Isolation of *Mycobacterium paratuberculosis* from apparently Healthy Sheep and Goats

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Abstract

A total of 260 tissue specimens (from the last portion of the small intestine, ileo-cecal valve and corresponding lymph nodes) were collected from apparently healthy sheep and goats in the slaughter houses. The specimens were prepared and inoculated on Middle Brook 7H10 agar base M199 media with and without mycobactin J. All the specimens were pre-treated with 5% oxalic acid. Direct smears were also prepared from intestinal scrapings and lymph nodes parenchyma and stained with Zeihl Neelsen (acid-fast stain). The cultures were monitored carefully and results were recorded throughout the incubation period, among which 23 (9%) of them showed growth after the 16th week. The culture Results were recorded considering the long incubation period, colony appearance and acid fastness of the bacteria. The direct smear result revealed 55 (21%) positivity. Sensitivity and specificity of the culture method was determined taking histopathological examination as a reference. It is concluded that the conventional culture method is laborious and time consuming for sub clinical paratuberculosis in small ruminants.

Keywords: Johne's disease, Mycobacterium culture, *Mycobacterium paratuberculosis*, mycobactin J

Introduction

Mycobacterium paratuberculosis is an acid-fast, short, thick rod bacteria with 0.5µ in diameter and 1-2µ in length. In smears, the organism is found in clumps or single cells scattered all over the preparation. The organism is aerobic at optimum temperature of 37ºC (Marchant 1983). Transmission electron microscopy reveals the waxy rough cell wall and the intracellular vacuole. The cell is composed of thick waxy mixture of lipid and polysaccharides (Johne's information Centre, 2001).

Fact sheet (1996) reported that there are about 71 species of Mycobactericeae family in which 32 of them are known to be pathogenic for humans or animals. They are divided as slow and fast growing Mycobacterium. *M. paratuberculo-*

sis belongs to the slow growing group and it is within *Mycobacterium avium* complex (atypical mycobacteria) which includes *M. avium,* subspp. *Silvaticum* and *M. intracellularae,* within this complex there are over 20 recognized serotypes. They grow well at 25ºC and many strains will grow at 42ºC and 45ºC.

M. paratuberculosis is a difficult bacterium to isolate. The conventional culture methods are not sensitive, but the modified Roche culture or double incubation with radiometric culture is the best to get the growth early and is more sensitive (Mc Donald *et al*., 1999)

Carter, (1995) reported that in smears organisms appear as short, thick, small, acid-fast rods similar to the avian tubercle bacillus. Identification is based on cultural (including growth rate and mycobactin dependency), and morphologic and staining characteristic. Jubb *et al.,* (1993) suggested that the examination of faeces for organisms is a useful diagnostic aid in clinical cases, even though the slow growth of the bacteria makes difficult the diagnosis using culture. *M. paratubercullosis* can also be detected using culture in animals that are apparently healthy (Whitelock *et a*l., (2000).

The objective of this study is to isolate and characterize *M. paratuberculosis* from apparently healthy sheep and goats in Jordan.

Materials and Methods

Study Area

Site for sample collection was selected according to small ruminant's distribution in Jordan. Amman and Sweleh abattoir were being selected representing the central part of the country and Botchers around Irbid as the northern part.

Study Animal

The study was carried out in Awassi sheep and Baladi (local breed) goats that were brought from semi intensive or mixed farming system between the age of 8-24 months and 6-12 months respectively.

Sampling

A total of 260 samples were collected from sheep and goats slaughtered in the two abattoirs (Amman and Sweleh). Mucosal scrapings and portions of lymphnodes were collected from the Ileum and Ileo-cecal valve portions of the small intestine and the surrounding lymph nodes.

Sample preparation and Inoculation

Approximately 3-5 grams of scrapped intestinal mucosa and lymph node parenchyma was taken and grounded by using a mortar, and incubated with trypsin 0.5% at 4 °C over night at pH of 7.4 (adjusted using 4% NaOH). After 16-18 hours the mixture was filtered using gauze (folded), and centrifuged at 400xg for 20 minutes (PK110, ALC, Italy). The supernatant was decanted and decontaminated with 5% oxalic acid and the samples were allowed to stay without disturbance for 24-30 hours at room temperature. Inoculum of 0.1ml (100µl) was taken carefully from the bottom of the tube and inoculated in middle Brook 7H10 agar base M199 (Himedia Laboratories, limited Mumbai (Bombay) 400086, India) with a supplement as a slant in tubes of 20ml. The media was prepared with and without mycobactin J (2mg/lt) according to OIE (2000). Each sample was inoculated in three tubes (one tube without mycobactin J and 2 with mycobactin J) and was evenly distributed on the media. The tubes were kept inclined with loosened screw to facilitate the evaporation of excess moisture and inoculum fluid for one week. After one week, the tubes were placed vertical with tightened screw and incubated for 16 weeks.

In parallel to culturing, direct smear was done from the scrapping and stained with Zeihl Neelsen (ZN) for acid-fast bacilli. The smears were examined under microscope (100 x magnifications) and results were recorded. The ZN staining was performed based on Quinn et al., 1999.

Culture reading

Reading of cultures was made starting from the 8th week of inoculation for the presence of any growth. By the end of the 16th week, smears were taken from cultures that showed growth, and stained with ZN. Cultures were considered positive based on the colony characteristics (white spot colonies) and presence of acid fast bacilli in the smear.

Results

Starting from the 8th week of inoculation, cultures were examined every week for the presence of bacterial growth. On the 16th week some samples showed growth. (8.8%) (Table1). The colonies were characterized by small white-yellowish spots with irregular border. Smears from colonies cultured in tubes with mycobactin J showed acid fast bacilli, in clump and dispersed form (Fig1). The rest of the samples were kept in the incubator for 24 weeks but no more samples showed growth of bacteria. Out of 260 direct smear samples, 55 (21%)

of them revealed acid-fast bacilli similar in appearance to that made from cultures (Table 1).

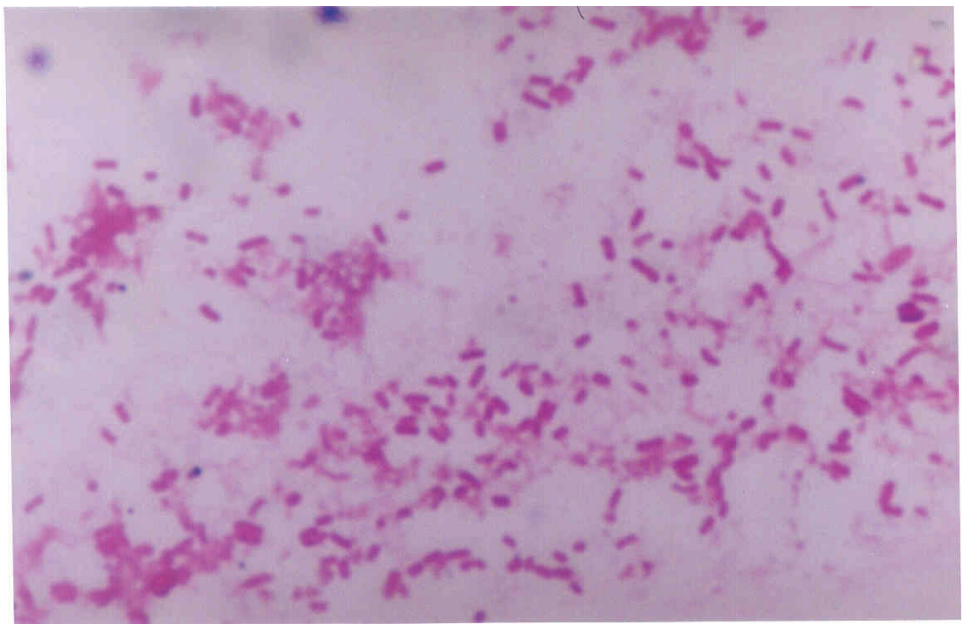

Fig. 1: Clumps & dispersed form of *Mycobacterium paratuberculosis from culture*

Histopathological examination is a confirmatory diagnostic method for Johne's disease. Sensitivity and specificity of culture technique for the diagnosis of *M. paratuberculosis* was compared with the histopathological examinations. The result showed a sensitivity of 14.8 % and specificity of 100%. Likewise, the direct smear showed 31.5% sensitivity and 50% specificity. The predictive value of culture and direct smear was also determined and were 100% and 95.8% respectively (Table 2).

Table 1.Culture and direct smear result from intestinal scrapping and lymph node parenchyma

		Culture		Direct smear	
Animal spp.	**No. of specimens**	**Positive**	**%**	**Positive**	**%**
Sheep	219	19	9	53	24
Goats	41	4	10	2	5
Total	260	23	8.8	55	21

The results of culture and direct smear examination on intestinal scrapings and lymph node is show on figure 2.

Table 2: Sensitivity, specificity and predictive value for direct smear and culture

Type of test	Sensitivity (%)	Specificity (%)	Predictive value (%)
Direct smear	31.5	50	95.5
Culture	14.8	100	100

Discussion

It was very difficult to culture *M. Paratuberculosis* as it needs special condition which differentiates the species from other *Mycobacterium* group in which all of them grow at 37ºC but *M. paratuberculosis*, is dependent on the presence of mycobactin J.

In this study samples were collected from apparently healthy animals and the finding of positive samples indicate that apparently healthy animals can shed the bacteria as suggested earlier by Whitelock *et al*., (2000). Ellis *et al* (1998) mentioned that culture results in cattle and goats depend on the stage of infection and disease prevalence within the herd, where the sensitivity is low (50%.) in subclinical cases Mc Donald *et al*., (1999) suggested that culture results during sub-clinical phase depends on the limited distribution of intestinal lesion with small number of acid fast organism. However, unlike that of cattle and goats, *M. paratuberculosis* (bovine strain) is easier to isolate from sheep even using the conventional culture method (Muskens *et al.,* 2001). The sensitivity of culture is also noted to depend on the medium used and the bacterial strain (Greig 2000).

Eamens *et al* (2000) compared different types of culture method for the ovine strain and suggested the radiometric method is the best to have a result in short time (8 weeks) compared to the conventional method. It was explained that the conventional method is the least sensitive and growth can take 12-16 weeks or even some times growth fails. Stable *et al* (1998) suggested that accurate diagnosis of paratuberculosis is often laborious and by culture may require up to 12 weeks for detection. The present study is in agreement with the report in that culturing *M. paratuberculosis* by using the conventional method is very laborious and time consuming.

Menzie (2001) explained that a negative result means failure to grow or that the animal is still in the early stage of the disease. The sensitivity of bacterial

culture in sheep even when showing severe clinical disease is only 8%. Jakobsen *et al.*; (2000) indicated that for the sheep strain there are no methods for definitive diagnosis, and the sensitivity of culture for this strain is only about 10% but it is reported that it is highly specific (no false positive). This is in agreement with the present findings.

Martin and Aitken, (1991) suggested that confirmation of the presence of the disease is obtained by the finding of acid-fast bacilli with the morphology of *M. johnei* in smears from the ileal mucosa or mesenteric lymph nodes, stained by the Zeihl Neelsen staining method. However acid- fast organisms may be absent even although enteric lesions are present. In live animals the finding of clumps of acid-fast organisms in the faeces provides evidence of the presence of the disease but here again negative result does not rule out the possibility of the disease being present.

Direct smear stain is fast diagnostic method in clinical cases. In a deer with chronic diarrhea *M.paratuberculosis* was identified easily by fecal direct smear stained with ZN (Godfroid *et al.,* 2000). In clinically affected animals with histological lesions in the intestines, examination of feces for bacilli in Ziehl-Neelson stained smears will identify up to 57% of infected animals (Greig, 2000). In our case scrapping smears from intestinal mucosa and lymph node reveald 24% positive in sheep.

Investigation of paratuberculosis from slaughter sheep showed that among different diagnostic method applied, high number of samples revealed positive results using direct smear from feces 2%, and 11.1% smear from ileum and 10.3% from caecum (Huchzermeyer and Bastianello, 1992)

Conclusion: As only 9% of the cultured samples showed growth of *M. paratuberculosis* the conventional culture method is laborious and time consuming for sub clinical paratuberculosis in small ruminants. The specificity of culture method is high (100%) but is less sensitive for *M. paratuberculosis.*

Acknowledgement

I would like to thank the Agricultural Research Training Project that had been under the Ethiopian Institute of Agricultural Research for the research fund to carry out this study. I would like to acknowledge Dr. Nabil Hailat and Dr. Ahmed Al Majali for their generous help in all aspects without whom it would have been impossible to finish this project successfully.

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