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Study on Bovine Tuberculosis in the Holeta Dairy Farm, Central Ethiopia

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Abstract: In light of the direct correlation between *Mycobacterium bovis* (*M. bovis*) infection in cattle and the disease in humans, control measures need to be applied to reduce the prevalence of tuberculosis in developing countries. To this effect, generation of epidemiological is of paramount importance. This study was undertaken on 243 heads of cattle using the comparative intradermal tuberculin test (CIT), gamma-interferon (IFN-γ) test and bacteriology. Twenty-two percent (n=243) of the herd tested positive for *M. bovis* by CIT test. In addition, bacteriological culturing of the milk and nasal discharges reconfirmed 38% (16/42) of the positively diagnosed subjects by the CIT test. An agreement (kappa=0.6) was recorded between the results of the CIT test and the IFN-γ test. Moreover, a positive correlation (r = 0.73) was found between the subjects deemed positive by both diagnostic tests. It was recommended the Farm management apply test and removal of positive animals regularly and strictly so that the disease is controlled in the Farm.

Key words: Bovine tuberculosis, Gamma-interferon test, Tuberculin test, Mycobacterium bovis, Incidence

INTRODUCTION

According to the World Health Organization, tuberculosis is one of the most widespread infectious diseases and the leading cause of death due to a single infectious disease among adults in the world[1]. Although *Mycobacterium tuberculosis* (*M. tuberculosis*) generally affects humans, *M. bovis* infect cattle and also can be transmitted to humans through drinking (or handling) contaminated milk, by aerosol, or through ingestion of undercooked or raw meat. After infection, the bacterium can spread throughout the body. Specific CD4 T-helper cells normally recognize Mycobacterium and release macrophage-activating IFN-γ. The activated macrophages kill the intracellular tuberculosis mainly through the generation of toxic nitric oxide, but when the macrophages are not activated in proportion to the rapidly multiplying bacteria, the disease can be overwhelming for the immune system[2]. Macrophages from susceptible strains also tend to have suppressor effects on T-cell proliferation to mycobacterial agents[2].

Thus, BTB has severe implications for animal welfare and animal health, since it causes reduced productivity and premature death in cattle and the affected farms suffer severe economic losses[3]. The number of infected animals shedding the bacteria, the number of susceptible animals present within a herd and the size of the containment facilities can all have an impact on the transmission of the bacteria. In many developing countries, the disease is widely distributed because control measures are not being applied or are applied sporadically and pasteurization is rarely practiced. Many of the cattle that are found to be positive are separated and slaughtered, which in turn causes severe financial losses to the farms.

In Ethiopia, BTB has been an epidemic since 1967, with prevalence ranging from 15.6% to 50% reported in dairy farms around the country[4]. In developing countries like Ethiopia, it is important to implement proper testing and control methods to reduce the transmission of the disease to humans.

The standard tests used for BTB detection in Ethiopia include the CIT test and the IFN-γ test. The CIT test compares the animal's reactivity to an injection of bovine tuberculin and avian tuberculin, distinguishing the infection from that of other species of mycobacterium[5]. The IFN-γ test measures the release of IFN-γ in a whole blood culture system as a means of determining *M. bovis*. The amount of IFN-γ produced in response to mycobacterial antigen is determined by an enzyme immunosorbent assay (EIA) using a monoclonal antibody specific for bovine IFN-γ[6]. The IFN-γ test has been evaluated in different places[7-9]. Bacteriological culturing is often used as a positive reinforcement and for further observation of *M. bovis*.

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The investigation BTB in the Holeta Farm has been started three years ago. But although the Farm has been divided into two; a negative herd and a positive herd, re-testing of the negative herd had not been conducted for approximately two years. Thus, this study was formulated to re-test the herd that was classified as a negative herd two years ago.

MATERIALS AND METHODS

Study farm: The Holeta Farm is located 43 km west of Addis Ababa in Holeta Town, on the main road to western Ethiopia. The main objective of the Farm is to serve as a source of semen and bull for the nation which are critical for the improvement of the genetic potential of the indigenous cattle breed. Besides, it serves as a source of milk for the public. Positive and negatively-tested cattle had been previously separated based upon skin test-results (three years ago). Tests had not been conducted since June of 2004. This study tested 243 of the proposed negative cattle using the CIT skin test. Sixty-two of the 243 cattle were milking cows and 37 were bulls. Of those 243 subjects, 18 positive and 18 negative were also tested using the IFN-γ test.

Comparative intradermal tuberculin test: Comparative Intradermal Tuberculin Test (CIDT) was used mainly to differentiate between animal infected with *M. bovis* and those sensitized to tuberculin due to exposure to other mycobacterium or related genera. Two sites at the middle of the neck were shaved and cleaned 10.cm apart on the same side of the neck, the areas were examined for the presence of any gross lesion. The skin fold at the two sites was measured by a caliper and recorded. Each animal was then injected 0.1 mL (2500IU mL) avian tuberculin PPD (Veterinary Laboratories Agency, Addlestone,Surrey KT153NB) and 0.1mL(2500IU mL) bovine PPD (Veterinary Laboratories Agency Addlestone Surrey KT153NB) intradermally at the anterior and posterior parts respectively. The sites were examined and the skin thicknesses were measured 72 h post injection. The interpretation was made in the following ways:

- When the skin thickness is increased by 4 mm or more at bovine PPD injection site regardless of the increase at avian site, the animals was considered as positive for BTB.
- When the skin thickness is increased at both sites, the difference of increase at Bovine (B) and increase at Avian (A) sites were considered. Thus, when B-A was less than 2mm, between 2mm and 4mm, or 4mm and above, the animal was considered as negative, doubtful or positive, respectively.

Blood collection, stimulation and gamma interferon assay: Blood sample were collected from the jugular vein into heparinized vacutainers before the administration of tuberculin for skin testing. The samples were transported to the laboratory with in 8 hours of collection. Whole blood was stimulated by avian purified protein derivative (PPD-A) and, bovine purified protein derivatives (PPD-B) Culturing was made for 48 hours at 37° C in 5% CO_2 humid atmosphere. Subsequently, the supernatant was collected after 48 h culturing and frozen (-20° C). Interferon gamma secretion by peripheral blood was measured using the bovine gamma interferon (BOVIGAM™) test kit (Common Wealth Serum Laboratories, Victoria, Australia). The plasma supernatant was thawed and transferred into 96 wells plate pre-coated with anti bovine gamma interferon antibody and incubated for 1h at room temperature. The plates were washed six times with a washing buffer and enzyme conjugated anti bovine gamma interferon secondary antibody was added into the wells of the plates. After incubation for 1h at the same condition, the plates were washed six times and enzyme substrate and chromogen were added and after 30 min of incubation, Optical Density (OD) was measured at 450nm. Results were interpreted as per the manufacturer's instruction.

Bacteriology: Twenty ml of milk sample was drawn from four quarts of the positively-tested milking cows and processed for isolation of mycobacterium[5]. Similarly, 3ml of nasal discharge was collected from the remaining positively-tested females and processed for mycobacterium isolation[10]. The samples were decontaminated using equal amounts of 4% NaOH solution. Thereafter, they were centrifuged at 3000 rpm for 15 min. The supernatant was discarded and the remains were suspended in Saline. The samples were neutralized by adding 1% HCl drop by drop until the color of indicator (phenol) turns from yellow. Lowenstein-Jensen media (one with pyruvate and one with glycerol) was used for inoculation and it was incubated in a slanted position at 37°C for one week and in an upright position for approximately 2 months. Acid-fast staining technique was used for mycobacterial identification of the acid-fast organisms.

Data analysis: Percentages were determined from the skin thickness of the CIT test and the optical density of the IFN-γ test using Microsoft Excel. Means were found for all the skin reactions and optical densities. Means were (mean±SEM) OD values for Avian PPD stimulation in the CIT positive and CIT negative animals were 0.487±0.068

and 0.289±0.04, respectively. On the other hand, the mean also determined for bovine minus avian (B-A) in the same manner. Standard Error of Mean (SEM) was ascertained from the standard deviation and the square root of the total number of samples. The significance of the skin thickness data at the avian and bovine sites was determined using the student's t test. A correlation coefficient (r) was found between the skin test and the IFN-γ test using Pearson's correlation coefficient. Agreement of the two tests was determined using Kappa statistics. Statistical significance for all tests was set at a p value of <0.05.

RESULTS AND DISCUSSION

Incidence of bovine tuberculosis: The incidence of BTB was found to be 22% using the CIT test in two years time Fig. 1. Testing of the Farm was begun in October 2002 and the incidence of BTB dropped in a year from 14% to 1% after three consecutive applications of testing the herd and removing the positive reactors[11]. However, for the last two years, testing had not been done on the negative herd. Thus, a significant rise (1% to 22%) in the incidence of BTB was observed in the herd in the past two years. In this study 28% of the negative herd reacted to avian tuberculin (≥2mm) Fig. 2. indicating the prevalence of *M. avium* and/or other species of *Mycobacterium*, other than the *M. tuberculosis* complex. The mean skin indurations (mm) to bovine PPD was significantly greater than the mean skin indurations to avian PPD, (2.048±0.189 vs. 3.906±0.443, $p< 0.001$) Fig. 3. Fourteen percent of the animals in the herd reacted to both avian and bovine tuberculin (≥4mm) signifying the presence of mixed infection with *M. bovis* and *M. avium*/*M. paratuberculosis*. These other species of *Mycobacterium* can be just has harmful to the cattle and also be transmittable to man. Of over 50% positive animals were milking cows, which could serve as the source of infection for humans through contaminated milk. Furthermore, many farm workers are at risk of infection by transmission through the air.

Bacteriology: Bacteriological culturing of the milk and nasal discharges reconfirmed 38% (16/42) of the positively diagnosed subjects by the CIT test. Out of the 23 milk samples collected, 9 were confirmed with colonies of *Mycobacterium* after acid-fast staining. Seven of the 19 nasal samples were confirmed for *Mycobacterium* growth. Bacteriological findings signify the risk of transmission of the bacilli to humans and animals from infected animals.

Table 1: Illustrates the agreement (kappa = 0.6) between the CIT test and the IFN-γ test as analyzed by kappa statistics.

		IFN-γ test		
		POS	NEG	Total
CIT test	POS	15	3	18
	NEG	9	9	18
	Total	24	12	36

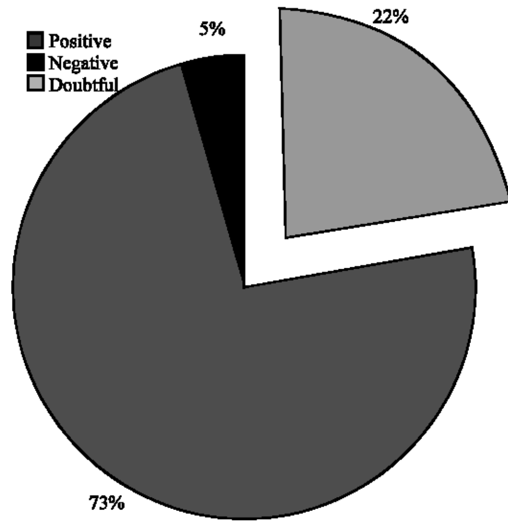

Fig. 1: Illustrates the prevalence of Bovine Tuberculosis in the negative head of the study Farm. Based on the CIT test results, 22% were determined positive for *Mycobacterium bovis*

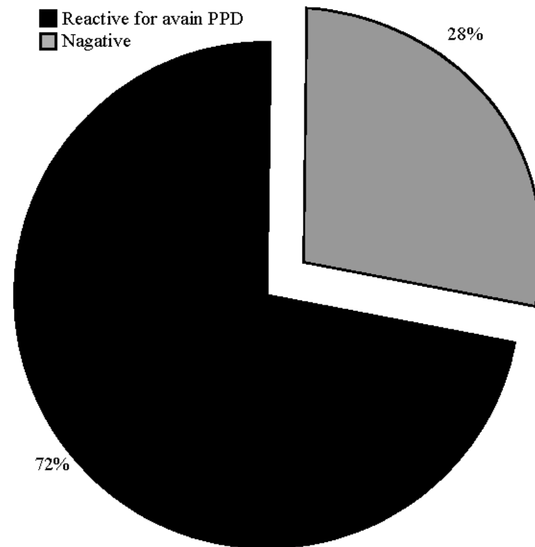

Fig. 2: Illustrates the prevalence of *M. avian* and/or other species of *Mycobacterium*, other than the *M. tuberculosis* complex, in the study Farm. Based on reactivity to avian PPD injection, 28% of the study farm reacted to the avian tuberculin with skin indurations of 2 mm or greater

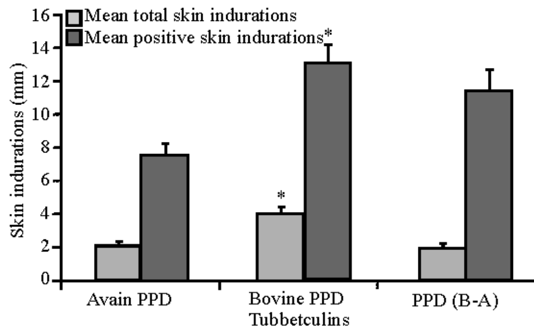

Fig. 3: Shows the mean reaction to avian PPD and bovine PPD. The mean reaction to bovine PPD is significantly greater than the mean reaction to avian PPD, (t test, $p< 0.001$). The mean of the total skin indurations (mm) at the bovine site was 3.906 ±0.443, indicating that the study farm had a high reaction to bovine PPD injection. The burgundy bars indicate the mean skin indurations of 4mm or greater after the tuberculin injection. The subjects were diagnosed for BTB when the skin indurations at the bovine site minus the skin indurations at the avian site (B-A) were 4mm or greater. The mean positive (burgundy) bar from Fig. 3 entitled PPD (B-A), indicates that on average, the positive skin indurations, which indicate BTB, were approximately 11.316±1.415 showing a considerable reaction to the PPD injections

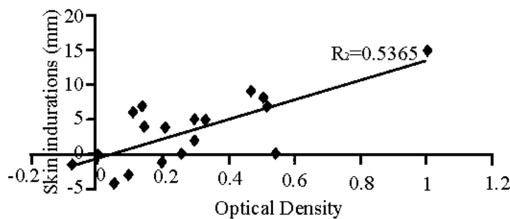

Fig. 4: indicates a positive correlation (r=.73) between the bovine minus avian (B-A) skin indurations of the CIT test and the B-A OD values of the IFN-γ test. The samples represent the 18 positive cattle tested by both diagnostic tests. Subjects were diagnosed for BTB in both the CIT test and the IFN-γ test when B-A was greater than or equal to 4 mm or 0.1, respectively

Correlation between CIT and IFN-γ tests: The mean (mean±SEM) OD values for bovine PPD stimulation in CIT positive and negative animals were 0.759±0.071 and 0.486±0.061, respectively. The difference in mean OD values of γ-IFN response to avian (0.487±0.068 and bovine PPDs (0.759±0.071) was significant ($p<0.001$). A moderate agreement (kappa = 0.6) was recorded between the CIT test and the IFN-γ assay Table 1. Furthermore, a positive correlation (r=0.73) was recorded between the CIT test and the IFN-γ test in the CIT-positive animals Fig. 4. Other workers have also reported similar findings[8,9,12]. Nine the CIT negative animals were found to positive by the IFN-γ assay. This shows that IFN-γ test has a higher sensitivity than the CIT test for detecting BTB in cattle[13-17]. On the other hand, three of the IFN-γ test negative cows were positive for CIT test. This suggests the use of both tests together for a better detection of the infection.

CONCLUSION

For many poor countries like Ethiopia, the method of test and slaughter can often be too expensive and cause severe economic losses. Vaccinations may be more cost efficient but are still at the experimental level. Test and segregation still serve a risk if infected animals are not fully isolated from the rest of the livestock. Nevertheless, the acceptable option for the Holeta Farm management is test and segregation supplemented with pasteurization of the milk and public education about the disease, how it is transmitted and how it can be prevented. Only repeated and regular testing will ensure that the disease will not spread in the negative herd in the near future. Furthermore, the newborn calves should be separated as early as possible after delivery and then tested for BTB.

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