte count

**3.8. Data Analyses**

Data from the laboratory results and questionnaires were stored in personal computer, using Microsoft Excel spreadsheet program. Descriptive statistical analyses of various risk factors and dependent variables were done using Intercooled STATA 9.0 (Stata Corporation 2008). Proportional analysis and multinomial logistic regression was used to interpret the data.