

Pathology of subclinical paratuberculosis (Johne's Disease) in Awassi sheep with reference to its occurrence in Jordan

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ABSTRACT: In this study, the pathological lesions and occurrence of subclinical Johne's disease in Awassi sheep is investigated using histopathological (HP) and immunohistochemical (IHC) examinations, Acid Fast Staining (AFS) of tissue sections, direct smears from tissues and culture. Histopathological examination of 202 ilea and the corresponding mesenteric lymph nodes (179) was conducted. In addition, IHC examination, using rabbit polyclonal antiserum, of 134 ilea and 123 mesenteric lymph nodes was also conducted. The occurrence of the disease was observed in 50% and 93% of the ilea examined using histopathology and IHC techniques, respectively. Fifty nine percent of lymph nodes were positive by IHC. The histopathological lesions were graded from I–IV, I being the least severe, based on the type of cellular infiltrate (lymphocytes, macrophages and epithelioid cells) and the severity of the lesions. Grades III and IV (SP) were considered positive while I and II were considered suspected. Analysis of the results also revealed that the majority of suspected cases (grades I and II) reacted positive with the IHC. Furthermore, the IHC reactions were classified into three categories depending on the number of stained cells and the intensity of the staining (I-mild, II-moderate and III-strong). Direct smears, and tissue sections obtained from the ilea and stained with AFS revealed that out of 202 tissue samples, 53 (26%) and 22 (11%) were positive, respectively. Results of the culture revealed that 22 (11%) out of 202 were positive. These results showed that the occurrence of paratuberculosis (Johne's disease) in Awassi sheep is very high in Jordan and needs further study in order to develop rational methods of control effective for the Jordanian sheep population.

Keywords: sheep; paratuberculosis; histopathology; acid fast stain; ileum; immunohistochemistry

Paratuberculosis (Johne's disease) is an infectious disease of ruminants, considered by many researchers to be one of the most serious infectious diseases currently plaguing the world's sheep, cattle and goat industries (Hope et al., 2000). An increasing number of publication highlights the importance of this animal infection with a zoonotic potential (Kaevska and Hruska, 2010). The disease caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), a gram-positive acid-fast organism shed in the faeces of infected animals, is characterized by chronic granulomatous enteritis (Jubb et al., 1993; Radostits et al., 1994; Adams et al., 1996).

Contaminated feed and water, bedding and soiled udders are thought to be the major routes for spread

of the organism and young animals less than six months of age are thought to be the most susceptible to infection. Cattle become infected early as young calves via faecal-oral transmission of the infectious agent (Stabel et al., 2002). Intrauterine infection in cattle has also been well documented (Martin and Aitken, 1991; Whittington and Windsor, 2009). Isolation of *M. paratuberculosis* from a sheep foetus has been confirmed, and antibodies to *M. paratuberculosis* have been demonstrated in colostrum-deprived lambs (Jones et al., 1997; Lambeth et al., 2004).

Focus on paratuberculosis has been intensifying as it is becoming an economically important disease for the livestock sector. It was reported that

the economic losses due to Johne's disease for the control and eradication program in one flock with a few cases would average \$650 with most of that cost going for serological tests. The cost of disease for ovines is approximately \$90 per clinical case and \$250 per bovine clinical case (Menzies, 2010). The economic effect of Johne's disease consists in the restriction of livestock marketing and contamination of land. In Iceland, for example, measures to be taken for the control of paratuberculosis are expensive, involving the fencing of hundreds of kilometres to restrict sheep movement from the infected area. Laboratory tests used to detect infected animals are also costly and notwithstanding all these expenses it is very difficult to eradicate the disease (Fridriksdottir et al., 2000).

In addition, consumers of animal products are becoming increasingly sensitive to the possible effect of livestock disease and residues on product quality and human health. The potential link between Crohn's disease (CD) and *M. paratuberculosis* is an example of such a potential relationship (Kennedy and Allworth, 2000; El-Zaatari et al., 2001). Recent data have strengthened the etiologic association between *M. paratuberculosis* and CD (Naser et al., 2000). Milk has been suggested as a possible vehicle of transmission of *M. paratuberculosis* to humans as *M. paratuberculosis* has been detected by PCR in raw milk as well as from pasteurized milk from cattle, sheep and goats, repeatedly in England (Millar et al., 1996; Ellingson et al., 2005; Hruska et al., 2005; Zoi Dimareli-Malli, 2010).

It has been suggested that the diagnosis of subclinical cases of paratuberculosis is still a problem (Kurade et al., 2004). It was also reported that the diagnosis of subclinical cases of paratuberculosis is more difficult by histopathology as lesions may be subtle and organisms may be low in number (Buergelt et al., 2000). In sheep, on the other hand, it has been reported that histopathology was found to be a better indicator of paratuberculosis than bacteriology (Kurade et al., 2004). Furthermore, it was suggested that histopathological studies of tissues from infected animals can be considered as the gold standard method for the diagnosis of Johne's disease and should be used to confirm the disease instead of other methods (Hope et al., 2000). However, expertise and diagnostic criteria may vary between institutions (Huda and Jensen, 2003). Therefore, accurate subclinical diagnosis needs more than one test to complement the histopathological diagnosis.

To estimate and understand the occurrence of Johne's disease in sheep, we conducted a field study of twenty sheep flocks, ranging from 50–500 heads and found that 80% of the flocks had a history of untreatable emaciation, bottle jaw and diarrhea which were associated with animal losses. Therefore, this investigation was carried out to study the associated pathological lesions of Johne's disease in the ilea and their corresponding mesenteric lymph nodes, and its prevalence in apparently healthy young Awassi sheep in Jordan, using gross/histopathological, immunohistochemistry (IHC), Acid Fast Stain (AFS) examinations and culture.

MATERIAL AND METHODS

Animals/Awassi sheep

Awassi sheep included in this study were located at 36°66'E, 32°04'N at about 616–686 m above sea level. During the period of this study, annual rainfall varied between 159 and 380 mm. Rainfall usually starts in November and ends by April. No precipitation occurs between May and November. Daily temperature is moderate with mean monthly maxima ranging between 12 and 32 °C and mean monthly minima between 2 and 14 °C. The mean monthly relative humidity ranged between 44 and 80%. The raising system of sheep in Jordan is mainly semi-intensive; farmers usually feed their animals chopped straw, barley and wheat bran during the winter season (October to February). During spring and summer seasons, the animals are maintained on grazing. Some farmers provide concentrate supplementation in seasons when the grazing grass is not adequate. The Awassi sheep breed is raised for meat, milk and wool production and it represents almost 98% of the sheep breeds in the country (Ministry of Agriculture, 2000).

Two hundred and seventy nine apparently healthy Awassi sheep of both sexes, 8–24 months of age, raised in different flocks in Jordan, were slaughtered and examined in the slaughterhouses of the Central (represents 32% of the national flock of 1.9 million sheep) and Northern (represents 45% of the national flock) parts of the country (Ministry of Agriculture, 2000). The animals had no signs of emaciation, bottle jaw and diarrhea, alone or in combination. The site for animal selection in the slaughterhouses was based on the sheep populations and distribution in Jordan. Animal movement

is free and the sheep are bought from the sheep farmers in local animal markets by the local butchers or animal traders twice a week; on Mondays and Thursdays. Sheep are either sent directly to the slaughter house or fattened for some time beforehand. Different sheep farmers bring their animals from different sites of the country; in some cases they transport them about 150 Km or more to bring them to the animal markets. In some cases, sheep traders visit sheep farmers and buy sheep from the farms directly before sending them to the slaughter house or for slaughter in local butcheries.

Tissue sample collection

During a period of five months (August to December 2001), a total of 279 apparently healthy Awassi sheep were subjected to postmortem examination in slaughterhouses where small and large intestines were examined thoroughly. The slaughterhouses were visited weekly to collect the samples. Before slaughtering, complete information about the animals regarding age, sex, significant clinical signs and health status were recorded. The age of the sheep ranged from 8 to 24 months. The majority of the sheep examined were males.

Gross examination and samples transportation

Samples from small and large intestines and respective lymph nodes of the 279 animals were thoroughly examined for any apparent gross lesions. Significant gross lesions including mucosal thickening, mucosal corrugations, prominent lymphatics and intestinal discolorations were recorded. The size, shape and discoloration of the respective lymph nodes were carefully described. Out of the 279 animals examined, 202 animals with some gross changes were sampled and tissues were collected from their ilea, ileocecal valves and respective lymph nodes based on tissue changes seen in these organs. In the few cases in which gross lesions were observed in the large intestine, tissue samples from the cecum and colon were collected. Tissues samples were trimmed to a smaller size (4 mm to 1 cm thickness), fixed in 10% buffered formalin, and subjected to histopathological examination (202 ilea and 179 lymph nodes) and immunohistochemical staining (134 ilea and 123 lymph nodes).

Other tissue samples were transported on ice to the laboratory for direct smear (202 ilea) and for bacterial culture (202 ilea). Samples for culture were kept at -20°C until needed.

Direct smear staining with acid fast stain

Scrapping of the intestinal mucosa and lymph nodes parenchyma were carried out using scalpel blade and smears were done on microscopic slides. Slides were dried by air, fixed with methanol (100%) and stained with AFS method according to Coles (1986). Each slide was examined for 30 min using oil immersion. The findings were registered according to the bacteria appearance, in which observation of bacteria's in clumps taken as positive, in dispersed form as suspected and as negative if neither of the two forms observed.

Culture

Media preparation. Middle Brook 7H10 agar base M199 (Himedia Laboratories, limited Mumbai (Bombay) 400086, India) was used with a supplement as a slant in tubes of 20 ml. The media was prepared as described in the OIE (2000). To 450 litres of distilled water, 9.73 g was added. Then, 5 ml of glycerol was added to this mixture which was boiled, autoclaved at 121°C , 15 psi, for 15 min, and then cooled at 45 to 50°C in a water bath (GFL 1083, Germany). Middlebrook OADC growth supplement (FD 018, India, 50 ml/l) was then added. For the suppression of contaminants, Penicillin (1000 000 IU) and the anti-fungal agent Nystatin (50 mg) were added to the media. The media containing mycobactin was prepared by adding mycobactin J 2 mg/l (Allied Monitor IAC, Fayette, MO, USA) of the medium dissolved in 4 ml of ethyl alcohol with the agar base before autoclaving.

Sample preparation and inoculation. Approximately 3–5 g of scraped intestinal mucosa and lymph node parenchyma was taken and ground together using mortar, and incubated with 0.5% trypsin at 4°C overnight at $\text{pH} = 7.2$ to 7.4 (adjusted using 4% NaOH). After 16 to 8 hours, the mixture was filtered using a gauze (folded), and centrifuged at $400 \times g$ for 20 min (PK110, ALC, Italy). The supernatants were decanted and decontaminated and 5% oxalic acid was added to the pellet. The samples were then left for 24–30 h at room temperature.

Finally, 0.1 ml (100 µl) of inoculum was taken carefully from the bottom of the tube and evenly distributed on the media. Each sample was inoculated in three tubes (one tube without mycobactin J and two with mycobactin J) and incubated at 37 °C (Binder GmbH, Germany) with a loss screw and inclined to facilitate the evaporation of excess moisture and inoculum fluid for one week. After one week tubes were returned to the vertical position with a tightened screw and incubated for 16 weeks.

Culture reading. Starting from eight weeks of inoculation, cultures were observed weekly for the presence of any growth. At 16 weeks, smears were taken from cultures that showed growth, and stained by AFS as described previously (Prophet et al., 1994). A culture was considered positive when white spot colonies were seen and this was confirmed by AFS. Slides were observed under 100 × objectives for the detection of acid fast bacilli. Results were recorded considering the long incubation period, the colony appearance, and acid-fastness of the bacteria. Nine tubes with micro-fungal growth and contamination were discarded.

Histopathological examination

All the trimmed samples were processed routinely for histopathological examination according to a protocol described previously (Bancroft and Stevens 1990). Sections (4–5 µm) were cut and stained with Haematoxylin and Eosin (H&E). Staining of tissue sections by AFS was performed as described previously (Prophet et al., 1994).

Grading criteria for histopathological lesions

The H&E stained sections from 202 ilea and the corresponding lymph nodes (179) were observed

under 4×, 10×, and 40× objectives. Pathological lesions were recorded. The lesions were classified and graded into grades I, II, III and SP, depending on the type and amount of the cellular infiltrates (lymphocytes, macrophages and epithelioid cells). The intestinal tissue sections were considered positive for paratuberculosis when there were macrophage infiltrations or epithelioid cells obvious in the lamina propria of the villi and between crypts, and there was involvement of the Peyer's patches as demonstrated by the presence of starry sky macrophages/tingible body macrophages or micro-granuloma. In cases where there was no microgranuloma, but macrophages and some epithelioid cells were present, these were considered suspected cases by histopathological criteria. The scoring of tissue lesions was as shown in Table 1. Therefore, grades I and II were considered suspected while grades III and SP were considered positive.

Immunohistochemical (IHC) staining

Tissue samples from paraffin-wax embedded blocks were sectioned at 2–3 µ prior to immunohistochemical staining. The sectioned tissue samples were laid on Vectabond (DAKO A/S.Glostrup, Denmark) coated slides, air-dried and then heated in an oven at 55 °C for two hours. Tissue sections were deparaffinized in xylene and hydrated by sequential immersion of slides in decreasing concentrations of ethanol (100%, 95% and 70%) for one minute each. Tissue sections were washed in distilled water for 5 min, then washed in PBS, followed by immersion in citrate buffer solution (pH= 6, 10mM). Antigen retrieval was carried out by autoclaving the tissue section at 120 °C, 15 psi for 15 min (Express, Italy). The sections were cooled at room temperature and washed in PBS for 5 min. All of the subsequent incubations were performed at room temperature, and all washes were in PBS (pH 7.4, 3×; 5 min each).

Table 1. Criteria used for grading pathological lesions found in the ilea and lymph nodes

Grade	Type of inflammatory cells				
	lymphocytes	macrophages	epithelioid cells	PP prol. and crypts replacement	microgranuloma
I	++++	++	+	–	–
II	+++	++++	+++	moderate	yes/no
III	+	+++	++++	severe	yes
SP	+	++++	++++	calcification, caseous necrosis in the LN	granuloma with giant cells

SP = special, PP prol. = Peyer's patches proliferation, LN = lymph nodes

Endogenous peroxidase was inactivated by immersion of the slides in a solution of 15% hydrogen peroxide in methanol for 30 min. After washing, non-specific adherence of proteins to tissue sections was blocked by incubating with 1% bovine serum albumin (BSA), (Sigma Chemical Co., St.Louis, MO) for 2 h. The solution was drained from the slides and the rabbit polyclonal *M. paratuberculosis* antiserum # 270, diluted 1 : 500 in PBS, was incubated on the slides for 2 h. The rabbit antibody was kindly provided by Dr. Stabel from the National Animal Disease Center, Ames, IA, USA. This was followed by further washing and incubation with biotinylated anti-goat, anti-rabbit, and anti-mouse immunoglobulin (DAKO A/S, Glostrup, Denmark), diluted at 1 : 20, as the secondary antibody for 15 min. After washing, streptavidin-biotinylated peroxidase conjugate (DAKO, A/S, and Glostrup, Denmark) was applied, and incubated on the tissue section for 15 min. The slides were washed and incubated with chromogen (3,3 diamino- benzidine-4HCL; DAB, DAKO) at 1 mg/ml in PBS supplemented with hydrogen peroxide (10 ul of 3% hydrogen peroxide for 2 ml of DAB). Samples were incubated at room temperature for 3–5 min, and slides were then washed in distilled water for five minutes. Slides were then counter stained in haematoxylin for 2–3 min. The sections were washed in water and dehydrated in graded alcohol and cleared in xylene and mounted using DPX for further observation.

Slides were observed using 4×, 10× and 40× objectives. Sections were considered positive according to the color observation that is an indication of antibody-antigen reaction, and manifested by intra-cytoplasm or extra-cellular brown coloration in different areas of the stained tissue sec-

tion. The findings were registered by counting the number of reactions at 10×, accordingly starting from one cell reaction recorded as positive; 1–10 as + (mild), more than 10 but less than 50% cells reaction as ++ (moderate), reaction in 50% cells from one tissue section was graded as +++ (strong as in Figure 6). And more that 50% reaction in the macrophages of one section as +++++, but because only one sample was +++++, it was added to the +++ group. Additionally the intensity of the reaction was considered and in all cases only a strong brown color was recorded as a positive reaction. If the above criteria were found in at least one field, it was considered positive. At least one slide from each tissue section from the ilea sampled from each animal was tested. In some cases, more slides were taken from each tissue preparation.

In all cases, positive and negative control slides were processed together from the same known group of tissue sections, in order to avoid false positive and negative reaction. The positive control tissue was obtained from a sheep clinically suffered from MAP as diagnosed by histopathology, AFS staining, IHC and culture. The negative control was obtained from a three-month old lamb which was healthy and negative by all the four tests.

RESULTS

In this study, gross/histopathological, immunohistopathological examinations, and AFS (tissue sections and scrapping) of the ilea and corresponding lymph nodes of 202 clinically healthy Awassi sheep, which were slaughtered during the study period, were used to assess, describe and grade the histopathological picture and examined the tissue

Table 2. Grade of histopathological findings, from ilea and corresponding lymph node H&E tissue sections in apparently healthy Awassi sheep aged 8 to 24 months in Jordan, 2002

Type of tissue	Samples processed	Grade of the lesions					
		I	II	III	SP	Total	
						+ve	–ve
Ileum	202	23	70	98	4	195	7
	%	(11)	(35)	(48)	(2)	(97)	(3)
Lymph nodes	179	10	35	76	4	125	54
	%	(5.5)	(19.5)	(42)	(2.2)	(69)	(31)

The percentage was taken by considering the decimal number > 5, adding one in all the results SP = special lesion, numbers in parentheses are percentiles

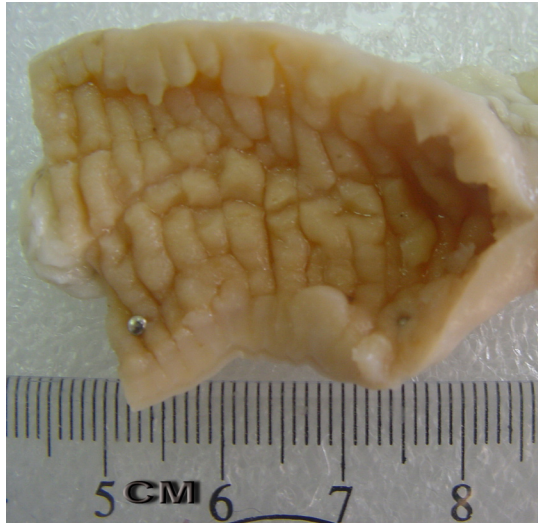


Figure 1. Gross pathology of an ileum section from apparently healthy sheep. Corrugations and thickening of the mucosal wall are prominent

reaction regarding the presence of *Mycobacterium avium* subsp. *paratuberculosis* for the purpose of diagnosing the disease in its subclinical presentation. Culture was also used to confirm the diagnosis.

When the intestines and the corresponding lymph nodes were examined grossly in the slaughterhouses, we found that 202 intestines or lymph nodes out of the 270 animals examined harboured some sort of pathological changes. These changes ranged from very mild to severe congestion in the mucosa and thickening in the wall of the ilea. The wall of the ilea was very thick with corrugation in 13 animals (Figure 1). The surrounding mesenteric lymph nodes

were enlarged and oedematous in few cases, and they were connected with each other as a result of their tissue reaction and appeared as cords.

Histopathological examination of H&E tissue sections from the 202 ilea and corresponding lymph nodes revealed that 195 (97%) ilea had variable increases in the thickness and congestion of the mucosa due to inflammatory cell infiltrations (Table 3). The mucosa and less frequently the sub mucosa were infiltrated primarily with variable numbers of macrophages, lymphocytes and plasma cells (Figures 2 and 3). Occasional numbers of epithelioid cells were also seen. They were present either as a scattered form in the lamina propria of the villi and between the crypts or as nests. Multinucleated giant cells were rarely seen in the affected tissues. In some cases the cellular infiltrates were associated with caseous necrosis and/or mineralization. Variable degrees of Peyer's patch hyperplasia were noted in many tissue sections with prominent starry sky macrophages/tingible body macrophages (Figure 3). In most cases, granulomatous lymphangitis with or without dilated lacteals were present (Figure 4). The villi exhibited different changes including: villous distortion and thickening by inflammatory cell infiltrations, villous atrophy and fusion. The lymph node changes consisted primarily of lymphofollicular hyperplasia and infiltration of mononuclear cells predominately with epithelioid cells (Figure 5). The most severely affected lymph nodes were characterized by granulomas formation with mineralization. In less severely affected lymph nodes, epithelioid cells mixed with

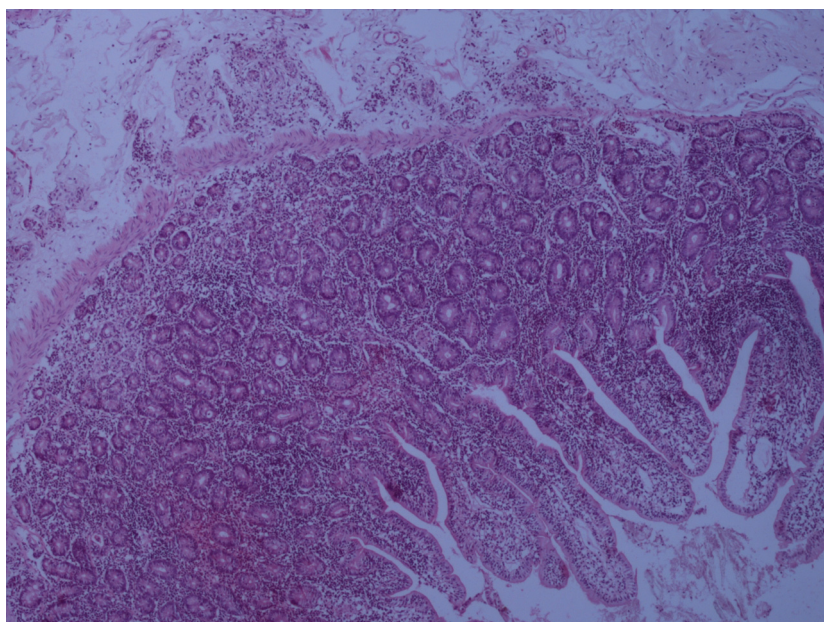


Figure 2. Histopathological section of the ileum from apparently healthy/subclinically infected sheep. Payer's patches lymphoid hyperplasia is seen throughout the section. H&E stain, 4×

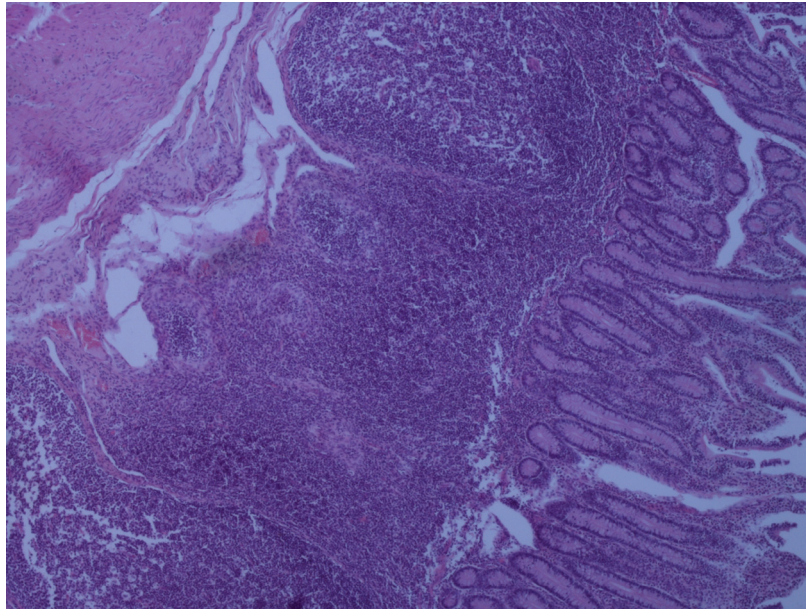


Figure 3. Histopathological section of the ileum from apparently healthy/sub-clinically infected sheep. Mononuclear cells infiltrate primarily the mucosa, and less frequently the submucosa. Dilation of some of the lacteals in the villi is also seen. H&E stain, 4×

other mononuclear cells and a few neutrophils were scattered throughout the lymph node sections.

Table 2 shows the grading of the lesions seen in the intestine and the lymph nodes. Out of the 202 intestinal H&E tissue sections examined, 23 (11%), 70 (35%), and 98 (48%) were graded as I, II and III, respectively, based upon the grading criteria described; namely the degree of lymphocyte, macrophage, and epithelioid cell infiltration; cellular proliferation within the Peyer's patches and crypts; and the presence of microgranuloma. Four cases were considered as a special category where calcification with severe reaction and typical granulomatous reaction with multinucleated Langhans giant cells were observed in the lymph nodes. The cases in grades III and SP (IV) were considered positive while the cases in grades I and II were considered suspected for the disease by histopathological evaluation.

To further understand the cellular reaction in the intestine and in the respective mesenteric lymph nodes and to investigate whether this reaction was associated substantially with the presence of *M. paratuberculosis*, immunohistochemical examination was used. A positive reaction was manifested by the presence of brown colour around the macrophage nucleus (perinuclear and intracytoplasmic reaction) (Figures 6 and 7). Table 3 shows the grading of the lesions seen in the intestine and the lymph nodes as the tissue samples were examined by immunohistochemistry. We examined only 134 ilea tissue sections and 123 tissue sections from the corresponding lymph nodes, and because of the limitation in the reagents, we did not examine all tissue specimen. We found that 124 (93%) tissue sections from the ilea were positive, ranging from mild (+) moderate (++) and strong (+++); 108 (81%), 9 (7%), 6 (4%) and 1 (1%) were graded as +, ++, +++.

Table 3. Immunohistochemical stain results from intestinal samples and lymph nodes in apparently healthy Awassi sheep, 8 to 24 months, in Jordan 2002

Type of tissue	Number of samples processed	Immunohistochemical stain				
		+	++	+++	total	
					+ve	–ve
Ileum	134	108	9	7	124	10
	%	(81)	(7)	(5)	(93)	(7)
Lymph node	123	55	10	7	72	51
	%	(44)	(8.1)	(5.6)	(58.5)	(41.4)

Numbers in parentheses are percentiles

Table 4. Distribution of Histopathological (HP) (H&E), Immunohistochemical (IHC) and Acid Fast Stain (AFS) used on histopathological tissue sections (AFS/HP) and AFS Direct Smears (AFS/DS) and Culture results from intestinal specimens of apparently healthy Awassi sheep, 8 to 24 months, in Jordan 2002

Type of test	Number of samples			Total
	positive	suspected	negative	
HP/H&E	102	93	7	202
%	(50)	(46)	(4)	(100)
IHC	124	0	10	134
%	(92)	(0)	(8)	(100)
AFS/HP	22	0	180	202
%	(11)	(0)	(90)	(100)
AFS/DS	53	99	50	202
%	(26)	(49)	(25)	(100)
Culture	22	0	180	202
%	11	0	89	(100)

and +++++, respectively, based upon the number of cells which reacted positively with the antibody (Table 3). Because only one sample was in grade 4, it was added to grade 3. Out of the 123 tissue sections from the lymph nodes tested by IHC, 72 (58.5%) sections reacted positive (Table 3). Fifty five (44%) out of 123 lymph node tissue sections showed mild 1+, 10 (8%) moderate 2+ and 7 (6%) strong 3+. Interestingly, seven lymph node samples which

were negative by IHC were graded as 2+ and 3+ in the corresponding intestine by IHC. In either tissue sections of the ilea or their corresponding lymph nodes, if the tissue section shown 1+ or more, the tissue section (sheep) was considered positive for Johne's disease by IHC.

The IHC staining of the tissue sections from the ilea was studied also to find the distribution of the stained macrophages in different histological parts

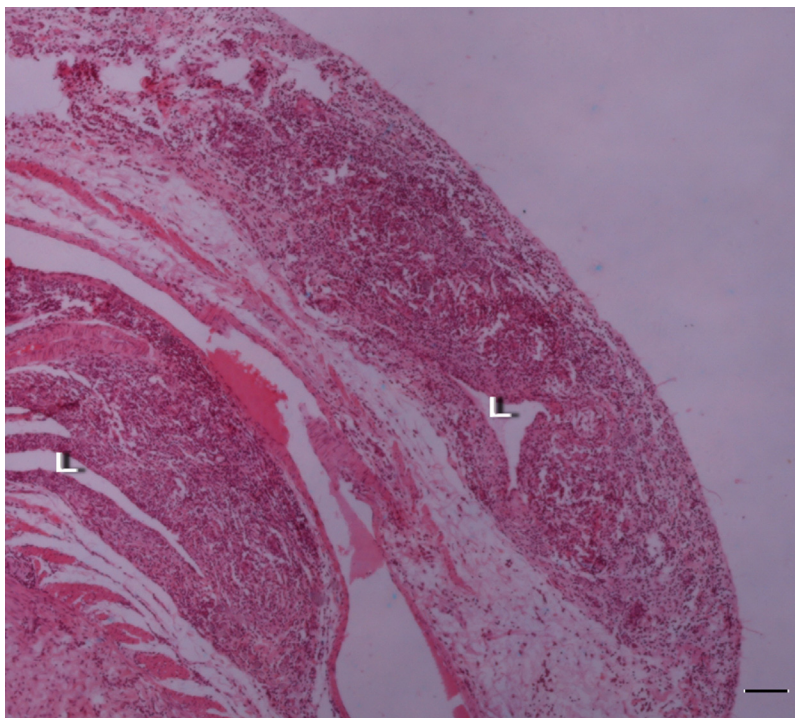


Figure 4. Histopathological section of the ileum (muscularis and serosa) from apparently healthy/subclinically infected sheep. Granulomatous lymphangitis is shown where mononuclear cells infiltrate the wall of the lymphatics. H&E stain, L = lymphatic vessels, bar = 100 μ

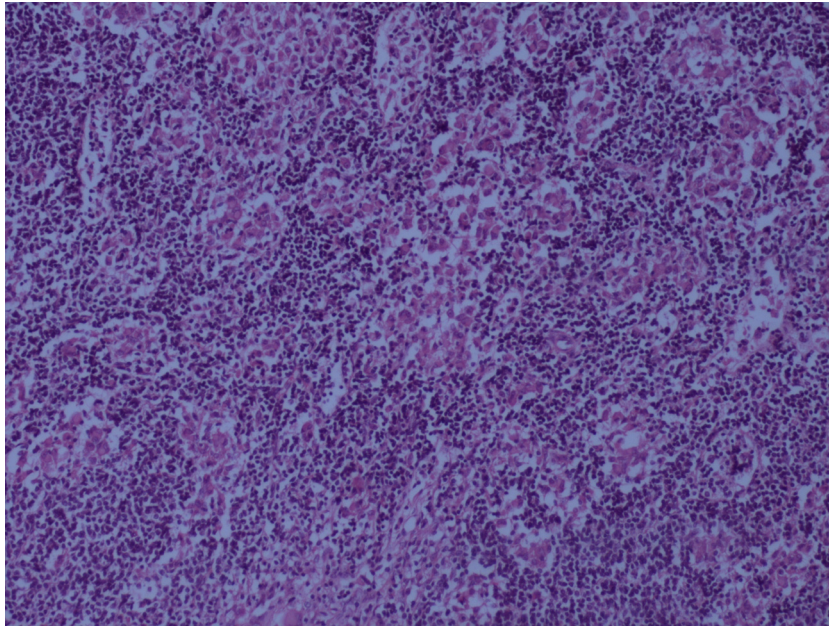


Figure 5. Histopathological section of the lymph node from apparently healthy/subclinically infected sheep. The lymph node is heavily infiltrated with mononuclear/epithelioid cells. H&E stain, 10×

of the tissue. In the intestinal tissue sections, the reaction was observed more in the macrophages infiltrated in the lamina propria between the crypts. The Peyer's patches showed less staining than the LP, while reactions were rarely seen in the LP of the villi. The reactions in the lymph nodes were commonly observed in the paracortex area dispersed between the cortex and medulla.

Analysis of the results pertaining to the AFS of direct smears from the 202 intestine samples revealed that 53 (26%) tissue samples were positive as the bacteria appeared in clumps. About 99 tissue

samples (49%) were suspected, where the bacteria were seen in the dispersed form and 50 (25%) slide smears were negative. When the tissue samples from the ilea (202 samples) were sectioned and stained by AFS, acid fast bacilli were seen only in 22 (11%) samples. The bacilli were seen either inside the cytoplasm of the macrophages or free outside the cells. Furthermore, out of the 202 intestine samples cultured, only 22 (11%) were positive. When these samples were traced, we found that they were in grade II and III by histopathological examination and 1+ by immunohistochemical staining.

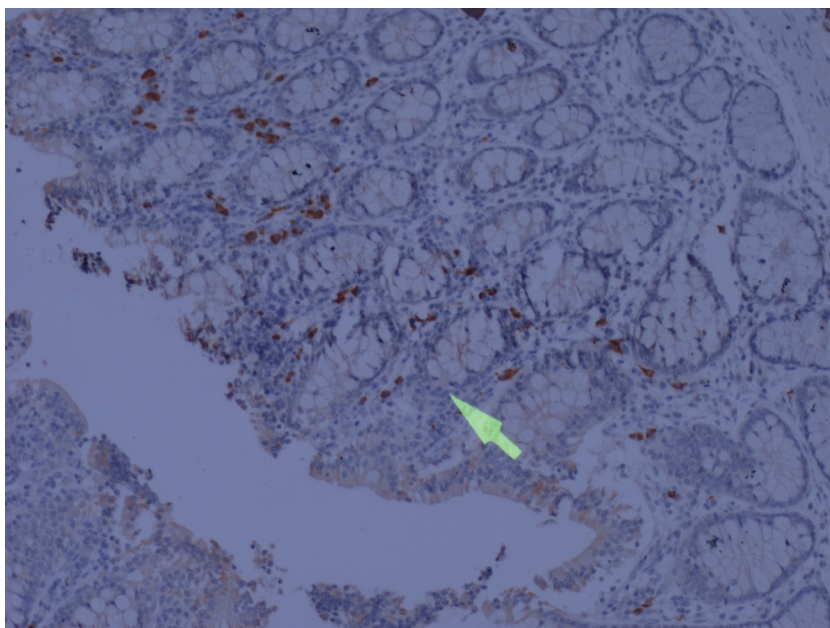


Figure 6. Histopathological section of the ileum from apparently healthy/subclinically infected sheep. The section was stained using an immunohistochemistry staining technique with a rabbit polyclonal anti-body against *M. paratuberculosis*. Bacterial antigens were detected in the cytoplasm of macrophages as dark brown staining. IHC staining, 10×

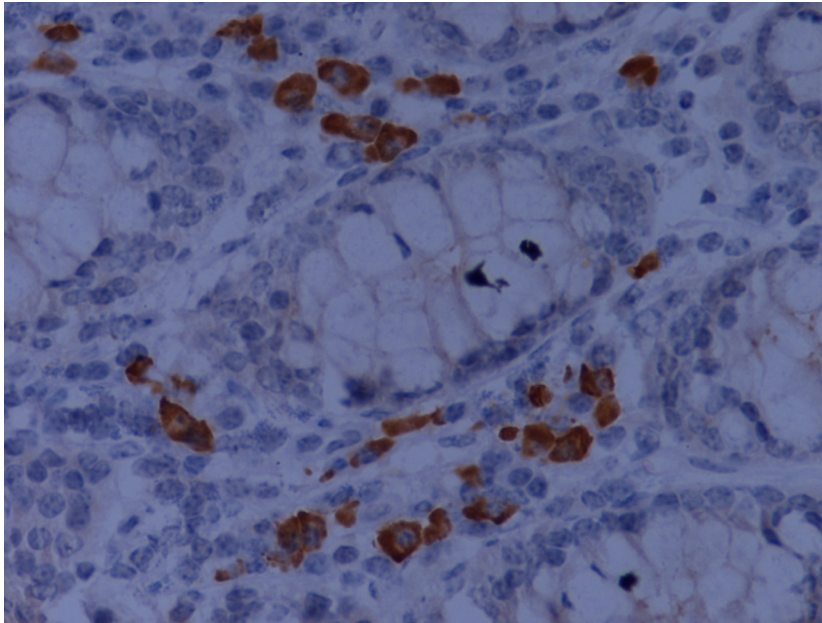


Figure 7. Histopathological section of the ileum from apparently healthy/subclinically infected sheep. This is a higher magnification view of figure six. IHC staining, 40×

DISCUSSION

In the present study, the pathological lesions and the occurrence of subclinical Johne's disease was studied in apparently healthy Awassi sheep between the ages of 8 to 24 months and with no history of clinical Johne's disease using multiple diagnostic tests, histopathological and immunohistochemical examination, AFS and culture. Studies reporting previously on subclinical cases of Johne's disease have demonstrated the importance of tissue sample selection from different sites and it was found that sampling from limited foci can influence the results (Fraser et al., 1999; McDonald et al., 1999). Therefore, the selection of tissues from different sites along with the adjacent lymph nodes should be done to confirm the diagnosis of subclinical Johne's disease, with the ileocecal valve as the first site to be selected. It was also reported that histopathological examination should be considered as the first option for the diagnosis of subclinical cases. In our study, the last portion of the ileum and the ileocecal valve with their adjacent lymph nodes were utilized as the sample site and were used for all the implemented tests in this study.

Examination of the ilea and the corresponding lymph nodes from the clinically healthy Awassi sheep in the slaughter houses revealed that seventy-two percent (202 out of 279 examined) of them showed very mild to severe thickening and congestion in the intestine, especially in the last portion of the small intestine (Figure 1). Inflamed, oede-

matous, enlarged and corded mesenteric lymph nodes around the ileum and ileocecal valve were also evident. The histopathological findings such as the infiltration of the mucosa and submucosa with lymphocytes, macrophages, replacement of the crypts with macrophages, Peyer's patches proliferation extending towards the mucosa and the presence of microgranuloma is partially in agreement with the results of others (Perez et al., 1996; Watkins et al., 2002). The grade 1 lesions noted in our study (11%; dominated by lymphocyte infiltration) is in agreement with the asymptomatic form and to some extent with the paucibacillary form, while grades 2 and 3 (83%) are in agreement with the multibacillary form previously reported, keeping in mind that about 30% and 70% of the last two forms affected clinical cases, respectively (Watkins et al., 2002). This is in contrast to our study where the Awassi sheep were young and apparently healthy with no clinical signs in spite of the presence of marked macrophage infiltration and granulomatous reactions (42% of ilea) and with granuloma, Langhan giant cell formation and calcification (2% of animals) (Table 2). It has been reported that the paucibacillary form of the disease occurs particularly in sheep and in such animals *M. paratuberculosis* cannot be detected in the tissue microscopically and usually cannot be cultured (Hermon-Taylor, 1998). The grade 1 lesion in this study is close to the type 1 lesion in a study reported from South Africa although that study did not focus on lymphocyte infiltration and concentrated more

on the epithelioid cell nest in the mucosa and sub-mucosa (Micheal and Bastianello, 2002).

Paratuberculous lesions have been classified into three types (1, 2 and 3a, 3b, 3c) disregarding the clinical signs (Perez et al., 1996, 1999). In these classifications, epithelioid cells were not encountered, and lymphocyte infiltrations were associated mainly with lesion type 3c. This classification was based mainly upon the presence of macrophages and acid-fast bacteria. In our study, only 22 (11%) of histopathological tissue sections revealed the presence of acid-fast bacilli and 26% were positive on the direct smears (Table 4). Most of the 22 samples were grade 3 and a few were grade 2, with one, two or three intra-cytoplasmic bacilli observed within the macrophages. In some cases, extracellular acid-fast bacilli were also observed. In clinical cases it was reported that the presence of acid fast bacilli might be demonstrated intra- and extracellularly using the AFS stain (Kahn, 1997). However, in subclinical cases, it was reported that it may not be possible to find bacilli in tissues. Each tissue section was examined for a minimum of 30 min for the presence of bacilli as reported by others (Carbonell, 1998). Also, it was reported that it is very difficult in sub-clinical cases to detect bacteria by light microscopy due to the possible changes in the cell wall during the tissue preparation and processing (Micheal and Bastianello, 2002).

In a previous study, polyclonal antiserum which was raised by inoculating heat-killed *M. paratuberculosis*, was shown to be useful for the detection of Johne's disease in infected bovine tissue (Stabel et al., 1996). The specificity and the sensitivity of the immunohistochemical stain were high and can be used as a diagnostic tool. It was also evaluated for cross-reactivity with *M. bovis* antigens by immunohistochemical staining of tissues from infected pigs and cows. Tissues were devoid of positive reactivity when evaluated at the same dilutions that demonstrated positivity in *M. paratuberculosis*-infected tissues. In addition, cross-reactivity of the polyclonal antibody with *M. avium* infected tissue in pigs showed little positive staining. Furthermore, *M. avium* infection does not generally target the intestinal tract in ruminant species. Since we used a polyclonal antibody, it is unfair to consider this antibody completely specific for *M. paratuberculosis*. This is the first study where we use this polyclonal antibody in Awassi sheep and the tissues are from young and apparently healthy animals; it would be very interesting to find out how this

antibody reacts with tissues from sheep clinically infected with MAP. The 22 animals which were found by culture to be positive were also positive 1+ with this antibody by IHC. In tissues sections of ilea from apparently healthy cattle however, we found a significant correlation between the results of histopathological and IHC examinations; 66% of the ilea examined had lesions compatible with Johne's disease and 65% reacted positive with the polyclonal antibody (Hailat et al., in preparation). Furthermore, others have also reported that polyclonal and monoclonal antibodies could be used for the diagnosis of paratuberculosis in tissue sections (Coetsier et al., 1999). Thus, it was concluded that the production of a polyclonal antibody to cell wall proteins of *M. paratuberculosis* resulted in a highly sensitive, species-specific tool for the detection of paratuberculosis in tissue sections. In addition, it was suggested that histopathological and immunohistopathological methods produced evidence of infection, which can be comparable with bacteriological examinations (Sigurdardottir et al., 1999).

In the present study, we used the same antisera, where a high percentage of the sheep tissue sections were reactive within the infected macrophages and antibody reaction was also seen extracellularly (Table 3 and Figures 6 and 7). These results indicate that it is appropriate to use an immunohistochemical stain with a specific antiserum for the detection of *M. paratuberculosis*. The ultimate goal in determining the etiologic relationship of an organism to a disease state is to demonstrate the association of the organism with the lesion. In our study, 93% of the ilea tissue sections (81% 1+) and 58% of the lymph node tissue sections (44% 1+) reacted with the polyclonal antiserum. Interestingly, 97% of the tissue sections from the ilea had mononuclear cellular infiltrates (50% grades III and SP) strongly suggesting an etiological relationship of the organism to the intestinal and lymph nodes lesions of Johne's disease. In this study, it is also very obvious that the presence of an antigenic reaction by immunostaining in 108 (75%) out of 134 samples demonstrated by 1+, was characterised by high cellular infiltration in the LP and between the crypts with Peyer's patches proliferation involving its border towards the mucosa. This indicates that at early stage of the disease or in subclinical cases, cell-mediated immune reactions could start with only few bacteria present in the intestinal mucosa, as demonstrated by our results in these young animals.

We conclude that the occurrence of paratuberculosis (Johne's disease) is very high in Awassi sheep in Jordan. About 50% of clinically healthy Awassi sheep, 8 to 24 months of age, tested positive as they had histopathological lesions compatible with the disease. In addition, these cases were tested positive by IHC using a specific rabbit polyclonal antibody to *M. paratuberculosis* antigens. Thus, we consider paratuberculosis (Johne's disease) to be a significant problem in Awassi sheep in Jordan and believe there is a great need for further studies on the prevalence and epidemiology of the disease in order to develop rational methods of control effective on the Jordanian sheep population.

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Editor-in-Chief quotes from the reviewer's final recommendations

"I do believe that if the authors wish to pursue further work in this area, either further work to determine the specificity of this polyclonal antibody or additional confirmatory tests should be done to convince readers that their test results do identify MAP infected animals. Since they have cultured MAP from some sheep, it would be useful for them to try to determine the strain type (or types) that are represented."

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