CHAPTER-1

INTRODUCTION

Tuberculosis (TB) is a worldwide problem present in all regions of the world. It is a highly communicable disease and ranked second highest killer after HIV. For the last 200 years, the only disease that caused the most deaths than any other infectious disease is tuberculosis. The disease is caused by *Mycobacterium*, which is a rod shaped aerobic, non-motile bacillus In 2014 about 9.6 million new disease cases were reported, from which male cases were 5.4 million, while female were 3.2 million along with 1 million children. From diseased people 1.5 million deaths were reported from the same year (WHO, 2015). Pakistan is included in the 22 high-burden TB countries, those bears almost 80% burden of the whole world in tuberculosis. Pakistan is ranked No. 7 among 11 high burden tuberculosis countries. From Pakistan in 2014, total reported cases were 316577 from which 122357 were confirmed by bacteriological examination, in the same year Pakistan was positioned No. 5 among 6 countries with highest TB incidence rate (WHO, 2015). Clinically diagnosed pulmonary cases were 120350 while extra-pulmonary were 57463 (WHO, 2015). Pakistan is not even behind in bovine tuberculosis; caused by *Mycobacterium bovis (M. bvois)* and it is classified as a risk 3 pathogen for public health and is a trade barrier (OIE, 2009). Tuberculosis caused by *M. bovis* in humans is a very old story which has just unfolded in the recent past. The main source of zoonotic disease transmission from animals to humans is the infected milk or meat. The risk of human infection has been linked through the use of unpasteurized, un-boiled milk, so the people related with raw milk selling, processing and drinking are at high risk. Different studies in Pakistan on bovine tuberculosis in cattle, buffalo, sheep and goat have reported its presence in animals which in general vary from 5- 10% (Javed *et al*., 2006). In 2013 the disease was estimated to, vary from, 6.62 to 11.96% in buffalo while 5.53 to 11.71% in cattle.

Typing of strain is very useful for knowing the transmission pattern of *Mycobacterium* geographically and disease origin may also be predicted from it, so it is a useful tool for controlling strategies of disease and origin of disease may also predict from it.

Mycobacterium tuberculosis complex (MTC) is a group of genetically related

Mycobacterium species, 99.9% similar at the nucleotide level, but, different as host and pathogenicity. The primary host of *Mycobacterium tuberculosis* are humans and of

Mycobacterium bovis are bovines, but additionally *M. bovis* may cause zoonotic disease in humans and at the same time it is also present in other large numbers of mammals, *Mycobacterium caprae* naturally affects goats and *Mycobacterium microti* dominantly found in rodents, however, it is well documented that all the members of MTC are known to infect humans as well (Soolingen *et al*., 1998).

Zoonotic TB and TB cause by other members of MTC can't be distinguished clinically and even by using conventional identification methods like Ziehl-Neelsen stain and culture isolation (Brosch *et al*., 2012). That is the reason, in-spite of putting all the efforts TB is on the increase, with advancing time multiple drug resistant (MDR) strains are being introduced. More- over poor understanding about the molecular epidemiology and origin of disease is a matter of concern (Abbadi *et al*., 2007). Molecular characterization of the organism is the need of the time, several methods are being used for amplification of DNA and strain differentiation, spoligotyping is the modern, fast and reliable method to differentiate all the members of MTC with high accuracy (Neonakis *et al*., 2008).

Multiple drug resistance (MDR) is also a major problem and is increasing, even in 2014, 3.3% new MDR cases have been identified and among this proportion of relapse was even about 6 times higher, so it is another major hindrance to control the disease. Pakistan has positioned No.6 from nine high burden drug resistance TB countries (WHO, 2015). From the year 2014, 3.7% new MDR-TB cases were reported from Pakistan (WHO, 2015). Typically, TB, that is resistant to the two most commonly used first line drugs, i.e. rifampicin (RIF) and isoniazid (INH) is called as multiple drug resistant TB (MDR-TB) (WHO, 2014). If MDR-TB occurs to a patient, it requires almost 500 UD Dollar per patient for treatment of MDR-TB, which is very much costly for a low income country like Pakistan (WHO, 2015). Additionally, no study has been conducted in Pakistan on zoonotic TB drug resistance this factor also requires an insight. Pakistan is positioned at no. 6 from nine high burden drug resistant TB countries (WHO, 2015). Rifampicin drug have most common use for curing tuberculosis by inhibiting the protein synthesis. A mutation in RNA polymerase beta-subunit (*rpoB*) gene is a common cause of drug resistance against rifampicin (McCammon *et al*., 2005). Isoniazid acts as an anti-TB drug by disturbing the mycolic acid synthesis to forming the cell wall of bacteria; catalase-peroxidase (*KatG*) is the major gene, which convert the pro-drug into active drug, its mutation will lead to drug resistant TB. In the past, the most

common way to determine the drug resistance was drug sensitivity Testing (DST) on a culture medium, but it requires almost 3-4 weeks. Molecular methods targeting genes associated with resistance are more quick and accurate for DST purpose and take only a day or so to produce reliable results (Afanas'ev *et al*.,2007). Mutated gene contains polymorphism, which, can, easily be detected by PCR restriction fragment length polymorphism (RFLP) or single nucleotide polymorphism (SNP) analysis and it is a relatively inexpensive technique and fast in action.

Genetic susceptibility against certain diseases is an old but newly unfolded story; in the case of TB, it has been studied that only 10% people those have intact immunity develop clinical disease, remaining can take control over disease due to a hold of innate immunity. Major cells involved in immunity are phagocytes. Naturally-resistance associated macrophage protein1 (Nramp) is transcribed by *NRAMP1* gene; this gene is associated with transportation of cations across the cell membrane of macrophages at time of activation (Gruenheid *et al*., 1997). A polymorphism in the *NARAMP1* gene at *3'UTR* and *INT4* has a close association between TB and genetic resistance against disease. This polymorphism will certainly increase the risk of developing active tuberculosis in patients and it is very good marker to observe the genetic susceptibility of local population (Bellamy *et al*., 1998).

Tuberculosis is consistently present and is even with the increase in the local population, so the genetic insight is needed. Drug resistance and its association with specific strains is a hidden story, molecular genetic susceptibility may be used as a predictor before the disease is a question mark and needs an advanced approach. Considering all these factors the study was planned in both animals and humans with the objectives;

Objectives:

- To study the disease and its associated factors, both in animals and humans.
- To study the pathological lesions in different organs of slaughtered animals.
- Molecular identification and characterization of isolates from animals and humans.
- Analysis of selected gene polymorphism of selected strains involved in drug resistance in local population strains.
- Evaluation of genetic susceptibility in local population by analyzing patterns of *NRAMP1* gene.

CHAPTER-2 REVIEW OF LITERATURE

(i) Tuberculosis

Tuberculosis is a very old story, caused by Mycobacterium, which is a weekly gram positive slow growing rod shape bacteria which may affect all types of mammals. The causative agent of both bovine and human TB is related to almost genetically similar species group called *Mycobacterium tuberculosis* complex (MTC) organism, Primarily *M. tuberculosis* causes disease in human and *M. bovis* causes disease in bovine and other animals. But both of them and other members may cause disease in humans or animals, that is the reason this bacterium is present from ancient time to date (Soolingen *et al*., 1998).

Tuberculosis is a very ancient disease and *Mycobacterium tuberculosis complex* organism was present in a conservative DNA region, even since17000 years. It was supported in a survey led by Rothschild *et al*. (2001) in the USA. Amplification of DNA was successful and even spoligotyping technique was feasible on all the specimens received from an extinct bison. In an advanced study it was confirmed that major test to detect TB was acid fast bacillus staining but it can only detect active tuberculosis. The cultural isolation on selected media has been a reference standard test for identification of active disease, but these both tests along with many can't differentiate the TB caused by bovine origin or caused by other members of the MTC group (Brosch *et al*., 2012).

(ii) In Animals

Ahmed *et al*. (1999) observed the presence of tuberculosis bacilli in bovine semen in India by using PCR. As shedding of the organism in semen is a potential threat to females that receive infected semen either by natural breeding or artificial insemination, so they carried out the study. They selected three healthy and suspects bulls and collected 20 semen samples from each bull. PCR was applied to detect microorganism based on insertion sequence IS 1081 for detection of *Mycobacterium tuberculosis complex*, both from fresh and frozen semen samples. 20 samples from one infected bull gave positive results, while 40 samples from other bulls did not produce positive results. The sensitivity of this method proved 100% by analysing those results. Amplified products were confirmed by applying southern blot hybridization on all the samples. The results concluded that PCR is an efficient

technique for the detection of bacilli in semen with remarkable precision. PCR can detect 20-100 acid fast bacilli from spiked semen in a short time.

In an experiment conducted with the aim of isolation and identification of *Mycobacterium* from milk and clinical samples in Brazil. 128 milk samples were collected from local markets; along with 22 clinical samples from different animals suspected to tuberculosis. Confirmation of organism was done by using PCR on isolated DNA. Results confirmed that 23 (18%) milk samples and 15 (68.2%) caseous lesions were positive for *Mycobacterium*, in which 11 were *M. bovis*, and 27 were recognized as non-tuberculosis mycobacterium (NTM). The study confirmed that animal products like milk and meat are reservoirs of *Mycobacterium* and there is a continuous threat of passing this disease from animal to human (Leite *et al*., 2003).

The prevalence of tuberculosis and related risk factors in buffalo and cattle were assessed at two different farms in Pakistan (Javed *et al*., 2006). The comparative intradermal tuberculin test was a common diagnostic tool with considerable detection rate was applied to all animals to check the response of disease in animals. The lower detection rate was 2.45%, while the highest detection rate was up to 8.48%, along with some unclear reaction results in 8.58% animals at farm number 2 with lower disease rate. More strong positive results were observed in buffalos in comparison with local cattle. There was an association observed between disease and associated epidemiological factors by which age of the animal was found a risk factor. The disease was observed with high frequency in animals that had around 7 liters production of milk, body weight not more than 550 kg and the animals had minimum three parturitions also had a high frequency of disease. The study revealed that tuberculin test is a valid technique for diagnosis of disease and the disease is present with a consistent pattern in Pakistan.

Another experiment was carried out to know about the tuberculosis status in Nili Ravi buffalo in Punjab, Pakistan. Application of comparative intradermal tuberculin test showed that 10.06% animals were positive for tuberculin test. Bacteriological studies depicted that 9 samples out of 16 were positive. *Mycobacterium bovis* was isolated from four milk samples, *M. tuberculosis* from two milk and one faecal sample, while atypical *Mycobacterium* were isolated from 2 milk samples. High yield of milk and older age were associated factors with

the prevalence of disease (Khan *et al*., 2008).

To check the reliability of polymerase chain reaction from processed milk samples an investigative study was conducted from Lahore, Pakistan (Mumtaz *et al*., 2008). Milk samples were directly obtained from animals and different sale points. Multiplex PCR was performed in a single tube. DNA was amplified targeting two different genes, one specific for MTC and other specie specific region of *M. bovis*. The results confirmed 35% MTC organism and 29% *M. bovis.* Milk was proved an active vehicle for transmission of TB organism.

Cardos *et al.* (2009) carried out a study for the detection of *Mycobacterium bovis* from slaughtered organs and lymph nodes in Brazil. It was an abattoir based study conducted in North west state Parana. From slaughtered animal, organs were observed for the presence of different lesions, lymph nodes were also collected from animals along with infected organs. Total 35 organs were collected from different animals. All tissue samples were decontaminated by using modified Petroff's method. Processed samples were subjected to Zeihl-Neelsen staining method for the detection of organisms. The samples were cultivated on different culture medium like Stonebrink's and LJ. From culture isolates, DNA was extracted for PCR. The obtained results indicated highest detection rate with PCR, i.e., 54.5%, followed by 51.5% culture positive cases. PCR was confirmed as highly specific test for confirmation of bacilli in milk samples.

A surveillance based study was carried out at 11 different experimental stations in Pakistan (Javed *et al*., 2011). Single comparative cervical intradermal tuberculin test was performed on adult animals to check the status of the disease. They found 7.6% cattle reactive against tuberculin testing. The percentage of tuberculin positive animal was higher in older age as compared to young one. The author observed that the prevalence of disease /'was associated with certain other factors like; more calving number and high production of milk per lactation length.

In 2012, a survey was conducted to record the prevalence of tuberculosis and its associated factors in zoo animals from Islamabad, Pakistan (Shahid *et al*., 2012). To screen out the diseased animals the intradermal tuberculin testing was conducted. Overall, 3.3% zoo animals were declared positive. Highest number of positive cases were observed in the

animals of the *Bovidae* family (3.6%), while, lesser number of positive cases were in the animals of the *Cervidae* family. No reactive animal was observed from *Equidae* family. Certain epidemiological factors were associated with the prevalence; like live weight of the animals, number of calving. Certain haematological parameters were also assessed, there was significant difference observed in haematological parameters including; total leukocyte count, haemoglobin concentration, total erythrocyte count, basophil and eosinophil %age between reactive and non-reactive animals. It was confirmed that tuberculosis was also present in zoo animals in Pakistan and frequency of disease was different in different families of wild animals.

(iii) In Humans

Cohen *et al*. (1998) carried out a study for early identification of *Mycobacterium* using two types of amplification assays within 24 hours of patient's admission in hospital in Chicago. Eighty-five patients were admitted for diagnosis purpose; sputum was taken as clinical specimen; 3 sputum samples were collected for each patient after 4-hour interval. All samples were processed using Sodium-N-acetyl-NAOH method for decontamination. Sediments concentrate were used for isolation. DNA was extracted by the heat boiling method. DNA amplification was done by targeting IS6110 gene sequence of MTC using in house PCR assay. Other amplification was based on 16s ribosomal RNA gene by Roch Amplicor PCR assay. Out of 85 enrolled patient's culture positive were 27 in numbers, while 12 patients were reported smear positive. Sensitivity showed by both Roch technique and in house PCR was 74% and 85% and specificity of 93% and 88%, respectively in first 24 hour's collected sputum samples. In smear negative samples, specificity and sensitivity of both assays was 53% and 73%. The detection rate was 95% for Roche Amplification and 100% for in-house technique. They concluded that both PCR assays were equally useful for early diagnosis of tuberculosis.

Bruchfeld *et al*. (2000) performed an experiment with the aim to improve the diagnosis of tuberculosis by increasing the sensitivity of the basic diagnostic technique; they used the sputum concentration method in the patients with a high load of HIV disease in Ethiopia. In resource poor countries, sputum smear microscopy is a valuable and cost effective tool for the diagnosis of tuberculosis, but, sensitivity of smear microscopy is a

major problem. To increase the sensitivity, certain modifications were made. 512 patients with clinical signs of tuberculosis were selected for the study, 3 sputum samples were collected from each patient. With standard ZN staining method 54.2 percent positive cases were recorded. All the sputum samples were stored at 4˚C. Processing of stored sample was performed with 5% Sodium-hypochlorite. Centrifugation was done for the separation of supernatant leaving behind a concentrated mass. Slides were made directly from this concentrated portion. With these little modifications the positive detection rate was increased up to 63.1 %age. The study suggested that sediment concentration is far better method to improve the sensitivity of sputum smear microscopy.

Bakshi *et al*. (2005) performed multiplex-polymerase reaction (mPCR) for rapid identification and differentiation of *M. tuberculosis* (Mtb) and *M. bovis* in a single tube. PCR was reported useful as it amplified DNA sequence conserved for *M. bovis* and *M. tuberculosis* with considerable precision. The samples were collected from different TB patients. Specific sets of oligonucleotide primers were used to perform mPCR in a single run. Designed primers amplified successfully the 168 bp DNA band specific for *M. bovis* and 337 bp DNA band specific for *M. tuberculosis*. The entire sample amplified were either positive for Mtb or for *M. bovis* , so the sensitivity of the method was 100%. Moreover, this method was excellent to detect as low as 20 pg DNA from direct clinical samples. Finally, it was concluded from the study that m-PCR was a highly sensitive, very useful and rapid test for the detection of TB organism, and it was proved a highly useful method to differentiate species of Mtb and *M. bovis* , from culture isolates and clinical samples accurately.

Maria *et al*. (2006) conducted a study to assess the usefulness of direct PCR for the detection of *Mycobacterium* DNA from blood and urine. Samples were drawn from susceptible patients of TB. The patients were divided into two groups, one, the diseased and the other control. A comparison was made between these two groups. Fifty-seven patients were added in diseased group, while, 29 were kept as control. Samples were processed for culture cultivation on selective medium and DNA amplification in the later stages with the help of PCR. Positive culture was observed from 19% suspected patients, while a positive PCR was observed in 42% cases. Out of total confirmed PCR results, 41% results were obtained from pulmonary patients, 36%, from extra-pulmonary patients and 50% of disseminated TB cases. It was concluded from the study tuberculosis was detectable from

clinical samples like blood and urine, but the detection rate varied in between two samples. PCR was an applicable method to diagnose the organism from clinical samples effectively with variable sensitivity. Better handling and storage conditions can increase the sensitivity of the PCR.

Tiwari *et al*. (2007) worked out to evaluate the most accurate and modern method for rapid diagnosis of tuberculosis in India. They compared most common methods for their utilizations, advantages and disadvantages in terms of sensitivity, specificity and time elapsed. High performance liquid chromatography (HPLC) was practiced for rapid detection of mycolic acid pattern, it was proved rapid and highly sensitive method but still there were chances of false negative results. Phage amplified biological assay were proved very useful to demonstrate the viability of *Mycobacterium* and gave good results for susceptibility tests of anti-tubercular drugs. But are not equally available at all places. Different immune based assays were analysed, had different antigen and antibody combinations, reported useful and could be applied in all conditions. Culture methods were remained the gold standard in the isolation of live Mycobacterium but still had the problem of time due to the long incubation period. Molecular amplification methods are new, rapid and have considerable sensitivity and specificity; are most promising techniques, have been used in all the world, these methods still proved best among all, although those required highly trained persons and adequate contamination free environment to avoid false positive results.

For the confirmation of active tuberculosis in early stages, an experiment was planned from suspected tuberculosis patients on clinical samples in India. The patients were selected on the basis of clinical signs and radiological images (Basil *et al*., 2010). For the molecular identifications; DNA amplifications along with restriction length polymorphism (RFLP) assay was used. More than 200 patients were included in the study, freshly voided sputum samples were collected from each patient. DNA was extracted directly from sputum and then direct PCR with RFLP was applied to all samples targeting hsp65 gene. PCR was able to detect bacterial gene from 84.5% sputum samples among all acid fast bacilli smear-positive samples. Even 11% samples those were initially found negative with smear microscopy, were declared positive by this method. It was proved that active tuberculosis was consistently present. With the use of this method, more reliable and accurate results were obtained as compared to the conventional methods. PCR-RFLP proved itself most rapid and sensitive test

for the detection of tuberculosis as compared to the other methods. It was recommended as a valid screening and a conformation test for the clinician for early identification for patients.

To assess the magnitude of disease in relation to certain risk factors a study was carried out in Arkansas (Sarah *et al.,* 2011). Samples were collected from suspected patients; at the same time little information regarding with certain epidemiological factors was collected. Samples were collected in cluster formation in which, larger cluster with 10 numbers of cases and a smaller cluster with minimum 5 numbers of cases were made. The genomic insight was helpful to assess the route of transmission. The study continued for almost one year. Obtained results confirmed that the maximum number of cases were under the age of 65 years and dominant gender was male. A relatively large number of Smear positive cases were observed in HIV positive male as compared to females. Excessive drinking of alcohol was also a risk factor. Genetic based transmission of active tuberculosis was linked with the presence or absence of 25 unique gene sequences present in the genome of circulating organism.

A planned investigatory study was performed to assess the relative efficacy of two laboratory-based PCR methods for the diagnosis of lung tuberculosis in Brazil. The aim of the study was to search out the new diagnostic tests that could replace the old conventional methods like ZN staining in the early confirmed diagnosis of TB. For this purpose, two PCR based methods were used for amplification and rapid identification, including PCR agarose gel electrophoresis (PCR-AG) with 2% agarose gel and PCR dot-blot methodology (PCR dot-blot), both were exercised on study population. Two hundred seventy-seven suspected pulmonary tuberculosis (PTB) patients were included in the study. In the morning time, first expectorated sputum was collected from all the patients (Scherer *et al*., 2011). All samples were processed for further procedures, including; ZN stained sputum, culture isolation of bacteria on LJ medium and final amplification of DNA with two different PCR based assays as mentioned above. According to the results, the overall prevalence of disease was 46%, while this percentage was increased up to 54% in HIV positive patients. Both molecular methods were applicable, but dot blot method was more sensitive as compared to PCR-AG in this experiment. It was suggested that after obtaining genetic material from *Mycobacterium*, amplification based molecular techniques increased the detection rate of disease organisms and dot-blot assay is more accurate and fast.

From Pakistan a study was conducted in 2013, major area of investigation was identification of animal and human tuberculosis using modern diagnostic technique (Ali *et al*., 2013). Molecular identification was performed in two-way operation. In the first stage bacterial DNA containing MTC targeted region was successfully amplified, later on specie specific PCR was conducted on the same samples. Sputum, lymph node aspirates and plural effusions were the sample of choice. The study confirmed the 56.79% samples as MTC while, 7.45 were *M. bovis*. Percentage of PCR positive samples were much higher as compared to AFB stained samples. Extra pulmonary tuberculosis was a dominant form of TB. The study confirmed that tuberculosis was present in the local community and modern techniques were most useful and accurate for diagnosis of disease.

Jabbar *et al*. (2015) from Kohat, Pakistan carried out a study to differentiate *Mycobacterium tuberculosis* and *Mycobacterium bovis* from human sputum samples on the molecular basis. ZN staining and culture isolation on two different mediums (LJ and SB) was done initially before performing PCR on the processed samples. Results of PCR were also compared with biochemical tests; the outcome was favourable. Out of 100 isolates 96 were confirmed as *Mycobacterium tuberculosis* while, 4 were confirmed as *M. bovis* with specie specific primer. Detection range was increased with the use of multiplex PCR in a single run. The study confirmed the presence of *M. bovis* as causative agent of pulmonary tuberculosis in humans.

(iii) Molecular Characterization

Hogan *et al*. (1998) attempted an investigative study to trace the transmission of *Mycobacterium tuberculosis* in the local population of the West Indies. Two highly specific DNA fingerprinting methods were selected to enhance the efficacy of the results. They were successful to type the bacterium with IS6110 RFLP giving 10 clusters while with Spoligotyping 12 clusters and 45 patterns were observed. Thirty-two patients were common in both clusters of RFLP and spoligotyping. Forty percent clusters showed the recent transmission of organisms. Spoligotyping was still fast technique requires less amount of DNA. With the combined use of both techniques it was useful in tracing active transmission of disease. It was suggested that for effective control of disease tracing the active transmission is a nice parameter.

Muller *et al*. (2008) performed spoligotyping in an abattoir based studies on slaughter cattle in Mali, where large number of positive cases of tuberculosis had been reported previously. No molecular characterization of isolates was observed before this study. From slaughtered animal's different organs like liver, lung, peritoneum was collected having lesion of tuberculosis. Decontamination of samples and processing was done, then incubated on LJ medium. 8 The weeks old positive culture isolates were used for molecular identification of different strains of *M. bovis* through Spoligotyping. Seven different Spoligotyping patterns were observed, from which three were new (not reported before). From maximum strains spacer 30 was not present, It is a common characteristic of African countries strains. Certain other strains had absence of spacer 6; these strains were found specifically in the Mali region only.

From Greece in 2008, a study was conducted to categorize the best molecular tool used for identification and characterization of *Mycobacterium*. The IS-6110 RFLP method with spoligotyping and MIRU-VNTR were the methods of choice (Neonakis *et al*., 2008). In the detailed observation, it was found that although IS6110 RFLP method has high discrimination power for strain identification, but it can't be performed on clinical samples accurately as it requires large amounts of DNA from culture isolated organisms. Spoligotyping was proved more economical, fast and had highest stability with reliable results. The MIRU-VNTR was most laborious method which, required lots of time. Spoligotyping was also proved best in tracing epidemiological links of different strains

Cicero *et al*. (2009) confirmed the presence of *Mycobacterium bovis* as the causative agent of extra-pulmonary tuberculosis in humans from, Mexico. They further performed the spoligotyping to confirm the genotype of *Mycobacterium*. Patients suffered with ganglionar, meningeal, pericardial and peritoneal tuberculosis were selected for the study. Urine, (cerebro-spinal fluids) CSF, peritoneal fluids and different aspirates were collected for identification and molecular characterization of *Mycobacterium*. Species included in MTC organism were confirmed with hsp65-RFLP. Spoligotyping patterns were obtained from species of MTC. The dominant lineage observed within the species of *the M. bovis* were BOV1 following by BOV2 as the second major lineage. The study confirmed the involvement of *M. bovis* as cause of human infection. Spoligotyping was excellent in sub typing of *M. bovis* successfully.

From Colombia in 2012 molecular characterizations of MTC organism was carried out to detect the genetic based population structure of *Mycobacterium tuberculosis,* by using the spoligotyping as a basic genotyping method, All the members of MTC were frequently distinguished on the genomic basis with the use of 43 spacers on a nitrocellulose membrane. The spoligotyping was proved excellent for separation and identifications of different strains of *M. tuberculosis* along with their transfer pattern. Most commonly observed strains were Latin American strains (Cerezo *et al*., 2012).

From Mexico in 2013 an attempt was made to distinguish different strains of *Mycobacterium* along, with, drug resistance profiling in the local population. *M. bovis* from humans as causative agent was the main focus (Guarneros *et al*., 2013). The method of choice was spoligotyping due to its rapid and accurate results and high discriminatory power for the members of the MTC. The drug resistance patterns were estimated by conventional and modern molecular method that successfully revealed the positive results. The spacer oligonucleotide pattern analysis was proved excellent and accurate method of genotyping, even spoligotyping was highly sensitive for sub typing of the same species. Isoniazid and rifampicin were the most resistant drugs among all.

Eldholm *et al*. (2006) performed genotyping of *Mycobacterium tuberculosis* for the first time in Tanzania Dar-Es-Salam. From 147 consecutive patients of tuberculosis clinical samples were obtained. DNA was isolated by boiling method. Genotyping was performed by using spoligotyping method due to its fast and accurate results and it is cost effective methods as had been written in the past literature. A 43 spacer bounded membrane was utilized to obtain different strain patterns. For the conformation purposes strain pattern signatures were compared with the recently updated database, i.e. SpolDB4 to put the strains in different clusters and groups. They obtained 64% those patterns which were never reported before. From already discovered patterns; the most dominant strains were included in Central Asian strains (CAS) followed by a Latin American Mediterranean (LAM) on the 2nd. The least number of isolates were grouped into family East-African Indian (EAI). The study validated the use of spoligotyping for genotyping of tuberculosis isolates.

Oloya *et al*. (2007) did molecular characterization of the isolates recovered from slaughtered organs of cattle in Uganda. Sixty-one organs suspected for tuberculosis based on clinical lesions observed were selected for the study. All samples were cultured on selective

medium. Isolates were typed by spoligotyping and IS-1311 restriction length polymorphism analysis. As a result, 19 isolates were confirmed as *M. bovis*, 1 as *M. intracllulare* and 3 *M avium subsp.hominissuis,* while, 11 other than MTC were also observed. In further sub typing of *M. bovis,* ten different spoligotyping patterns were observed, those were classified into three different clusters. The study revealed that subtypes of *M. bovis* were also involved in the presence and transmission of disease beyond the species level. Spoligotyping was quite capable to subtype the species involved with high accuracy.

(iv) Drug Resistance

Piana *et al*. (2003), from Italy conducted a study to check the involvement of *KatG* and *rpoB* gene mutation in the drug resistance against tuberculosis. They locally collected isolates of *Mycobacterium tuberculosis*. The results were obtained by application of two different molecular typing techniques, one single strand conformation polymorphism (SSCP) and the other polymerase chain reaction- Restriction length polymorphism (PCR-RFLP). *BstuI* and *HaeIII* endonuclease enzymes were used for restriction of *KatG* and the *rpoB* gene respectively. The 6.12% strains were resistant to isoniazid, while 2% were resistant to rifampicin. It was concluded that selective gene mutation was associated with drug resistance and both the methods were precise and rapid in action. It was suggested from the study that these methods can be used as rapid screening methods in the future studies.

Viader-Salvado *et al*. (2003) turned out for the identification of drug resistance associated genes against two key antibacterial drugs, i.e., rifampicin (RIF) and isoniazid (INH). Out of total 48 INH and RIF resistant strains 19, INH and 9 RIF already known resistant strains were randomly selected to determine the mutation in *KatG* for Isoniazid and *rpoB* gene for rifampicin by amplification with PCR followed by restriction fragment length polymorphism analysis. The obtained results were comparable with the results of the line probe assay. PCR-RFLP was excellent at screening of drug resistance in selected isolates. The method was proved fast and accurate for early diagnosis of drug resistance tuberculosis.

Ranmaswamy *et al*. (2004) in a detailed observation confirmed the genetic based multiple drug resistant tuberculosis. An association was also observed between genotypes of organism with a type of drug resistance. 40 isolates were obtained from the study population,

from, which 37 were MDR. On the basis of IS6110 RFLP, 25 different patterns were observed. From cultivated organisms on a culture medium, all the isolates were isoniazid resistant, while, 97.3% were rifampicin resistant. A mutation in the gene had been similar to already reported genes, i.e., *KatG* and *rpoB* genes, additionally two new mutation sites were also observed from above described gene, i.e., *KatG* (n ¼ 5) to INH and *rpoB* (n ¼ 1).

Arnold from UK, in 2004 standardized a technique with the name pyrosequencing to confirm the mutation in the genome of *Mycobacterium tuberculosis* - complex; these mutations were associated with drug resistance against rifampicin and isoniazid. DNA from *Mycobacterium* was isolated from sputum samples of patients. Single nucleotide polymorphism (SNP) was checked in mutated genes. The SNP was observed in 68% of isoniazid, while 92% of rifampicin resistant isolates. Moreover, pyrosequencing was equally capable to differentially diagnose different species of *Mycobacterium tuberculosis* - *complex* organism. The method was proposed for future studies of MDR tuberculosis.

MDR is a term that describes the members of *Mycobacterium* species that are resistant to two very important and widely used first line drugs, i.e., rifampicin and isoniazid. Both drugs have a very important role in the treatment of TB. The mode of action of rifampicin is to inhibit transcription of essential proteins by acting on RNA polymerase, so it causes hindrance in the growth of bacteria. The *rpoB* gene is the responsible gene, which mostly harbor mutations in the genetic sequence, ultimately leads to resistance against this important drug. The most common sites of mutations in *rpoB* gene have been reported from codon 507 to 533 by substitution of codon (McCammon *et al*., 2005).

Brown *et al*. (2006) developed a technique named low density oligonucleotide arrays (macroarrays) to identify multiple drug resistance among MTC isolates in the UK. The mutations in the *rpoB* gene were associated with rifampicin resistant tuberculosis. Two candidate genes associated with isoniazid resistance are randomly screened from all the isolates. The mutation was observed from all the isolates. It was proved that in comparison with laborious and time consuming proportion methods of drug sensitivity, this procedure was more accurate and less time consuming. Both *KatG* and I*nha* genes were found associated with drug resistance against isoniazid.

The isoniazid (INH)) does bactericidal effects of stopping the mycolic acid synthesis, which causes interruptions in the cell wall synthesis, but the drug has to convert into an

active form to perform its bactericidal activity. The resistance against INH developed due to induction of mutation in *KatG* and *inh*-promoter regions, ultimately leads to over expression of activated INH targets, the major genomic portion that is prone to mutation in the is promoter region -15 nad -8 (Afanas'ev *et al*., 2007).

Abbadi *et al*. (2009) worked for the molecular identification of the existence of MDR-TB associated with mutation of the genome. The study was carried out on patients of pulmonary tuberculosis in Egypt. Isoniazid and rifampicin resistant strains were the main focus. Initially conventional drug susceptibility testing was performed by proportion method. The molecular identification of mutated gene was done by the use of single strand conformation polymorphism. *KatG* and *rpoB* genes were the target genes for isoniazid and rifampicin resistance has been reported from many studies earlier. Out of 155 isolates 16.1% were confirmed as multiple drug resistant tuberculosis cases, the *rpoB* gene mutation was involved in 76% cases, while, resistance against isoniazid was found in 47.1% cases. The study confirmed the presence of MDR-TB along with successful identifications of mutated sites in the genome of *Mycobacterium* at the molecular level.

Ahmed and Mokaddas, (2010) highlighted the status of MDR-TB cases across the globe, along with some possible reasons of resistance development. They compared the usefulness of different methods for the early and confirmed diagnosis of drug resistant tuberculosis. Inappropriate patient therapy and poor case detection were two main risk factors observed. Short time therapy was another problem for resource poor countries. Conventional susceptibility testing methods were found to be slow and biased as compared to molecular based genetic detection methods. It was confirmed by a study that molecular methods could also be effectively applied on clinical samples. Combination of HIV and TB was proved very lethal. Some recommendations were made to minimize future outbreaks, including optimization of drugs along with a rapid early diagnosis of disease. Susceptibility testing for both first line and second line anti-TB drugs were suggested to be must be a regular laboratory practice to minimize the danger in the future.

A comparative study was carried out to check the efficacy of 2 different PCR based methods, i.e., PCR-RFLP and PCR- single strand conformation polymorphism (PCR-SSCP) to detect the mutation in the *KatG* gene that is a reliable indicator of isoniazid resistant tuberculosis (Unissa *et al*., 2011). Total 105 resistant isolates were collected from, which,

54% were positive with PCR-RFLP while 51% were positive with PCR-SSCP. PCR-RFLP method was proved most reliable method with sensitivity of 84% and specificity up to 100%. The study results confirmed the validity and usefulness of PCR-RFLP, in confirmation of drug resistant tuberculosis.

Rahmo *et al*. (2012) made an attempt to confirm the role of *rpoB* gene mutation and its possible use as a marker of rifampicin resistant tuberculosis. A total of 56 Rifampicin-resistant strains were obtained from the biotechnology section of Syria. DNA was extracted from all isolates. Amplification was done for *rpoB* gene using specified primers. A standard pyrosequencing method was applied to detect mutation associated with hot spot regions within *the rpoB gene*. Out of 97 observed modified condos, 35% mutation sites were new, never reported before. Within codons, maximum changes were observed were in between codons 526 and 531. The results confirmed the involvement of *the rpoB gene* in the development of resistance against rifampicin. The authors further suggested that *rpoB* gene mutation was a good indicator of rifampicin resistant tuberculosis.

In a published report from the World Health Organization in 2004, it was reported that multiple drug resistance tuberculosis (MDR-TB) against most common first line drugs was a major problem, still present and it is on increase; even in 2013, 3.5% of new MDR-TB cases were reported. Frequency for development of MDR-TB cases was reported 3 times more in relapse TB cases in the same year, so it was reported to be another major hindrance to treat and control the disease (WHO, 2014).

(v) Genetic Susceptibility

Genetic susceptibility against certain diseases is an old but now unfolded story. In case of TB it has been studied that only 10% people having intact immunity can develop clinical disease, remaining can take the control over disease due to a hold of innate immunity. The major cells involved in the immunity are phagocytes. Naturally resistant associated macrophage protein1 is transcribed by *NRAMP1* gene; this protein is associated with transportation of certain cations across the cell membrane of macrophages at time of activation (Gruenheid *et al*., 1997).

A polymorphism in the *NRAMP1* gene at *3'UTR* and *INT4* has a close association with TB and natural genetic resistance against tuberculosis. This polymorphism in the host

genome will certainly increase the risk of developing active tuberculosis in the patients. Hence, it is a very good marker to observe the genetic susceptibility of local population (Bellamy *et al*., 1998).

A study was carried out to find a genetic susceptibility association in tuberculosis patients. Genetic polymorphisms of the *NRAMP1* gene region, especially *3'UTR* region was the main focus (Ryu *et al*., 2000). Two groups were made having 193 numbers in each; first one diseased and the other control. Blood was collected and DNA was separated, deletion mutation regions were identified with the use of polyacrylamide gel electrophoresis following amplification with region specific polymerase chain reaction. The results confirmed the direct association between genetic susceptibility and smear positive tuberculosis. The genetic polymorphism in the selected genes was found as a reliable marker to detect genetic susceptibility patterns in the population.

From Mexico a scientific investigation was made by Estrada-Chavez *et al*. (2001) to trace the clue between active animal tuberculosis and involvement of *NRAMP1* gene. Peripheral blood was collected from bovines. Immunohistochemistry was also performed to detect the epithelioid and macrophage cells directly in the granulomatous lesions. Nramp proteins were analysed and quantified. Results confirmed that with a positive tuberculosis affected animals *NRAMP1* gene was actively involved as maximum gene expression was observed. Active tuberculosis was still found in a persistent manner in-spite of such huge gene expression of *NRAMP1* gene.

Taiwanese were able to detect the genetic susceptibility pattern variation among patients of tuberculosis by using PCR-RFLP technique, targeting *NRAMP1* gene. Forty-nine samples were taken with active tuberculosis and comparison was made with the same number of the healthy group. The *D543N* and *3'UTR* were most sensitive regions involved in the mutation and thus produced heterogeneous results. The pattern observed by RFLP typing was observed on 2% agarose gel. Different band patterns were specifically for healthy and diseased groups. The obtained results were satisfactory. PCR-RFLP method was excellent in differentiating genome based heterogeneity on the *NRAMP1* gene (Liaw *et al*., 2002).

Baghdadi *et al*. (2003) from Morocco, worked out and investigated study to confirm the relationship between chronic diseases and variation in *NRAMP1* gene. Family based

samples were collected having at least one confirm positive member. Nuclear families were selected having samples both from parents and children to find out genetic linkages of diseases. DNA was extracted from whole blood. PCR following restriction length polymorphism was used to observe the different genetic pattern. No association was found in active tuberculosis and genetic polymorphism in a *NRAMP1* gene in this region.

The Chinese were intended to confirm the presence of pulmonary tuberculosis. A study was conducted by Liu *et al*. (2004). To know the genetic variation in the host DNA they performed a case control study in the Han population of china. The patient was called diseased that had a positive slide or culture or had typical clinical signs of tuberculosis, persons with negative test and no clinical signs were considered as a control. Variation in *NRAMP1* gene was assessed at *INT4*, *D543N* and *3'UTR* regions. PCR-RFLP was practiced to separately type susceptible and resistant types. The method was successful in differentiating genetic based heterogeneity in *NRAMP1* gene, sequences both in disease and healthy group. The DNA mutation was confirmed at above described sites, i.e., *INT4*, *D543N* and *3'UTR*. A significant difference was observed in the two groups at *D543N* and *3'UTR* in *NRAMP1* gene, while, no difference was observed at *INT4*. It was concluded from the study that genetic susceptibility against tuberculosis was a real story. PCR-RFLP was a useful and reliable method to indicate genetic variation at the nucleotide level. The study proved that the *NRAMP1* gene polymorphism was a useful marker for susceptibility testing against tuberculosis.

Vejbaesya *et al*. (2007) in the Thailand planned a case control study for the confirmation of polymorphism in the genetic sequence of the *NRAMP1* gene in association with susceptibility against tuberculosis. From two groups 147 healthy patients were included in the control group, while 149 confirmed tuberculosis patients were included in the disease group. The DNA was isolated from whole blood using commercial kits. PCR along with sequence specific oligonucleotide analysis were used to detect the genetic variation between two groups in the same gene. They confirmed that there was no genetic difference observed in treatment and control group for *NRAMP1* gene polymorphism as same genetic profile was observed in the local population.

Eldholm *et al*. (2006) performed genotyping of *Mycobacterium tuberculosis* first time in Tanzania Dar-es-Salam. From 147 consecutive patients of tuberculosis isolates were

obtained. DNA was isolated by boiling method. Genotyping was performed by using Spoligotyping method which is fast and require a comparatively minimum cost of operation. A 43 spacer membrane was utilized for hybridization and identification of strain pattern. Recently updated database, i.e. SpolDB4 was selected for identification of families and variation. As a result, they obtained 64% pattern those were not reported previously, out of already discovered patterns; the most dominant family was Central Asian strains (CAS) following by Latin American Mediterranean (LAM) on the 2nd. Least isolates were observed in family East-African Indian (EAI). The study validated the use of Spoligotyping for genotyping of tuberculosis isolates.

CHAPTER-3 MATERIALS AND METHODS

The study was, carried out on cattle and buffaloes, humans and milk collected from milk selling points. The study focuses on tuberculosis. For this purpose, cattle and buffalo, humans and milk samples were evaluated for the causative agent of tuberculosis.

3.1 Targeted population

3.1.1 Animals

The study was carried out on two buffalo/cattle colonies, including one on Satiana road and the other on Aminpur road, Faisalabad along with two public livestock farms and a local abattoir.

A total of 132 cattle and buffalos on Satiana road and 133 cattle and buffaloes on the Aminpur road of above two years of age were randomly selected for screening of animals against tuberculosis through tuberculin testing. This number of 132 and 133 was ascertained on the basis of formula given by Thrusfield (2007), as follows:

$n=1.96^2 P_{exp}(1- P_{exp})/d^2$

Where:

n= required sample size

P_{exp} = expected prevalence

d= desired absolute precision

All the cattle and buffaloes as of above 2 years of age present on Livestock Production Research Institute Bahadur Nagar and dairy farm of University of Agriculture Faisalabad were also screened through comparative intradermal tuberculin testing (OIE, 2009).

A total of 400 consecutive animals, including 200 cattle and 200 buffalos being slaughtered in Faisalabad abattoir were also included in the study.

3.1.1.1 Age and body weight estimation of animals

At two livestock farm a written record was available regarding age and weight of animals so it was taken as granted. At two buffalo/ cattle colonies age of the animal was estimated with dentition method as previously described by Kikule, 1953, the weight of

animals was estimated with heart girth measurement as early detected by Goe *et al*., 2001.

3.1.2 Humans

Four hundred consecutive patients having clinical signs and symptoms and positive chest radiographs suspected for tuberculosis were included in the study. Those patients were selected from Faisalabad, in addition, people working as attendants on livestock farms, cattle/buffalo colonies, people involved in selling milk on milk sale points and those involved in slaughtering of cattle and buffaloes were also tested for tuberculosis through standard Mantoux intradermal testing.

3.1.2.1 Survey Entry Criteria

Those patients having typical clinical signs of disease along with positive X-ray radiographs were included in the study as suspects. A verbal question was asked about occurrence of disease in the past, medication etc.

3.1.3 Milk selling points

A total of 20 different milk selling points was randomly selected with highest sale. Those milk samples were taken for the presence of *Mycobacterium.*

3.2 Sampling

a) Animals:

The milk and nasal swab samples were collected from tuberculin positive animals at two livestock farms and two buffalo/cattle colonies. The comparative cervical interadermal test was performed on all animals above two years of age at two livestock farms. A total of 265 cattle/buffalo were also tested by the tuberculin in two cattle/buffalo colonies. The sampling was done by observing all precautionary measures for collection of sample to maintain sterile conditions. The sterile containers/swabs were used for collection of samples and they were numbered corresponding with identity of animals. They were placed in ice buckets and transported to the molecular pathology laboratory, Department of Pathology, Faculty of Veterinary Science, University of Agriculture Faisalabad. Similarly, samples were also collected from the same number of animals as being positive by the tuberculin test from apparently healthy and tuberculin negative cattle/buffalo from each of these locations from the same number of animals.

The sampling from slaughter house included nasal swabs, lung tissues and thoracic lymph nodes. A part of the sample was placed into 10% buffered formalin, while the

rest of the sample was placed in sterile plastic bags for culture isolation, ZN microscopy and PCR. All the samples were carried to the molecular pathology laboratory, FVS, UAF.

b) Humans

The sputum samples and fine needle aspirations were collected into sterile screw capped leak proof containers, while blood was collected in EDTA mixed blood collection tubes. Samples after collection were placed in the refrigerator till further use. Equal number (as of T.B. suspected) of blood samples were also collected from an apparently healthy group of people.

b) Milk Selling Points

Fifty ml of raw milk was collected from pooled large milk container present at milk sale points in a sterile screw capped bottle and was placed into the ice bucket. The samples were transported to the molecular pathology laboratory for isolation, identification and confirmation of *Mycobacterium*.

Processing of samples:

3.3.1 From animals

Following protocol was adopted for processing of milk and nasal specimens.

Different solution used were prepared as under:

1. Normal Saline solution (0.85%)

Nacl	0.85 g
Distilled water	100 ml

Prepared normal saline solution was autoclaved at 121 °C for 15 minutes.

2. Sodium hydroxide solution (4%)

NaOH	40g
Distilled water	1000 ml

NaOH pellets were dissolved in a liter water and was autoclaved at 121 °C for 15 minutes.

3. **Hydrochloric acid solution (91.7%)**

Concentrated	917 ml
Distilled water	1000 ml

Sterilized distilled water was used for preparation of HCL solution

3.3.1.1 Milk samples

- 2 ml milk sample was taken in a sterile test tube.
- Equal amount of NaOH solution was added into it.
- One to two drops of phenol red were added as an indicator.
- A whole mixture was incubated at 37 °C temperature for 30 minutes.
- Neutralization was done by adding HCL solution to it.
- Centrifugation was done of the whole solution @ 1000 RPM for 15 minutes.
- After discarding the supernatant, sediment was used for DNA extraction and inoculation on culture medium.

3.3.1.2 Nasal samples

- Sterile swabs were used for collection of nasal samples.
- The swabs were dipped into a normal saline solution for 30-40 minutes.
- Swabs were well squeezed against the walls of the test tubes to extract maximum solution from it.
- Centrifugation was done of solution @ 1000 RPM for 15 minutes.
- The supernatant was removed and the pellet was used for DNA extraction and inoculation on culture medium.

3.3.1.3 Slaughter House

A cross sectional survey was carried out between May-2015 and January 2016. Four hundred consecutive cases 200 each for buffalo and cattle were screened in the local slaughter house. The sample was considered as case having visible nodular lesions on the lungs and liver along with swollen lymph

nodes in the area.

3.3.1.3.1 Slaughtered Organs

- Tissues were homogenized using stomacher/blender on first.
- Decontamination of tissues was done by 2-4% sodium hydroxide solution.
- Neutralization of whole solution was done by using HCL solution.
- The specimens were centrifuged @ 1500 RPM for 15 minutes.
- Supernatant was discarded and the pellet was used for ZN staining, inoculation on culture medium and DNA extraction for PCR (OIE, 2009).

3.3.2 From Humans

3.3.2.1 Sputum

3.3.2.2 Ziehl-Neelsen staining:

The Ziehl-Neelsen solution was prepared following the method as described by Ellis *et al*. (1993) which is given below:

ZN Carbol Fuchsine Solution:

Carbol Fuchsine	10 g
Ethanol (95-100%)	100 ml
5% Phenol solution	1000 ml
(5 gm phenol →	100 ml)

Fuchsine was dissolved in phenol placing them in a flask over a boiling water bath for about 15 minutes with occasional shaking. Then ethanol was added and mixed thoroughly.

Sulphuric acid (20%):

80 ml distilled water was taken into a flask and 20 ml sulfuric acid was slowly added in to it.

Methylene Blue solution:

Methylene blue chloride	0.3 g
Distilled water	100 ml

Observation of positive slides:

The bacillus was stained bright pink in color having 5-10 µm in size and appeared as slender rods against a blue background at 100X with immersion oil. However, they may also appear curved or bent coccobacillary or even filamentous. Sometimes, bead and round pattern was also observed.

Slides were graded according to the number of bacteria present;

1 positive: if 1-9 AFB present in 100 microscopic fields

2 positive: if 1-9 AFB present in ten fields

3 positive: If 10 AFB present in one microscopic field,

4 positive: above 10 AFB in one microscopic field were considered as 4 positive.

3.3.2.3 Processing of sputum samples

Sputum was digested and decontaminated using sodium hydroxide (Modified Petroff) method,

Procedure:

The 5 ml sputum was taken into a plastic sterilized centrifuge tube overlaid with 10 ml 4% NaOH, incubated at room temperature for 15 minutes, centrifuged at 3000 rpm for 15 minutes. Supernatant was poured off, 15 ml sterile saline was added and sediments were re-suspended into it. Again centrifuged at 3000 rpm for 15 minutes, the supernatant was removed. The pH was neutralized with HCl before inoculation on to the culture media.

3.3.2.4 Isolation on culture media:

Two solid egg based culture media were prepared for isolation and identification of *Mycobacterium*. Lowenstein Jensen (LJ) with glycerol which is a selective medium for *M. tuberculosis,* Stonebrink's (SB), which is a selective medium for *M. bovis* and 0.2 ml of finally processed samples were inoculated onto slants of both the media. Sputum and LN aspirates were inoculated on both LJ and SB medium, while samples from animal origin were only inoculated on SB medium.

Composition of LJ medium: (Martin *et al*., 1975) in tuberculosis control)

KH2PO4 anhydrous	2.40 g
MgSO4	0.24 g
Magnesium citrate	0.60 g
Asparagine	3.60 g
Malachite green	0.40 g
Glycerol	12.0 ml
Potato flour	30 g
Beaten eggs	1000 ml
Distilled water (sterile)	600 ml

Composition of Stone Brink's medium: (Horris *et al*., 1993)

KH2PO4 anhydrous	3.5 g
Na2HPO4	2.0 g
Sodium pyruvate	6.3 g
Distilled water	500 ml
Whole eggs	1000 ml
Malachite green solution (Sterile)	20 ml

- All mixture had final PH of 6.9 at 25°C.

Slants were prepared by placing the media bottles in horizontal (little inclined) in a water bath submerged in water at 75-80°C for 1 hour. The solidified media was then stored in a refrigerator till further used. After inoculation, media were incubated for up to 8 weeks at 37°C.

Observation of positive culture:

Typical rough crumby cream colored colonies were regarded as positive for *M. tuberculosis*; white dysgenic flat colonies were identified as positive for *M. bovis* . AFB was confirmed by ZN staining, culture and PCR from the colonies obtained (M**aurice, 1961).**

3.4 Hematological studies

3.4.1 In Animals

The blood samples were analyzed for haematological parameters including in red blood cell (RBC) counts, haemoglobin (Hb) concentration, packed cell volume (PCV), white blood cell (WBC) counts and differential leukocyte counts following the methods described by Benjamin, (1978). Erythrocyte indices, including MCV, MCHC and MCH were also calculated.

3.4.1.1 Erythrocyte Count

Erythrocytes were counted by hemocytometer method as described by Benjamin, (1978). Blood was drawn exactly up to the 0.5 mark in a Thoma erythrocyte diluting pipette.

Then the normal saline was drawn up to 101 marks, in this way the dilution was 1:200. Fluids were mixed thoroughly in the pipette. A hemocytometer chamber with a cover glass was set under microscope. Two or three drops were discarded from the pipette before filling the counting chamber and waited approximately two minutes to settle the cells. The erythrocytes were counted under high power (X40) in five out of 25 small squares in the central area. Calculations were done by the following formulas:

$$\text{Total erythrocytes Count} = \frac{X}{80} \times 400 \times 200 \times 10 = \frac{}{} \times 10^{12}/\text{ I}$$

Where

X = cells counted in 80 small squares

80 = subdivision of small squares

400 = total number of small squares

200 = Dilution 1:200

10 = Depth of chamber (0.1mm)

3.4.1.2 Haemoglobin concentration

Haemoglobin concentration was determined by the Sahli's method (Benjamin, 1978). Briefly, 0.1N HCL was poured into the graduated measuring tube up to 2 marks, 20 µl EDTA mixed blood was mixed and stirred thoroughly. With the help of a dropper, distilled water was added drop by drop with continuous mixing and looking for color matching with the standard color. On exact matching, reading was noted in g/dl.

3.4.1.3 Haematocrit (Pack cell volume- PCV)

The PCV was determined by using plain capillary microhaematocrit tubes (75mm × 1.0mm). Approximately two third of the tube was filled with blood. The blood from outside of the tube was wiped and the tube was sealed at one end by holding it in the flame of a burner. The tube was centrifuged in a microhaematocrit centrifuge machine at 10,000 rpm for six minutes. The tube was taken out and the percentage of the PCV was noted by using a haematocrit chart.

3.4.1.4 Erythrocyte Indices

Erythrocyte indices, including MCV, MCHC and MCH were calculated as described by Benjamin (1978).

3.4.1.5 Total Leukocyte count (TLC)

Leukocytes were counted by haemocytometer method (Benjamin, 1978). Blood was drawn exactly up to the 1.0 mark of a leukocyte-diluting pipette.

The composition of a diluter is:

Glacial Acetic Acid	2.0 ml
Gention Violet (1% aqueous)	1.0 ml
Distilled water	100ml

The diluter was drawn up to the mark 11 in the leukocyte-diluting pipette. The diluter caused lysis of erythrocyte. Haemocytometer chamber with cover glass was set under the microscope. Two/three drops were discarded from the pipette before filling the counting chamber, and waited for approximately two minutes to settle the cells. The leukocytes were counted under the low power (X 10) in four large squares. Calculations were carried out by the following formula:

$$\text{Total Leukocyte Counts} = X/4 \times 20 \times 10 = \text{----------} \times 10^9/1$$

Where

X = Cells counted in 4 large squares

4 = Large squares from where cells were counted

20 = Dilution 1: 20

10 = Depth of chamber (0.1 mm)

3.4.1.6 Differential Leukocyte counts

Blood smears were prepared using a drop of fresh blood, air dried and fixed with methanol. The fixed smears were stained with Wright Giemsa stain (Benjamin, 1978).

Composition of Wright Giemsa stain:

Wright stain powder	300mg
Giemsa stain powder	30 mg
Methyl Alcohol (Absolute)	100ml

The fixed smears were completely covered with Wright Giemsa stain and allowed to react for three minutes. An equal amount of buffered distilled was added for three minutes. The slides were then washed with distilled water. Stained slides were dried and examined under high power (X 100) by placing a drop of oil immersion on the slides. One

hundred leukocytes were counted and proportion of neutrophils, lymphocytes, monocytes, eosinophils and basophils were calculated as a percentage.

3.4.2 From Humans

3.4.2.1 Erythrocyte Sedimentation Rate (ESR):

ESR test was performed on all blood samples. 3 ml anticoagulant mixed blood was sucked into westergen tubes. All tubes were placed in an upright position for 1 hour. Falling down of RBC level was observed taking readings in mm/ hr.

3.4.2.2 Rapid Test

A commercially available rapid test cassette was used for rapid test of human tuberculosis, based on the presence of IgG, IgA or IgM. Serum was separated from freshly collected blood. 3 drops of serum were put into well of the cassette. After 10 minutes if T band (test band) along with C band (control band) appeared, it was the indication of a positive result. Absence of T band confirmed the negative results.

3.4.2.3 Gamma Interferon Release Assay (γIRA)

The gamma interferon release assay was performed on selected human blood samples by using TB- IGRA kit. (Wntai Myco-TB). Detailed procedure is as follows:

- 3 ml fresh blood was collected by venous puncture; blood was collected into heparinized vacutainer tubes.
- Mixing of blood was done by gentle rotation of the tube on a rolling platform.
- 1 ml of heparinized blood was mixed into a culture tube coated with specific TB antigen.
- After gentle shaking of tubes incubation was done at 37 °C for 24 hours.
- Centrifugation of tubes was done @ 3000 rpm for 10 minutes and plasma was collected for further procedure.
- 96 well coated plate was used for evaluation of interferon gamma concentrations.
- 3 wells were kept as standard and one well as blank.
- 20 ul sample dilution buffer was added in all the wells except the blank well.

- 50 ul of the standard was put in respective wells, while nothing was added into blank well.
- In remaining all wells 50 ul of the specimen was put into respective wells, according to the specified number.
- The 96 wells plate was incubated at 37 °C for 60 minutes.
- Except blank well, 50 ul of conjugate was added in all the wells. Plate was incubated again at 37 °C for 60 minutes.
- Each well was washed five times.
- 50 ul of chromagen-A and 50 ul of chromagen-B were dispensed in all wells including blank. Plate was incubating at 37 °C for 15 minutes.
- Stop solution was added with a volume of 50 ul in each well, mixing was done.
- Optical density was measured at the absorbance of 450 nm.
- Concentration of gamma interferon was obtained by a specific formula.

3.4.2.4 Histopathology

The histopathological examination was performed to observe different lesions after hematoxylin and eosin staining as described by Bancroft and Gamble (2002). The detailed procedure is given as under:

Washing

5 mm thick fixed tissues in 10% buffered formalin were subjected to washing under tap water overnight to remove fixative agents.

Dehydration

Tissues were then dehydrated into ascending grades of ethyl alcohol as follows

Alcohol 70%	8 hours
Alcohol 85%	4 hours
Alcohol 95%	4 hours
Absolute Alcohol-I	2 hours
Absolute Alcohol-II	2 hours

Clearing

For elimination of dehydrating agents and substitution with some fluids miscible with dehydrating agent clearing of samples was done as follows:

Tissue samples were put in:

Xylene+ Absolute Alcohol (50+50)	30 minutes
Xylene-I	15 minutes
Xylene-II	15 minutes

Infiltration

Infiltration procedure was done by putting tissues into liquid paraffin at 60 °C temperature as mentioned below

Paraffin-I	2 hours
Paraffin-II	2 hours
Parafin-III	2 hours

Embedding

Treated tissues were embedded in melted wax, shaped by a steel mold. The wax was solidified at the temperature of -1 to -5 °C. On solidification of paraffin steel molds were removed from block.

Sectioning and Mounting

Form embedded blocks, 3-4 µm thick tissues were sectioned with the help of the microtome (Microm) and were placed into 50°C warm water. Extremely thin smear of Meyers egg albumin was made onto glass slides. The slides were mounted by dipping the slides under the tissue section floating in water. Slides were dried in an incubator, keeping temperature 45-55°C for 30 minutes to remove fragments of paraffin.

3.4.2.5 Hematoxylin and Eosin (H & E) Staining:

A detailed procedure **(**Lillie and Fuller, 1976)

- Slides were dipped into Xylen-1 for 3 minutes

- Slides were shifted into xylene-II
- Slides were put into absolute alcohol-I.
- Slides were dipped into absolute alcohol-II and put for 3 minutes.
- Slides were shifted into alcohol 70% and stained for 3 minutes.
- Slides were placed under running water for 4- 5 minutes (with no direct contact with the stream of water)
- Slides were carried to hematoxylin containing jar and stained for 5-8 minutes.
- Again slides were placed under running water for 5 minutes (with no direct contact with the stream of water).
- Only 1-2 dips were given into acid alcohol.
- Again slides were brought under running water for next 3 minutes.
- Slides were placed in ammonia alcohol for 3 minutes.
- Moved back into running water and placed there for 3 minutes.
- Slides were placed into Eosin-Y solution for 1-2 minutes.
- For next 3 minutes these were placed into Alcohol 70%, followed by absolute Alcohol-I and II for 3 minutes each.
- Staining procedure was ended with a final dip into Xylene-I and II for 3 minutes each.

After completion of staining procedure, a drop of DPX was put on the coverslip and coverslips were gently placed on the stained section (any bubble formation between section and coverslip was strictly avoided).

3.5 DNA extraction and molecular Identification

- Phenol Chloroform method was used for extraction and purification of genomic DNA. Detailed procedure is as follows:
- 1.5 ml eppendorf containing samples were heat boiled for 15 minutes
- 0.5 ml freshly prepared phenol was added in 1 ml of processed and heated samples.
- Vortexed for a while
- Centrifugation was done @ 2000 rpm for 5 minutes

- The aqueous phase was removed into another sterilized tube.
- An equal amount of chloroform-isoamyl alcohol (24:1) was added in the aqueous phase.
- Again centrifuged @ 12000 rpm for 5 minutes.
- The aqueous phase was carefully removed into another sterilized eppendorf tube.
- Freshly prepared 3M sodium acetate (pH 5.5) was added and vortexed, then two volumes of absolute ethanol was added and whole material was incubated at -40 °C for 1 hour.
- Centrifugation of tubes was done @ 10000 rpm for 15 minutes.
- Supernatant was removed, leaving, behind a pellet of DNA.
- The pellet was dried into an incubator having temperature 50 °C.
- Finally, pellet was suspended into sterile Tris- EDTA buffer, depending upon the size of pellet and preserved into -20 °C till the start of amplification.

PCR amplification was done by using the primer of genus, MTB-complex and *M. boivs* with respective amplification conditions as indicated in the table.

Primer name with sequence	**Thermal cycle conditions**
Genus ***Mycgen-F*** 5′-AGAGTTTGATCCTGGCTCAG-3′ ***Mycgen-R*** 5′-TGCACACAGGCCACAAGGGA-3′	Cycle 1 1 time 94°C/2 min Cycle 2 35 times 94 °C/30 Sec 65 °C/ 2 min 72 °C/3 min Cycle 3 1 time 72°C/10 min.
MTB-complex ***TB-1 F*** 5′-GAACAATCCGGAGTTGACAA-3′ ***TB-1 R*** 5′-AGCACGCTGTCAATCATGTA-3′	Cycle 1 1 time 94°C/4 min, Cycle 2 30 times 94°C/1 min

	55°C/1 min 73°C/1 min) Cycle 3 74°C 10 min
M. bovis ***JB21*** 5′-TCGTCCGCTGATGCAAGTGC-3′ ***JB22*** 5′-CGTCCGCTGACCTCAAGAAG-3′	Cycle 1 1 time 95°C/10 min Cycle 2 30 times 94°C/1 min, 67°C/1 min and 7°C/1min Cycle 3 72°C/10 min 1 time

3.6 Spoligotyping

All *Mycobacterium tuberculosis* complex confirmed positive samples were used for amplification of multiple, direct repeat region of about 36 b length, interspersed with spacers that are specific for each member of MTC including *M. tuberculosis* and *M. bovis*.

The DNA was amplified by using biotin labeled primers pair named DRa and DRb with following sequence and thermal cycler conditions.

Primer sequence	**Thermal cycle conditions**
	Cycle 1 1 time Denaturation 96^0 C / 3 m Cycle 2 29 times 96^0 C / 1 m 55^0 C / 1m 72^0 C / 3 m Cycle 3 1 time 72^0 C / 5 m Store 4^0 C
***Dra* F** 5’-GGTTTTGGGTCTGACGAC-3’ ***DRb* R** 5’-CCGAGAGGGGACGGAAAC-3’	

Method, as described originally by Kamerbeek *et al*., 1997.

Briefly,

- The commercially available activated membrane was used with 43 bound oligonucleotides specific for each type.
- The membrane was fixed into miniblotter having each well straight to perpendicular lines of the membrane.
- PCR product was put into miniblotter and membrane was placed into hybridizer at 43 °C for 1 hour.
- After incubation membrane was put into solution containing streptividin into rolling fashion.
- The membrane was dipped in a solution containing ECL detection reagent.
- The membrane was covered in plastic sheets overlaid by x-ray hyper film for recording of signals.
- The X- ray film was developed in a dark room; oligonucleotides pattern was visualized.
- Obtained profile was matched with the online Spoligotyping database.

3.7 Drug Resistance

3.7.1 Susceptibility Testing by proportion method

The clinical samples collected from patients were initially cultured on LJ medium. Growth was obtained. The growth of 1:100 dilution of the MTC-organism isolate on media without drug with growth of the undiluted suspension on media containing each drug was compared. If the undiluted suspension grows faster or more abundantly in the presence of the drug than does the 1:100 dilutions in the absence of the drug, the isolate is considered contain a resistant population greater than 1% and is reported as resistant. The drug concentrations used in culture medium were 1.0 µg/ml isoniazid and 40 µg/ml of rifampicin (Dinesh *et al.*, 2012).

3.7.2 Molecular Identification

3.7.2.1 Isoniazid

For Isoniazid drug resistance *KatG* gene was amplified with following a set of primer (Varela *et al*., 2008)

Primers with sequence	Thermal cycle conditions	
Isoniazid ***KatG*-F**(5'-AGCTCGTATGGCACCGGAAC-3') ***KatG*-R**(5'-TTGACCTCCCACCCGACTTG-3'	Cycle 1 95°C/5 min Cycle 2 95 °C/40 sec 53 °C/ 2 min 75 °C/40 sec Cycle 3 95 °C/40 sec 50 °C/ 40 sec 72 °C/40 sec Cycle 4 95 °C/40 sec 48 °C/ 40 sec 72 °C/40 sec Cycle 5 72°C/10 min	1 time 10 times 10 times 10 times 1 time

Visualization of amplified product was done by running on 1% agarose gel electrophoresis stained with ethidium bromide, bands were seen under ultraviolet light translaminar.

Restriction of Amplified Products

Amplified product was digested with *MspI* restriction endonuclease for 4 hours at 37°C (Varela *et al*., 2008). The restricted product was run on 2% agarose gel stained with ethidium bromide. After digestion, the product yielded four digested fragments were considered as wild type, while more or less number of fragments in comparison with wild type were considered as mutant (due to gain or loss of DNA from mutation).

3.7.2.2 Rifampicin

For rifampicin multiple allele-specific PCR was done targeting three mutated codons of the *rpoB* gene.

Primers with sequence	Thermal cycle conditions
MAS primers **(*rpoB* 516)** CAG CTG AGC CAA TTC ATG GA TTG ACC CGC GCG TAC AC **(*rpoB* 526)** CTG TCG GGGTTG ACC CA TTG ACC CGC GCG TAC AC **(*rpoB* 531)** CAC AAG CGC CGA CTG TC TTG ACC CGC GCG TAC AC	Cycle 1 1 time 96°C/3 min Cycle 2 23 times 95 °C/50 sec 68 °C/ 40 Sec 72 °C/1 min Cycle 3 1 time 72 °C/ 7 min (Yang *et al*., 2005)

Visualization of amplified product was done by running on 2.5 % agarose gel electrophoresis stained with ethidium bromide, bands were seen under ultraviolet light translaminar. Absence of specific band at particular site was noted for the mutation of specific codon (Yang *et al*., 2005).

3.8 Genetic Susceptibility

For genetic susceptibility testing an equal number of positive and negative patients were selected. Five ml blood was collected from each patient and healthy suspects. DNA was extracted with blood DNA extraction kit (Favorgen®). For *NRAMP1* (Naturally resistance-associated macrophage protein1) gene amplification following sequence and thermal cycle conditions were used as described by Liaw *et al*. (2002) as describe below

Primer Sequence	Thermal Cycler Conditions
***NRAMP1* primer** **F** 5'-GCA TCT CCC CAA TTC ATG GT-3' **R** 5'-AAC TGT CCC ACT CTA TCC TG-3'	Thermal cycler conditions Cycle 1 — 1 time 95^0 C / 5 mint Cycle 2 — 35 times 95^0 C / 1 mint 56^0 C / 1 mint 72^0 C / 1 mint Cycle 3 — 1 time 95^0 C / 1 mint 56^0 C / 1 mint 72^0 C / 5 mint Store — 4^0C

Visualization of amplified product by running on 1% agarose gel electrophoresis stained with ethidium bromide, bands were seen under ultraviolet light translaminar.

Restriction of Amplified Products

Amplified product was digested with *AvaII* (Fermentas) restriction endonuclease for 2 hours at 37°C. The restriction product was run on 2% agarose gel stained with ethidium bromide. Heterogeneity was observed.

3.9 Data Analysis

Frequency analysis was carried out on data obtained. Where appropriate 95% CI and odds ratio were also worked out. Logistic regression analysis was also carried out to find out association of risk factors with disease. Cluster analysis and strain frequency were tested by Lisrel software.

CHAPTER-4 RESULTS

4.1 STUDY AT TWO CATTLE/BUFFALO COLONIES

This study was carried out on total of 132 and 133 animals at Malkhanwala (Satiana road) and Paroka (Aminpur road) cattle and buffalo colonies, respectively to ascertain the prevalence of Tuberculosis**.**

4.1.1 Prevalence of Tuberculosis at two cattle/buffalo colonies

The results of the prevalence of tuberculosis at two cattle/buffalo colonies on the basis of tuberculin test are presented in Table 4.1**.** An overall prevalence of 10.56% was observed in two colonies at the animal level, while herd prevalence was recorded. The prevalence was 9.09% of Satiana road colony and 12.03% of Aminpur road colony.

Table: 4.1 Prevalence of Tuberculosis at two cattle/buffalo colonies

Parameters	**Tuberculin**		
Colony	**Negative**	**Positive**	**95% Confidence Limit**
Satiana+ Aminpur	237	28(10.56%)	7.28 to 14.71

4.1.2 Prevalence of Tuberculosis in Cattle and Buffaloes

The results of prevalence of Tuberculosis in the species of cattle and buffalo at two cattle/buffalo colonies on the basis of tuberculin test are presented in the Table 4.2. The prevalence in buffaloes was 11.04%, while, in cattle was 9.67%.

Table: 4.2 Prevalence of tuberculosis in different species at two cattle/buffalo colonies

Specie based prevalence of tuberculosis at two cattle/buffalo colonies			
Parameters	**Tuberculin**		
Species	**Negative**	**Positive**	**95% Confidence Limit**
Buffalo	153	19 (11.04%)	6.99 to16.41
Cattle	84	9 (9.67%)	4.82 to 17.01

	M.H Chi Square P= 0.729		
Specie based prevalence of tuberculosis at two cattle/buffalo colonies			
Parameters	**Tuberculin**		
Species	**Negative**	**Positive**	**95% Confidence Limit**
Buffalo	153	19 (11.04%)	6.99 to16.41
Cattle	84	9 (9.67%)	4.82 to 17.01
	M.H Chi Square P= 0.729		

4.1.3 Prevalence of Tuberculosis in herds

The prevalence of tuberculosis in eleven herds at two cattle/buffalo colonies on the basis of tuberculin test are presented in the Table 4.3. The 100% were found positive for tuberculosis by tuberculin test. The prevalence ranged from 3.8 -15.7%. The chi-square and 95% confidence interval results revealed non-significant difference in prevalence between eleven herds.

Table 4.3: Prevalence of tuberculosis in herd at two cattle/buffalo colonies

Parameter	**Tuberculin**		**95%**
Herds	**Negative**	**Positive (%)**	**Confidence Limit**
1	16	3(15.7)	4.18 to 37.21
2	19	2(9.5)	1.63 to 28.05
3	23	2(8)	1.36 to 24.0
4	18	1(5.2)	0.26 to 23.33
5	25	1(3.8)	0.19 to 17.54
6	20	3(13)	3.43 to 31.53
7	18	2(10)	1.71 to 29.29
8	26	4(13.3)	4.38 to 29.10
9	27	3(10)	2.61 to 24.85
10	18	2(10)	1.71 to 29.29
11	27	5(15.6)	5.96 to 31.29
	MH Chi Square P= 0.561		

4.1.5 Tuberculosis in Cattle and Buffaloes of Different Weight Groups

The results on prevalence in different weight groups are presented in Table 4.4. On the basis of live body weight, cattle and buffaloes were divided into two groups, i.e.<500 and >500 kg. The chi-square analysis and 95% confidence interval revealed non-significant difference in prevalence of these two weight groups.

Table: 4.4 Weight based prevalence of tuberculosis at two cattle/buffalo colonies

Parameter	**Tuberculin**		**95% Confidence Limit**
Weight groups (Kg)	**Negative**	**Positive (% age)**	
<500	39	2 (4.8)	0.83 to 15.19
>500	198	26 (11.6)	7.89 to16.31
		M-H Chi Square P= 0.198	

4.1.6 Tuberculosis Relationship with Status of Cattle and Buffaloes

The results on prevalence of disease in different status groups are presented in Table 4.5. The animals were divided into two groups on the basis of status, i.e. dry and lactating. The chi-square analysis and 95% confidence interval revealed non-significant difference in prevalence of these two weight groups.

Table 4.5: Status based prevalence of tuberculosis at two cattle/buffalo colonies

Parameter	**Tuberculin**		**95% Confidence Limit**
Status	**Negative**	**Positive (% age)**	
Dry	44	2 (4.34)	0.74 to13.63
Lactating	193	26 (11.87)	8.07 to 16.67
		M-H Chi Square P= 0.131	

4.1.7 Tuberculosis prevalence with reference to milk yield of Cattle and Buffaloes

The results on prevalence of disease in different milk yield groups are presented in Table 4.6. The animals were divided into four groups on the basis of milk yield,

i.e., 1-4.9, 5-9.9, 10-15 liters. The chi-square analysis and 95% confidence interval revealed non-significant difference in prevalence of these four milk yield groups.

Table 4.6: Milk Yield based prevalence of tuberculosis at two cattle/buffalo colonies

Parameter	Tuberculin		
Milk Yield (Liters)	**Negative**	**Positive (%)**	**95% Confidence Limit**
1-4.9	8	1(11.1)	0.56 to 43.86
5-9.9	131	23(14.93)	9.95 to 21.23
10-15	53	3(5.36)	1.38 to 13.89
	MH Chi Square P=<0.120		

4.1.18 Bivariate Logistic Regression in Cattle and Buffaloes at Two cattle/buffalo colonies

The bivariate logistic regression analysis in cattle and buffaloes revealed that age showed significant association with the occurrence of tuberculosis (Table 4.7). The results showed that with the increase in one-year age, there will be 56.4% more chance of occurrence of tuberculosis.

Table 4.7: Bivariate logistic regression of cattle and Buffaloes at two cattle/buffalo colonies

Parameter	Odd Ratio	95% Confidence Limit	P-Value
Age	1.564	1.137 to 2.152	0.006

4.9.1 Multivariate logistic regression after controlling the Age as a constant Factor,

After controlling the age as constant factor, the logistic analysis revealed that specie and status of animals showed significant association with occurrence of tuberculosis (Table 4.8). The chances of occurrence of tuberculosis in buffalo are 2.32 times higher than in cattle, while, the same is 9.19 times higher in lactating than dry animals.

Table 4.8: After controlling age as constant bivariate logistic regression of Cattle and Buffaloes at two cattle/buffalo colonies

Parameter	Odd Ratio	95% Confidence Limit	P-Value
Specie	2.32	0.926-6.074	0.050
Status	9.19	1.162-72.70	0.035

4.10.1 Multivariate logistic regression with backward elimination procedure.

Multivariate logistic regression analysis with backward elimination procedure revealed that, age, body weight and status showed significant association with occurrence of tuberculosis (Table 4.9). With one-year increase in age, there will be 56.4% more chances of occurrence of tuberculosis and with the increase in one kg body weight there will be 0.008% more chances for occurrence of tuberculosis. The results also revealed that the chances of occurrence of tuberculosis in buffalo are 5.72 times higher than in cattle.

Table: 4.9 Multivariate logistic regression with backward elimination procedure in cattle and Buffaloes at two cattle/buffalo colonies

Parameter	Odd Ratio	95% Confidence Limit	P-Value
Age	1.564	1.137-2.152	0.006
Body Weight	1.008	0.99-1.016	0.050
Status	5.72	0.783-44.84	0.050

4.1.11 Comparison between PPD and ZN, Considering Direct PCR as Gold Standard at two cattle/buffalo colonies

The results of PPD and ZN tests in PCR positive and negative cases are presented in Table 4.10. Considering PCR as a gold standard, the PPD sensitivity and specificity were 77.8% and 100%, while ZN sensitivity and specificity were 86.1% and 99.1%, respectively.

Table 4.10: Relationship between PPD and ZN at tow cattle/buffalo colonies

Parameters	Direct PCR			
	Positive		Negative	
ZN	PPD Positive	PPD Negative	PPD Positive	PPD Negative
Positive	28	3	0	2
Negative	0	5	0	227

PPD Sensitivity = 77.8%

PPD Specificity = 100%

False +Ve = 0%

Both tests Sensitivity = 77.8%

ZN is more sensitive, PPD is more specific.

ZN Sensitivity = 86.1%

ZN Specificity =99.1%

False +Ve = 0.9%

Both tests Specificity = 100%

4.2 STUDY AT TWO LIVESTOCK FARMS

This study was carried out on a total of 115 animals at Livestock Experiment station Bahadurnagar and 140 animals of dairy farm at University of Agriculture, Faisalabad (UAF), all above two years of age, respectively to ascertain the prevalence of Tuberculosis.

4.2.1 Prevalence of Tuberculosis at two livestock farms

The results of the prevalence of tuberculosis in the cattle and buffaloes at two livestock farms on the basis of tuberculin test are presented in Table 4.11**.** An overall prevalence of 15.72% was observed on two livestock farms at the animal level. The prevalence was 19.13% at Bahadurnagar (BN) farm and 12.14% of UAF, dairy farm in cattle and buffalo.

Table 4.11 Prevalence of Tuberculosis at two Livestock Farms

Farms	PPD		95% Confidence Limit
	Negative	Positive (%)	
BN Farm	93	22(19.13)	12.71 to 27.09
UAF Farm	123	17(12.14)	7.48 to 18.36
MH Chi Square P= 0.123			

4.2.2 Prevalence of Tuberculosis in Cattle and Buffaloes

The results of the prevalence of Tuberculosis in cattle and buffalo at two livestock farms on the basis of tuberculin test are presented in the Table 4.12. The prevalence in buffaloes was 17.7%, while, in cattle was 13.5%.

Table: 4.12 Prevalence of tuberculosis in different species at two Livestock Farms

Parameter	PPD		95% confidence Limit
Specie	Negative	Positive	
Buffalo	88	19(17.7)	11.37 to 25.87
Cattle	128	20(13.5)	8.69 to 19.75
	MH Chi Square P= 0.353		

4.2.3 Tuberculosis in Different Age Groups of Cattle and Buffaloes

The results on prevalence of tuberculosis in different age groups are presented in Table 4.13. The cattle and buffaloes were divided into two groups on the basis of age, i.e., 3 - 5 and >5 years. The statistical analysis, i.e., the chi-square test and 95% confidence interval showed significant difference in prevalence of tuberculosis in cattle and buffaloes between two age groups, with age group more than 5 years had significantly higher prevalence.

Table: 4.13 Age based prevalence of tuberculosis at two Livestock Farms

Parameter	PPD		95% Confidence Limit
Age (years)	Negative	Positive (%)	
3-5	146	17 (10.4)	6.40 to 15.85
>5	70	22 (23.9)	16.03 to 33.41
	MH Chi Square P = 0.004		

4.2.4 Tuberculosis in Cattle and Buffaloes of Different Weight Groups

The results on prevalence of tuberculosis in different weight groups are presented in Table 4.14. On the basis of live body weight, cattle and buffaloes were divided into two groups, i.e., <500 and >500 kg. The chi-square analysis and 95% confidence interval revealed non-significant difference in prevalence of these two weight groups.

Table: 4.14 Weight based prevalence of tuberculosis at two Livestock Farms

Parameter	PPD		95% Confidence Limit
Weight groups (Kg)	**Negative**	**Positive (%)**	
<500	188	36 (16.7%)	11.69 to 21.32
>500	28	3 (9.67%)	2.52 to 24.12
	MH Chi Square P = 0.354		

4.2.5 Tuberculosis Relationship with Status of Cattle and Buffaloes

The results on prevalence of disease in different status groups are presented in Table 4.15. The animals were divided into two groups on the basis of status, i.e. dry and lactating. The chi-square analysis and 95% confidence interval revealed significant differences in prevalence (P<0.001) between these two groups. Significantly higher prevalence was recorded in lactating than dry animals.

Table 4.15: Status based prevalence of tuberculosis at two livestock farms in cattle/buffalo

Parameter	PPD		95% Confidence Limit
Status	**Negative**	**Positive (%)**	
Dry	53	4 (7.01)	2.27 to 16.06
Milking	163	35 (17.6)	12.83 to 23.46
	MH Chi Square = 0.049		

4.2.6 Tuberculosis prevalence with reference to milk yield of Cattle and Buffaloes

The results on prevalence of disease in different milk yield groups are presented in Table 4.16. The animals were divided into four groups on the basis of milk yield, i.e., 1-4.9, 5-9.9, 10-15 liters. The chi-square analysis and 95% confidence interval revealed non-significant difference in prevalence of these three milk yield groups.

Table 4.16: Milk Yield based prevalence of tuberculosis at two Livestock Farms

Parameter	**Tuberculin**		
Milk Yield (Liters)	**Negative**	**Positive (%)**	**95% Confidence Limit**
1-4.4.9	18	3(14.28)	3.77 to 34.14
5-9.9	100	28(21.87)	15.35 to 29.66
10-15	45	4(8.16)	2.65 to 18.53
	MH Chi Square P=0.204		

4.2.7 Bivariate Logistic Regression in Cattle and Buffaloes at Two Livestock Farms

The bivariate logistic regression analysis in cattle and buffaloes revealed that age showed significant association with the occurrence of tuberculosis (Table 4.17). The results showed that with the increase in one year age, there will be 14.9% more chance of occurrence of tuberculosis.

Table 4.17: Bivariate logistic regression of cattle and Buffaloes at two Livestock Farms

Parameter	**Odd Ratio**	**95% CI**	**P-value**
Age	1.149	1.025 to 1.288	**0.01**

4.2.8 Bivariate logistic regression after controlling the specie as a constant Factor,

After controlling the specie as a constant factor, the logistic analysis revealed that the body weight of animals showed significant association with the occurrence of tuberculosis (Table 4.18). The chances of occurrence of tuberculosis in cattle and buffaloes are 0.007 % higher in light than heavy animals.

Table 4.18: After controlling specie as constant bivariate logistic regression of Cattle and Buffaloes at two Livestock Farms

Parameter	Odd Ratio	95% CI	P-value
Body weight	1.007	1.00 to 1.014	**0.04**

4.2.9 Comparison between PPD and ZN, Considering Direct PCR as Gold Standard at two Livestock Farms

The results of PPD and ZN tests in PCR positive and negative cases are presented in Table 4.19. Considering PCR as a gold standard, the PPD sensitivity and specificity were 90.5% and 99.5%, while ZN sensitivity and specificity were 95.2% and 99.1%, respectively.

Table 4.19: Relationship between PPD and ZN at tow Livestock Farms

Parameters	Direct PCR			
	Positive		Negative	
PPD	**ZN Positive**	**ZN Negative**	**ZN Positive**	**ZN Negative**
Positive	37	01	01	0
Negative	03	01	01	211

PPD Sensitivity = 90.5 %

PPD Specificity = 99.5%

False +Ve = 0.5 %

Both tests Sensitivity = 81.5 %

ZN Sensitivity = 95.2 %

ZN Specificity =99.1 %

False +Ve = 0.9 %

Both tests Specificity = 99.5 %

*ZN is more sensitive while PPD more specific test

Fig:1 Results of skin fold thickness showing tuberculin positive animals

Fig: 2 Results showing Tuberculin Positive in cattle

4.3 Slaughterhouse

A total of 200 consecutive cattle and 200 consecutive buffaloes were examined for gross abnormalities in different organs, especially with reference to tuberculosis. Tuberculosis suspected morbid samples from 27 buffaloes and 21 cattle were collected which included, lungs, liver and associated lymph nodes. These samples were further processed for ZN staining, culture, histopathology and PCR.

4.3.1 PCR Based Prevalence of Tuberculosis in Different Species of Animals

The results of prevalence of tuberculosis in cattle and buffaloes at the slaughterhouse of Faisalabad are presented in Table 4.20. The results showed that on the basis of PCR, out of total 200 buffaloes and 200 cattle, 3.5% buffaloes and 2.5% cattle were found positive for tuberculosis. The chi-square test and 95% confidence interval revealed non-significant difference in prevalence between cattle and buffaloes. The odds ratio indicated that there are 41% chances of occurrence of tuberculosis in buffaloes than cattle.

Table 4.20: Tuberculosis suspected with respect to species in cattle and buffaloes

Parameter	**PCR**		**95%**
Specie	**Negative**	**Positive (%)**	**Confidence Limit**
Buffalo	193	7(3.5)	1.54 to 6.80
Cattle	195	5 (2.5)	0.92 to 5.45
		M-H Chi-square P=0.558	**OR=1.41**

4.3.2 Prevalence of Tuberculosis with Respect to Sex in Both Cattle and Buffaloes

The results of the prevalence of tuberculosis with respect to sex in both cattle and buffaloes on the basis of PCR are presented in Table 4.21**.** The PCR revealed that 2.66% male and 4.57% females were found positive for tuberculosis. The chi-square analysis and 95% confidence interval revealed non-significant difference in prevalence between male and

females. The odds ratio indicated that there are 75% more chances of occurrence of tuberculosis in female than male.

Table 4.21: Tuberculosis suspected with respect to sex in both cattle and buffaloes

Parameter	DPCR		95% Confidence Limit
Sex	**Negative**	**Positive (%)**	
Male	219	8 (2.66)	1.09 to 5.46
Female	167	6 (4.57)	2.15 to 8.49
M-H Chi-square P= 0.304			

4.3.3 Prevalence of Tuberculosis in Different Age Groups of Animals in Both Cattle and Buffaloes

The results on prevalence in different age groups in both cattle and buffaloes on the basis of PCR are presented in Table 4.22. Animals were divided into three groups on the basis of age, i.e., 1-3, 4-7 and >7 years. The PCR showed that 3.38% animals were positive for Tuberculosis between 1-3 years of age, while 2.54% animals were between 4-7 years of age and 3.03% were in age group >7 years. The statistical analysis, i.e., chi-square test and 95% confidence interval showed non-significant difference in prevalence of Tuberculosis in cattle and buffaloes between three age groups. However, relatively higher prevalence was found in animals of 1-3 years of age.

Table 4.22: Tuberculosis suspected with respect to age in both cattle and buffaloes

Parameter	PCR		95%
Age groups (years)	**Negative**	**Positive (%)**	**Confidence Limit**
1-3	171	6(3.38)	1.39 to 6.92
4-7	153	4(2.54)	0.81 to 6.03

>7	64	2 (3.03)	0.51 to 9.65
		M-H Chi-square P=0.788	

4.3.4 Prevalence of Tuberculosis with Respect to Weight of Animals in Both Cattle and Buffaloes

The results of prevalence of Tuberculosis in different weight groups of both cattle and buffaloes on the basis of PCR are presented in Table 4.23. On the basis of body weight, animals were divided into two groups, i.e., <350 and >350. The chi-square analysis and 95% confidence interval revealed a significant difference (P<0.05) in prevalence between two weight groups. Higher prevalence was found in the group having weight<350kg. The odds ratio indicated that there are 3.43 times more chances of occurrence of tuberculosis in <350 kg group than >350 kg group.

Table 4.23: Tuberculosis suspected with respect to body weight in both cattle and buffaloes

Parameter	**PCR**		**95%**
weight groups	**Negative**	**Positive**	**Confidence Limit**
<350	181	9 (4.73)	2.33 to 8.51
>350	207	3 (1.42)	0.36 to 3.84
		M-H Chi-square P=0.049	**OR=3.43**

4.3.5 Comparison between Growth PCR (GPCR) and ZN, Considering Direct PCR as Gold Standard.

The results of Growth PCR and ZN tests in PCR positive and negative cases are presented in Table 4.24 (out of total suspects 48). Considering PCR as a gold standard, the GPCR sensitivity and specificity were 29.5% and 100%, while PPD sensitivity and specificity were 90.9% and 99.1%, respectively.

Table 4.24: Relationship between Growth PCR (GPCR) and ZN in Slaughtered animals.

Parameters	Direct PCR			
	Positive		Negative	
ZN	GPCR Positive	GPCR Negative	GPCR Positive	GPCR Negative
Positive	5	3	0	1
Negative	1	3	0	35

ZN Sensitivity = 66.7%
ZN Specificity =97.2%
False +Ve = 2.8 %
Both tests Sensitivity = 41.7 %

GPCR Sensitivity = 50 %
GPCR Specificity = 100%
False +Ve = 0%
Both tests Specificity = 100%

*ZN is more sensitive while, GPCR is a more specific test

4.3.6 Tuberculosis with Respect to Pathology of Organs

The results on prevalence of Tuberculosis with respect to gross lesions in lungs/liver on the basis of PCR are presented in Table 4.25. The lungs/liver in 48 cases showed nodular lesions suspected for tuberculosis, out of these 10 cases were found positive by PCR for *Mycobacterium bovis.*

Table 4.25: Pathological lesions of organs in confirmed cases of Tuberculosis

Parameters	PCR		95%
Lungs/liver	Negative	Positive (%)	Confidence Limit
Normal	352	0(0.00)	0.00 to 0.85
Nodular/Caseous	38	10(20.83)	11.10 to 34.00
M-H Chi-square P = <0.001			

4.3.7 Tuberculosis with Respect to Pathology of Lymph Nodes

The results on prevalence of Tuberculosis with respect to gross lesions in lymph nodes on the basis of PCR are presented in Table 4.26. The lymph nodes were found swollen in 19 cases, out of these two cases yielded positive PCR for *Mycobacterium bovis*

Table 4.26: Pathological lesions in lymph nodes in confirmed cases of Tuberculosis

Parameters	**PCR**		
Lymph Node	**Negative**	**Positive**	**95% Confidence Limit**
Normal	381	0(0.00)	0.00 to 0.0078
Swollen	17	2(10.52)	1.80 to 30.63
	M-H Chi-square P = <0.001		

4.3.8 Multivariate Logistic Regression in Cattle and Buffaloes slaughtered cattle/buffaloes

The multivariate logistic regression analysis in cattle and buffaloes revealed that age and body weight showed significant association with the occurrence of tuberculosis (Table 4.27). The results showed that with the increase in one-year age, there will be 11.92 times more chances of occurrence of tuberculosis and with increase one kg body weight 0.009% more chance of occurrence of tuberculosis will be there.

Table 4.27: Multivariate logistic regression of cattle and Buffaloes at slaughtered house

Parameter	**Odd Ratio**	**95% Confidence Limit**	**P-Value**
Age	11.92	5.72 to 23.87	**<0.001**
Body weight	1.009	1.004 to 1.234	**0.002**

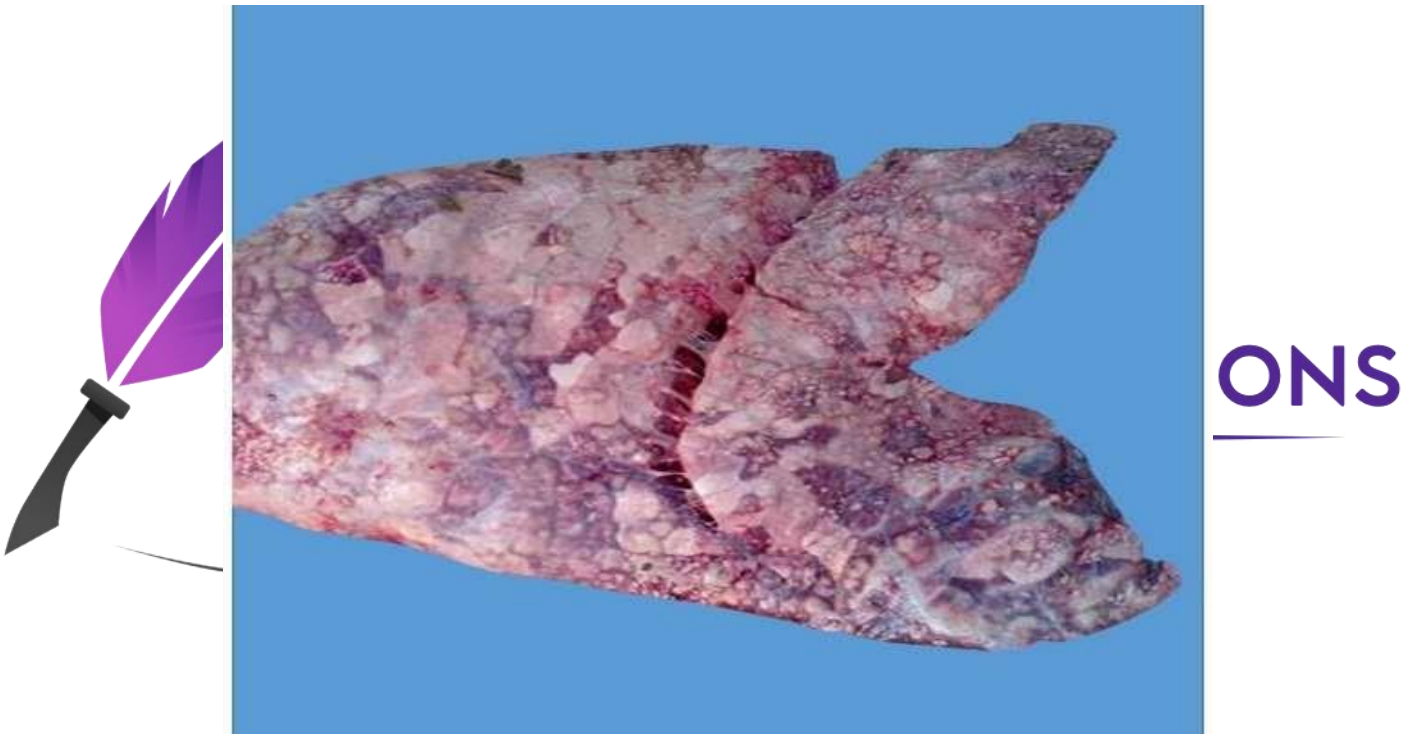

Fig: 3 Gross Pathological Lesions in lungs

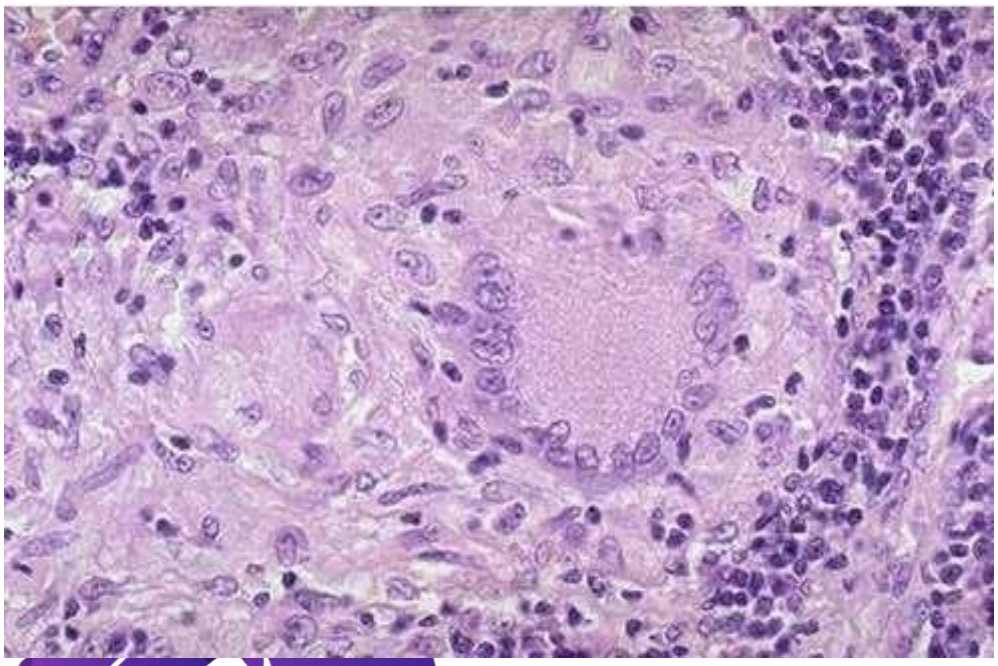

Fig:4 Photomicrograph of Lymph node showing cellular infiltration

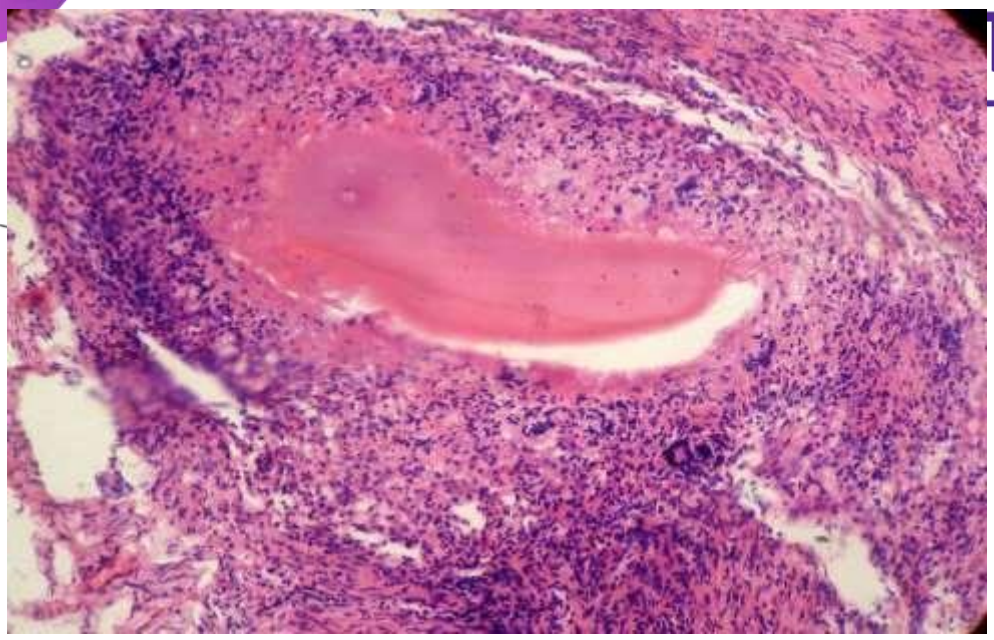

Fig:5 Photomicrograph of Lung Showing infiltration of inflammatory cells along with Granuloma formation. Caseous Necrosis is visible with huge amount of mononuclear cells, Multinucleated giant cells also visible

4.4 Haematological Parameters

Haematology

The results revealed significant difference between positive and negative control on most parameters, the WBC count, PCV, Neutrophils, lymphocytes and monocytes were significantly higher in positive infected animals, while only haemoglobin concentration was significantly lower in positive infected animals (Table 28).

Table 28: Comparison of hematological parameters in tuberculin positive and negative reactor cattle and buffaloes

Parameters	Negative mean ± SD	Positive mean ± SD
RBC	7.01±1.89	6.19±1.77
WBC	4.46±0.58B	6.03±0.21 A
HGB	12.36±2.15 A	10.95±0.77 B
PCV	32.11±0.94	32.49±0.55
NEUT	35.87±5.08 B	39.32±4.29 A
LYMPH	49.56±6.35 B	55.43±5.87 A
MONO	3.60±0.47 B	5.12±1.37 A
EIOS	6.10±0.53	5.22±0.26
BAS	0.24±0.04	0.39±0.06

4.5 Milk selling points

Results of Mycobacterium observed from milk selling points confirmed that 1(5%) out of 20 was declared positive for MTC-organism and *M. bovis* specific PCR.

5 STUDIES FROM HUMANS

5.1 Different TB Hospitals

This study was carried out on a total of 515 human patients having clinical signs symptoms and positive chest radiographs suspected for tuberculosis, visited different hospitals of Faisalabad

5.1.1 Univariate Frequency Analysis of different Variables Studied in Humans from Different TB Hospitals.

The results of univariate analysis are presented in Table 29. Out of 515 suspected patients 88.3% had raised ESR levels and 73.98% had suspected positive chest x-rays, while 63.5% had positive ZN results, and 77.09% had positive PCR results for Mycobacterium Genus. In 216 cases, growth on culture medias could be obtained, of it 188 was on LJ and 28 cases on stonebrink's medium. All the growths on Stonebrink's medium was confirmed as *Mycobacterium tuberculosis* complex (MTC) organisms and 21 were confirmed as *Mycobacterium bovis*. The 92.55% of growths on LJ medium were confirmed as MTC-complex growths, while 7.44% as other kind of Mycobacterium. The 359/515 patients were confirmed to have MTC-organisms, *M. bovis* in 21 cases and other types of Mycobacterium in 38 cases.

Table 29: The results of univariate frequency analysis of different variables studied in humans.

Parameter	Frequency (%)	95% Confidence Limit
ESR		
< 20	60 (11.65)	9.09 to 14.64
20 – 40	89 (17.25)	14.20 to 20.73
40 – 80	289 (56.1)	51.80 to 60.36
> 80	77 (14.95)	12.07 to 18.23
Disease Frequency		
Once	477 (92.62)	90.11 to 94.65
Re-occurred	38 (7.38)	5.35 to 9.89
ZN Results		
Negative	188 (36.50)	32.43 to 40.73
Positive	327 (63.50)	59.27 to 67.57
Growth result on LJ medium		

Negative	327 (63.50)	59.27 to 67.57
Positive	188 (36.50)	32.43 to 40.73
Growth on Stonebrink's medium (n=28)		
No	0 (0.00)	0.00 to 10.15
Yes	28 (100.0)	89.85 to 100.00
PCR results for *Mycobacterium tuberculosis* complex on Stonebrink's growth		
No	7 (25.0)	11.64 to 43.30
Yes	21 (75.0)	56.70 to 88.36
PCR Results for *Mycobacterium tuberculosis* complex isolates on LJ growths		
No	14 (7.44)	4.30 to 11.90
Yes	174 (92.55)	88.10 to 95.70
Direct PCR for Genus Mycobacterium		
Negative	118 (22.91)	19.44 to 26.69
Positive	397 (77.09)	73.31 to 80.56
Tuberculosis complex organisms confirmed by PCR		
No	156 (30.29)	26.44 to 34.37
Yes	359 (69.71)	65.63 to 73.56
Type of Mycobacterium Confirmed by PCR by using specific primers		
Other type of Mycobacteria	38 (9.57)	6.96 to 12.77
M. tuberculosis complex alone	330 (83.12)	79.20 to 86.57
M. bovis alone	21 (5.29)	3.39 to 7.84
Mixed Infection by *M. bovis* and *M. tuberculosis* complex	8 (2.02)	0.94 to 3.79

5.1.2 Bivariate Frequency Analysis of Different Variables Studied in Humans from Different TB Hospitals.

The results of the bivariate frequency analysis are presented in Table 5.2. Among MTC confirmed cases, 70.93% were males and were 68.7% females. The odds ratio revealed that there would be 11% more chance of positive MTC-organism PCR in male patients than female patients. Age of the patient was found significantly associated with positive MTC-organism PCR results. A total of 16 samples were from human less than 15 years of age and of these 80% were confirmed to have *Mycobacterium tuberculosis* complex organisms. The highest confirmation percentage was in people with 15-30 years of age. Of the people had animals at home, 65.09 were confirmed as infected with the MTC-organism, while this percentage was 71.08% in those did not have animals at home. The odds ratio showed that there would be 32% more chance of positive MTC-organism PCR results in the people not having animal at home than those kept animals at home. The results also revealed that all the people who had milk animals at home, from these 66.3% were confirmed to have MTC-organisms, while those people who had pet animals or pet and milk animals, 10 cases could be diagnosed of having MTC-organisms. Of the 106 cases, where people had animals at home, 57 had very frequent contact with animals and of these 66.7% were confirmed to have infected with MTC-organisms. Of the 27 people who had less frequent contact with animals, of these 63.0% were also confirmed to have been infected with MTC-organisms. Among the 46 people who were involved in very frequently with the milking of animals, 63.0% of them were found infected with MTC-organisms and of the 17 people who were rarely involved in milking of animals, of these 82.4% were also confirmed to have infected with the MTC-organism.

Only 18 people reported to have consumed raw milk very frequently and of these 77.8% were confirmed to have been infected with the MTC-organism, while of the 17cases who took less frequently the raw milk, 60% were confirmed to have been infected with MTC-organisms. Of the 487 cases where pulmonary infection was suspected, 69% were confirmed to have infected with MTC-organisms. Of the 20 cases where extra-pulmonary tuberculosis were suspected, 85% of them were confirmed to have been infected with MTC-organisms and all the 8 cases where both types of tuberculosis were suspected, 75% were found positive to have MTC-organisms. Duration of disease was significantly associated

with the rate of positive MTC-organism PCR results. Of 109 cases where MTC-organisms were confirmed by PCR disease duration was less than 31 days. Similarly, of the 128 cases where MTC-organisms were confirmed by PCR, the disease duration was 32-90 days. Of the 40 cases where MTC-organisms were confirmed by PCR, the disease duration was 91-170 days, while of the 34 cases where MTC-organisms were confirmed by PCR, the disease duration was 171-290 days. Of the 24 cases where MTC-organism was confirmed by PCR the disease duration was 291-365 days and of the 24 cases where MTC-organism was confirmed by PCR the disease duration was more than 365 days. Of the 477 cases where the disease occurred for the first time, 70.44% were confirmed to have been infected with MTC-organisms, while of the 38 cases where the disease was supposed to have reoccurred, 60.53% were confirmed to have been infected with MTC-organisms. The odds ratio revealed that there would be 55% more chance of positive MTC- organism's PCR in new diseased patients than re infected patients. Of the 381 cases where the chest x-rays were suspicious, 73.22% were confirmed to have been infected with MTC-organisms, while of the 134 cases where the chest x-ray was not suspicious, 59.70% were confirmed to have been infected with MTC-organisms**.** The odds ratio showed that there would be 80% more chances of MTC-organisms positive PCR in chest x-ray positive patients than x-ray negative patients. Level of ESR was found significantly associated with positive PCR results of the MTC-organism. Of the 7 cases where ESR was less than 20 mm/hr, 71.4% were confirmed to have been infected with MTC organisms and of the 508 cases where ESR was higher than 20mm/hr, 68.0% were confirmed to have been infected with MTC-organisms. Direct PCR for the genus was significantly associated with positive PCR results of MTC-organism. Out of the 397 PCR positive cases of genus Mycobacterium, 90.43% were confirmed to have been infected with MTC-organisms and the rest were supposed to be other Mycobacterium circulating in the environment. The ZN results were found significantly associated with positive PCR results of the MTC-organism. Of the 214 cases where ZN negative results were obtained, 48.60% of these were confirmed by PCR that they were infected with MTC-organisms and of the 301 cases, where ZN was positive, 84.72% of the cases were confirmed to have been infected with MTC-organisms. The odds ration revealed that there would be 5.86 times more chances of MTC-organism positive PCR for ZN positive patients than ZN negative patients.

Table 30: The results of bivariate frequency analysis of various parameters studied in humans.

Parameters/Variable	Tuberculosis complex confirmed		95% Confidence Limit	Statistic
Gender	**No n**	**Yes n (%)**		
Male	66	161 (70.93)	63.22 to 73.91	**P = 0.594**
Female	90	198 (68.75)	64.76 to 76.55	
Age groups				
<15years	4	16 (80.0)	58.51-93.30	**P = 0.006**
16-30	44	151 (77.4)	71.17 to 82.89	
31-50	64	95 (59.7)	51.96 to 67.16	
51-70	31	74 (70.5)	61.23 to 78.60	
>70years	13	23 (63.9)	47.39 to 78.23	
Animals Kept				
No	118	290 (71.08)	66.53 to 75.32	**P = 0.232**
Yes	37	69 (65.09)	55.66 to 73.71	
Animal Type				
No animals	118	290 (71.1)	66.53 to 75.32	**NS**
Milk animals	30	59 (66.3)	56.01 to 75.53	
Other animals	8	10 (55.6)	32.66 to 76.79	
Contact with animals				
No	127	304 (70.5)	66.10-74.70	**NS**
Very frequent	19	38 (66.7)	53.72 to 77.95	

Less frequent	10	17 (63.0)	43.85-79.42	
Milking of animals				
No Milking/No milk animals	136	316 (69.9)	65.56 to 74.08	**NS**
Very Frequent Milking	17	29 (63.0)	48.49 to 76.00	
Less Frequent Milking	3	14 (82.4)	59.11 to 95.31	
Raw Milk Taken				
No	144	333 (69.8)	65.58 to 73.80	**NS**
Very Frequent	4	14 (77.8)	54.69 to 92.51	
Less Frequent	8	12 (60.0)	37.89 to 79.39	
Type of Tuberculosis				
Pulmonary	151	336 (69.0)	64.78 to 72.98	**NS**
Extra-pulmonary	3	17 (85.0)	64.39 to 96.04	
Both (pulmonary + extra-pulmonary)	2	6 (75.0)	38.83 to 95.57	
Duration of Disease				
<31 days	24	109 (82.0)	74.73 to 87.80	
32-90 days	27	128 (82.6)	76.00 to 87.95	
91-170 days	58	40 (40.8)	31.42 to 50.75	**P =< 0.001**
171-290	20	34 (63.0)	49.56 to 75.01	
291-365	17	24 (58.5)	43.12 to 72.78	
>365 days	10	24 (70.6)	53.81 to 83.09	
Disease Frequency				
First time occurrence	141	336 (70.44)	6623 to 74.41	
Re-occurrence	15	23 (60.53)	44.48 to 75.02	**P=0.211**

X-ray Results				
Negative	54	80(59.70)	51.23-67.76	**OR=1.85**
Positive	102	279(73.22)	68.61-77.49	**P=0.003**
ESR				
< 20	2	5 (71.4)	33.02 to 94.90	**P = 0.040**
20 – 40	24	65 (73.0)	63.12 to 81.47	
40 – 80	96	246 (71.9)	66.99 to 76.50	
> 80	34	43 (55.8)	44.64 to 66.62	
Direct PCR for Genus Mycobacterium				
Negative	118	0 (0.00)	0.00 to 2.51	**P = 0.000**
Positive	38	359 (90.43)	87.23 to 93.04	
ZN Result				
Negative	110	104 (48.60)	41.94 to 55.29	**OR = 5.86**
Positive	46	255 (84.72)	80.32 to 88.45	**P=<0.001**

5.1.3 Growth on Lowenstein Jensen (LJ) Medium and Direct PCR (*Mycobacterium tuberculosis* complex) from Humans Samples in Different TB Hospitals.

The results on LJ media and the direct PCR carried out by using MTC-organism's primers are presented in Table 31. The LJ growth was found significantly associated with positive MTC-organism PCR results. A total of 328 cases did not show the growth on the culture media, while 188 showed positive growth on the culture media. From the negative cases on LJ media, 55.05% were found positive by direct PCR, while 95.21% were found positive by direct PCR where culture growth was also found positive. Odds ratio showed that there would be 16.4 times more chances of positive MTC-organism PCR in LJ growth positive than growth negative patients.

Table 31: The results on the growth of LJ medium and the direct PCR carried out by using MTC primers.

LJ Growth	Direct PCR		95% Confidence Limit	
	Negative	**Positive (%)**		
Negative	147	180 (55.05)	33.98 to 83.97	**OR = 16.4**
Positive	9	179 (95.21)	91.40 to 97.64	**P=<0.001**

5.1.4 Raw Milk Taken and Types of TB in Humans from Different TB Hospitals

The results of Raw milk taken and the type of TB are presented in Table 32. From 515 suspected patients 359 MTC-organism's positive PCR cases were observed. Of these, 333 were those where no raw milk was taken, while 14 cases were those where raw milk was taken very frequently and 12 cases were those where raw milk was taken less frequently. Of the 333 where no raw milk was taken, 97.89% had pulmonary TB and 2.11% had extra-pulmonary. Of the 14 cases where raw milk was taken frequently, 78.57% cases had pulmonary, 14.28% had extra-pulmonary, while 7.15% had both types of TB. Of the 12 cases, where the raw milk was taken less frequently, 50% had pulmonary TB, 33.33% had extra-pulmonary, while 16.67% had both types of TB.

Table 32: The results of Raw milk taken and the types of TB in humans.

	TB Type		
Raw Milk Taken	**Pulmonary (%)**	**Extra-pulmonary (%)**	**Both (%)**
No	326(97.89)	7(2.11)	0(0.00)
Very Frequent	11(78.57)	2(14.28)	1(7.15)
Less Frequent	6(50.0)	4(33.33)	2(16.67)

5.1.5 Raw Milk Taken and *M. bovis* PCR Confirmed Cases from SB Growth in Humans from Different TB Hospitals

The result of raw milk taken and the *M. bovis* confirmed by PCR on Stonebrink's growths are presented in Table 33. A significant association was observed between raw milk taken and *M. bovis* positive culture PCR. The raw milk was not taken by 480 cases, in 20 cases it was taken very frequently, while in 17 cases it was taken less frequently. Of the 480 cases where it was not taken, 1.25% cases were found positive for *M. bovis* on stonebrink's growth medium, while of the 20 cases where very frequently raw milk was taken 50% were found positive by PCR on growth on stonebrink's medium and of the 17 cases, where raw milk was taken less frequently, 29.41% were confirmed as *M. bovis* on Stonebrink's growth medium.

Table 33: Results of raw milk taken and *M. bovis* PCR confirmed cases from SB growth in humans.

Raw Milk taken	*M. bovis* on Stonebrink's confirmed by PCR		95% Confidence Interval	P Value
	Negative	Positive (%)		
No	472	6 (1.25%)	0.51 to 2.59	P = 0.000
Very Frequent	10	10 (50%)	28.86 to 71.14	
Less Frequent	12	5 (29.4%)	11.66 to 53.68	

5.1.6 Types of Animal Kept at Home and TB Types in Humans from Different TB Hospitals.

The results of types of animals kept at home and the types of TB are presented in Table 34. From 515 suspected patients 359 MTC-organism's positive PCR cases were observed. Of these 290 were those where no animal was at home, while 59 cases were those where milking animals were at home and 10 cases were those where other than milking animals were at home. Of the 290 cases where no animal was kept at home, 97.50% had pulmonary TB, 2 and 11% had extra-pulmonary. Of the 59 cases where milking animals were

present at home, 83.05% cases had pulmonary, 6.77% had extra-pulmonary, while 10.16% had both types of TB. Of the 10 cases, where the other than milking animals were at home, 90% had pulmonary TB, 10.0% had extra-pulmonary, while no case one had both types of TB.

Table 34: The results of types of animals kept and TB types in humans.

Animal type	**TB Type**		
	Pulmonary (%)	**Extra-pulmonary (%)**	**Both (%)**
No	283(97.58)	7(2.42)	0(0.00)
Milk animal	49(83.05)	4(6.77)	6(10.16)
Other animals	9(90.0)	1(10)	0(0.00)

5.1.7 Contact with Animals and TB Types in Humans from Different TB Hospitals

The results of animal contact and TB type are presented in Table 35. From 515 suspected patients 359 MTC-organism's positive PCR cases were observed. Of these, 305 were those where no contact was there with animals, while 38 cases were those where very frequent contact was there with the animals and 17 cases were those where less frequent contact was found. Of the 305 cases where no animal contact was there, 97.69% had pulmonary TB and 2.30% had extra-pulmonary TB. Of the 38 cases where very frequent animal contact was there, 71.05% had pulmonary TB, 15.78% had extra-pulmonary, while 13.5% had both types of TB. Of the 17 cases where less frequent animal contact was there, 88.3% had pulmonary TB, 5.88% had extra-pulmonary and both types of TB each.

Table 35: The results of contact with animals and TB types in humans.

Animal Contact	**TB Type**		
	Pulmonary (%)	**Extra-pulmonary (%)**	**Both (%)**
No	297(97.69)	7(2.30)	0(0.00)
Very Frequent	27(71.05)	6(15.78)	5(13.15)
Less Frequent	15(88.23)	1(5.88)	1(5.88)

5.1.8 Milking of Animals and TB Type in Humans from Different TB Hospitals.

The results of milking of animal and Type of TB are presented in Table 36. From 515 suspected patients 359 MTC-organism's positive PCR cases were observed. Of these 316 cases were those where no milking was done, while 29 cases were those where very frequent milking and 14 cases were those where less frequent milking of animal was done. Of the 316 cases where no milking was done, 97.15% had pulmonary TB and 2.84% had extra-pulmonary TB. Of the 29 cases where milking was done very frequently, 75.85% had pulmonary TB, 10.34% had extra-pulmonary and 13.79% had both types of TB. Of the 14 cases where milking was done less frequently, 85.71% had pulmonary TB, 7.14% had extra-pulmonary and same percentage had both types of TB.

Table 36: The results of milking of animals and TB types in humans.

Milking of Animal	TB Type		
	Pulmonary (%)	Extra-pulmonary (%)	Both (%)
No	307(97.15)	9(2.84)	0(0.00)
Very Frequent	22(75.86)	3(10.34)	4(13.79)
Less Frequent	12(85.71)	1(7.14)	1(7.14)

5.1.9 Animal Kept at Home and *M. bovis* PCR Confirmed Cases from SB Growth in Humans from Different TB Hospitals.

The results of *Mycobacterium bovis* confirmed by PCR on stonebrink's growth and the animal kept at home are presented in Table 37. A significant association was observed between animal kept at home and positive *M. bovis* PCR on culture growth. Of the 493 cases, where *M. bovis* was negative on stonebrink's growth, 18.46% had animals at home. Of the 21 cases where *M. bovis* was positive on stonebrink's growth, 71.43% had animals at home. The odds ratio revealed that there would be 11.04 times more chances of positive *M. bovis* culture PCR in the patients having animal at home.

Table 37: The results of animal kept at home and *M. bovis* PCR confirmed cases from SB growth in humans.

***M. bovis* confirmed by PCR on Stonebrink's growth**	**Animal kept at home**		**95% Confidence Limit**	P value / OR
	No	**Yes (%)**		
Negative	402	91 (18.45%)	15.22 to 22.07	P=<0.001
Positive	6	15(71.42%)	49.77 to 87.52	OR=11.04

5.1.10 Growth on Stonebrink's Medium and *M. bovis* PCR Confirmed Cases in Humans from Different TB Hospitals

The results of PCR for *M. bovis* on stonebrink's growths are presented in Table 38. A significant association was observed between positive growth on stonebrink's medium and *M. bovis* PCR on culture growth. Of the 479 cases, PCR for *M. bovis* was negative for 1.46% growths, while in 100% cases PCR was positive for *M. bovis* positive growths on stonebrink's medium.

Table 38: The results of growth on Stonebrink's medium and *M. bovis* PCR confirmed cases from SB growth in humans.

Stonebrink's PCR for *M. bovis*	**Stone Brink growth**		**95% Confidence Limit**	
	Negative	**Positive (%)**		
Negative	472	7(1.46%)	0.64 to 2.87	**P=<0.001**
Positive	0	21(100%)	86.71 to 100	

5.1.12 Growth on Lowenstein Jensen (LJ) Medium and *Mycobacterium tuberculosis* complex PCR Confirmed Cases in Humans.

The results of LJ growth and the PCR for *M. tuberculosis* complex are presented in Table 39. A significant association was observed between positive growths on LJ medium and MTC-organism's PCR on culture growth. Of the 327 cases, no growth could be obtained, while in 188 cases where the growth was obtained, 92.5% of the growths were confirmed by PCR as *M. tuberculosis* complex organisms.

Table 39: The results of LJ growth and *Mycobacterium tuberculosis* complex PCR confirmed cases in Humans.

LJ Growth *Mycobacterium tuberculosis* complex PCR on	**LJ growth**		**95% Confidence Limit**	P value/OR
	Negative	**Positive (%)**		
Negative	327	0 (0.0%)	0.00 to 0.91	P = <0.001
Positive	14	174(92.5%)	88.10 to 95.70	

5.1.14 GAMMA INTERFERON RELEASE ASSAY FROM HUMAN

The results of gamma interferon release assay in ZN positive and negative cases are presented in Table 40. A total of 200 blood samples were collected from the 100 confirm ZN positive and 100 ZN negative cases. 100% ZN positive cases were also positive with gamma interferon assay. Additionally, gamma interferon assay was also positive in 20% ZN negative cases.

Table 40: The results of gamma interferon in relation with ZN test

	Gamm Interferon Results		**95% Confidence Interval**	**P-value**
ZN	**Negative**	**Positive**		
Negative	80	20(20%)	13.02 to 28.69	P=<0.000
Positive	0	100(100%)	97.05 to 100.00	

The results of gamma interferon release assay in rapid test positive and negative cases are presented in Table 41. A total of 200 blood samples were collected. Of these 100% rapid test positive cases were also positive with gamma interferon assay. Additionally, gamma interferon assay was also positive in 20% rapid test negative cases.

Table 41: The results of gamma interferon in relation with rapid test.

	Gamm Interferon Results		**95% Confidence Limit**	**P-value**
Rapid Test	**Negative**	**Positive**		
Negative	90	8(8.16%)	3.86 to 14.91	P=<0.000
Positive	0	102(100%)	97.11 to 100.00	

Fig. 6 Results of Rapid Test positive case T band positive

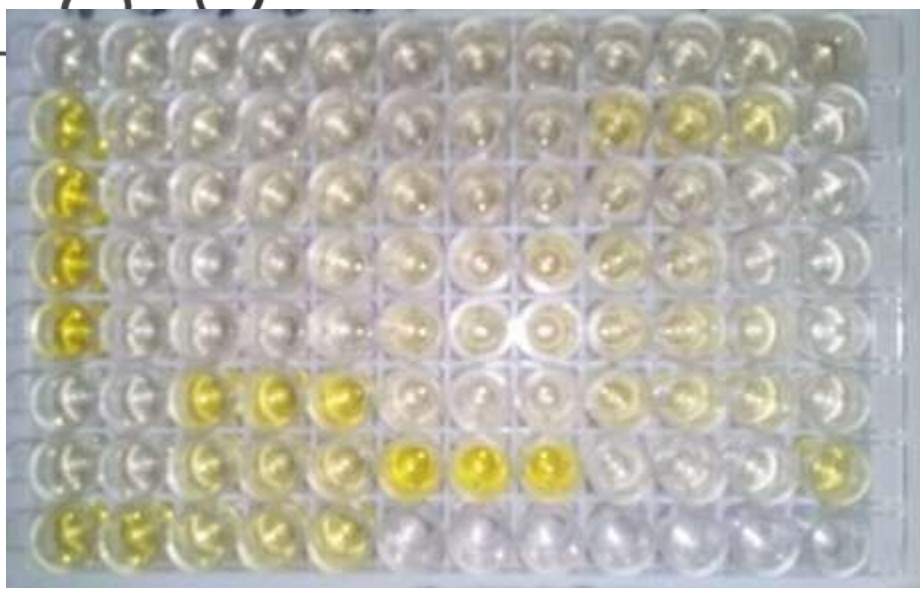

Fig. 7 Results of γ interferon Release Assay

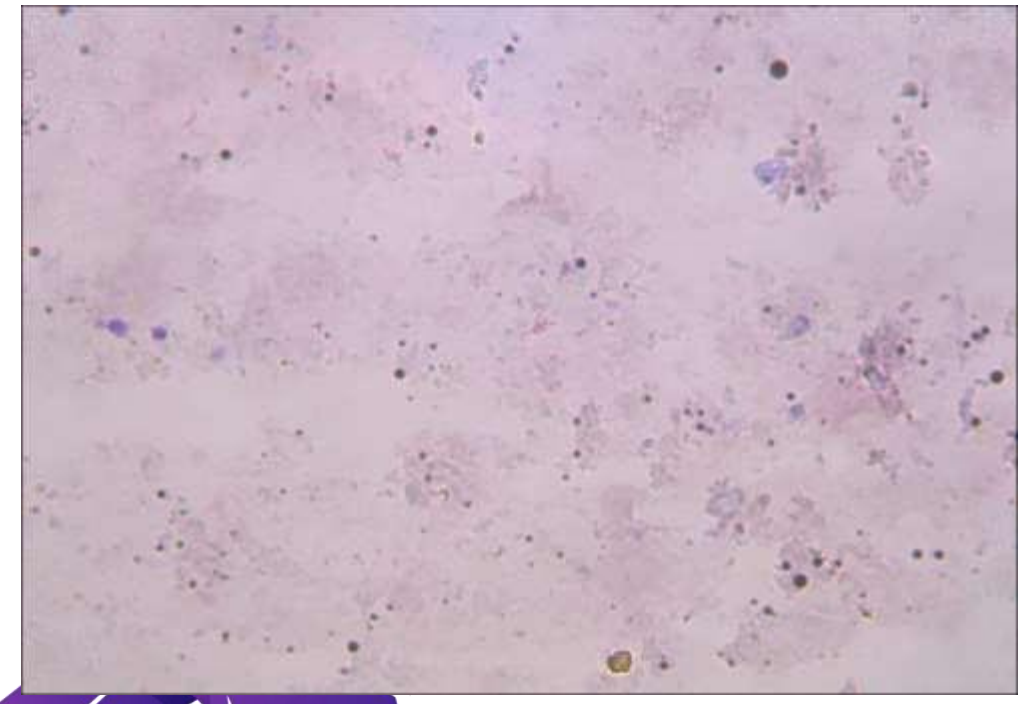

Fig.8: Ziehl Neelsen stained smear

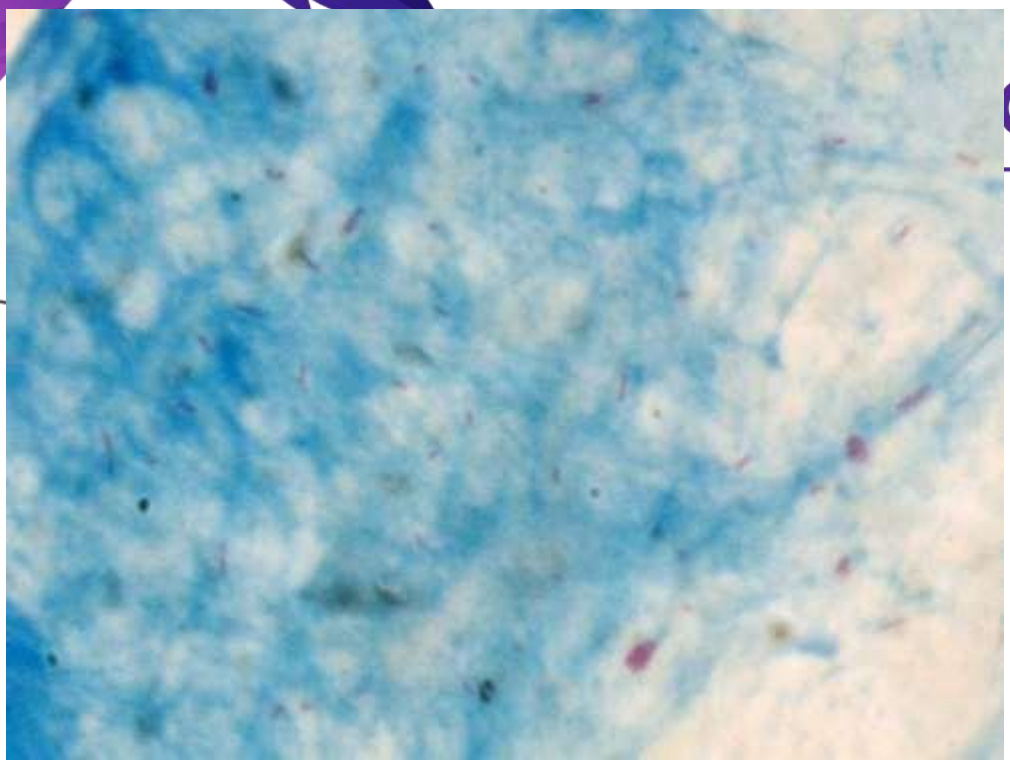

Fig.10: Ziehl Neelsen Staining smear

Fig. 10: Positive growth of Mycobacterium on LJ medium

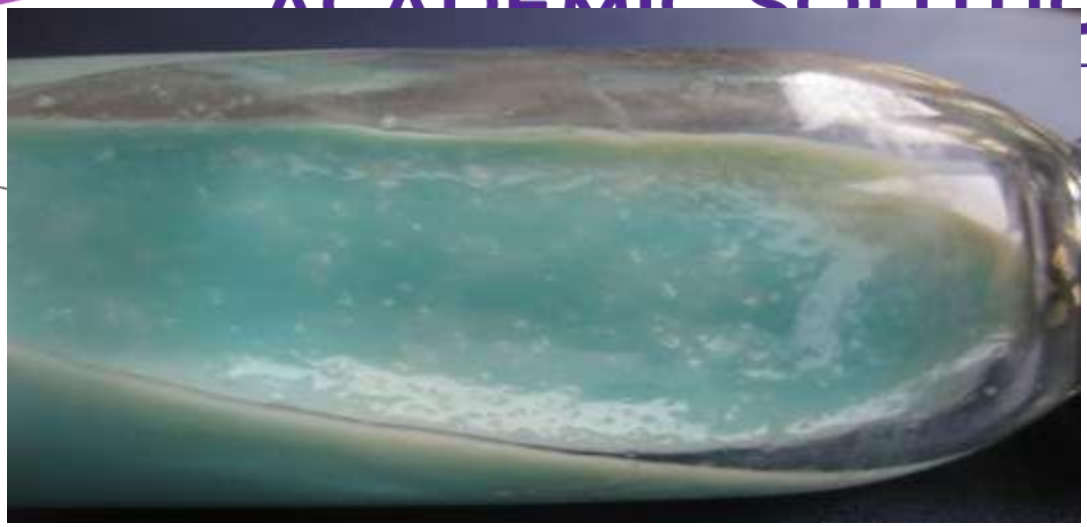

Fig. 11: Positive growth of Mycobacterium on Stonebrink's medium

5.2 TB IN FARM ATTENDANTS

The results of univariate analysis of different parameters of farm attendants are presented in Table 42. The results showed that the culture growth was obtained in 10/128 cases, 7 on LJ medium and 3 on Stonebrink's medium. The colony PCR for MTC-organism was positive in 9/10 cases with 6 cases of LJ and 3 cases of Stonebrink's. The direct PCR by MTC-organism was found positive in 9 cases, ZN test was also found positive in 9 cases, while the Mantoux test was positive in 8 cases.

The results of different parameters relate to host and different test results, including the direct PCR results for the MTC-organism are presented in Table 43. The results revealed that highest PCR positive cases were in age between 41-50 (12.77%), the second being the age greater than 50 years (7.69%), while it was lower in other age groups. The results also revealed that the TB was higher in attendants having frequent contact with animals and also where that contact duration was more than 10 years. The disease duration was less than 1 year in 2 cases, while it was between five months to one year in 5 cases. The TB could be diagnosed by PCR in 1.67% negative cases by Mantoux test. While where rapid test was positive it was, it could be diagnosed in 87.5% cases. Similarly, TB was diagnosed in 0.84% ZN negative cases, while in 88.89% cases of ZN positive. The pulmonary tuberculosis was confirmed by PCR in 5/6 cases, while it was confirmed in all the 4 cases of extra-pulmonary tuberculosis of the cultural growth, 90% were confirmed as an MTC-organisms. All the cases where colony PCR was positive, they were also positive by direct PCR on samples collected.

The results of colony PCR for MTC-organisms and type of TB and the growth of culture media are presented in Table 44. The results revealed that the colony PCR was positive on 9 out of 10 growths. The culture growths were obtained from 6 cases of pulmonary TB while in 4 cases of extra-pulmonary TB.

Table 42: Univariate analysis of various parameters of farm attendants.

Parameter	**Negative**	**Positive (%)**	**95% Confidence Limit l**
Colony PCR	121	7	
Direct PCR	119	9(7.0)	6.2 to 7.7
ZN result	119	9	
Mantoux Test	120	8(6.3)	5.4 to 7.1

Culture	118	10(7.8)	7.0 to 8.5
LJ growth	121	7(5.5)	4.6 to 6.3
Stonebrink's growth	125	3(2.3)	0.6 to 6.2

Table 43: Results of different host/farm factors and direct PCR for MTC-organisms

	Negative	Positive (%)	CI or Odd Ratio
Age Groups			
<30	14	1(6.67)	0.33 to 28.73
30-40	52	1(1.89)	0.09 to 8.95
41-50	41	6(12.77)	5.34 to 24.67
>50	12	1(7.69)	0.38 to 32.48
Contact Type			
Less Frequent	35	2(5.14)	0.92 to16.73
Very Frequent	84	7(7.69)	3.43 to 14.62
Contact Duration (years)			
<5	39	1(2.50)	0.13 to 11.72
5-10	42	4(8.70)	2.82 to 19.66
>10	38	4(9.52)	3.10 to 21.39
How old disease(years)			
0.5	1	2(66.67)	13.20 to 98.33
0.6-1	0	5(100)	54.93 to 100.00
>1	0	2(100)	22.36 to 100.00
Mantoux Test			
Negative	118	2(1.67)	0.28 to 5.40
Positive	1	7(87.50)	51.97 to 99.37
ZN results			
Negative	118	1(0.84)	0.04 to 4.07
Positive	1	8(88.89)	56.14 to 99.44
TB Type			
Pulmonary	1	5(83.33)	40.91 to 99.17

Extra-pulmonary	0	4(100)	47.29 to 100.00
Culture Results			
Negative	118	0(0.0)	0.00 to 2.51
Positive	1	9(90.0)	59.65 to 99.50
Colony PCR for *M. tuberculosis* complex			
Positive	119	0(0.00)	0.00 to 2.49
Negative	0	9(100)	71.69 to 100.00

Table: 44 The results of colony PCR for *M. tuberculosis* complex organism and type of TB and growth of culture medium

Parameter	**Culture on Media**		**95% Confidence Limit**
	Negative	**Positive (%)**	
Colony PCR			
Negative	118	1(0.84)	0.04 to 4.07
Positive	0	9(100)	71.69 to 100.00
TB Type			
Pulmonary	0	6(100)	60.70 to 100.00
Extra-pulmonary	0	4(100)	47.29 to 100.00

Fig.12: Pic Showing Multiplex PCR for Genus(1080bp) and MTC-organism (370 bp)

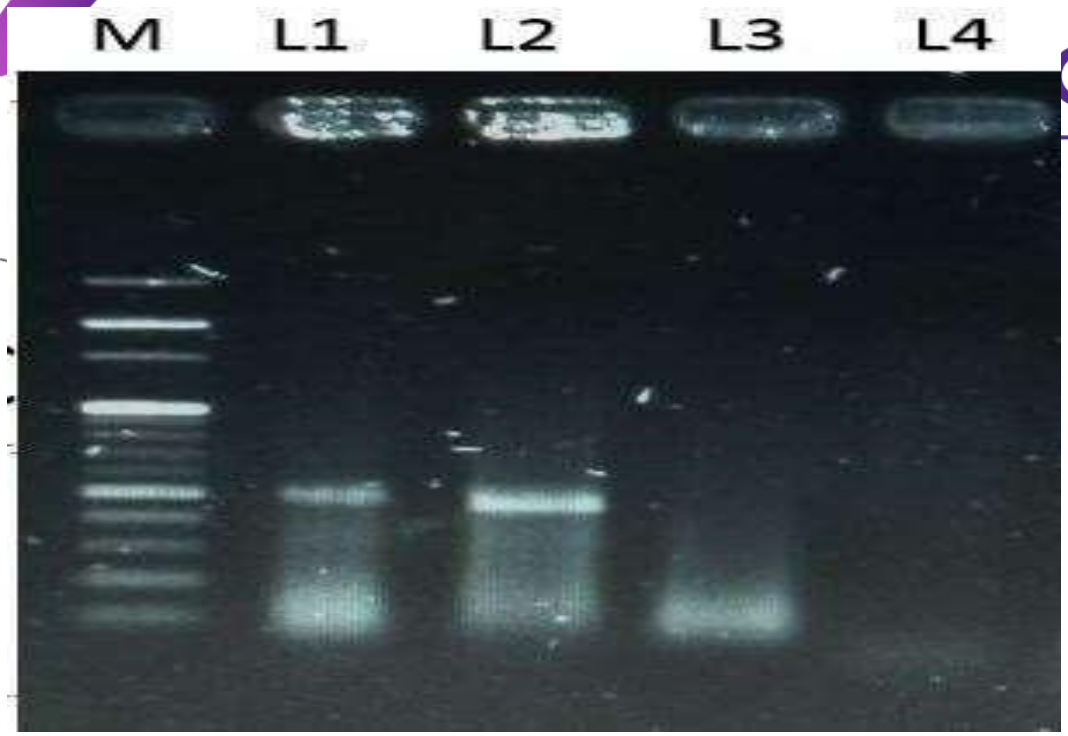

Fig 13. PCR results for *M. bovis* specie specific primer 500 bp amplification. M= Marker well number. Wells L1-L2 showing 500 bp band of *M. bovis*

6 .1 SPOLIGOTYPING

A total of 210 strains obtained from different culture medium could be used for spoligotyping purpose. The results revealed a total of 51 different response pattern (different strains) as indicated in Table 45. Out of these 39 were identified as new while 12 were already present in the international databases accessed on 09 -06-2016. (http:www.pasteur-guadeloupe.fr:8081/SITVITDemo/trouverSouchesParSpoligo.jsp).

The already strain present in the database were strain 126 (Belgium, Bangladesh, India, Tanzania, US, Uganda), Orphan (India), 141 (Indian and New York, USA), 26 (UK, India, Pakistan, Austria, Bangladesh, Somalia, Turkey , China, Georgia, Italy, Nepal), 236 (Ethiopia, UK, Vietnam, Bangladesh, Somalia, Georgia, India, US, Cambodia), 482 (Austria, Argentina, Bulgaria, Belgium, Germany, Denmark, France, Spain, Iran, Italy, Mexico, New Zealand, USA, South Africa), 777 (Armenia, Brazil, Georgia, Kazakhstan, Russia, Saudia), Orphan (Madagascar), 54 Armenia, Eritrea, Brazil, Georgia, India, Italy, Poland, Russia, US, Sadia, Egypt, Zambia and 100 (India, Saudia, Haiti and Tanzania). The new strain identified will be submitted to the database. It was also noted that in the database no strain from Pakistan have been submitted, while the name of Pakistan has been seen as strains identified from the people of Pakistan origin working or living in other countries.

Table 45. Molecular Characterization (Spoligoty)

Strain	Patt. Freq.	Pattern Observ	Octal Code	Graphic Profile	SIT/Clade	Countries Identified
1	4	nnnnnnn nnnnnnn nnnnnnn nnnnnnn nnnnnon nnnnnnn n	7777777 7777377 1		100 / MANU1	India, Haiti, UK, France, Malaysia, Thailand, USA, Vietnam, South Africa, Tanzania, Saudia
2	5	Nnnnnn nnnnnnn nnnnnnn nnnnnnn nnnnnoo nnnnnnn nn	7777777 7776377 1		54 / MANU2	Armenia, Eritrea, Bangladesh, Brazil, Georgia, India, Italy, Poland, Russia, Saudia, USA, South Africa, Zambia, Egypt, Madagascar, Iran,

3	3	nnnnnnn nnnnnnn nnnnnnn nnnnnnn nnnnoon onnnnnn n	777777 777762 771			Brazil, USA
4	2	Nnnnnnn nnnnnnn nnnnnnn nnnnnnn nooooon nnnnnnn	777777 777603 771		No	
5	4	nnnnnnn nnnnnnn nnnnnnn nnnnnnn onoonon	777777 777513 771		ORPHAN/FAMILY33	
6	3	nnnnnnn nnnnnnn nnnnnnn nnnnnnn ooonoon	777777 777423 771		Orphan / MANU2	Madagaskar

7	5	nnnnnnnnn nnnnnnnnn nnnnnnnnn nooonoooo nnnnnnn	77777777 7420771	[redacted]	777 / H3	Armenia, Brazil, Georgia, Kazakhstan, Russia, Saudia, Turkey, USA, Sweden, Holland,
8	6	nnnnnnnnn nnnnnnnnn nnnnnnnnn nooooononn	77777777 7413771	[redacted]	236 / EAI5	Ethiopia, UK, Vietnam, Bangladesh, Somalia, Georgia,
9	2	nnnnnnnnn nnnnonnn nnooooooo oooonoonn nnnnnnn	77776760 0023771	[redacted]	No	
10	3	nnnnnonnn nnnnnnnnn nnnnnnnnn nnnnnoonn nnnnnnn	76777777 7763771	[redacted]	No	

11	3	nnnnoonnn nnnnnnnnn nnnnnnnnn nnnnnoonn nnnnnnn	7477777 7776377 1	[redacted]	No	
12	3	nnnnooonn nnnnnnnnn nnnnnnnnn ooooooonn nnnnnnn	7437777 7700377 1	[redacted]	No	
13	4	nnnnooonn nnnnnnnnn nnnnnnnno ooooooonn nnnnnnn	7437777 7600377 1	[redacted]	No	
14	2	nnnnooonn nnnnnnnnn nnnnnonno ooooooonn nnnnnnn	7437777 6600377 1	[redacted]	No	

15	2	nnnnooonn nnnnnnnnn nnnnonnnn ooooooonn nnnnnnn	74377775 7003771	■■■■□□□■■■■■■■■■■■■■■□■■■■□□□□□□□■■■■■■■■■	No	
16	2	nnnnooonn nnnnnnnnn nnnnonooo ooooooonn nnnnnnn	74377775 0003771	■■■■□□□■■■■■■■■■■■■■■■□■□□□□□□□□□■■■■■■■■■	No	
17	3	nnnnooonn nnnnnnnnn nnnnoonno ooooooonn nnnnnnn	74377774 6003771	■■■■□□□■■■■■■■■■■■■■■■■■□■□□□□□□□□□■■■■■■■■■	No	
18	5	nnnnooonn nnnnnnnnn nnnnooooo ooooooonn nnnnnnn	74377774 0003771	■■■■□□□■■■■■■■■■■■■■■■■■□□□□□□□□□□■■■■■■■■■	No	

19	**3**	nnnnooonnnn nnnnnnnnnno ooooooooooo onnnnnnnnn	74377770 0003771	[redacted]	No	
20	**2**	nnnnooonn nnnnnnnnn nnnooooo ooooooooo onnnnnn	74377770 0000371	[redacted]	No	
21	**3**	nnnnooonn onnnnnnnn nnnnnonnn noonooonn nnnnnnn	74337776 7443771	[redacted]	No	
22	**2**	nnnnooono onnnnnnnn nnnnonnnn ooooooonn nnnnnnn	74237775 7003771	[redacted]	No	

23	3	nnnononnn nnnnnnnnn nnnnnnnon nnnnnoonn nnnnnnn	72777777 5763771	[redacted]	No	
24	3	nnnononnn nnnnnonnn nnnnnnnon nnnnnoonn nnnnnnn	72776777 5763771	[redacted]	No	
25	3	nnnononnn onnnnnnnn nnnnnnnnn nnnnnoooo nnnnnnn	72737777 7760771	[redacted]	No	
26	7	nnnoooonn nnnnnnnnn nnnnooooo ooooooonn nnnnnnn	70377774 0003771	[redacted]	26 / CAS1-Delhi	UK, Inida, Sri Lanka, Myanmar, Pakistan, Austria, Bangladesh, Turkey, China,

27	4	nnnooooonn nnnnnonnn nnnnnnnnn nnnnnoonn nnnnnnn	70376777 7763771	[redacted]	No	
28	3	nnnooooonn nnnnnonnn nnnnnnnoo ooooooonn nnnnnnn	70376777 4003771	[redacted]	No	
29	5	nnnooooonn nnnnnonnn nnnnooooo ooooonnn nnnnnnn	70376774 0007771	[redacted]	No	
30	10	nnnooooonn nnnnnonnn nnnnooooo ooooonnn nnnnnnn		[redacted]	141 / CAS1-Delhi	India, France, Holland, Pakistan, Saudia, USA,

31	2	nnnoooonn nnnnnonnn nnnooooooo ooooooooo onnnnnn	70376770 0000371		No	
32	3	nnnoooonn nnnnnonnn nnooooooo ooooooonn nnnnnnn	70376760 0003771		No	
33	3	nnnooooono onnnnonnn nnnooooooo ooooooooo ononnnn	70236770 0000271		No	
34	4	nnnooooono onnnnoonn ooooooooo ooooooooo onnnnnn	70236300 0000371		No	

35	2	nnnooooon onnnnonnn nnnnnnnon oonnnoooo nnonnnn	70136777 5160671	[redacted]	No	
36	3	nnnooooon onnnnonnn nnnnooooo ooooooonn nnonnnn	70136774 0003671	[redacted]	Orphan / CAS	India
37	2	nnnooooon onnnnonnn nnnoooooo ooooooooo ononnnn	70136770 0000271	[redacted]	No	
38	3	Nnnooooo onnnnnonn nnnnnoooo ooooooon nnnnnnn	70076774 0003771	[redacted]	No	

39	22	Nnonnnnn onnnnnnon nnnnnnnnn nnnnnnnnn nnnooooo	676773777777600	[illegible]	482/ SB0120	Austria, Argentina, Bulgaria, Belgium, Germany, Denmark, France, Spain, Iran, Italy, Mexico, New
40	3	nonoooooon nnnnnonnn nnoooooooo ooooooooo nonnnnn	501767600000571	[illegible]	No	
41	4	noonnnnnn nnnnnnnnn nnnnnnnnn noooononn nnnnnnn		[illegible]	126 / EAI5	Bangladesh, India, UK, Malaysia, Saudia, Holland, Sudan, USA, Uganda, Senegal,
42	4	noonnnnnn nnnnnonnn nnnnnnnnn nonnnnonn nnnnnnn	477767777573771	[illegible]	No	

43	4	oonooooon ooonnnnnn nnnnooooo ooooooooo nnnnnnn	10107774 0000771	[redacted]	No	
44	3	Oooonoon ooooooon ooooooooo ooooooooo ooooooo	02200200 0000000	[redacted]	No	
45	3	ooooooono onnonoono ooooooooo ooooooooo ooooooo	00232200 0000000	[redacted]	No	
46	5	ooooooono onooooono ooooooooo ooooooooo ooooooo	00220200 0000000	[redacted]	No	

47	3	Ooooooon ooooooon ooonooooo ooooooooo ooooooo	00200210 0000000		No	
48	16	ooooooono ooooooono ooooooooo ooooooooo ooooooo	00200200 0000000		No	
49	3	ooooooooo onnnnonnn nnnooooooo ooooooooo ooooooo	00036770 0000000		No	
50	3	ooooooooo oooooonooo ooooooooo oooooonnoo ooooooo	00001000 0014000		No	

51	7	Oooooooo oooooooon oooooooo oooooooo oooooooo	00000200 0000000	□□□□□□□□□□□□□□□■□□□□□□□□□□□□□□□□□□□□□□□□□□□	No	

Clades: **EAI** = East African Indian, **CAS** = Central Asian, **H3** = Haarlem 3, **MANU** = ancestral strain

6.2 Euclidean distance of different strains on the basis of unweighted pair-group (UWPG) average

Euclidean distance of different strains on the basis of unweighted pair group average are showed in the Fig. (5.19)

The results confirmed that at:

Linkage Distance 1: Forty-three clusters were identified having 9 clusters with 2 strains, while all others each with a single strain.

Linkage Distance 2: Twenty clusters were identified having 13 strains as independent, while other strains were clustered with related strains, one cluster had 6 strains, other cluster had 4 strains another had 8 strains, while 6 strains in a separate cluster, 6 strains in another cluster and 8 strains in the separate cluster.

Linkage Distance 3: One cluster had 7 strains, another had 6 strains, another cluster with 17 strains, another cluster with 16 strains, while all 5 strains were appeared independently.

Linkage Distance 4: Two major clusters were obtained. One cluster had 8 strains, 42 strains in 2nd cluster, while one strain was appeared independently.

Linkage Distance 4.5: Two major strains were obtained. One cluster had 8 strains and 2nd cluster had 43 strains.

Linkage Distance 5.5: In this linkage all the 51 strains produced a single cluster.

Tree Diagram for 51 Variables Unweighted pair-group average Euclidean distances

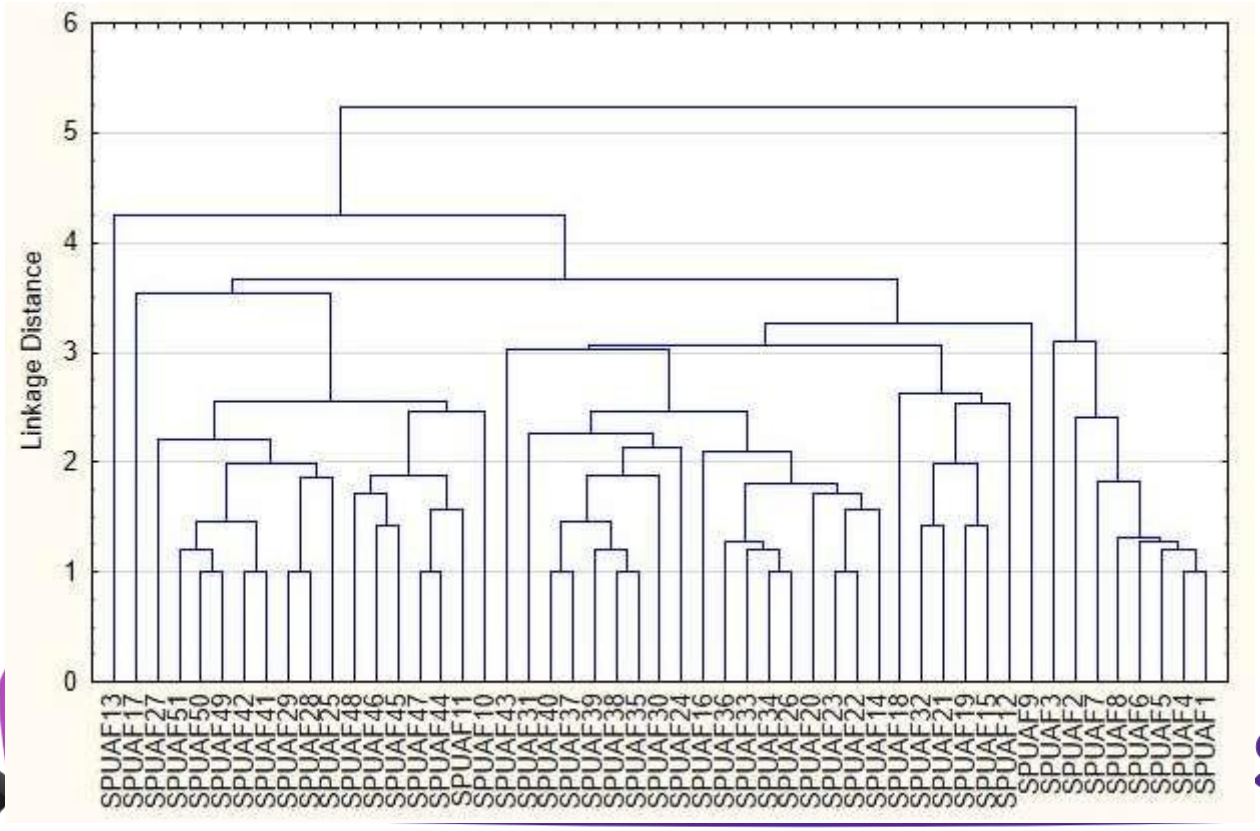

Fig 4.14: Unweighted pair-group average Euclidean distances

6.4 Drug Resistance

6. 4.1 Results of drug resistance ascertain by drug susceptibility testing on culture medium

The results of frequency analysis of isoniazid (INH) and rifampicin (RIF) resistant cases ascertained by drug susceptibility testing are presented in Table 46. Out of total suspected 70 isolates those were not responding to drug 22 (31.42%) were resistant to isoniazid and 13 (18.57%) were found resistant to rifampicin. The chi-square analysis and 95% confidence interval revealed non-significant difference between both drugs resistance.

Table 46: Results of INH and RIF resistant cases with drug susceptibility testing

Parameter	**No of cases (N)**	**Frequency %age**	**95% Confidence Limit**
Isoniazid			
Resistant	22	31.42	21.39 to 42.97
Sensitive	48	68.57	57.03 to 78.61
Rifampicin			
Resistant	13	18.57	10.74 to 28.96
Sensitive	57	81.42	71.04 to 89.26
			MH Chi-Square P = 0.079
MDR (INH+RIF)			
Resistant	10 (14.28)	7.49 to 23.99	
Sensitive	60 (85.71)	76.01 to 92.51	

6.4.2 Frequency of INH+ RIF resistance cases among all cases of drug resistance with drug susceptibility testing

The results of frequency analysis of INH+RIF (MDR) resistant cases ascertained by drug susceptibility testing are presented in Table 47. The results showed that out of 22 INH resistant cases 10 (45.45%) cases were also resistant with RIF.

Table 47: Frequency of INH+ RIF resistance with drug susceptibility testing

RIF sensitive			RIF Resistant	
INH	**No. of cases (N) %**	**95% confidence interval**	**No. of cases (N) %**	**95% Confidence Limit**
Sensitive	45 (93.75)	83.93 to 98.39	12 (54.54)	33.84 to 74.12
Resistant	03 (6.25)	1.61 to 16.07	10 (45.45)	25.88 to 66.16
MH Chi-Square P = <0.001				**OR= 12.50**

6.4.3 Results of drug resistance ascertain by molecular identification methods

The results of frequency analysis of isoniazid and rifampicin resistant cases ascertained by molecular identification methods are presented in Table 48. Out of total 70 isolates 18 (25.71%) were resistant to isoniazid and 9 (12.85%) were found resistant to rifampicin. The chi-square analysis and 95% confidence interval revealed significant difference between both drugs resistance.

Table 48: Results of INH and RIF resistance with molecular identification methods

Parameter	**No of cases (N)**	**Frequency %age**	**95% Confidence Limit**
Isoniazid			
Resistant	18	25.71	16.52 to 36.89
Sensitive	52	74.28	63.11 to 83.48
Rifampicin			
Resistant	9	12.85	6.46 to 22.28
Sensitive	61	87.14	77.72 to 93.54
MH Chi-Square P = 0.050			**OR= 2.35**
MDR			
Positive	04	5.71	1.84 to 13.21
Negative	66	94.28	86.79 to 98.16

6.4.4 Frequency of INH+ RIF resistance cases among all cases of drug resistance with molecular identification methods

The results of frequency analysis of INH+ RIF (MDR) resistant cases ascertained by drug susceptibility testing are presented in Table 49. The results showed that out of 18 INH resistant cases 4 (44.45%) cases were also resistant with RIF.

Table 49 : Frequency of INH+ RIF resistance with drug susceptibility testing

INH sensitive			INH resistant	
RIF	**No. of cases (N) %**	**95% confidence interval**	**No. of cases (N) %**	**95% Confidence Limit**
Sensitive	47 (90.38)		14(77.77)	18.24 to 92.65
Resistant	05 (9.61)		04 (22.22)	16.05 to 75.96
				MH Chi-Square P = 0.172

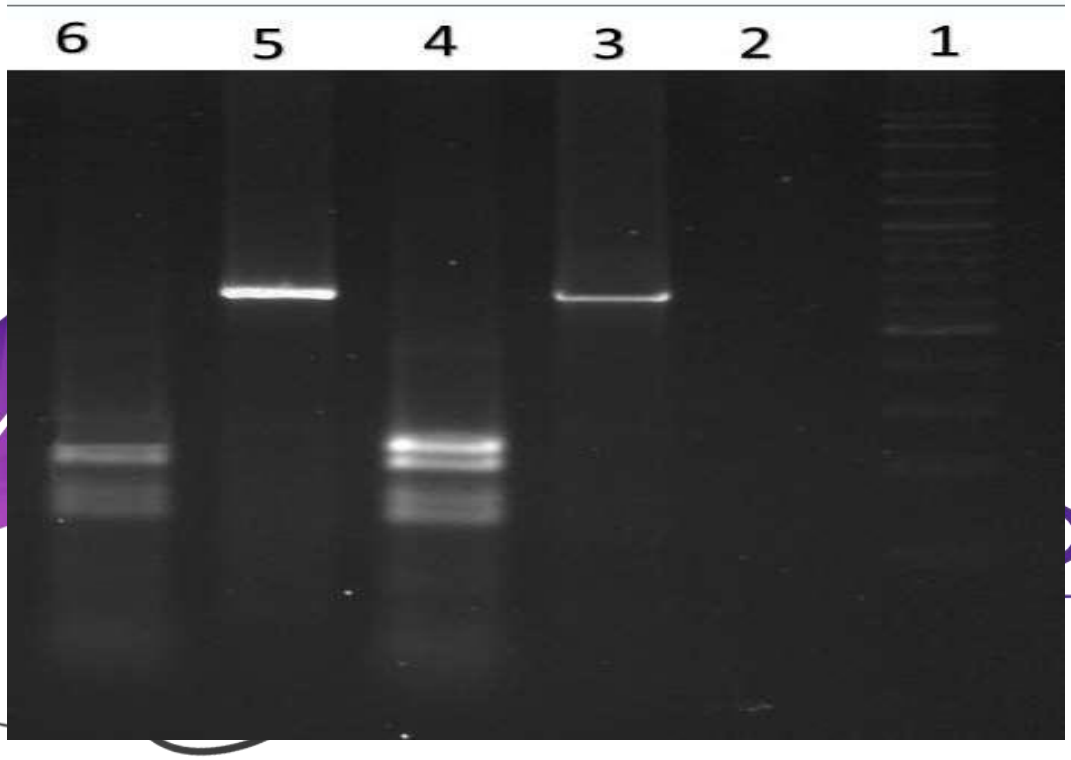

Fig 15: Pic Showing PCR Results of drug Resistance, L1=Marker; L3 undigested fragment of *KatG* gene 4,5,6 showed digested PCR RFLP product of different sized *KatG* gene fragments with *Mspl.*

6.7 Results of Genetic Susceptibility testing in animals and humans.

The results of genetic susceptibility testing in animals and humans on the basis of polymorphism in *NRAMP1* gene are presented in Table 50. From two groups of healthy and diseased people, 6 (20%) healthy people and 7 (23.33%) diseased patients were found positive with *NRAMP1* polymorphism. The chi-square analysis and 95% confidence interval revealed non-significant difference between healthy and diseased group. From animals from

in diseased and control group not a single case of genetic polymorphism was observed.

Table 50: The results of genetic susceptibility testing in animals and humans on the basis of *NRAMP1* polymorphism

Parameter	**Polymorphism**	**Frequency %age**	**95% Confidence Limit**
***NRAMP 1*Genotype**			
Humans			
Healthy	6/30	20.00	
Diseased	7/30	23.33	
			P = 0.756
Animals			
Healthy	0/10	0	0.00 to 31.23
Diseased	0/10	0	0.00 to 31.23

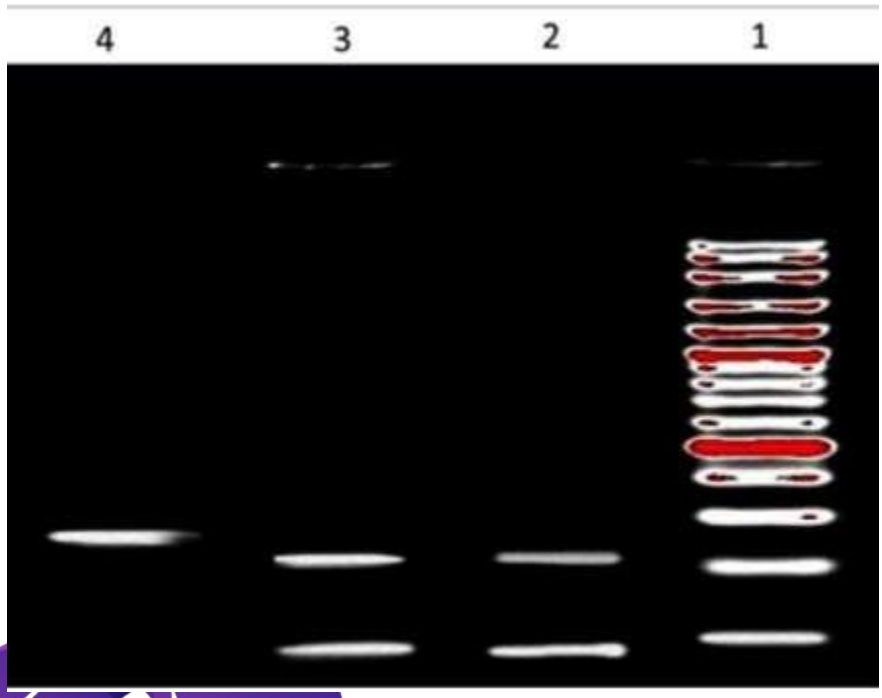

Fig 16: PCR-RFLP of *NRAMP1* Gene Polymorphism

L1=DNA ladder; L2-3 polymorphic bands of 212 and 39 bp; L4 solid band of 245 bp.

CHAPTER-5 DISCUSSION

Two Buffalo/cattle colonies

This study was carried out on a total of 132 and 133 animals at Malkhanwala (Satiana road) and Paroka (Aminpur road) cattle and buffalo colonies, respectively to ascertain the prevalence of Tuberculosis**.** Nasal swabs and milk samples were collected from all the positive animals and from the same number of negative animals. These samples were further processed for ZN staining, culture isolation and PCR. The blood samples were collected for haematological studies. The present study revealed an overall prevalence of 10.56% at two livestock colonies. Individually, the prevalence was 9.09% at Satiana road colony and 12.03% at Aminpur road colony. The prevalence was high in buffaloes (11.04 %), while low in cattle (9.67%). The study conducted by Ghumman *et al*. (2013) revealed that buffaloes were found to be more positive for TB than cattle. Different prevalence based studies in Pakistan has been carried out in cattle and buffaloes with varied prevalence. A study showed the prevalence of bovine tuberculosis in buffaloes to be10.6 % (Khan *et al*., 2008), whereas; Javed *et al*. (2011) reported 8% disease prevalence in cattle. The disease was present in all herds with varying frequency ranging from 3.8% as minimum to 15.7% as highest number of cases at two colonies. Cattle and buffaloes were divided into two groups; prevalence was more in heavy animals (>500 kg) than lighter one. A prevalence based study conducted by Javed *et al*. (2012) in Pakistan also showed that the occurrence of bovine tuberculosis was more in heavy animals (>500 kg body weight). On the basis of milking status, prevalence was more in lactating animals. On the basis of milk yield, animals were divided into 3 groups, i.e., 1-4, 9.5- 9.9 and 10-15 liters, maximum prevalence was observed in group having 5-9.9 liters milk production in a day. Similar type of findings was also observed by Imtiaz *et al.* (2008) as higher number of positive reactors were having milk production >7 liters. The probable reason of higher prevalence in high producing animals could be due to the reason that high milk yield enhance the disease load and animals can become immuno-compromised due to constant production stress. Multivariate logistic regression analysis with backward elimination procedure at two buffalo/cattle colonies revealed that, age, body weight and status showed significant association ($P<0.005$) with occurrence of tuberculosis, while the

bivariate logistic regression analysis in buffalo and cattle revealed that after controlling the age as constant factor, specie and status of animals showed significant association with occurrence of tuberculosis. From Pakistan an earlier study conducted by Javed., *et al*. (2013), reported the association of disease with age and body weight of animals, additionally total number of animals were also associated with occurrence of disease.

In addition, with tuberculin testing, ZN staining, culture isolation and PCR was also performed. Considering PCR as a gold standard, the PPD sensitivity and specificity were 77.8% and 100%, while ZN sensitivity and specificity were 86.1% and 99.1%, respectively.

Two Livestock Farms

This present study was carried out on a total of 115 animals at Livestock Experiment station Bahadurnagar and 140 animals of dairy farm at University of Agriculture, Faisalabad (UAF), all above two years of age, respectively to ascertain the prevalence of Tuberculosis. On the basis of tuberculin an overall prevalence of 15.72% was observed on two livestock farms at the animal level. The prevalence was 19.13% at Bahadurnagar (BN) farm and 12.14% of UAF, dairy farm in cattle and buffalo as together. Specie specific prevalence of tuberculosis in cattle and buffalo revealed that 17.7% buffaloes and 13.5% cattle were positive by tuberculin test. Similar findings were observed in a study conducted by Zahid *et al*. (2014) where prevalence was high in buffalo than cattle. The reason may be buffaloes like to live in hot and humid environment, swimming for long time in a pond also facilitate the organism to transfer from one animal to others. Cattle and buffaloes together were divided into two groups on the basis of age, i.e., 3-5 and more than 5 years. Significant statistical difference ($P<0.005$) was observed with the high prevalence in the age group >5 years of age. Javed *et al*. (2006) carried out a study which also showed high prevalence of tuberculosis in older aged buffaloes (>6 years). Similarly, according to a study conducted by Imtiaz *et al.* (2008) more disease positive buffaloes had age 8 years or above. Another study conducted by Rodwell *et al*. (2000) also supported the concept where prevalence was found associated with older aged. The possible reason of higher rate of infection in older age is due to poor feeding increase susceptibility of host to get infection with the passage of time (Cagiola *et al*., 2004), another reason can be long time stay in the contaminated environment with minimal movement. On the basis of milking status, the chi-square analysis and 95% confidence

interval revealed significant differences (P<0.005) between two groups with a higher prevalence in lactating animals. Noorrahim *et al*. (2015) also observed higher TB in lactating females than that of dry animals. The animals were divided into three groups on the basis of milk yield, no significant difference observed between all three. In contrast Javed *et al*. (2006) observed that higher prevalence of tuberculosis was in animals with milk production of 5-7 litres/day. The lactating animals are at the risk of nutritional imbalance which can disturb the immune status of the infected animals that may lead to occurrence of disease. The bivariate logistic regression analysis in cattle and buffaloes revealed that age showed significant association (P<0.005) with the occurrence of tuberculosis Many early studies has also reported the same pattern where age and positive tuberculosis cases were directly associated (Pollock et al., 2013). After controlling the specie as constant factor, the body weight of animals showed significant association with the occurrence of tuberculosis (P<0.005). In addition, with tuberculin testing, ZN staining, culture isolation and PCR was also performed. Considering PCR as a gold standard, the PPD sensitivity and specificity were 90.5% and 99.5%, while ZN sensitivity and specificity were 95.2% and 99.1%, respectively.

Slaughterhouse

A total of 200 consecutive cattle and 200 consecutive buffaloes were examined for gross abnormalities in different organs, related with tuberculosis. Tuberculosis suspected samples from 27 buffaloes and 21 cattle were collected which included, lungs, and associated lymph nodes. These samples were further processed for ZN staining, culture, histopathology and PCR. The results on the basis of positive PCR for tuberculosis at slaughter house revealed that, 3.5% buffaloes and 2.5% cattle were positive. The prevalence was higher in buffaloes than cattle; similar findings were also recorded by Javed *et al*. (2015). With respect to sex, in both cattle and buffaloes, the PCR revealed that 2.66% male and 4.57% females were positive for tuberculosis. No significant difference was observed between male and female. However relatively higher prevalence was observed in males than females. Regarding the positive PCR in different weight groups of both cattle and buffaloes, animals were divided into two groups, i.e., <350 and >350 kg. The chi-square analysis and 95% confidence interval revealed a significant difference (P<0.05) between two weight groups with higher prevalence in weight group of <350kg body weight group. Javet *et al*. (2006)

also suggested that the tuberculosis was more in animals with <500 kg body weight. The reason is that, mostly the debilitated and weak animals are sold for slaughtering. The similar findings were also observed by Kwaghe *et al*. (2015), who stated that mostly the sick and emaciated animals are brought for slaughter. Keeping direct PCR as gold standard the sensitivity and specificity of GPCR were 29.5% and 100%, while sensitivity and specificity of PPD test were 90.9% and 99.1%, respectively. The lungs/liver in 48 cases showed nodular lesions suspected for tuberculosis, out of these 10 cases were found positive by PCR for *Mycobacterium bovis.* Most positive PCR were obtained from lungs, as most dominant form of TB is pulmonary tuberculosis in animals. In 19 swollen lymph nodes were collected, out of these, only two cases yielded positive PCR for *Mycobacterium bovis.* Some earlier studies conducted on the abattoir samples in search of TB, showed the 6.7% positive PCR from lungs and only 2.9% from other tissues and lymph nodes (*Shitaye et al., 2006)*. The multivariate logistic regression analysis in both cattle and buffaloes revealed that age and body weight showed significant association ($P<0.005$) with the occurrence of tuberculosis. In different studies it has been reported that the chances of Mycobacterium infection were high with the increase in animals (Phillips *et al*., 2002). Similarly, the disease has been reported higher in old aged cattle than the young calves (Kazwala *et al*., 2001)

Studies from Humans

This study was carried out on a total of 515 human patients having clinical signs, symptoms and positive chest radiographs suspected for tuberculosis, visited different hospitals of Faisalabad. Sputum (from pulmonary) and lymph node aspirates (from extra-pulmonary) from suspected patients were collected. People working as attendants on livestock farms, buffalo/cattle colonies and people involved in selling milk on milk sale points (n=128) were also tested for tuberculosis through the standard Mantoux intradermal test. The collective samples were further processed for ZN staining, culture isolation and PCR. Blood samples were used for ESR, rapid on-site test and Interferon gamma release assay.

The results of the bivariate frequency analysis showed that among MTC confirmed cases, 70.93% were males and 68.7% were females. Results revealed that the chances of occurrence of tuberculosis were 11% higher in males than females. Chi square and 95%

confidence revealed that there was no significant difference between two genders. Similarly, WHO report showed that more number of males were reported diseased as compared to females, the male/female ratio was 1.96 for the global reporting rate of tuberculosis that year (WHO, 2009). Among the patients maximum number of confirmed cases were observed in the age group 16-30 years of age (P<0.005). Tuberculosis is said to be the disease of old people, but it can occur at any age. These findings were supported in an investigative study, where maximum cases were observed in the age group 16-30 years (kamal *et al*., 2016). Duration of disease was significantly associated (P<0.005) with the prevalence of tuberculosis. Highest percentage was observed in the patients had duration of disease ranged from 32-90 days. Many studies have revealed that most of the signs in the patients of tuberculosis appeared after 2-12 weeks of incubation period (Bronze and Greenfield, 2005). More than 90% patients were encountered with disease first time, while less than 10% cases were those where tuberculosis reoccurred, there was no significant difference observed in confirmed cases of TB and frequency of occurrence of disease. Similar type of findings has been reported by WHO that number of newly diagnosed TB patients were more than reoccurred tuberculosis cases (WHO, 2009). The X-ray is a very common practice to detect pulmonary tuberculosis and it is being done all over the country, from suspected samples 73.98% were found X-ray positive. Chi Square and 95% confidence limit revealed that there was a significant association observed (P<0.005) between X-ray positive cases confirmed by PCR. Even 59.70% X-ray negative cases were declared positive with PCR. Odds ratio showed that there would be 1.85 times more chances of positive PCR of MTC-organisms in X-ray positive patients than X-ray negative patients. Another study conducted by Kivihya-Ndugga *et al*. (2004) reported the same findings where PCR was proved more sensitive and specific test for the detection of tuberculosis than positive chest radiographs. Level of erythrocyte sedimentation rate (ESR) was found significantly associated (P<0.005) with positive PCR results of the MTC-organism. More than 90% MTC positive PCR was observed for the patients had raised levels of ESR (>20mm/HR). Raised ESR has been associated with many infectious diseases including tuberculosis. From India, Garg and Somvanshi, (2011) reported the same trend, i.e., elevated ESR level was highly associated with the active farm of tuberculosis. The ZN stained smear microscopy is a very common practice in resource poor countries for the diagnosis of tuberculosis, it has certain issues if

there is an improper specimen collection and low number of bacilli in clinical specimen may not allow to produce favorable results. The overall detection rate for all types of sample was 63.50%, a significant association (P<0.005) was found between ZN positive slide smears cases and MTC positive PCR (84.72%). Even PCR was able to detect the 48.60% ZN negative cases as MTC-organism. Another study was conducted with the similar aim where, 80.1% ZN positive cases were found positive by PCR, while 28% ZN negative cases were declared positive. It showed PCR was a more specific method than ZN, but to use PCR as a routine practice is not cost beneficial, however, combined use of both the methods can increase the detection rate of tuberculosis (Lee *et al*., 2011). The organism was cultivated on two different medium LJ, for MTB and Stonebrink's for *M. bovis.* Data revealed that from study population, overall 41.94% samples were culture positive (both of LJ and SB) and from positive growths 87.03% growths were observed on LJ slants, while 12.09% growths were observed on SB medium. In a study conducted in Turkey replicate results were obtained where, 81.2% growths were observed on LJ medium and lesser number of growths on SB medium (Ciftci *et al*., 2015). From positive growths MTC-organism specific PCR was positive in 92.55% cases of LJ growth and 75% of Stonebrink's growth indicated that the majority of organism were belonged to a diversified group of genetically related organisms called *Mycobacterium tuberculosis* complex. In a similar study, MTC-organism was observed in 68% cases, while 4% organisms other than MTC were observed. The study confirmed the involvement of a little proportion of types, other than MTC-organism in the occurrence of tuberculosis (Lee *et al*., 2011). The results showed that there was non-significant statistical association observed between animal kept at home, types of animal, frequency of contact with animals and raw milk taken in relation with MTC-PCR positive confirm cases of tuberculosis. While, from above mentioned parameters, animals kept at home and frequent raw milk taken had significant association with *M. bovis* specific culture PCR. The 72.42% *M. bovis,* culture PCR positive cases were obtained from the patients had animal at home, while 50% Stonebrink's culture positive patients those had the history of frequently taken raw milk were also positive for *M. bovis* culture PCR. These findings revealed that probably a transmission of TB from animals to humans occurred through aerosol route. Transmission of disease via raw milk was observed in lesser number than aerosol route, as maximum cases of zoonosis belonged to pulmonary tuberculosis, one case

of extra-pulmonary tuberculosis was observed in the patients had the history of raw milk taken. Different earlier studies revealed that tuberculosis due to *M. bovis* in humans was a consistent problem in the areas where tuberculosis was endemic in animals. It was reported in a study that 4.45% samples were those where *M. bovis* was the causative agent of pulmonary tuberculosis in human patients (Acha *et al.*, 1987). In a study conducted in Argentina, similar findings were observed where *M. bovis* was detected with a range of 0.34% to 1%, a large proportion of this percentage contained patients frequently worked with animals (de-Kantor *et al*. 2008). For the rapid diagnosis of tuberculosis in resource poor countries, maximum rely is based on ZN staining smear microscopy but it can only detect the organism during the active phase of shedding. Moreover, it can't diagnose the latent form of tuberculosis (Mazurek *et al*., 2010). A newly adopted test is immunoglobulin based rapid cassette test, but it has certain issues of specificity, γIFRA is a test used based on the detection of IFγ released from sensitized T cells from infected patients (Sester *et al*., 2010). The IFγRA was able to detect 20 % of ZN negative and 8.1% of rapid test negative tests as TB positive. In routine practices, we can't use γ IFRA because of its high cost and lengthy procedure, but it can be used with other tests to increase the detection ratio.

Farm attendants/co-workers

Tuberculosis is a very old disease and has a diversified host range. The areas where animal tuberculosis is endemic, there are continues reports of transfer of zoonotic TB in humans from infected animals. A Limited number (n=128) of people, those had direct contact with animals including farm attendants, milkers, raw milk seller and abattoir worker were tested by the intradermal skin test. The results showed that 10 (7.81%) people had positive skin test. From positive patients, sputum/lymph node aspirates were collected for the conduction of different tests like, ZN, isolation on culture medium and PCR, by which 9 (90%) were ZN positive. Total eight (80%) growths, were observed, 6 (75%) on LJ and two (25%) on SB medium. All the growths on both culture media were confirmed as *Mycobacterium tuberculosis* complex organism with PCR. Both the growths on Stonebrink's medium were confirmed as *M. bovis*. From Canada, a study revealed higher level of prevalence in animal handlers and abattoir workers. Later on, all the isolates were characterized as *M. bovis.* The chief source of infection was direct contact with live animals and meat (Liss *et al*., 1994). Similar studies were conducted in England those confirmed two

cases of *M. bovis.* Both the patients had a closer contact with animals, history showed that no raw milk was taken. This predicted that source of infection was direct contact with infected animals (Smith, 2004).

Molecular Characterization (Spoligotyping):

Spoligotyping is a finger printing technique being used all over the world for the molecular characterization of Mycobacterium, and molecular epidemiological studies. In Pakistan, a very little work is done with spoligotyping so a very limited information is available regarding origin and transmission of disease within different populations (Filliol *et al*., 2003). A total of 210 strains obtained from different culture medium could be used for spoligotyping purpose. The results revealed a total of 51 different strains pattern. Out of these 39 (76.47%) patterns were identified as new, while 12 (23.52%) patterns were already present in the international databases (spodb4). Most prevalent clade was Central Asia strain (CAS1) or simply called Delhi strains. Within this clade three different spoligotypes CAS ST-141 with a percentage of 20%, ST-26 CAS/Delhi strain had a share of 14.58% and orphan-CAS (6.18%). It is well documented that Central Asian strains or Delhi strain remained prevalent in many studies conducted in south Asian region. Different experiments confirmed that the CAS1 was a dominant strain in south Asia region. A study was conducted in India, where CAS was the dominant form of spoligotypes in Delhi region, among all (Singh *et al*,. 2004). In the year of 2013, another study was performed in India, 39% of all spoligotypes were identified as Delhi type in the Mumbai region (Almeida *et al*., 2005). In the next year, (2006) a province based study from Pakistan also revealed that, 39% spoligotypes were CAS1 being dominant to all, but in that study Beijing type was the 2 nd most prevalent strain which is not observed in our study, may be due to small geographical area included in our study (Hasan *et al*., 2006).

The second most prevalent clade was East-African Indian (EAI) with two different spoligotypes with varying frequencies were observed, ST-236 in (12.5%) and ST-126 in (8.33%) respectively. These findings are supported with earlier studies conducted in Delhi, India where 8% isolates were identified as EAI strains (Singh *et al*., 2004). Similar results with lower percentage were observed from Pakistan, where 4% of all identified strains belonged to EAI family. The prevalence was more in Sindh district that had a consistent long

border with India (Tanveer *et al*., 2009). From Bangladesh, EAI strains were reported with a varying frequency of 25-50% within all known strains (Banu *et al*., 2004). The MANU is the ancient type of mycobacterial family, it has very old history. It is sub-divided into three separate spoligotypes MANU1 (ST100; loss of spacer 34), MANU2 (ST 54; loss of spacers 33, 34) and MANU3 (ST1378; deletion of spacers 34–36), these have been reported from many countries (Helal *et al*., 2009). In our study, third most prevalent strain was MANU the ancestral clades, within this clade three further spoligotyping pattern were observed, MANU2/ST-54 in 10.41% and MANU1/ST-100 in 8.33% and orphan MANU-2 in 6.81% cases, respectively. From Pakistan, an earlier study showed that varying frequency of both MANU1 and MANU2 was observed in Karachi, Pakistan (Tanveer *et al*., 2008). From India a study was conducted in Andhra Pradesh region which confirmed that 8% strains belonged to ancient, while other 8% as MANU strains along with some other modern strains. In a report from north India confirmed that 4.95% of all spoligotypes were MNAU, ancestral strains (Varma-Basil *et al*., 2011). Our findings confirmed that a highly diversified population of *M. tuberculosis* genotypes were circulating in local patients, as both new and ancient types were observed at same place, it is also an indicator of evolution process being continue in the local strains. The Haarlem clade contained only 10.41% cases among all the known cases. In a study conducted in Iraq, out of 270 isolates, 7% of isolates were found associated with Haarlem family (Ali *et al*., 2014). Within known strains of *M. tuberculosis* (8.33%) cases were observed with the spoligotyping pattern of orphan-33. The new strain identified will be submitted to the database to get identification number according to region. It was also noted that in the database no strain from Pakistan have been submitted. The isolates obtained from animal sources and all the isolates those were confirmed as *M. bovis* from human Stonebrink's culture were subjected for molecular characterization through spoligotyping isolated obtained from humans and animals depicted a same spoligotyping pattern compared with database named 482/SB0120, had a characteristics of lacking spacers 3, 9, 16, and 39−43. A virulent strain of *M. bovis* was observed in all the isolates obtained from, both animals and humans. The study revealed that high genetic homogeneity was observed in the strain circulating in the local environment. These findings confirmed that zoonotic tuberculosis was being transferred from animals to humans and was threat to public health. Many studies around the world confirmed the presence of same strain

in the animals and its zoonotic potential. Likewise, a study was conducted in Zambia, where two isolates from humans and 22 isolates from animals in the region yielded a single spoligotype that was observed in our study, i.e., 482/SB 0120 same spoligotyping pattern (Malama *et al*,. 2014). From India an earlier reports confirmed the presence of this spoligotyping pattern in tuberculin positive cattle, However this strain was not checked for zoonotic TB (Thakur *et al*., 2012). Same pattern has been observed from certain other countries like Algeria (Sahraoui *et al*., 2009), Brazil (Parreiras *et al*., 2012) in the animals and from Germany (Kubica *et al*., 2003) and Italy Lari *et al*., 2006) in the humans. The results of study again reconfirmed the presence of strain in Pakistan which were reported earlier in the different studies conducted in the surrounding countries. As 76.47% observed new patterns proved its well tractability in the local conditions. Another achievement to trace the zoonosis transmission from animals to humans, that was done from both animals and clinical samples.

Drug Resistance

A total of 70 isolates were included in the study, on the basis of proportion methods overall 35.71% cases were confirmed as drug resistant, while with molecular based identification methods an overall prevalence of 32.85% was observed. The results of present study revealed a lower percentage of drug resistance as compared to other reports as Khan *et al*. (2013) reported an overall prevalence of 57% in Punjab province of Pakistan; Khoharo and sheikh reported an overall resistance of 71.92% while Akhtar *et al.* (2007) reported an overall prevalence of 60.5% in Sindh province of Pakistan, Assad and Alqahtani (2012) reported that 38% patients were resistant to one or more drugs in Saudi Arabia. Sagavara *et al.* (2005) reported an overall resistance of 51.5% in China, Ndungu *et al.* (2012) reported an overall resistance of 30%, Purwa *et al.* (2011), reported an overall resistance of 47.3% in India and Khan *et al.* (2013), who reported an overall resistance of 63% in Punjab, Pakistan. On the basis of susceptibility testing, out of 70 isolates 22 (31.42%) were resistant to isoniazid and 13 (18.57%) were found resistant to rifampicin. Out of 22 INH resistant cases 10 (45.45%) cases were also resistant with RIF. On the molecular basis 18 (25.71%) isolates were resistant to isoniazid and 9 (12.85%) were found resistant to rifampicin individually. Our results showed, out of 18 INH resistant cases 4 (44.45%) cases were also resistant with RIF (MDR-TB). Xue-Qiong *et al.* (2006) reported isoniazid resistance in 66.4% cases. Similarly, (Kandi *et al.*, 2013) reported that among 44 rifampicin resistant isolates 80% were

also resistant to INH in Belarus. However, the resistance observed during present study was higher than the results reported by Tripathi *et al.* (2012), as the resistance to INH was 37.87% in India. In contrast, a much lower, only 7% INH resistance has been reported from khan *et al*. (2013). Among 41 isolates in Punjab Pakistan. The Moaddab *et al.* (2011), reported 50% INH resistance isolates in Iran. Ndungu *et al.* (2012) reported that 12.9% cases were resistant to INH in Kenya. The results of drug susceptibility testing based on MAS-PCR assay revealed that out of 70 isolates (12.85%) were found resistant to rifampicin (RIF), within this 65% were mono resistant. The MAS-PCR assay has widely been used all over the world, A report from Vidwai *et al*. (2012) declared 97.9% cases as rifampicin resistant. Similarly, 79.2% RIF resistant cases were observed in another study conducted by Sui *et al*. (2011). In our study all the drug resistant cases were obtained within genotypes specific for *Mycobacterium tuberculosis*. The Haarlem (H) family was the dominant genotype associated with drug resistance in our study, similar results have been obtained previously from Karachi a biggest city of Pakistan, where Haarlem strains had significant association with drug resistance. Association of (H) strain with MDR has been confirmed from Iran and Afghanistan in some earlier studies (Farnia *et al*., 2006). Not a single drug resistant case was observed within the genotype of *M. bovis* strains, these findings strengthen the concept that mostly *M. bovis* isolates are resistant to pyrazinamide because the organism does not produce the enzyme pyrazinamidase which is needed to convert pyrazinamide into pyrazinic acid, the active form of the antimicrobial agent (Barouni *et al*., 2004), this might be the reason that no drug resistance either RIF or INH was observed in *M. bovis* strains.

Genetic Susceptibility

The results of genetic susceptibility testing in animals and humans on the basis of polymorphism in *NRAMP1* gene showed that, from two groups of healthy and diseased people, 6 (20%) healthy people and 7 (23.33%) diseased patients were found positive for *3'UTR* region polymorphism. Two types of genetic polymorphism were observed in human genome, i.e., TGTG+/TGTG+ being the dominant form with a very less number TGTG-/TGTG- and non-significant association was observed between both of polymorphism and active tuberculosis. Similar findings were observed in a study conducted by Tiksnadi and Herman (2013) in Thailand similar no significant association was observed between

tuberculosis and *NRAMP1* gene polymorphism. However, a study conducted by Hsu *et al*. (2006). Genetic polymorphism at *INT4* region was significantly associated with the occurrence of tuberculosis in the local population, but, no effect of *3'UTR* gene polymorphism was observed within diseased and non-diseased groups. In another report published from Japan, it was confirmed that there was no connection found between tuberculosis susceptibility and *NRMAP1* gene polymorphism, especially when they targeted *3'UTR* (Abe *et al*., 2003). In contrast with this Korean population had a direct relationship of *3'UTR* gene polymorphism and occurrence of tuberculosis as previously described by Ryu *et al*. (2000). Genetic susceptibility due to genetic polymorphism always remained a debate able topic many people believed that these are other factors those increase susceptibility against disease than that of genetic polymorphism at certain alleles this was mentioned in an earlier report by Rieder *et al*. (1989) where diabetes was associated with increase susceptibility of tuberculosis. Another study from Thailand confirmed that not only *NRAMP1*/*3'UTR* but, TNFα had no association with the susceptibility against tuberculosis. Almost cloned results have been obtained from Taiwan, where no relationship was estimated in *NRAMP1* polymorphism and active disease in the patients (Liaw *et al*., 2002). As mentioned above identical reports has been observed as we got from our study. To enhance this concept other studies had also linked to certain other factors have also been reported associated with *NRAMP1* gene polymorphism like a Chinese study revealed that *NRAMP1* polymorphisms were associated with TB progression but not with host susceptibility (Zhang *et al*., 2006). We could not find the relationship between active disease and genetic polymorphisms in our region, the reason may be we targeted only one allele, our sample number was very small. The Literature support our findings like in a study, it has been described that 3UTR or *D543N* variants might not be the direct cause of susceptibility, rather it was another functional polymorphism exists in *NRAMP1* (Bellamy *et al.*, 1998). From animals no genetic polymorphism was observed. As very little reports have been observing from animal for *3'UTR* polymorphism. A report for *NRAMP1* gene polymorphism has been observed in Africa (Zebu cattle) but it was found in very low incidence cases (Kadarmideen *et al*., 2011).

CHAPTER-6 SUMMARY

This study was carried out on the animals at Malkhanwala (Satiana road) and Paroka (Aminpur road) cattle and buffalo colonies and two livestock farms, respectively, to ascertain the prevalence of tuberculosis in the animal, and different TB hospitals were selected for the screening and sample collection from humans. Additionally, animal handlers and some milk selling points were screened for the presence of Mycobacterium. The present study revealed an overall prevalence of 10.56% at the two livestock colonies and 15.72% at two livestock farms. Individually, the prevalence was 9.09% at Satiana road colony and 12.03% at Aminpur road colony, while it was 19.13% at Bahadurnagar (BN) farm and 12.14% at UAF dairy farm. The prevalence was higher in buffaloes, while low in cattle at both colonies and two cattle/buffalo farms. The disease was present in all herds with varying frequency ranging from 3.8% as minimum to 15.7% as highest number of cases at two colonies and farms. The significant statistical difference ($P<0.005$) was observed in different age groups of animals and the highest prevalence was observed in the age group >5 years. On the basis of milking status, prevalence was higher in lactating animals in both colonies and farms, the maximum prevalence was observed in high milk producing animals (5 -9.9 liters milk per day). Multivariate logistic regression analysis with the backward elimination procedure at two buffalo/cattle colonies revealed that, age, body weight and status showed a significant association ($P<0.005$) with the positive skin test. The bivariate logistic regression analysis in buffalo and cattle at two colonies revealed that after controlling the age as a constant factor, the specie and status of animals showed significant association with the positive skin test. The bivariate logistic regression analysis at two farms revealed that age showed a significant association ($P<0.005$) with the occurrence of tuberculosis. After controlling the specie as a constant factor, the body weight of animals showed significant association with the occurrence of tuberculosis ($P<0.005$). Considering PCR as a gold standard, the PPD sensitivity and specificity were 77.8% and 100% at two colonies and it was 90.5% and 99.5%, respectively at two animal farms, while ZN sensitivity and specificity were 86.1% and 99.1%, respectively at colonies and 95.2% and 99.1%, respectively at two farms.

The abattoir study was conducted on the morbid tissues, a total of 200 consecutive cattle and 200 consecutive buffaloes were examined for gross abnormalities in different organs, related to tuberculosis. Based on lesions, 27 apparently suspected samples from buffaloes and 21 from cattle were collected which included, lungs, and associated lymph nodes. The positive PCR for tuberculosis at the slaughter house revealed that, 3.5% buffaloes and 2.5% cattle were positive for tuberculosis. The prevalence was higher in buffaloes than cattle. However, the relatively higher prevalence was observed in males than females. The PCR showed more number of cases of young animals (between 1-3 years), than older ones (4-7 years). Among different weight groups, a significant difference ($P<0.05$) was observed between two weight groups with higher prevalence in the weight group <350kg body weight. Keeping direct PCR as gold standard, the sensitivity and specificity of GPCR were 29.5% and 100%, while sensitivity and specificity of PPD test were 90.9% and 99.1%, respectively. The lungs/liver were the chief morbid organs (48 cases) with the visible nodular lesions. Ten/48 cases were confirmed by PCR as *Mycobacterium bovis.* Among 19 swollen lymph nodes, only two cases yielded positive PCR for *Mycobacterium bovis.* The multivariate logistic regression analysis in both cattle and buffaloes revealed that age and body weight showed a significant association ($P<0.005$) with the confirmed cases of tuberculosis. The results of hematology in the animals revealed significant difference between diseased and control (healthy) groups of animals in the most parameters like, WBC count, PCV, neutrophils, lymphocytes and monocytes were significantly higher in the TB infected animals, while only haemoglobin concentration was significantly lower in positive infected animals.

From Humans, the results of the bivariate frequency analysis showed that among *Mycobacterium tuberculosis* complex confirmed cases, 70.93% were males and 68.7% were females. Results revealed that the chances of occurrence of tuberculosis were 11% higher in males than females. Similarly, Among the patients, maximum number of confirmed cases were observed in the age group 16-30 years ($P<0.005$). Highest percentage was observed in the patients had duration of disease group 32-90 days. More than 90% of patients encountered the disease first time, while less than 10% cases were those where tuberculosis reoccurred. Odds ratio showed that there would be 1.85 times more chances of positive PCR of MTC-organisms in X-ray positive patients than X-ray negative patients. More than 90%

MTC positive PCR was observed for the patients had raised levels of ESR (>20mm/HR). With ZN-stained smear microscopy the overall detection rate for all types of sample was 63.50%, a significant association (P<0.005) was found between ZN positive cases and MTC positive PCR. Of the study population, overall 41.94% samples were culture positive (both of LJ and SB) and from positive growths 87.03% growths were observed on LJ slants, while 12.09% growth were observed on SB medium. From positive growths, MTC-organism specific PCR was positive in 92.55% cases of LJ growth and 75% of SB growth There was non-significant statistical association observed between animal kept at home, types of animal, frequency of contact with animals and raw milk taken in relation with MTC positive-PCR. From above mentioned parameters, animals kept at home and frequent raw milk taken had significant association with *M. bovis* specific culture, PCR. The 72.42% *M. bovis* culture, PCR positive cases were obtained from the patients had animal at home, while 50% SB culture positive patients, those had the history of frequently taken the raw milk were also positive for *M. bovis* culture PCR. The γIFRA was able to detect 20 % of ZN negative and 8.1% of rapid test negative tests. To assess the chances of zoonosis, transfer from animals to humans, a limited number (n=128) of people, those had direct contact with animals, including farm attendants, milkers, raw milk sellers and abattoir worker were tested by the intradermal skin test. The results showed that 10 (7.81%) people had positive skin test from, which 9 (90%) cases were ZN positive and 8(80%) samples were those where positive growths were observed; 6 (75%) on LJ and 2(25%) on SB medium. All the growths on both culture media were confirmed as MTC-complex organisms with PCR and all the growths on SB medium were confirmed as *M. bovis*.

A total of 210 strains obtained from different culture medium could be used for spoligotyping purpose. The results revealed a total of 51 different strains pattern. Out of these 39 (76.47%) patterns were identified as new, while 12 (23.52%) patterns have already been present in the international databases. Most prevalent clade was Central Asia strain (CAS1) or simply called Delhi strains. Within this clade three different spoligotypes were observed with the difference percentage, i.e. CAS ST-141 (20%), ST-26 CAS/Delhi (14.58%) and orphan-CAS (6.18%). The second most prevalent clade was an East-African Indian (EAI) with two different spoligotypes ST-236 (12.5%) and ST-126 (8.33%) respectively. In our study third most cases were observed with MAANU ancestral clades, within this clade three

further spoligotyping pattern were observed, MANU2/ST-54 in 10.41% and MANU1/ST-100 in 8.33% and orphan MANU-2 in 6.81% cases, respectively. Our findings confirmed that a high diversified population of *M. tuberculosis* genotypes were circulating in local patients as both new and ancient types were observed at the same place. Haarlem clade contained only 10.41% cases among all the known cases. Within known strains of *M. tuberculosis* 8.33% cases were observed with the spoligotyping pattern of orphan-33. It was also noted that in the database no strain from Pakistan have been submitted. The isolates obtained from animal sources and all the isolates those were confirmed as *M. bovis* from human SB culture were subjected for the molecular characterization through spoligotyping isolates obtained from humans and animals. The results showed a same spoligotyping pattern compared with database named 482/SB0120, which had a characteristic of lacking spacers 3, 9, 16, and 39−43. To find out drug resistance, a total of 70 isolates were included in the study. In the first phase on the basis of proportion methods overall 35.71% cases were confirmed as drug resistant, while with molecular based identification methods an overall prevalence of 32.85% was observed. Out of total suspected 70 isolates, 22 (31.42%) were resistant to isoniazid (INH) and 13 (18.57%) were found resistant to rifampicin (RIF). From twenty-two INH resistant cases, 10 (45.45%) cases were MDR. With the molecular methods, drug resistance was observed by observing a genetic based polymorphism. Out of total 70 isolates 18 (25.71%) were resistant to isoniazid and 9 (12.85%) were found resistant to rifampicin. The results showed that out of 18 INH resistant cases, 4 (44.45%) cases were also resistant against RIF (MDR). Genetic susceptibility testing was done both in animals and humans on the basis of polymorphism in *NRAMP1* gene. From humans 06 (20%) healthy people and 07 (23.33%) diseased patients were found positive with *NRAMP1* gene. From animals in diseased and control group not a single case of genetic polymorphism was observed.

Conclusions

- ❖ It is concluded from the study that tuberculosis is present both in animals and humans in the local population with a great prevalence.
- ❖ From the animals, higher prevalence was found in buffalo than cattle.

From two colonies

- ❖ Overall 10.56% prevalence was observed while herd prevalence was 100%.
- ❖ The multivariate logistic regression analysis with backward elimination revealed that age, body weight and status showed significant association with occurrence of tuberculosis.
- ❖ The bivariate logistic regression revealed that by controlling age as constant factor specie and status of animals showed significant association with occurrence of tuberculosis.

Two livestock Farms

- ❖ An overall prevalence of 15.72% was recorded on two livestock farms.
- ❖ Bivariate logistic regression confirmed that age showed significant association with the occurrence of tuberculosis.
- ❖ After controlling the specie as constant factor the body weight of animals showed significant association with occurrence of disease.
- ❖ Slaughterhouse
- ❖ A varying prevalence of 3.5% buffalo and 2.5% of cattle was recorded in the so called normal animals comes for slaughtering.

Human Studies

- ❖ A huge percentage of confirmed Tb cases were obtained from suspected patients. More disease was observed in males than female.
- ❖ A large proportion of young people had more disease.
- ❖ Pulmonary tuberculosis was more than extra-pulmonary TB.
- ❖ MTC-organism was frequently present in the local environment.
- ❖ A very small portion of zoonotic TB was also observed in humans.
- ❖ Farm attendants/ co-workers.
- ❖ Zoonosis was a threat to animal workers/ attendants, as two pulmonary (via aerosol route and one extra-pulmonary TB was found infected via milk route.

Molecular Characterization

- Spoligotyping confirmed that a highly diversified genetically related organism was circulating in the local environment.
- Delhi, EAI, MANU, Haarlem were the most dominant strains observed with a large number of not identified strains.
- Only one pattern of *M. bovis* was obtained from animals and humans, confirmed that zoonotic TB was being transferred with animals and Humans.

Drug Resistance

- Maximum resistance was observed against isoniazid than rifampicin, a little proportion of MDR was also observed.
- All the drug resistant strains were identified as *M. tuberculosis* genotypes, no case of drug resistant had *M. bovis*.

Genetic susceptibility

- A little polymorphism was found but no association with occurrence of disease was observed both in animals and humans.

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