**Chapter-2**

**RESEARCH METHODOLOGY**

**2.1 Study areas, size and sample collection**

A total of 60 samples (20 Goats) were purchased from retail outlet of different parts of Chittagong metropolitan area (Jhautola Bazar, Reajuddin Bazar and Kornafuly Market Chittagong) during 10.01.2015 to 20.06.2015. For goats samples were taken from thigh muscle (20), liver (20) and kidney (20) separately. These samples were screened detected for antibiotic residue in meat by Thin Layer Chromatography (TLC).

**2.2 Preparation of Phosphate buffer (pH 6.5)**

For the Preparation of Phosphate buffer solution 1.76 gm Disodium Hydrogen Phosphate and 2.46 gm Sodium Dihydrogen Phosphate were added with distilled water upto 1000ml.

**2.3 Preparation of 30% Trichloroacetic acid**

30 gm of crystal Trichloracetic acid was diluted with distilled water up to 100 ml mark level with proper care.

**2.4 Silica plates for sample running**

TLC Silica plates with 0.25 mm. thickness (Merck, Germany), were activated in 120°C for two hours before use.

**2.5 Standard preparation and selected antibiotics**

For comparison of extracted residues with routinely used four antibiotics such as Tetracycline, Amoxicillin, Ciprofloxacin and Sulfanilamide penicillin were prepared by dissolving 0.1 gm of powder/ 0.1 ml of solution in 4 ml methanol.

**2.6 Method used for Thin Layer Chromatography**

***2.6.1. Antibiotic Extraction***

 For the detection of antibiotic byThin Layer Chromatography, these samples were stored in deep fridges at -20°C until further advanced procedures were performed. Samples (Muscle tissue, Liver and Kidney) were blended with a food processor (Macro Food Processor) properly for three to five minutes. Running of blender was continued for one minute and a pause of five to eight seconds followed by running again. This technique was repeated until tissues were blended properly. These mashed/ blended samples were taken into properly cleaned and sterilized petridishes with proper care as well as covering. From this 4 gm of aliquoted sample was taken into beaker with the help of electric balance and spatula. Then homogenization was done with addition of 10 ml phosphate (pH 6.5). After proper mixing, protein content of these samples were precipitated with the addition of 2 ml Trichloroacetic acid (30%) maintaining sufficient care and attention. Then these mixtures samples were taken into properly cleaned and sterilized test tubes for centrifugation. Then centrifugation was done / performed at 3000 rpm for 25 minutes with the help of automatically time regulated centrifuged machine (Labofuge, 200). 2 ml supernatant was mixed with 100 µL of formaldehyde for 45 minutes at 100°C water bath. The supernatant was extracted with equal volume of diethyl ether and mixing was done properly in order to perform defatation. Then mixture was kept for 10 minutes to become a separate layer, a upper oily layer and bottom layer. Then by using cleaned and sterilized separating funnel, these mixture were separated from each other and upper oily layer was discarded but only the bottom layer was collected. This extraction of supernatant was repeated twice with diethyl ether. Then the extracts were evaporated until dryness. The dried sample was reconstituted within 2 ml of mobile phase methanol, acetone (1:1). Then, Extracts were collected into screw cap vial with proper care and kept into refrigerator for further advanced analysis. Total procedure was performed as the reference cited by Popelka et al., (2005).

In order to performance of Thin Layer Chromatography (TLC) along with stationary phase, a mobile phase or solvent preparation was done as directed in the references (Thangadu et al., 2002). Here 50 ml of methanol and 50 ml of acetone was mixed properly and was use as mobile phase.

**2.6.2 Pointing of Thin Layer Chromatography plate**

TLC plates were cut according to the shape of TLC tank with scissors. At first a line was drawn with the help of a pencil and scale as much above of the solvent. Then pointing of standard solution was done with capillary tube on this line 2cm distance from each other. All spots had dried up plates were placed in the TLC tank and allowed for running.

**2.6.3 Running of TLC**

For ensuring this study perfectly continuous development of chromatogram was done. The duration of mobile phase to run was one (1) hour for each plate placed in the TLC tank.

**2.6.4 Examination of Chromatogram under UV detector**

In this TLC, the chromatogram was examined under the ultraviolet lamp at 256 nm for spots i.e., spot that fluorescenced. The outline of the spot was marked with a series of dots using a sharp pencil.

**2.6.5 Determination of RF (Retardation factor) value**

The calculation of RF values was done using the following equation:

**2.6.6 Data Collection**

All information regarding questionnaire and results of the above mentioned test was kept systematically and well organized form over the study period.

**2.6.7 Statistical Analysis**

The obtained data were entered into MS Excel-2007 and exported to STATA 9.0 for data analysis.