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Identification of Mycobacterium avium subspecies paratuberculosis in Faecal and Tissue Smears from Small Ruminants

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Abstract

Paratuberculosis (Johne's disease) is a contagious chronic incurable disease of ruminants, can reduce the productivity, and difficult to diagnose and control. It is caused by *Mycobacterium avium* sub species *paratuberculosis* (MAP). Ruminant paratuberculosis is pathologically similar to human inflammatory bowel disease (IBD) which includes three pathological forms; Crohn's disease (CD), ulcerative colitis (UC) and indeterminate colitis or unclassified IBD. The zoonotic concern is much to be detained since MAP is heat resistant and is capable of hiding inside white blood cells. In the current study the presence of acid-fast bacilli compatible with MAP in faecal and tissue smears were microscopically examined using Ziehl-Neelsen stain technique. During the period from the 9th of November 2017 to 7th of March 2018, a total of 27 faecal smears and 14 tissues smears gathered from 12 sheep and 15 goats were tentative infected with paratuberculosis (depending on the clinical features). These animals representing19 commercial herds in various locations in Northwestern Libya. Eighteen (66.6%) faecal smears demonstrated clumps compatible with acid-fast bacilli. Ten animals (71.4%) were positive depending on the presence of the same microorganism in tissue smears. Moreover, positivity of combination of both faecal and tissue smears was 85.7%. Additionally, our results indicate the importance of ileocecal lymph node as a target tissue for the detection of MAP. The findings of the present study reveal that Ziehl-Neelsen stain procedure is indicated for the rapid identification of MAP existing in faeces and tissues. Furthermore, this high occurrence showed a require for the applications of a vigorous programme to control of small ruminants paratuberculosis, established on more sensitive tests, improving juvenile livestock administration, and rising biosecurity procedures.

Key words: *Mycobacterium avium*; Paratuberculosis; Sheep; Goats; Zoonotic; Crohn's Disease; Ulcerative colitis

1. Introduction

Paratuberculosis, or Johne's disease, is an infectious disease caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP) which is an extremely slow-growing, gram positive, facultative intracellular, mycobactin J dependent and acid-fast bacteria. It mainly affects domestic and wild ruminants, causing chronic granulomatous enteritis (Maroudam *et. al*., 2015). Paratuberculosis has a long incubation period and is characterised by progressive and fatal weight loss, accompanied by diarrhoea (Chiodini *et. al*., 1984). The public health concern like a correlation of MAP with Crohn's disease (CD) has been thrashed out debatable (Rosenfeld and Bressler 2010). This argument

was commenced by a clinical and pathological similarity between two chronic inflammatory bowel diseases (IBD); paratuberculosis in cattle and CD in humans (Dalziel 1989). As predisposing factors in patients with CD, deficiencies in antibacterial defense mechanisms, genetic polymorphisms associated with disorders in autophagy processes, activation of autoimmune components, and loss of integrity of the epithelial barrier have been documented (Autschbach *et. al*., 2005; Lowe *et. al*., 2008; Klionsky 2009). Among the possible aetiological agents of CD, bacteria such as MAP, Pseudomonas spp, *Helicobacter* spp and *Yersinia* spp, in addition to infection with the Epstein-Barr virus and other intestinal viruses, have been described. A factor that makes the causal association with such microorganism's complex is the significant modification that the intestinal commensal microbiota of patients with CD presents with respect to healthy people, a situation that can contribute to the severity of the disease and determine the success of the treatment (Lowe *et. al*., 2008; Frank *et. al*., 2007; Kirkwood *et. al*., 2009; Sutton *et. al*., 2000; Laharie *et. al*., 2009).

Ziehl-Neelsen staining method (ZN) is conventionally used in the laboratory for the diagnosis of mycobacterioses due to its simplicity and speed (Steingart *et. al*., 2006). As for the identification of MAP in smears stained with Ziehl-Neelsen technique from faeces, intestinal mucosa or visceral lymph node samples are considered a useful method for rapid and economical way to obtain the diagnosis, depending on the visualization of acid-fast bacilli (AFB) aggregates, which can be excreted intermittently (Stewart *et. al*., 2004; Gerish *et. al*., 2018).

In the more advanced cases of the disease, the diagnosis offers no doubt, since the bacilli are very abundant and adopt a characteristic arrangement in clumps, due to the maintenance of the structure that they have inside the macrophages (Cheng *et. al*., 2020). In cases where the number of bacilli is lower, the diagnosis becomes more difficult, since the sample under examination under the microscope may not allow visualization of the mycobacterium due to its small amount. In the cattle, the appearance of bacilli with characteristics similar to *Mycobacterium avium* subspecies *paratuberculosis*, with the only difference that they do not form aggregates, and may induce false positive qualifications (Gerish *et. al*., 2018). However, this technique only provides a presumptive diagnosis of mycobacteriosis (Collins *et. al*., 1993), presenting low sensitivity in the early stages of the disease, although when the individual presents itself in the clinical phase, it reaches practically 100% sensitivity (European Commission 2000). Still, in relation to sensitivity, it hardly provides information in subclinical cases. When faecal samples are included from animals with clinical and subclinical infections from farms with a history of paratuberculosis, the sensitivity has been estimated at 36.4% (Zimmer *et. al*., 1999). The present research objected to ascertain the epizootiology of *M. avium* subsp. *paratuberculosis* in faecal and tissue

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smears from ovine-caprine individuals are suspect infected with this aetiology, predominantly, in simple-equipped laboratory, lacking of sophisticated infrastructure.

2. Materials and Methods

In the period between November 9th, 2017 and March 7th, 2018, samples of faeces and tissues were collected from 27 mature sheep and goat (of both sexes, and different breeds), whose presumptive infected with Johne's disease based on the clinical manifestations (diarrhoea, loss of body weight "up to cachexia in some cases", submandibular oedema "bottle jaw", and decrease of milk production). These animals represent nineteen commercial herds in numerous places in Northwestern area, Libya.

Faecal samples were collected aseptically and individually by direct extraction from the rectum with a sterile glove. Subsequently, they were introduced into a sterilized polypropylene tube, properly labelled. Tissue samples obtained immediately after death or sacrifice of moribund cases, and eviscerated tissues (ileocecal valve, ileocecal and mesenteric lymph nodes) of the animals were also reserved in sterile polypropylene containers. The specimens were kept refrigerated at 4°C. On the day of the samples receipt, a faecal smear was carried out in the laboratory, in order to determine the presence of mycobacteria. The smear was prepared on a glass slide occupying the entire surface of the same, with the help of a lingual depressor of wood, without any previous dilution. In the tissues, the scalpel was scraped with a scalpel blade, and in the case of the lymph nodes, by direct impression of the portion of the sample on the slide.

The preparations were stained according to the method of Ziehl-Neelsen (Zimmer *et. al*., 1999). The smears were fixed to the flame and then covered with the phenolic fuchsin dye. The slides were heated for five minutes, until vapors were released without boiling. After being washed in running water, the discoloration was done for 90 seconds with alcohol-acid solution. It was again poured into running water, and the contrast was assessed for three minutes with the malachite green dye. They were allowed to dry on filter paper and examined in at least 100 fields with an oil immersion objective (1000x). The smears were classified as negative or positive, according to the presence or absence of characteristic AFB (Gerish *et. al*., 2018): negative - absence of AFB compatible with *M. avium* subsp. *paratuberculosis*; and positive - presence of AFB of atypical morphology or large amount of AFB alone or presence of AFB aggregates compatible with *M. avium* subsp. *paratuberculosis*. Altogether, two laboratory technicians were included in the microscopic inspection of the specimens. To enhance the perfect reproducibility of the examination, every positive or suspicious smear was re-inspected microscopically by a second technician before a definitive conclusion on the analysis result was executed.

The data were evaluated and statistical comparisons were achieved by SPSS (SPSS for Windows, Version 17.0, Rainbow Technologies®). To compare the efficacy of the

techniques in the different samples, Choen's kappa coefficient (κ), which measured the non-random proportional concordance between two tests according to sample type [faecal or tissue smears] (Altman 1991) on the results obtained in two methods was used. A value of κ of 0.5 indicates a moderate level according to the techniques. A value of κ> 0.80 represents excellent non-random proportional agreement. For this calculation, WIN Episcope 2.0® program (Blas *et. al*., 2004) was used.

3. Results

Out of the 27 faecal smears examined, 18 (66.6%) showed clumps compatible with acid fast bacilli, being classified as positive. From 14 tissue smears examined, 10 (71.4%) presented acid fast bacilli compatible with MAP. The simultaneous application of the method in faeces and tissue samples presented better diagnostic value, analysing 12 animals as positive (85.7%). The results are shown in Table 1.

Table (1) Results of faecal and tissue smears of 27 animals demonstrate clumps compatible with acid-fast bacilli on microscopic examination after Ziehl-Neelsen staining

Result	Faecal smear		Tissue smear		Combination of both faecal and tissue smears	
	n	%	*n*	%	*n*	%
Positive	18	66.6	10	71.4	12	85.7
Negative	9	33.3	4	28.5	2	14.2
Sensitivity (%)		**66.6**		**71.4**		**85.7**

The technique revealed the largest number of positive samples in the ileocecal valve smear, ranking 4 of 5 smears (80%) as positive. From the mesenteric lymph nodes, 3 of 4 smears (53.8%) positive results were found. Moreover, the ileocecal valve presented a positive percentage of 33.3%. The results of the tissue samples are shown in Table 2.

Table (2) Positivity of *Mycobacterium avium* subspecies *paratuberculosis* in different tissue samples microscopic examination after Ziehl-Neelsen staining

Tissue	Positive/No. of Sample	%
Ileocecal valve	1/3	33.3
Ileocecal lymph node	4/5	80
Mesenteric lymph node	3/4	75
Total	10/12	83.3

According to the finding of combination of both faecal and tissue smears, the overall prevalence of paratuberculosis in sheep and goats was 7 (87.5%) and 5 (83.3%), respectively Table 3.

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Table (3) Illustrate the positivity rates (%) of paratuberculosis in sheep and goat according to different smear methods.

Method / Animal species	Faecal smear		Tissue smear		Combination of both faecal and tissue smears	
	No. of examining animals	No. of infected animals (%)	No. of examined animals	No. of infected animals (%)	No. of examined animals	No. of infected animals (%)
Sheep	14	10 (71.4)	8	6 (75)	8	7 (87.5)
Goat	13	8 (61.5)	6	4 (66.6)	6	5 (83.3)
Total	27	18 (66.6)	14	10 (71.4)	14	12 (85.7)

Table 4 shows the percentage of agreement and the result of the kappa index for the comparison of Ziehl-Neelsen method in the two types of samples. In 9 animals, the results of faecal smear and tissues were coincident. It is also possible to observe that the two faecal smear of an animal had compatible bacilli that were not observed in the tissue smear. Conversely, there was one animal had a positive result with Ziehl-Neelsen staining tissues, but it was not possible to observe compatible bacilli in faeces. The observed agreement was 71.4%, and the result of the index was equal to 0.45. A value between 0.41 and 0.60 represents a moderate agreement (Altman 1991).

Table (4) Comparison between results of ZN staining method in faecal smears and ZN staining method in tissue smears.

ZN staining method in faecal smears	ZN staining method in tissue smears		
	+ (n)	- (n)	Total
+ (n)	9	2	11
- (n)	1	2	3
Total	10	4	14

4. Discussion

Although the number of samples was limited, the sensitivity presented individually and in combination by Ziehl-Neelsen method in faeces and tissue smears was elevated (87.5% in sheep, and 83.3% in goat), higher than the 36.4% observed in faeces by Zimmer *et. al.*, (1999), in a study of infected cattle. The diagnosis of paratuberculosis in small ruminants is particularly difficult. There is no benchmark examination to revealing all infected individuals. MAP cultivation is regarded as to be the most consistent reference standard procedure. However, the bacterial growth is slow, so the results of examination need a longer period up to at least several weeks to determine the infection. Serological tests combined with clinical, bacteriological examinations (Pavlik

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et. al., 2000; Gerish *et. al*., 2018) and histopathology (Perez *et. al*., 1997) have been used conventionally. In subsequent years, there has been an increase in molecular techniques in the diagnosis of paratuberculosis to solve the identification approach problem (McKenna *et. al*., 2005). However, compared to Ziehl-Neelsen (Acid-fast) method, these expensive and time-consuming techniques require specialised personnel. Ziehl-Neelsen stain method is fast, cheap and allows a first diagnostic approach that should not be ruled out, being within the extent of simple equipped laboratory (Gerish *et. al*., 2018). Ziehl-Neelsen staining of faecal/ tissue smears from suspected animals is used as a substitute option for reaching to diagnose with the alike restriction of least sensitivity. Hence, the determination of MAP in faeces or faecal cultures has been likely just in the excretors of individuals with restricted sensitivity (Gerish *et. al.,* 2018). The presence of isolated AFB from environmental sources is relatively common, which makes diagnosis difficult due to the low specificity of the technique (Gerish *et. al.,* 2018). The visualization of bacilli in faecal and tissue swabs is a rapid and sensitive test that provides good results in small ruminants. In the more advanced cases, there is no doubt when mycobacteria adopt a characteristic association of clumps, attributable to the preservation of the structure they had inside the macrophages (Cheng *et. al*., 2020). Although in cases where the bacterial load is low, it is possible that no bacilli were detected on microscopic examination, which could explain the negative results to Ziehl-Neelsen staining and positive results in other techniques.

Comparing the results obtained from the microscopic examination of faeces and tissues by Ziehl-Neelsen method, we observed differences that can be elucidated in numerous approaches. The demonstration of a positive sample in the faecal smear, and negative tissue smear, may reveal non-specificity of the microscopic examination. It may also be explained by the scraping for the impression to be done in the wrong place due to incorrect collection of the lesion fragments to be cultured in the case of diffused lesions (Gerish *et. al*., 2018). The observation of compatible bacteria in the tissues, but not in the faeces can be attributed to the intermittent excretion of small amounts of microorganisms in the faeces (Pavlik *et. al*., 2000) or because the bacterial load is greater in the tissues than in the faeces, facilitating observation of MAP from this material (Huda and Jensen 2003). As MAP is not homogeneously distributed in feces, faecal smear results may vary, especially in poor excretors (Visser 1999). The results of our study, exhibit that the faecal smear was the method with the lowest sensitivity (66.6%), detecting the lowest number of positive samples. From the tissues (ileocecal valve, ileocecal lymph node, and mesenteric lymph node), 10 animals (71.4%) were classified by Ziehl-Neelsen method as positive, and 4 as negative (28.5%). In spite of 14.2% of the animals were not detected by Ziehl-Neelsen method in different examined smears and despite the small sample size, the findings of this report strengthen the value of rapid microscopic examination in sheep and goats with disease-compatible semiology

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as a first technique easily performed. It is suggested to be used as a routine diagnostic method regardless of the use of other microbiological techniques. These results also emphasise the need to use Ziehl-Neelsen technique as a method of rapid confirmation of clinical cases submitted to necropsy (Gerish *et. al*., 2018). The faecal smear is an examination within the reach of the veterinarian who may make an initial observation of the animal under field conditions in the advanced stages of the disease. Furthermore, in combination with tissue smears during the perform post mortem techniques, it is very important to diagnose the infection in the herd. The findings of the current study reveal that Ziehl-Neelsen staining method is indicated for the fast detection of MAP existing in faeces and tissue smears. In the present study, Ziehl-Neelsen staining was superior for the diagnosis of tissue smears in ileocecal lymph node (80%). The preparations of this tissue gave the greatest number of positive results. This study attends on Ziehl-Neelsen stain method for detection of paratuberculosis. The PCR technique for straight identification of MAP in the faeces could provide a substitute option for the evidencing proof of a clinical tentative judgment, offering prompt results and a diagnostic sensitivity equal to microbiological cultivation (Alinovi *et. al*., 2009). Conversely, the expenses of a PCR technique are habitually relatively elevated than those of a serological test, as well as requiring a special equipment and trained persons. Furthermore, reach to a certified PCR analysis for standard diagnostic examining faecal specimens is until now unavailable in all countries where paratuberculosis is enzootic. In those situations, the serological tests present an inexpensive and credible, useful option to confirm of clinical paratuberculosis. Revealing of the infection relies on the existing of *Mycobacterium avium* subspecies *paratuberculosis* in the faeces or tissues by cultivation, serological or nucleic acid recognition methods. The choice of examination depends on the availability and rate of sensitivity needed at individual animal or herd level. The particular main trouble with paratuberculosis combat is the hardness of revealing the subclinically "shedder" infected animals. From this current study, we conclude that Ziehl-Neelsen stain method is suitable for use in emerging situation and is a good alternative to more sensitive testing at distant inaccessible locations, as well as in traditional laboratories. Furthermore, the high prevalence of paratuberculosis in examined animals that demonstrates a necessity for the application of paratuberculosis control plan in countrywide, based on testing, improving young stock administration, and intensification hygiene procedures. The hypothesis of MAP as a causal agent of CD arose from the identification of this pathogen by direct isolation from affected intestinal parts of CD patients. This clinical strain was also pathogenic for laboratory animals and was able to reproduce paratuberculosis in experimentally inoculated ruminants (Chiodini *et. al*., 1984). Nevertheless, since then multiple studies have been carried out to verify this association, with variable results where the identification of MAP has not constantly been achieved in immunocompromised

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patients and conversely, it has also been detected in healthy individuals (Grant 2005). With this background, obtaining definitive conclusions has become complex and has generated controversy regarding the association of biological agents and the development of this disease. Regardless of this, several research both at the molecular and epidemiological fields are motivating to consider in order to elucidate these questions.

Competing interests

The authors declare that there is no conflict of interest.

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التعرف على المتفطرة الطيرية النوع الفرعي نظيرة السُليّة في مسحات روث و أنسجة من مجترات صغيرة

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الملخص

داء نظير السل (مرض جون) هو مرض معدي مزمن غير قابل للشفاء يصيب المجترات، يمكن أن يقلل من الإنتاجية، و يصعب تشخيصه و مكافحته. هو ناتج عن عدوى بكتريا *المتفطرة الطيرية* النوع الفرعي *نظيرة السُليّة* (MAP). مرض نظير السل في المجترات يشبه من الناحية المرضية مرض البطن الإلتهابي Inflammatory Bowel Disease (IBD) في البشر، الذي يتضمن ثلاثة أشكال مرضية؛ مرض كرون Crohn's Disease (CD)، إلتهاب القولون التقرحي Ulcerative Colitis (UC) و التهاب القولون الغير محدد Indeterminate Colitis أو مرض التهاب الأمعاء غير المصنف Unclassified IBD. يجب الانتباه عن كون بكتريا *المتفطرة الطيرية* النوع الفرعي *نظيرة السُليّة* من مسببات الأمراض المشتركة حيوانية المنشأ Zoonotic، و هو أمر مقلق كثيرًا نظرًا لأن هذه البكتريا مقاومة للحرارة و قادرة على الاختباء داخل خلايا الدم البيضاء. ضمن الدراسة الحالية، تم فحص وجود عصيات صامدة للحمض Acid-Fast Bacilli متوافقة مع بكتريا *المتفطرة الطيرية* النوع الفرعي *نظيرة السُليّة* MAP في مسحات روث و أنسجة أغنام باستخدام تقنية صبغة زيل–نيلسن-Ziehl Neelsen Stain خلال الفترة من 9 نوفمبر 2017 و حتى 7 مارس 2018، ما مجموعه 27 مسحة روث، و 14 مسحة من الأنسجة مأخوذة من 12 رأسًا من الضأن، و 15 رأسًا من المعز، أشتبه بإصابتها بمرض نظير السل (اعتمادًا على العلامات الإكلينيكية). هذه الحيوانات تمثل 19 قطيعًا تجاريًا في مواقع مختلفة بشمال غرب ليبيا. أظهرت ثمانية عشر (66.6٪) مسحة روثية وجود تكتلات متوافقة مع العصيات الصامدة للحمض. عشرة حيوانات (71.4٪) كانت ايجابية اعتمادًا على وجود نفس الكائنات الحية الدقيقة في مسحات الأنسجة. بالإضافة إلى ذلك، كانت نسبة إيجابية مزيج كلاً من مسحات الروث و الأنسجة 85.7٪. إضافة إلى ذلك، تشير نتائجنا إلى أهمية العقدة الليمفاوية اللفائفية Ileocecal lymph node كنسيج مستهدف للكشف عن بكتريا *المتفطرة الطيرية* النوع الفرعي *نظيرة السُليّة*. نتائج الدراسة الحالية كشفت أن إجراء صبغة زيل–نيلسن موصى به للتعرف السريع على هذه البكتريا الموجودة في الروث وا لأنسجة. علاوة على ذلك، أظهر معدل الانتشار المرتفع هنا الحاجة إلى تطبيق برنامج محكم للسيطرة على مرض نظير السل في المجترات الصغيرة، يؤسس على اختبارات أكثر حساسية، تحسين رعاية الأغنام اليافعة، و زيادة إجراءات الأمن الحيوي.

الكلمات المفتاحية: *المتفطرة الطيرية*– داء نظير السل – الضأن، المعز – الأمراض المشتركة حيوانية المنشأ – مرض كرون – إلتهاب القولون التقرحي.