Comp Clin Pathol (2012) 21:879–888
DOI 10.1007/s00580-011-1192-9

ORIGINAL ARTICLE

Investigation on the occurrence and pathology of paratuberculosis (Johne's disease) in apparently healthy cattle in Jordan

Nabil Hailat · Houari Hemida · Wael Hananeh · Judy Stabel · Feth Eddine Rezig · Saied Jaradat · Abed-Alrahman Al-Saleh

Received: 17 October 2010 / Accepted: 8 February 2011 / Published online: 1 March 2011

Abstract Paratuberculosis (Johne's disease) is an infectious, incurable, chronically progressive granulomatous enteritis which affects domestic and exotic ruminants. The causative agent is *Mycobacterium avium paratuberculosis (Mycobacterium johnei)*, a slow growing mycobactin-dependent acid-fast bacillus. We investigated the occurrence of Johne's disease in apparently healthy cattle, using 263 ileum and corresponding mesenteric lymph nodes, by histopathological examination, and 170 ileum and 120 mesenteric lymph nodes by immunohistochemical examination. The occurrence of the disease was 65% and 66% using immunohistochemistry and histopathology techniques, respectively. When Ziehl-Neelsen (ZN) and ELISA techniques were implemented, the occurrence was 1% (4/120) and 3% (8/278), respectively. Grading from I–IV of histopathological lesions based on type of cellular infiltrate and severity of lesions revealed that most of the positive cases were in grade I and II. Furthermore, staging I–III of immunohistochemistry results, has presented a high number of positive cases in stage I. ZN stain showed a very low occurrence; however, it is still used as a confirmatory test for clinical cases. On the other hand, ELISA technique showed a low occurrence of the disease (3%) in this study reflecting the low sensitivity of the technique in diagnosis of subclinical Johne's disease (JD). These results showed that histopathology is a very good diagnostic method for subclinical paratuberculosis in cattle. From the present study, we conclude that the occurrence of JD in cattle is high in Jordan. It is interesting to note that this is the first study of JD in cattle in Jordan, and the results strongly suggest alarming fears of severity of the disease at the national level. The urgent need for national control strategies are well founded due to the economical importance of the disease.

Keywords Cattle · Enzyme-linked immunosorbent assay · Histopathology · Ileum · Immunohistochemistry · Paratuberculosis

N. Hailat (✉)
Department of Pathology and Animal Health,
Faculty of Veterinary Medicine,
Jordan University of Science and Technology,
Irbid, Jordan
e-mail: hailatn@just.edu.jo

H. Hemida · W. Hananeh · F. E. Rezig · A.-A. Al-Saleh
Molecular Pathology Laboratory,
Department of Pathology and Animal Health,
Irbid, Jordan

J. Stabel
USDA-ARS, National Animal Disease Center,
Ames, IA 50010, USA

S. Jaradat
Department of Molecular Biology and Genetic Engineering,
Jordan University of Science and Technology,
Irbid, Jordan

Introduction

Johne's disease (JD) is an infectious, incurable, chronically progressive granulomatous enteritis which affects domestic and exotic ruminants (Coetsier et al. 1998). The causative agent is *Mycobacterium avium paratuberculosis*, a facultative intracellular acid-fast bacillus. *M. avium paratuberculosis* is an extremely slowly growing mycobactin-dependent organism that replicates within macrophages of both the

gastrointestinal tract and associated lymphoid tissues (Chiodini et al. 1984; Reichel et al. 1999; Klausen et al. 2003). Cattle become infected as young calves, via fecal–oral transmission of the organism and may remain subclinically infected for long periods of time, shedding low numbers of organisms before progression to the terminal stage of infection (Stabel et al. 2002).

Early diagnosis of infected cattle is essential for avoiding the spread of infection, and eradication is dependent on detection and culling of infected animals as early as possible (Collins et al. 1989; Green et al. 1989). Diagnostic tests for JD are divided into two categories: those that detect the organism and those that assess the host response to infection. The first category includes acid-fast stain on fecal smear, culture, and polymerase chain reaction tests. The second category, detection of host response, includes clinical signs in combination with gross and microscopic pathology and immunologic markers of infection (Riedout and Brown 2003).

Johne's disease progresses through several stages and, in the majority of cases, takes several years from infection to manifest clinical signs (Buergelt and Ginn 2000). Buergelt et al. 1978 showed that infected animals pass through three main disease stages classified as: (1) subclinical, non-shedding; (2) subclinical, shedding; (3) clinical and intermittently or permanently shedding. Each of the stages is marked by specific pathologic changes which are best recognized at the microscopic level (Collins et al. 2000). It has also been stated that there is no challenge for the pathologist to diagnosis JD when it is in the clinical stage; however, subclinical disease is difficult to diagnose (Buergelt et al. 1978).

Paratuberculosis induces a significant economic and health problem worldwide, especially in the cattle industry. Economic losses occur due to animal culling, lowered milk production, reduced carcass value, and poor reproductive performance, and are estimated to be about $200 per infected cow per year in herds with at least 10% prevalence.

Very limited studies on JD have been conducted in the Middle East and North African countries. JD was reported in cattle from Egypt, in sheep in Morocco and Saudi Arabia (Benazzi et al. 1995; Mahmoud et al. 2002; Salem et al. 2005). In Jordan, we conducted a study on apparently healthy sheep and goats, using histopathological and immunohistochemistry examinations and culture and found a high prevalence (Hailat et al. 2010). Therefore, we conducted this study with the objectives to describe the histopathological lesions and to investigate the occurrence of JD in apparently healthy cattle using histopathology, IHC, acid-fast stain, and serological screening tests, and to compare them as diagnostic methods in detection of paratuberculosis in Jordanian cattle.

Materials and methods

Cattle

A total of 263 cattle, slaughtered at the regional slaughter houses in the Northern part of Jordan, during a period of 6 months, May to October 2007, were used in this study. The ages of the animals ranged from 8 months to 6 years. Before slaughter, information was obtained concerning animals' age, breed, sex, and health status. Clinical signs were also recorded. The majority of the cattle in this study were Holstein Friesian lactating dairy cows raised in dairy farms at Ramtha, Irbid, and Dulail areas of the northern part of Jordan at 32°33′ N, 35°51′ E. Cows were housed in a free-stall barns provided with shades. These three areas have about 60% of the national herd which was estimated to be 65,000. Environmental data for mean maximum temperature (31.4°C and 20°C), minimum temperature (14.9°C and 4.9°C), and relative humidity (59.5% and 58.7%) were obtained from the Official National Station at Dulail area. All animals were derived from areas in which there was no recorded history of Johne's disease research or assessment.

Gross examination and tissue sampling

After slaughter, complete gross examination was performed with emphasis on the digestive system (small and large intestines) and regional lymph nodes. Information about corrugation, thickening, and hyperemia of the intestine were recorded. Furthermore, regional lymph node size, shape, and color were also described. Any other significant gross pathological findings were also recorded. Intestinal (ileum), ileo-cecal valve and respective lymph node tissues were collected, trimmed to small sizes (4 mm to 1 cm thickness), and fixed in 10% buffered formalin and transported to the pathology laboratory for histopathological examination and IHC staining.

Histopathological examinations

For histopathological examination, the formalin-fixed tissue samples were processed by routine methods, as described by Bancroft and Stevens 1990. Sections (4–5 μm) were cut and stained with hematoxylin and eosin (H&E) and Ziehl-Neelsen (ZN) method for acid-fast bacilli. The stained sections were examined histopathologically. Lesions were classified into four grading groups I, II, III, and VI/special grade, according to the type and amount of cellular infiltration regardless of the number of the acid-fast bacilli. The grading criteria of tissue lesions that were used are shown in Table 1.

Table 1 Histopathological criteria and grading of the lesions found in the last part of the ileum and the ileo-cecal valve and respective lymph nodes

Finding	Grade
Absent or very few macrophages and lymphocytes without apparent thickening of the intestinal mucosa	Negative
Many lymphocytes with some macrophages with no epithelioid cells	+
Additionally to the previous criteria, we can see many macrophages with an increased number of lymphocytes and a few scattered epithelioid cells	++
In addition to criteria of previous grade, a prominent number of epithelioid cells in nests or scattered could be observed	+++
Presence of multinucleated giant cells with or without epithelioid cells with granuloma formation	Special (SP)

Immunohistochemistry (IHC)

Tissues sections (3 μm) were placed on vectabond (DAKO A/S. Glostrup, Denmark) coated slides. The tissue samples, from paraffin-wax embedded blocks, were sectioned in 2–3 μm laid on vectabond (DAKO A/S. Glostrup, Denmark) coated slides, dried by air, and then heated at 55°C for 2 h. Tissue sections were deparaffinized in xylem and hydrated by sequential immersion of slides in graded concentration of ethanol (100%, 95%, and 70%) for 1 min each. Then, it was washed in distilled water for 5 min. After being washed in phosphate-buffered saline (PBS), the tissue sections were immersed in citrate buffer solution (pH=6), 10 mM, and antigen retrieval was carried out by autoclaving the tissue section at 120°C at 15 psi pressure for 15 min (Express, Italy). Endogenous peroxidases were inactivated by immersion of the slides in a solution of 15% hydrogen peroxide–methanol for 30 min. Non-specific adherence of proteins to tissue sections was blocked using 1% bovine serum albumin (Sigma Chemical Co., PO. Box14508, St. Louis, MO 63178) and incubated for 2 h. The solution was drained from the slides, and the polyclonal *M. avium paratuberculosis* antiserum, raised in rabbit, diluted 1:500 in PBS, was applied for 2 h. The rabbit antibody was kindly provided by Dr. Stabel from the National Animal Disease Center, Ames, AI, USA. Universal biotinylated anti-goat, anti-rabbit, and anti-mouse immunoglobulin (DAKO A/S, Glostrup, Denmark) diluted at 1:20 was applied as secondary antibody, and the slides were incubated for 15 min. After washing, streptavidin biotin complex peroxidases (DAKO, A/S, and Glostrup, Denmark) was applied, and incubated on the tissue section for 15 min. The slides were washed and were exposed to chromogen 3, 3 diaminobenzidin-4HCl (DAB, electron microscopic product, DAKO) 1 mg/ml in PBS supplemented with hydrogen peroxide (10 μl of 3% hydrogen peroxide for 2 ml of DAB) and incubated at room temperature for 3–5 min. The slides were washed in distilled water for 5 min followed by counter staining in hematoxylin for 2–3 min and immersing in bluing water for 30 s. Slides were dehydrated in graded alcohol (70%, 95%, and 100% three passes) 1 min each and cleared in xylene (three passes 1 min each) 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 extracellular brown coloration in different areas of the stained tissue section. The findings were registered by counting the number of positive cells 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% of the cells or more from one tissue section was graded as +++ (strong). Additionally, the intensity of the reaction was considered, and in all cases, only strong brown color was recorded as 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. Immunohistochemistry examination was performed on both tissues from the ilea and the associated mesenteric lymph nodes.

Collection of blood samples

A total number of 278 serum samples were collected. Out of this, 140 serum samples were collected in parallel of tissue samples from the cows slaughtered in the slaughter houses, while the rest of serum samples (138) were collected from four different farms where the status of infection was unknown taking a sampling ratio of 10% population size. Blood samples were centrifuged, and serum samples were frozen for further examinations.

Enzyme-linked immunosorbent assay (ELISA)

Frozen serum samples were subjected to a *Mycobacterium phlei*-absorbed enzyme-linked immunosorbent assay (*M. paratuberculosis* Antibody Test Kit, IDEXX Laborato-

ries Inc., Westbrook, USA). This test measures serum antibodies to *M. avium paratuberculosis*, using an absorption step to remove non-specific antibodies. Plates were read with an automated ELISA-plate reader (EL x 800 Universal Microplate Reader, Bio-Tek Instruments, Inc.). The serum samples were duplicated, and the means of the results were recorded.

Results

In the present study, we found that the nutritional body condition of 20 cows out of 263 examined animals (8%) were ranging from poor to emaciation with no history of chronic diarrhea. These cows were between 5 and 6 years old. Gross examination of cattle intestinal segments, mainly ileum, showed thickening of the mucosa with corrugation. This was seen in 19 cases (7%; Fig. 1). In three cases, prominent, white, enlarged, and thickened tube-like lymphatic vessels in the intestine were seen. No lesions were observed in intestine segments other than the ileum. Thirteen (5%) ileal and ileo-cecal mesenteric lymph nodes were enlarged five to sevenfold over the normal size, and in many cases, they were markedly edematous and congested.

The histopathological lesions were variable ranging from mild to severe. In mild cases, the lamina propria and submucosa of the ileum were mildly and loosely infiltrated with mononuclear cells, consisting primarily of lymphocytes with few numbers of macrophages and plasma cells (Figs. 2 and 3). Variable degrees of eosinophilic infiltrates were present in some of the examined intestinal sections (Fig. 4). Occasionally, a number of dead and degenerated nematodes were observed. Epithelioid giant cells were occasionally seen. In severe cases, extensive numbers of inflammatory cell infiltrates mainly macrophages and epithelioid cells with lymphocytes and plasma cells were present throughout the intestinal layers (Fig. 5). In some cases, the epithelioid cells forming sheets of inflammatory cells. Multinucleated Langhan's and foreign body giant cells were scattered throughout the mucosa and submucosa. Mild to severe granulomatous lymphangitis was a prominent finding. Other significant villous changes including disruption of the normal architecture of the villi, villous atrophy, and fusion were seen. Mild cryptitis was also preset. Prominent lymphoid follicular hyperplasia was evident. Areas of necrosis were rarely seen in the intestinal sections. By using acid-fast bacilli stain, acid-fast rods were seen either as aggregates or as scattered within the cytoplasm of the macrophage (Fig. 6).

Table 2 provides a summary of the occurrence of Johne's disease in subclinically infected cattle in Jordan using histopathological and immunohistochemical examinations, ZN stain, and ELISA technology. Out of 263 tissue

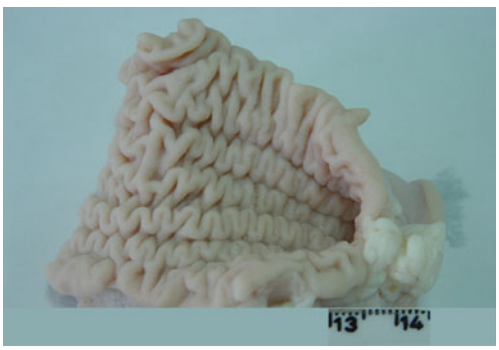

Fig. 1 Intestine (ileum); cattle. Corrugation of the intestinal mucosa and thickening of the intestinal wall sixfolds of normal size

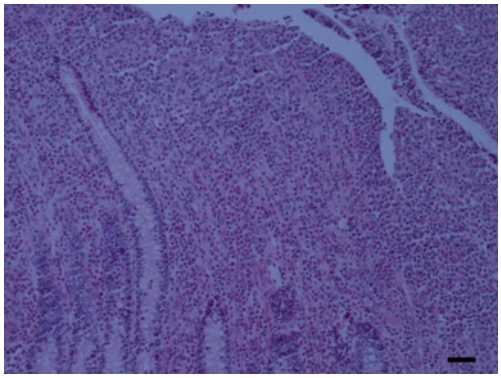

Fig. 2 Intestine (ileum); cattle. Grade I type lesion, thickening of the mucosa due to large infiltration with mononuclear cells. H&E. Bar=50 μm

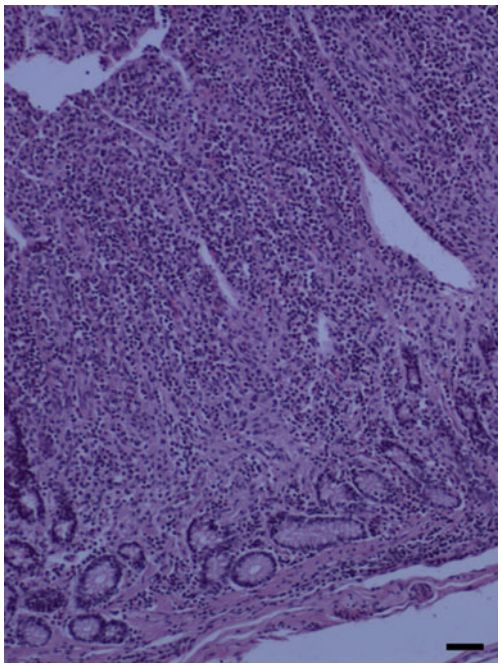

Fig. 3 Intestine (ileum); cattle. Grade III type lesion, thickening of the mucosa due to large infiltration with mononuclear cells mainly macrophages. H&E. Bar=50 μm

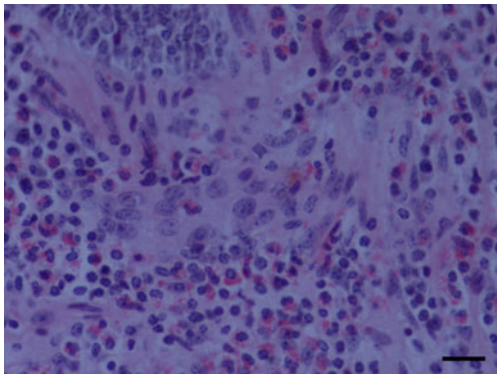

Fig. 4 Intestine (ileum); cattle. Grade II type lesion, scattered epithelioid cells with large numbers of eosinophils. H&E. Bar=20 μm

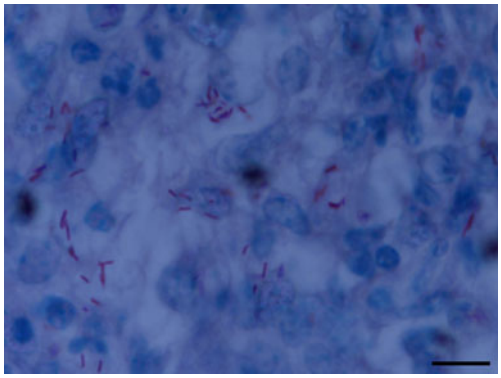

Fig. 6 Intestine (ileum); cattle. Scattered acid-fast bacilli. ZN. Bar=10 μm

specimens (ileum), 175 (66%) had histopathological lesions consistent with Johne's disease. Furthermore, out of 170 intestinal specimens examined 110 (65%) were positive using immunohistochemistry (Tables 1 and 2). Statistical analysis of the results showed no significant difference between the occurrence of the disease using histopathological and immunohistochemical examinations. However, when ZN staining on tissue sections was used, only eight (3%) samples out of 263 intestinal specimens examined were positive showing intracytoplasmic and extracellular red rods either in clump or scattered forms (Fig. 6). Furthermore, when ELISA technology was used, eight samples out of 278 bovine sera, tested positive (3%; Table 2). When we traced the sera samples, we found that seven positive samples were from the 140 sera samples collected from cows slaughtered in local slaughter houses. More specifically, these seven samples were from the 20 cows which had poor body condition. The H&E tissue sections of their ilea had consistent lesions with JD, had positive reaction by IHC, and four out of the seven specimens from the ilea had positive reaction with ZN staining. From the rest of the sera samples (138) collected from four dairy farms, only one sample was positive in Dulail area. The four dairy farms had no history of diagnosed JD.

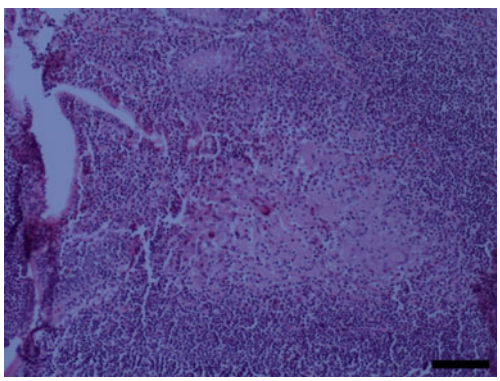

Fig. 5 Intestine (ileum); cattle. Grade IV type lesion, severe granulomatous enteritis. H&E. Bar=50 μm

To better understand the pathogenesis of the disease in cattle, corresponding regional mesenteric lymph nodes (MLNs) were examined by histopathological, immunohistochemical (Table 3), and ZN staining techniques. Analysis of the H&E results showed that out of 263 lymph nodes examined by histopathology, 67 (25%) showed granulomatous reaction consistent with JD, with numerous epithelioid and giant cells. Analysis of the results obtained by IHC examination of tissue specimens of 120 mesenteric lymph nodes revealed that 74 (61%) tissue specimens were positive. There was significant difference between the histopathological and immunohistochemical examinations of the MLNs. When lymph node tissue specimens were stained with ZN, four tissue specimens out 120 showed red bacilli or rods in the cytoplasm of macrophages or epitheloid cells. These lymph nodes belonged to the four ZN-positive intestinal specimens.

Table 4 shows the grading distribution of Johne's disease-positive cases in cattle using histopathological examination of ileum. The majority of the positive cows, 113, (43%) were in grade I (mild), representing tissue reaction with prominent lymphocytes infiltration, with some macrophages accompanied with no epithelioid cells. While the least number of cows, 14 (7%), were in grade IV (severe), representing tissue reaction with multinucleated giant cells with or without epithelioid cells with granulomatous reactions (Fig. 5). Grade II and III had 29 (11%) and 19 (7%) cows, respectively. In our study, we consider cows which were in grade I suspected (43%) for JD, while cows which were in grades II, III, and IV were considered positive (23%) for the disease.

Analysis of the immunohistochemical findings, using a polyclonal antibody specific for *M. avium paratuberculosis*, of the ileum tissue sections showed that out of 170 tested, 52 specimens (30.5%) were in stage I, 52 specimens (30.5%) in stage II, and six (3.5%) in stage III (Table 5;

Table 2 The occurrence of subclinical Johne's disease in cattle (ileum) using histopathological and immunohistochemical examinations, ZN, and ELISA techniques, Jordan, 2007

	Histopathology			IHC		ZN		ELISA	
	+tve	Suspect	−tve	+tve	−tve	+tve	−tve	+tve	−tve
Total	62 (23.5%)	113 (43%)	88 (33.5%)	110 (65%)	60 (35%)	08 (3%)	255 (97%)	08 (3%)	270 (97%)

IHC immunohistochemistry, *ELISA* enzyme-linked immunosorbent assay, *ZN* Ziehl-Neelsen's stain, *−tve* negative, *+tve* positive, *Susp* suspected

Fig. 7). Thus, the IHC staining revealed 65% of the examined ilea specimens to be positive. However, when the results of the IHC staining of the MLN were analyzed, 51% were positive. In addition, further analysis of the immunohistochemical findings revealed that, out of 120 of the lymph node specimens tested, 44 (36%) were in stage I, 23 (19%) were in stage II, and seven (6%) were in stage III (Table 4; Fig. 7). These were based on the criteria mentioned in the Materials and methods section.

Discussion

Diagnosis of subclinical cases of paratuberculosis is a recognized problem, either in the field or at postmortem (Kurade et al. 2004). In addition, it was observed that diagnosis of subclinical disease of paratuberculosis is more difficult by histopathology as lesions may be subtle and organisms may be rare (Buergelt et al. 2000). Furthermore, it was also reported that histopathology was found to be a better indicator of paratuberculosis than bacteriology in sheep (Kurade et al. 2004). In addition, 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.

We have shown previously that a polyclonal antiserum which was raised by inoculating heat-killed *M. paratuberculosis*, to be useful for the detection of JD in infected bovine tissue (Stabel et al. 1996). The specificity and the sensitivity of the antiserum were high, and thus, it can be used as a diagnostic tool. It was also evaluated for cross-reactivity with *Mycobacterium bovis* antigens by immunohistochemical staining of tissues from infected animals. Prefemoral and cervical lymph nodes and liver samples obtained from pigs intravenously infected with *M. bovis* were stained with the *M. paratuberculosis* polyclonal antibodies. Tissues were devoid of positive reactivity when evaluated at the same dilutions that demonstrated positivity in *M. paratuberculosis*-infected tissues. In the present study, we used the same antibody to complement our histopathological findings.

In the present study, the occurrence of subclinical JD in cattle was 1%, 3%, 65%, and 25% using ZN, ELISA, immunohistochemistry, and histopathology techniques, respectively (Tables 2 and 3). If the presence of *M. avium paratuberculosis* protein in the intestinal tissue, as seen by IHC, can be considered definitive of infection, then there is a 65% incidence in cattle at slaughter in Jordan. This is may be explained by the high occurrence of the disease in sheep (Hailat et al. 2010), the average age of the examined cattle which was 5 years old and lack of national control and prevention programs for the disease. It is worth noticing, however, that most of the dairy cattle and large portion of the sheep population especially for meat production in Jordan are imported.

Johne's disease has gained the attention of many countries. The disease may be considered as trade barrier where many countries request testing of cattle at the borders. Furthermore, it has been shown in many countries to cause significant economical losses. Many strategies and control programs were conducted on JD in different countries during the last decade such as Spain, Australia, The Netherlands, Dutch, and different states of USA (Aduriz et al. 1995; Benedictus et al. 2000; Sergeant et al. 2003; Stabel et al. 2002).

The disease has been reported in virtually every country that has agriculture and laboratory capability to diagnose

Table 3 Distribution of positive cases of intestine (ileum) and mesenteric lymph nodes (MLNs) of cattle examined by histopathology and immunohistochemistry, Jordan, 2007

Tissue	Histopathology			Immunohistochemistry		
	Tissue no.	+ve	(%)	Tissue no.	+ve	(%)
Ileum	263	175	66%	170	110	65%
MLNs	263	67	25%	120	74	61%

Table 4 Grade distribution of Johne's disease-positive cases in cattle using histopathological examination of ileum, Jordan, 2007

Tissue no.	Histopathology grades			
	I (%)	II (%)	III (%)	IV (%)
175	113 (64%)	29 (16%)	19 (11%)	14 (8%)

Table 5 Stage distribution of Johne's disease-positive cases in cattle using immunohistochemical examination of ileum, Jordan, 2007

Tissue	No. of tissues examined	No. of positive tissues	Immunohistochemistry stages		
			Stage I (%)	Stage II (%)	Stage III (%)
Ileum	170	110	52 (30.5%)	52 (30.5%)	6 (3.5%)
MLNs	120	74	44 (36%)	23 (19%)	7 (6%)

the disease. Prevalence of infection in most countries of the world is unknown, but studies from some countries indicate a herd prevalence ranging from almost 0% in Norway and Sweden to 22% in the USA (Sternberg and Viske 2003; Djonne et al. 2003; NAHMS 1997). Herd prevalence of bovine paratuberculosis in Europe ranges between 7% and 55%, and in USA, the disease affected 22% of the dairy cattle herds (Collins et al. 1994). It was reported that, in days, open dairy cattle in Michigan, the prevalence reached 41.8% using ELISA and radiometric fecal culture and approximately 40% of herds are infected (Johnson-Ifearulundu et al. 2000). Two recent Dutch studies have indicated a prevalence of 50–70% among the dairy herds in the Northern provinces of The Netherlands. The overall herd-level prevalence of paratuberculosis in the Danish dairy herds investigated was approximately 70% (Nielsen et al. 2000). Disease prevalence in Australia is unevenly distributed in the state of Victoria; infection in dairy herds reached 22%, while in New South Wales only 9% was reported (Collins 1994). In the Middle East region, JD was reported in cattle from Egypt with a prevalence of 16.7% in native cattle and 85.7% in Holstein cows reared in Egypt, and in sheep in Morocco; however, Mahmoud reported one case of sheep pigmented paratuberculosis in Saudi Arabia (Benazzi et al. 1995; Mahmoud et al. 2002; Salem et al. 2005). Saxegaard and Fodstad (1985) indicated that, in Norway in 1967, based on postmortem data (including clinical cases); the number of infected goats was 53%, which encouraged the control program (Benedictus et al. 2000). In the light of these data, subclinical Johne's disease in cattle in Jordan is considered to be high, and an urgent need for control program must be considered seriously. Stringent legislation regarding the importation of cattle from infected countries is also recommended.

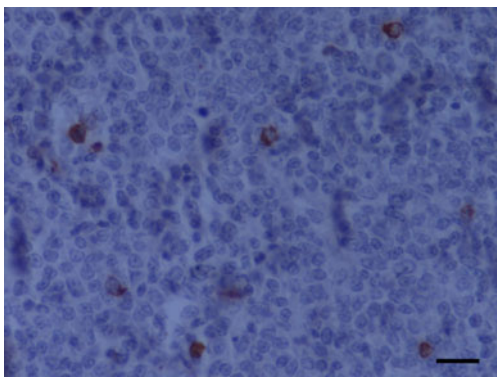

Fig. 7 Lymph node; cattle. Stage III type lesion, numerous strongly stained of macrophages. Streptavidin biotin complex peroxidases, hematoxylin counter stain. Bar=20 μm

In this study, the different tests indicated vastly different impressions of disease prevalence. When using IHC and histopathology, disease prevalence was high, reflecting the early reaction occurring in the intestine and associated lymph nodes. Also, in the immunohistochemical technique, this may due to the presence of small numbers of bacteria in the tissue. However, low occurrence was detected using ZN and ELISA, as ZN staining method detected only animals in advanced clinical stages with high numbers of organisms. The ELISA is related to the appearance of antibody titers in the blood of infected animals and that the humoral response occurs late in the course of JD infection.

It has been suggested that lesion grading according to different histological patterns can be used to classify the disease stage as it correlates with the subclinical state of animals at different phases of infection development in sheep, goats, and rabbits (Buergelt et al. 2000; Kurade et al. 2004). In sheep paratuberculosis, grading criteria were used in four independent studies including a study which we conducted previously (Perez et al. 1996, 1999). In a rabbit, natural infection study conducted in UK, Beard et al. (2001a) classified lesions in only severe and mild groups. Buergelt et al. (2000) studying paratuberculosis in North American bison, classified lesions in three categories as positive, suspicious, and negative (Buergelt et al. 2000). In a similar study conducted in Spain by Perez in naturally infected goats, lesions were categorized into five groups, focal, diffuse multibacillary, diffuse lymphocytic, diffuse mixed, and incidental lesions group. This classification was based also on a previous study conducted in Spain but of that in sheep (Perez et al. 1996; Corpa et al. 2000). All previous studies shared common classification criteria, the presence of granulomatous reaction located in different intestinal and lymph node histological compartments. In the present study, grading was based on the previous criteria described above with some modifications. These modifications were in regard to the severity

and type of cellular infiltration, considering infiltration with macrophages as a more advanced grade with reference to epithelioid cell infiltration, and grade one lesions were characterized by large infiltration of lymphocytes. Our grading criteria comply with all precedent classifications with particularities in being the more accurate and strengthened by immunohistochemistry and positive regional mesenteric lymph nodes. The combination of these two tests gave a particular sensitivity by narrowing the range of error. Our histopathological grading agreed with most of the researchers, where they were dealing with clinical cases and other species in diagnosis criteria, but we added some criteria such as the type of cellular infiltrate and severity of lesions (Perez et al. 1996; Corpa et al. 2000; Beard et al. 2001b). To the best of our knowledge, there was no previous investigation that deals with the grading of subclinical bovine paratuberculosis.

Results of mesenteric lymph nodes using histopathological examination (25%) correlated neither with those obtained with intestine (ileum; 66%) nor with results of immunohistochemical examination (65%; Table 3). This could be explained by the difficulties in histological evaluation of lymph nodes due to the natural cell population dominated by lymphocytes which makes real evaluation of infiltration more restrictive and also to the changes occurring during disease development. We conclude that lymph node examination by histopathology is not necessary when we have ileum sections.

It is interesting to report that most lymph nodes which were positive by H&E belonged to both grades I and II of ileum positive sections. When using immunohistochemistry, results correlated to those obtained by histopathology and reflected the association between the immunological reactions and histological sequential changes (Sigurdardottir et al. 1999; Kurade et al. 2004). Immunohistochemistry technique results showed the utility of this technique in detection of bacterial antigens. Immunohistochemistry results showed also a strong correlation between the two different tissue samples, ileum 65% and MLNs 61%, respectively (Table 5). These results are found for the first time, and these data may help in future studies giving choice in diagnostic methods by reducing time and cost of Johne's disease diagnosis. Statistical analysis also confirmed the correlation between the two techniques. Regarding the low sensitivity of immunohistochemistry and based on the present results, we conclude that histopathological examination can be used alone without immunohistochemical examination, although this technique is considered as a good diagnostic tool when associated with other immunological diagnostic methods especially of the cellular mediated type immunity (Stabel et al. 1996; Lee et al. 2001).

Although reported as a fast tool for paratuberculosis diagnosis, ZN technique showed no practical value in subclinical cases (eight cases out of 263 examined cases; Table 2). It is worth noticing also that the eight positive cases were positive in both the ileum and the corresponding lymph nodes. This is in agreement with the results reported by Salem who stated that this method is not trustable and shows a large percentage of false-negative reactions (Salem et al. 2005).

ELISA technique had detected eight of 270 examined sera (Table 2) resulting in a prevalence of 3%. This prevalence is considered low when compared with the prevalence found using other techniques, 23% and 65% for histopathology and immunohistochemistry, respectively. These results correlated with the low sensitivity of the technique reported by many authors (Nielsen 2002). By considering the "iceberg concept" of Johne's disease, we propose to divide our tests according to the disease development: ELISA-positive animals (as clinical cases) and the histopathology and immunohistochemistry results (as subclinical picture of the disease). Putting the composition of the target population in consideration, the ELISA technique is reported to be the best test for screening purposes. This was explained by Nielsen who suggested that, in an infected population with many latently infected animals, the sensitivity of ELISA is quite high, but the sensitivity of culture is quite low (Nielsen et al. 2002). In addition, our serology results are in agreement with the study reported recently by Al Hajri who reported 2–4.5% seropositive in subclinical cases of Johne's disease in cattle (Al Hajri and Alluwaimi 2007).

We conclude that the occurrence of subclinical Johne's disease in cattle in Jordan based on the IHC staining and histopathological examinations are high. We also conclude that using histopathology alone is very sensitive and cheap technique but requires experience and multiple reading times. In addition, examination of the ilea specimen is sufficient for JD diagnosis without examination of the MLN. Furthermore, control and prevention programs should be initiated in Jordan.

Acknowledgments We thank the deanship of research at Jordan University of Science and Technology for supporting this research work, project number 30/2004. The data presented here is part of a master thesis of a graduate student in the Faculty of Veterinary Medicine.

References

Aduriz JJ, Juste RA, Cortabarria N (1995) Lack of mycobactin dependence of mycobacteria isolated on Middlebrook 7H11 from clinical cases of ovine paratuberculosis. Vet Microbiol 45:211–217

Al Hajri SM, Alluwaimi AM (2007) ELISA and PCR for evaluation of subclinical paratuberculosis in the Saudi dairy herds. Vet Microbiol 121:384–385

Bancroft JD, Stevens A (1990) Theory and practice of histological techniques, Churchill Livingston, Edinburgh, London, Melbourne and New York, pp 21–119

Beard PM, Rhind SM, Buxton D, Daniels MJ, Henderson D, Pirie A, Rudge K, Greig A, Hutchings MR, Stevenson K, Sharp JM (2001a) Natural paratuberculosis infection in rabbits in Scotland. J Comp Pathol 124:290–299

Beard PM, Stevenson K, Pirie A, Rudge K, Buxton D, Rhind SM, Sinclair MC, Wildblood LA, Jones DG, Sharp JM (2001b) Experimental paratuberculosis in calves following inoculation with a rabbit isolate of *Mycobacterium avium* subsp. paratuberculosis. J Clin Microbiol 39:3080–3084

Benazzi S, Berrada J, Schliesser T (1995) First report of paratuberculosis (Johne's disease) in sheep in Morocco. Zentralbl Veterinärmed B 42:339–344

Benedictus G, Verhoeff J, Schukken YH, Hesselink JW (2000) Dutch paratuberculosis programme history, principles and development. Vet Microbiol 77:399–413

Buergelt CD, Ginn PE (2000) The histopathologic diagnosis of subclinical Johne's disease in North American bison (Bison bison). Vet Microbiol 77:325–331

Buergelt CD, Hall C, McEntee K, Duncan JR (1978) Pathological evaluation of paratuberculosis in naturally infected cattle. Vet Pathol 15:196–207

Buergelt CD, Layton AW, Ginn PE, Taylor M, King JM, Habecker PL, Mauldin E, Whitlock R, Rossiter C, Collins MT (2000) The pathology of spontaneous paratuberculosis in the North American Bison (Bison bison). Vet Pathol 37:428–438

Chiodini RJ, Van Kruiningen HJ, Merkal RS (1984) Ruminant paratuberculosis (Johne's disease): the current status and future prospects. Cornell Vet 74:218–262

Coetsier C, Havaux X, Mattelard F, Sadatte S, Cormont F, Buergelt K, Limbourg B, Latinne D, Bazin H, Denef JF, Cocito C (1998) Detection of *Mycobacterium avium* subsp. paratuberculosis in infected tissues by new species-specific immunohistological procedures. Clin Diagn Lab Immunol 5:446–451

Collins MT (1994) Clinical approach to control of bovine paratuberculosis. J Am Vet Med Assoc 204:208–210

Collins DM, Gabric DM, de Lisle GW (1989) Identification of a repetitive DNA sequence specific to *Mycobacterium paratuberculosis*. FEMS Microbiol Lett 51:175–178

Collins MT, Sockett DC, Goodger WJ, Conrad TA, Thomas CB, Carr DJ (1994) Herd prevalence and geographic distribution of, and risk factors for, bovine paratuberculosis in Wisconsin. J Am Vet Med Assoc 204:636–641

Collins MT, Lisby G, Moser C, Chicks D, Christensen S, Reichelderfer M, Hoiby N, Harms BA, Thomsen OO, Skibsted U, Binder V (2000) Results of multiple diagnostic tests for *Mycobacterium avium* subsp. *paratuberculosis* in patients with inflammatory bowel disease and in controls. J Clin Microbiol 38:4373–4381

Corpa JM, Garrido J, Garcia Marin JF, Pere V (2000) Classification of lesions observed in natural cases of paratuberculosis in goats. J Comp Pathol 122:255–265

Djonne B, Jensen MR, Grant IR, Holstad G (2003) Detection by immunomagnetic PCR of *Mycobacterium avium* subsp. *paratuberculosis* in milk from dairy goats in Norway. Vet Microbiol 92:135–143

GDYH MDC (1998) Progress in culture and subculture of spheroplasts and fastidious acid-fast bacilli isolated from intestinal tissues. J Clin Microbiol 26:1591–1600

Green EP, Tizard ML, Moss MT, Thompson J, Winterbourne DJ, McFadden JJ, Hermon-Taylor J (1989) Sequence and characteristics of IS900, an insertion element identified in a human Crohn's disease isolate of *Mycobacterium paratuberculosis*. Nucleic Acids Res 17:9063–9073

Hailat N, Hananeh W, Metekia AS, Stabel JR, Al-Majali A, Lafi S (2010) Pathology of subclinical paratuberculosis (Johne's disease) in Awassi sheep with reference to its occurrence in Jordan. Vet Med 55:590–602

Huda A, Jensen HE (2003) Comparison of histopathology, cultivation of tissues and rectal contents, and interferon-gamma and serum antibody responses for the diagnosis of bovine paratuberculosis. J Comp Pathol 129:259–267

Johnson-Ifearulundu YJ, Kaneene JB, Sprecher DJ, Gardiner JC, Lloyd JW (2000) The effect of subclinical *Mycobacterium paratuberculosis* infection on days open in Michigan, USA, dairy cows. Prev Vet Med 46:171–181

Klausen J, Huda A, Ekeroth L, Ahrens P (2003) Evaluation of serum and milk ELISAs for paratuberculosis in Danish dairy cattle. Prev Vet Med 58:171–178

Kurade NP, Tripathi BN, Rajukumar K, Parihar NS (2004) Sequential development of histologic lesions and their relationship with bacterial isolation, fecal shedding, and immune responses during progressive stages of experimental infection of lambs with *Mycobacterium avium* subsp. *paratuberculosis*. Vet Pathol 41:378–387

Lee H, Stabel JR, Kehrli ME (2001) Cytokine gene expression in ileal tissues of cattle infected with *Mycobacterium paratuberculosis*. Vet Immunol Immunopathol 82:73–85

Mahmoud OM, Haroun EM, Elfaki MG, Abbas B (2002) Pigmented paratuberculosis granulomata in the liver of sheep. Small Ruminant Res 43:211–217

NAHMS (1997) Johne's disease on U.S. dairies, 1991–2007. USDA-APHISVS, Fort Collins, CO

Nielsen SS (2002) Variance components of an enzyme-linked immunosorbent assay for detection of IgG antibodies in milk samples to *Mycobacterium avium* subspecies *paratuberculosis* in dairy cattle. J Vet Med B Infect Dis Vet Public Health 49:384–387

Nielsen SS, Thamsborg SM, Houe H, Bitsch V (2000) Bulk-tank milk ELISA antibodies for estimating the prevalence of paratuberculosis in Danish dairy herds. Prev Vet Med 44:1–7

Nielsen SS, Enevoldsen C, Grohn YT (2002) The *Mycobacterium avium* subsp. *paratuberculosis* ELISA response by parity and stage of lactation. Prev Vet Med 54:1–10

Perez V, Garcia Marin JF, Badiola JJ (1996) Description and classification of different types of lesion associated with natural paratuberculosis infection in sheep. J Comp Pathol 114:107–122

Perez V, Tellechea J, Corpa JM, Gutierrez M, Garcia Marin JF (1999) Relation between pathologic findings and cellular immune responses in sheep with naturally acquired paratuberculosis. Am J Vet Res 60:123–127

Reichel MP, Kittelberger R, Penrose ME, Meynell RM, Cousins D, Ellis T, Mutharia LM, Sugden EA, Johns AH, de Lisle GW (1999) Comparison of serological tests and faecal culture for the detection of *Mycobacterium avium* subsp. *paratuberculosis* infection in cattle and analysis of the antigens involved. Vet Microbiol 66:135–150

Riedout BA, Brown ST (2003) National Research Council: diagnosis and control of Johne's disease. The National Academies

Salem M, Zeid AA, Hassan D, El-Sayed A, Zschoeck M (2005) Studies on Johne's disease in Egyptian cattle. J Vet Med B Infect Dis Vet Public Health 52:134–137

Saxegaard F, Fodstad FH (1985) Control of paratuberculosis (Johne's disease) in goats by vaccination. Vet Record 116:439–441

Sergeant ES, Marshall DJ, Eamens GJ, Kearns C, Whittington RJ (2003) Evaluation of an absorbed ELISA and an agar-gel immuno-diffusion test for ovine paratuberculosis in sheep in Australia. Prev Vet Med 61:235–248

Sigurdardottir OG, Press CM, Saxegaard F, Evensen O (1999) Bacterial isolation, immunological response, and histopathological lesions during the early subclinical phase of experimental infection of goat kids with *Mycobacterium avium* subsp. *paratuberculosis*. Vet Pathol 36:542–550

Stabel JR, Ackermann MR, Goff JP (1996) Comparison of polyclonal antibodies to three different preparations of *Mycobacterium paratuberculosis* in immunohistochemical diagnosis of Johne's disease in cattle. J Vet Diagn Invest 8:469–473

Stabel JR, Wells SJ, Wagner BA (2002) Relationships between fecal culture, ELISA, and bulk tank milk test results for Johne's disease in US Dairy Herds. J Dairy Sci 85:525–531

Sternberg S, Viske D (2003) Control strategies for paratuberculosis in Sweden. Acta Vet Scand 44:247–249