

Distribution of *Mycobacterium avium* subsp. *paratuberculosis* in the gastrointestinal tract of shedding cows and its application to laparoscopic biopsy

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ABSTRACT: The gastrointestinal tract (GIT) is a major target for *Mycobacterium avium* subsp. *paratuberculosis* (*M. a. paratuberculosis*) in cattle. Culture examination was achieved in tissue samples obtained from 10 different regions of the GIT (proximal and distal parts of the duodenum, proximal, middle and distal parts of the jejunum, proximal and distal parts of the ileum, the ileocecal valve, the caecum and the rectum) and their adjacent lymph nodes. The culture results were statistically analysed to elucidate the distribution of *M. a. paratuberculosis* in the GIT. A total of 63 cows older than 24 months were diagnosed with paratuberculosis by faecal and tissue cultures. The better detection rate of *M. a. paratuberculosis* was found in the mucosae from the jejunum to the ileocecal valve and in the lymph nodes from the jejunum to the caecum. The mean number of colony forming units (CFU) in the mucosae and the lymph nodes of the distal jejunum and the proximal ileum was significantly higher than that in the mucosae of the duodenum, the caecum and the rectum, and in the lymph nodes of the duodenum and the rectum, respectively ($P < 0.05$). Laparoscopic biopsy attempted out on 4 animals to test its potential use for sample collection from the statistically optimal mesenteric lymph nodes; but resulted in an abortive attempt because these targets were encircled by the intestines, the pressure of which complicated the laparoscopic approach.

Keywords: Johne's disease; diagnostic; IS900; PCR

Shedding animals predominantly transmit *Mycobacterium avium* subsp. *paratuberculosis* (*M. a. paratuberculosis*) by faecal contamination of water and feed (Chiodini et al., 1984; Harris and Barletta, 2001; Ayele et al., 2001). Ingestion of *M. a. paratuberculosis* induces granulomatous lesions of the small intestine and the adjacent lymph nodes in cattle. Macroscopic lesions of the intestine characterised by thickening and corrugation are prominent especially in the ileum. Clinical signs, however, cannot be easily recognised up to 18 months of age (Clarke, 1997). Therefore, if latent infection cannot be excluded by proper diagnostic tools, *M. a. paratuberculosis* is allowed to be transmitted to other animals until paratuberculosis

infection can be realised by clinical signs or ended by slaughter. Subsequent spread of the causal agent over the gastrointestinal tract (GIT) of the host's body causes emaciation, drop in milk production and death, by which a great financial loss is incurred (Buergelt and Duncan, 1978; Benedictus et al., 1987; Ott et al., 1999). Notorious slow *in vitro* growth of *M. a. paratuberculosis* complicates early detection of the causal agent of paratuberculosis. Enzyme-linked immunosorbent assays (ELISA) have been widely accepted as fast and economical methods, but are not so beneficial on the early stage of infection because antibody response cannot fully develop until the later stages of the disease (Reichel et al., 1999;

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Eamens et al., 2000; Whitlock et al., 2000). All tests available up to now have limited value for detecting *M. a. paratuberculosis* (Anonymous, 2000).

Pavlik et al. (2000) showed that even in non-shedders, 25.6% of tissue culture from the GIT and its related lymph nodes became positive. In 9.7% of animals, *M. a. paratuberculosis* was detected only from non-GIT areas (liver, spleen, pulmonary lymph nodes etc.). In other words, there is a possibility about 90% of *M. a. paratuberculosis* infection are detected by GIT-related tissue culture. As the GIT and its associated lymph nodes are main sites of infection caused by *M. a. paratuberculosis*, it is also meaningful to elucidate how *M. a. paratuberculosis* distributes over the GIT area during infection. Such information will help the bacteriological examination of the GIT samples from slaughtered animals by routine inspection and from live animals by laparotomy or laparoscopy (Benedictus and Haagsma, 1986; Benedictus, 1987).

Although the endoscopic biopsy of the intestinal mucosa should be ideal, very long colon and large rumen prevent the endoscope from non-invasively reaching the jejunal and ileal mucosae in cattle. Surgical lymph node biopsy can also be used for sampling, but a wide flank incision has not been widely accepted by veterinary surgeons as routine examination (Benedictus, 1987). Laparoscopic biopsy requires only a few centimeter incisions to obtain abdominal tissue samples, e.g., mesenteric lymph nodes, so animals suffer less pain during and after the laparoscopic procedures (Klein et al., 2002). Therefore, laparoscopy appears amenable for the early diagnosis of paratuberculosis without exerting any serious and harmful effects on animals.

The purpose of this study was to provide information about the distribution of *M. a. paratuberculosis* in the intestinal mucosa and its associated lymph nodes of shedding cattle, and to apply our statistical data to a diagnostic method of laparoscopic biopsy. The laparoscopy was challenged to explore the possibility of sampling from the mesenteric lymph nodes with high detection rates of *M. a. paratuberculosis*.

MATERIAL AND METHODS

Tissue culture analysis

Animals. Paratuberculosis infection was confirmed in faecal culture described by Pavlik et al. (2000) before the cattle were slaughtered and

63 cows older than 24 months were diagnosed with paratuberculosis. Tissue samples were obtained at slaughter in the Czech Republic (26 cows) and the Slovakia (37 cows) during the control of paratuberculosis in infected cattle herds between 1995 and 2000 (Pavlik et al., 1999, 2000, 2001).

Sample collection. In each animal, 10 different regions of the GIT (proximal and distal parts of the duodenum, proximal, middle and distal parts of the jejunum, proximal and distal parts of the ileum, the ileocecal valve, the cecum and the rectum) were determined for mucosal collection. Parallel 9 adjacent mesenteric lymph nodes were selected for lymphoid collection (lymph nodes related to the distal ileum and the ileocecal valve were not distinguished because of their close location). Animals with 10 or more tissue samples were chosen for our analytical study. A total of 890 samples were obtained from

Table 1. Abbreviations used for sampling sites in the gastrointestinal tract

Gastrointestinal tract	Location	Description of the tissue sample	
		mucosa	lymph node
Duodenum	proximal	Md1	Ld1
	distal	Md2	Ld2
Jejunum	proximal	Mj1	Lj1
	middle	Mj2	Lj2
	distal	Mj3	Lj3
Ileum	proximal	Mi1	Li1
	distal	Mi2	Li2
Ileocecal valve		Micv	
Cecum		Mc	Lc
Rectum		Mr	Lr

*lymph nodes related to the distal ileum and the ileocecal valve were not distinguished because of their close location

Md1 = mucosa (M) of proximal duodenum; Md2 = M of distal duodenum; Mj1 = M of proximal jejunum; Mj2 = M of middle jejunum; Mj3 = M of distal jejunum; Mi1 = M of proximal ileum; Mi2 = M of distal ileum; Micv = M of ileocecal valve; Mc = M of caecum; Mr = M of rectum
Ld1 = lymph node (Ln) of proximal duodenum; Ld2 = Ln of distal duodenum; Lj1 = Ln of proximal jejunum; Lj2 = Ln of middle jejunum; Lj3 = Ln of distal jejunum; Li1 = Ln of proximal ileum; Li2 = Ln of distal ileum and ileocecal valve; Lc = Ln of caecum; Lr = Ln of rectum

1,197 sampling sites of 63 cows. Abbreviations used for sampling sites on this paper are shown in Table 1. Intestinal tissue samples (6 × 6 cm) and their related lymph nodes were carefully excised from the selected GIT regions at slaughter. All samples were kept at –20°C, transported to the laboratory under frozen conditions, and cultured within two months after delivery.

Culture examination. All tissue samples were cultured after 24 h decontamination with 0.75% HPC (hexadecylpyridium chloride: N-cetylpyridium chloride monohydrate, No. 102340 Merck), inoculated on three slants of Herrold's egg yolk media with Mycobactin J, and incubated at 37°C for 14 to 16 weeks (Pavlik et al., 2000). The mycobactin J-dependence test and IS900 PCR were performed to confirm paratuberculosis infection when culture results were suspicious (Svastova et al., 2002).

Statistical analysis. Mean number of colony forming units (CFU) was calculated from three slants of each sample. Among those data used for our analysis, 200 CFU were maximally counted on each medium, and if CFU were estimated over 200, it

was indicated as an uncountable number of CFU. In this case, arbitrary number of 300 CFU was given for analysis. Student's *t*-test, Welch's *t*-test and χ^2 test were performed to compare number of CFU and positive rates (%) in tissue culture of the mucosa and its associated lymph node (Snedecor and Cochran, 1967; Ishii, 1982). The level of significance was chosen as $P < 0.05$.

Laparoscopy

Animals and surgical methods. Biopsy was performed in two adult Holstein cows and two one-year-old Holstein bulls with a Wolf laparoscope (TCP5107, Germany). In upright position, lidocaine hydrochloride was injected into the right paralumbar fossa to induce a paravertebral nerve blocking. The same local anesthetic was also subcutaneously injected over the surgical area of the right flank, which was aseptically prepared after clipping and shaving. In left recumbency, caudal epidural anesthesia was additionally used to control the hind

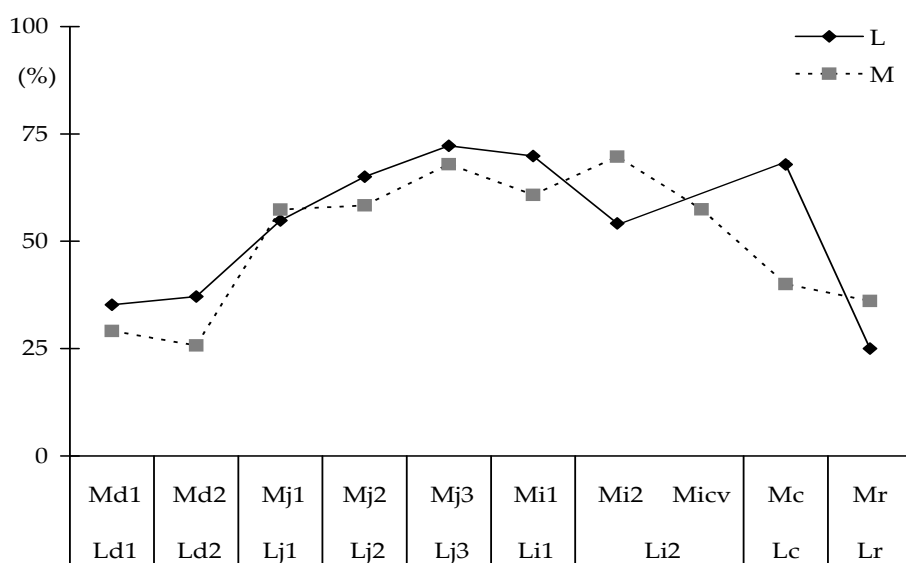


Figure 1. Positive rates of tissue culture of the mucosa and the lymph node in shedding cows. Black line and grey dotted line indicate lymph node (L) and mucosa (M), respectively

Md1 = mucosa (M) of proximal duodenum; Md2 = M of distal duodenum; Mj1 = M of proximal jejunum; Mj2 = M of middle jejunum; Mj3 = M of distal jejunum; Mi1 = M of proximal ileum; Mi2 = M of distal ileum; Micv = M of ileocecal valve; Mc = M of caecum; Mr = M of rectum

Ld1 = lymph node (Ln) of proximal duodenum; Ld2 = Ln of distal duodenum; Lj1 = Ln of proximal jejunum; Lj2 = Ln of middle jejunum; Lj3 = Ln of distal jejunum; Li1 = Ln proximal ileum; Li2 = Ln of distal ileum and ileocecal valve; Lc = Ln of caecum; Lr = Ln of rectum

legs. Three 2-cm incisions were made through the skin and followed by each trocar cannula assembly penetrating at a right angle through the abdominal musculature. The first trocar was replaced by laparoscope connected to the cool light source, the second by grasping forceps and the third by hook scissors (Maxwell and Kraemer, 1980). To enable visual observation, carbon dioxide (CO₂) gas was insufflated into the abdominal cavity. The last animal was kept pre-surgical starvation for 24 hours. The first two animals were euthanized soon after the laparoscopic experiment and effects of laparoscopic manipulation were examined at necropsy.

RESULTS

Tissue culture analysis

The rate of tissue culture positive is shown in Figure 1. It described bimodal distribution both in the lymph node and the mucosa. In the mesenteric lymph nodes, the highest detection rate of *M. a. paratuberculosis* was marked in Lj3 (72.2%), and the rate gradually declined with distance from this region and rose in Lc (67.9%) again. The cultivation of the intestinal mucosa showed that the positive rate of *M. a. paratuberculosis* infection had the first peak in Mj3 (67.9%) and the second peak in Mi2 (69.7%). The first peaks of the lymph node and the mucosa occurred in the distal jejunum, but the sec-

ond peaks were in the caecum and the distal ileum, respectively. The cecal lymph node and the distal ileum stand very closely each other.

The positive appearance rates did not prominently differ and stayed over the level of 50% between Lj1 and Lc, and between Mj1 and Micv (Figure 1).

The positive rates in tissue culture (%) were compared among 19 different regions. The detection rates in the regions between Lj1 and Lc were higher than those in the duodenum and the rectum ($P < 0.05$) except between Ld2 and Li2 ($P = 0.0624$). The regions of Lj3 and Li1 were additionally better than the regions of Lj1 and Li2 ($P < 0.05$). The regions from Mj1 to Micv were significantly different from the duodenum ($P < 0.01$), the caecum and the rectum ($P < 0.05$). Positive appearance rates in the regions from Mj1 to Micv had no critical differences one another ($P > 0.05$).

Mean number of CFU calculated from the culture of each GIT-related tissue did not clearly parallel the regional difference figured out in the detection rate of *M. a. paratuberculosis*. The mean number of CFU culminated at Li1 (117.7 CFU) and Lc (104.1 CFU) among the lymph nodes, and at Mi1 (102.3 CFU) and Micv (94.3 CFU) among the mucosae (Figure 2). The first peaks of the lymph node and the mucosa were seen in the proximal ileum, and the second peaks were in the caecum and the ileocecal valve, respectively. The mean number of CFU in the regions between Ld1 and Lc except Ld2 and the regions between Md2 and Mc was more than 50 CFU.

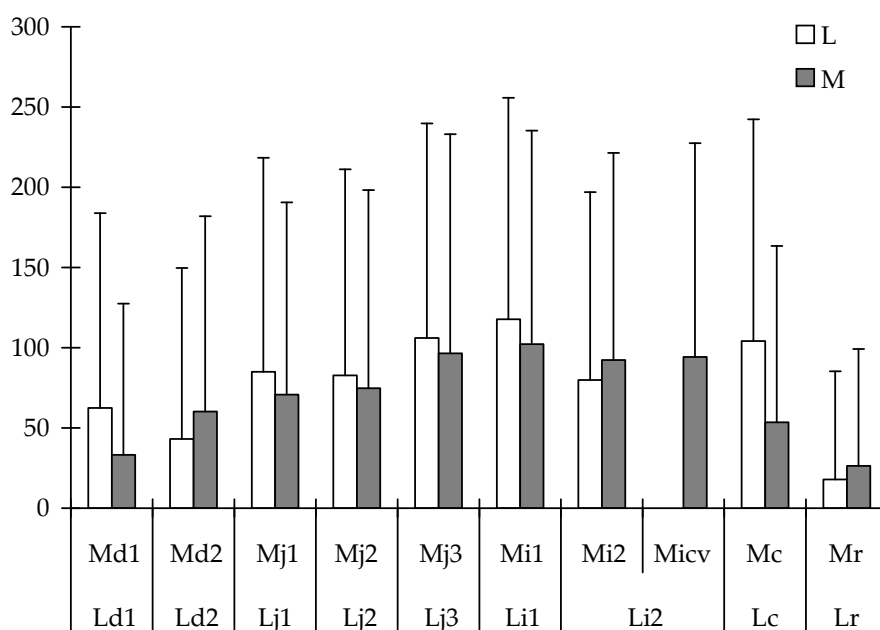


Figure 2. Mean number \pm SD of colony forming units (CFU) after cultivation of 14–16 weeks at 37°C. White and grey columns indicate lymph node (L) and mucosa (M), respectively. Vertical bar indicates number of CFU.

Abbreviations follow Figure 1

The comparison of each region of the intestinal mucosa and its related lymph node by the mean number of CFU proved that Lj3 and Li1 with the mean number over 100 CFU were higher than both the duodenum and the rectum ($P < 0.05$). Lj1, Lj2 and Lc were different from Lr ($P < 0.05$), but only Lc differed from Ld2 ($P < 0.05$). The regions from Mj1 to Micv were better than the proximal duodenum and the rectum ($P < 0.05$), but only Mj3 and Mi1 marking about 100 CFU were statistically higher than the caecum ($P < 0.05$). Comparative study of the positive rate and the mean number of CFU confirmed that there were no crucial differences between the lymph nodes related to the duodenum and the rectum and between the mucosae belonging to the duodenum, the caecum and the rectum ($P > 0.05$).

In Figures 3 and 4, the results of tissue culture by the positive appearance rate and the mean number of CFU were graphed by two levels of faecal culture results. One group with mean number of $n < 10$ CFU (39 cows) and another group with mean number

of $n \geq 10$ CFU (24 cows) in faecal culture were illustrated by the grey dotted line and the black line, respectively. Total average of positive rate and total mean number of CFU were crucially different between these two groups in the mucosa (M) (37.3% , 39.1 ± 94.8 CFU; 75.1% , 127.5 ± 140.1 CFU) and the lymph node (L) (45.3% , 45.3 ± 100.1 CFU; 74.8% , 150.3 ± 142.6 CFU) ($P < 0.01$). In a group of $n < 10$ CFU, the regions between Lj2 and Lc had better detection rates (over 50%) than other regions ($P < 0.05$) with exception of no difference between Ld2 and Li2 ($P = 0.0736$).

On the other hand, in a group of $n \geq 10$ CFU, there was no remarkable difference among the lymph nodes and all of regions marked more than 50%. The mean number of CFU could not prove any regional differences in both groups ($P > 0.05$), but all of regions in a group of $n \geq 10$ CFU except the rectum had very high mean number of CFU over 100. Only Lj3, Li1 and Lc in a group of $n < 10$ CFU had the mean number over 50 CFU. Maximum positive rate

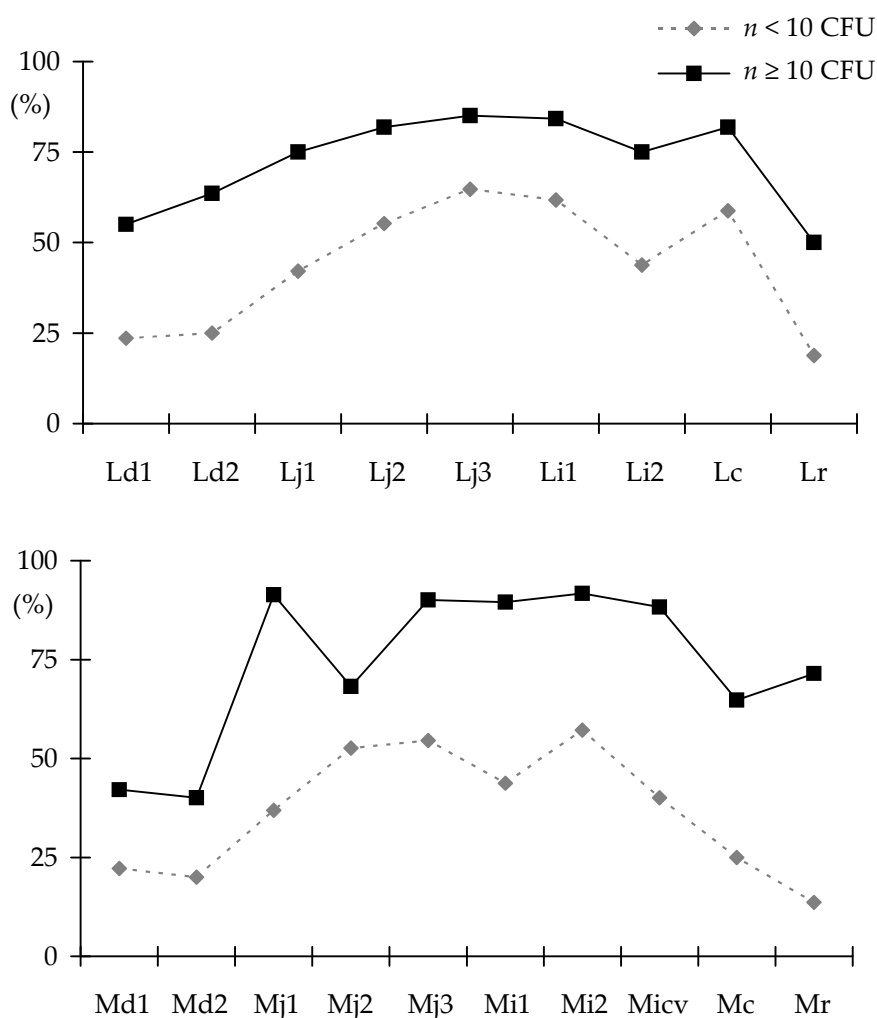


Figure 3. Positive rates in culture of the lymph node (L) and the mucosa (M) according to two different levels of faecal culture results. See Results for more detail

Abbreviations follow Figure 1

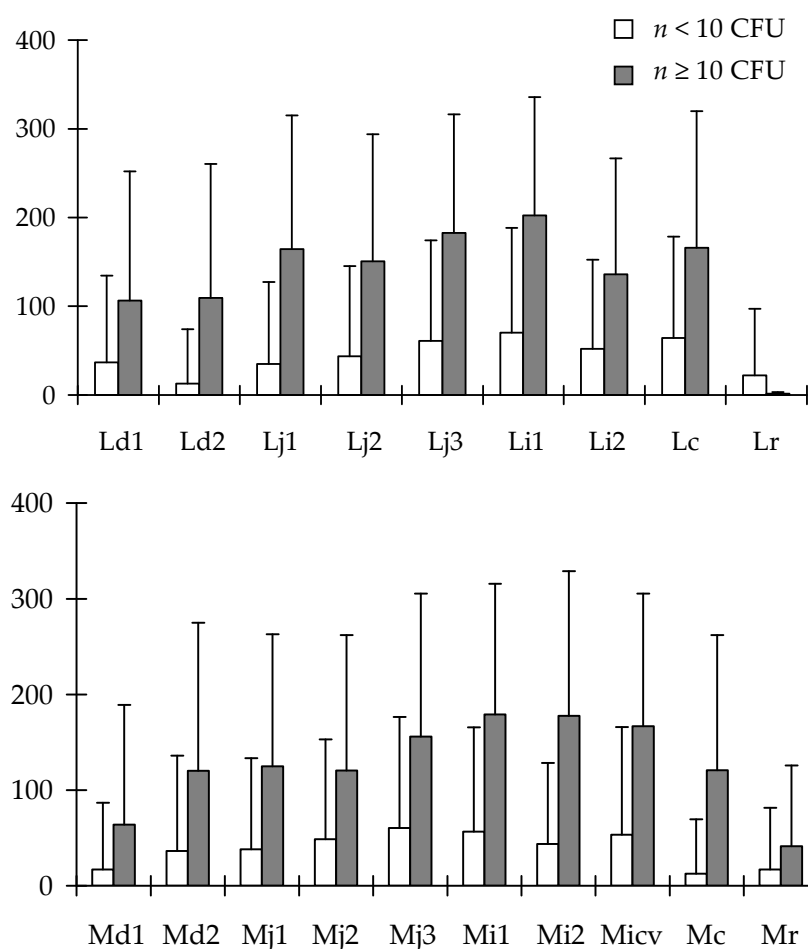


Figure 4. Mean number \pm SD of colony forming units (CFU) in culture of the lymph node (L) and the mucosa (M) compared by two levels of faecal culture results

Abbreviations follow Figure 1

and mean number in a group of $n < 10$ CFU were seen in Lj3 (64.7%) and in Lj1 (70.3%), respectively. In a group of $n \geq 10$ CFU, the analysis of the mean number of CFU proved that the regions from Lj1 to Lc were better than Lj1 in a group of $n < 10$ CFU ($P < 0.05$), but the positive rate did not show the advantage of any regions of $n \geq 10$ CFU over Lj3 of $n < 10$ except Lj3 of $n \geq 10$ CFU. The number of samples from the lymph node of the rectum was only 4 in a group of $n \geq 10$ CFU, so the rectum was excluded from the analysis of the lymph node.

The positive rates of Mj2, Mj3 and Mi2 were over 50% in a group of $n < 10$ CFU, and were statistically higher than those of the duodenum, the caecum and the rectum ($P < 0.01$). In a group of $n \geq 10$ CFU, the regions between Mj1 and Mc had the positive rate over 50% and the mean number over 100 CFU. Especially, the regions between Mj1 and Micv except Mj2 which marked about 90% by the positive rate were significantly better than the duodenum and the caecum ($P < 0.05$). The mean number of CFU showed that there was almost no difference among the mucosae in a group of $n < 10$ CFU, but in a group

of $n \geq 10$ CFU, the crucial lower mean number of CFU appeared in the proximal duodenum and the rectum, especially in comparison with the regions between Mj3 and Micv ($P < 0.05$). Maximum rate and mean number of CFU were observed in Mi2 (57.1%) and in Mj3 (60.3%) in a group of $n < 10$ CFU, respectively. The regions between Mj1 and Micv in a group of $n \geq 10$ CFU were significantly better than Mi2 of $n < 10$ CFU by the positive rate and Mj3 of $n < 10$ CFU by the mean number of CFU ($P < 0.05$), but the superiority of Mj2 of $n \geq 10$ CFU was denied by the positive rate ($P > 0.05$). The differences ($P < 0.05$) of the same region by both analyses were remarkable between two groups among the lymph nodes in all regions (excluding the rectum) except Lc by the positive rate and among the mucosae from the jejunum to the rectum except Mr by the mean number.

In Figures 5 and 6, the results of tissue culture were compared according to whether animals had macroscopic lesions in some of mesenteric lymph nodes or in some of intestines. Lesions in the GIT were scrutinised during sampling. If the lymph

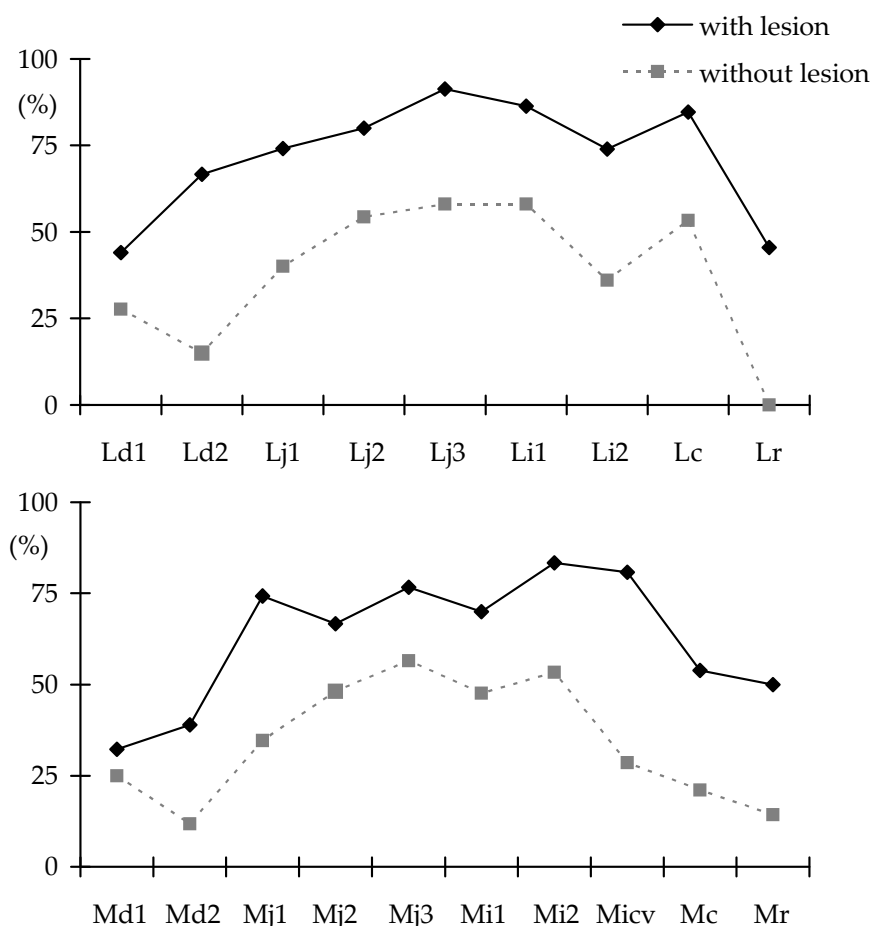


Figure 5. Positive rates in culture of the lymph node (L) and the mucosa (M) with (black line) and without lesions (grey dotted line) in the mesenteric lymph nodes. See Results for detail

Abbreviations follow Figure 1

node was enlarged and/or had inflammatory exudate in some of mesenteric lymph nodes, 27 animals were regarded as animals with lesions in the lymph node (described as “with lesion” in figures). If the intestinal wall increased in thickness in some of intestines, the animal was regarded as animal with lesions in the intestine or the mucosa (36 cows). When the lesions were found in one of mesenteric lymph nodes, all of lymph nodes collected from the same animal were grouped in “with lesion” even though they were macroscopically normal. The same went for the mucosa.

The differences ($P < 0.05$) in the same region between two groups were recognized by the positive rate and/or the mean number of CFU in all regions of the lymph node and the mucosa except Md1. In animals without lesions, Lj2, Lj3, Li1, and Lc (over 50%) were statistically higher than the duodenum ($P < 0.05$) and the rectum ($P < 0.01$), and the regions from Mj2 to Mi2 (about 50%) were better than the duodenum, the caecum and the rectum ($P < 0.05$). But the mean number of CFU did not prove any differences among the lymph nodes and among the

mucosae ($P > 0.05$) in the case of “without lesion”. The mean number over 50 CFU was seen only in Li1, Lc, and Mj3.

In animals with lesions, the regions between Ld2 and Lc and the regions between Mj1 and Mr had the positive rate over 50%. The regions from Lj1 to Lc with the positive rate over 74% and the mean number over 100 CFU were different from the proximal duodenum and the rectum, and Lj3 differed from the whole duodenum by the positive rate ($P < 0.05$). Lj3 and Li1 were higher than the duodenum ($P < 0.05$) and the rectum ($P < 0.01$) by the mean number. The positive rate in the regions from Mj1 to Micv (over 74%) except in Mj2 (66.7%) and Mi1 (70%) differed from that in the duodenum and the rectum ($P < 0.05$). The mean number of CFU between Mj1 and Micv (over 97 CFU) were higher than that of the proximal duodenum ($P < 0.05$) and the rectum ($P < 0.01$). Especially, Mi1, Mi2, and Micv (about 150 CFU) showed their superiority over the caecum as well ($P < 0.05$).

Maximum positive rates and mean number of CFU were seen in Lj1 (58.1%, 65.8 CFU) and Mj3

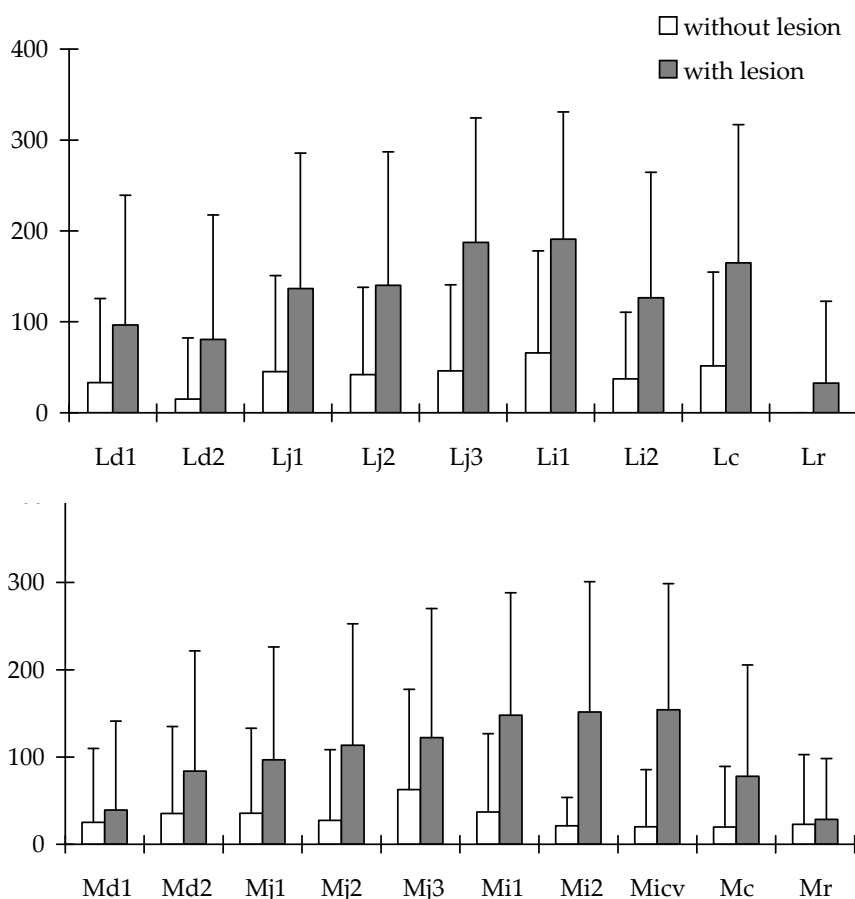


Figure 6. Mean number \pm SD of CFU in culture of the lymph node (L) and the mucosa (M) with (grey columns) and without macroscopic lesions (white columns)

Abbreviations follow Figure 1

(56.5%, 62.9 CFU) in animals without lesions. The regions from Lj1 to Lc with lesions were better than Lj1 without lesions by both analyses ($P < 0.05$) except Lj1 and Li2 with lesions by the positive rate. The regions of Mi2 and Micv with lesions by the positive rate and the regions from Mj3 to Micv with lesions by the mean number of CFU were higher than Mj3 without lesions ($P < 0.05$). Nine samples were obtained from the rectum in animals without lesions, and none of them yielded culture positive.

Laparoscopy

First trial. In the first trial, the laparoscope was directed towards the ileocecal area in an attempt to reach some lymph nodes situated around this area from direction of the dorsal border of the cecum because in standing position, this area seemed to be the most accessible from the right flank. Fat and connective tissues standing in the way of the target were removed by cutting with scissors, but continu-

ous secretion and mild bleeding from cut ends of the tissues and thick adipose tissue interfered with this approach. Post-surgical necropsy disclosed that the scissors used for the laparoscopic approach were not appropriate to cut a path through abundant adipose tissue.

Second trial. During a second trial, an approach through the ventral border of the caecum was attempted to circumvent an obstacle of the adipose tissue. A metallic tube with an adjuster of gas flow (50 cm long and 8 mm in diameter) was used to supply CO₂ gas into the abdominal cavity, but space produced by this device was not enough to keep visual observation and to enable manipulation of the laparoscope between the intestines. The laparoscopic approach was sometimes interrupted by intermittent fluid flow from dissected tissues pooled in narrow interspace between the intestines. The necropsy revealed that this approach had to face pressure from the intestine and might induce injury to or perforation of the intestinal wall.

Third trial. In the third trial, a bull was placed into left recumbency in order to access some lymph

nodes in the jejunal area. When the animal was placed on a surgical table, the large rumen moving from the left to the right side denied the creation of space within the abdominal cavity. All efforts to reach the target were in vain and in danger of penetrating the intestine.

Fourth trial. A fourth trial was performed after one-day starvation of the animal and the biopsy was carried out in standing position. In this position, the intestine gravitated to the bottom of the abdominal cavity, and more space was available in the dorsal part of the abdomen by decreasing the rumen volume. But strong pressure from twisted intestines did not allow the laparoscope to go through the intestines and to head into the target. Insufflation of CO₂ gas did not improve the space issue.

DISCUSSION

Nineteen different sampling sites shown in Table 1 were decided according to “Atlas of Topographical Anatomy of the Domestic Animals” edited by Popesko (1977). Whole intact GIT from the duodenum to the rectum was removed from the abdominal cavity and placed on a dissecting table so that anatomical location of each target sample was unequivocally understood and distinguished. Two samples were obtained from descending and ascending parts of the duodenum (about 1 m in length). The duodenum begins at the pylorus and ends at the jejunum, which is arranged in numerous close coils. The length of the jejunum (about 90% of the total length of the small intestine) was equally divided among three. Three samples of the jejunum were obtained according to their location (proximal, middle and distal parts). The jejunum is continued by the ileum with straight shape. The ileum (the last 4 or 5% of the length of the small intestine) was largely divided into half. Sampling was done from the anterior half and the posterior half of the ileum. One sample was picked up from the ileocecal valve which is a junction between the ileum and the caecum, and the others from the caecum with S-shape ending as a blind tube and the rectum at the end of the large intestine (Bone, 1988). Each lymph node, very close to these intestines, was selected and collected simultaneously. To achieve accurate sample collection, a few licensed veterinarians (some of our authors) with the anatomical knowledge of cattle were engaged in sampling.

Although the majority of prominent lesions in Johne’s disease were recognized in the distal ileum and the ileocecal valve during natural infections (Buergelt et al., 1978), the lesions were also observed throughout the GIT (from the duodenum to the rectum) in advanced infection stages (Taylor, 1953). The comparative analysis of the positive rate showed that the mucosae from the jejunum to the ileocecal valve and the lymph nodes corresponding from the jejunum to the caecum seemed to yield better detection of *M. a. paratuberculosis* in tissue culture. The superiority of Li2 over Ld2 could not be apparently proved by the positive rate, but Li2 was not different from Lj1, Lj2 and Lc, which had significantly higher positive rates than the duodenum and the rectum.

On the other hand, the comparison of the mean number of CFU in the lymph nodes and the mucosa could not clearly disclose significant regional difference as given by the positive rate. The mean number of CFU ranged very widely in each site of the GIT as high SD indicated. The mucosae and the lymph nodes only from the distal jejunum and the proximal ileum had absolute superiority over the mucosae from the proximal duodenum, the caecum and the rectum and over the lymph nodes from the duodenum and the rectum, respectively. Tissue samples from the duodenum and the rectum did not have any differences each other and should not be taken as the first choice of sampling except in the case of heavy infection.

The Payer’s patches mainly distributed over the jejunum and the ileum (Landsverk et al., 1991) are portal sites of entry for the causal agent of paratuberculosis (Momotani et al., 1988). Their location, structure, and development in young calves (until 3 months of age) which are more susceptible to *M. a. paratuberculosis* than older animals (Anonymous, 2000) reflect the distribution of paratuberculosis in the GIT and its related areas. The primary lesions of *M. a. paratuberculosis* infection are also focused on these regions. Taking our statistical data with this special nature of *M. a. paratuberculosis*, the mucosae in the jejunum and the ileum and their adjacent lymph nodes seem to be worth sampling and suitable for the detection of paratuberculosis. The ileocecal valve and the cecal lymph nodes residing in the vicinity of the ileum were statistically identical to the jejunum and the ileum. These regions may be involved in sampling sites to diagnose paratuberculosis. The positive rate and mean number were also high enough in samples from

the jejunum, the ileum, ileocecal valve and the cecal lymph node.

As different timing of tissue sampling produced different results of uptake of *M. a. paratuberculosis* in the intestine after infection (Momotani et al., 1988; Sigurdardottir et al., 2001), the sites mainly infected with *M. a. paratuberculosis* depend on the stages of infection. The following four stages have been proposed: (1) non-infected, (2) subclinical carriers, (3) asymptomatic shedders, and (4) clinically affected animals (Larsen, 1973), and (1) silent infection, (2) subclinical disease, (3) clinical disease, and (4) advanced clinical disease (Whitlock and Buergelt, 1996). If the results from tissue culture are classified according to these clinical stages, and if definitive regional susceptibility to *M. a. paratuberculosis* is proven following the stages, more specific sampling sites will be disclosed and higher detection of the microorganism will be expected using these optimal tissues.

Moreover, two types of pathology have been known in paratuberculosis, i.e., the paucibacillary form and the multibacillary form. The former is related to strong cell-mediated immunity, low antibody concentrations and predominant Th1-like cytokines. The latter is associated with weak cell-mediated immunity, high antibody concentrations and predominant Th2-like cytokines. These different host responses appear to exert their influence on the proliferation and distribution of *M. a. paratuberculosis* (Clarke, 1997; Burrells et al., 1998). Thus, many factors are involved in the distribution of the microorganism in the GIT, and make it more complex to specify the localisation of *M. a. paratuberculosis*.

The growth rate of *M. a. paratuberculosis* in faecal culture also affected the bacterial growth in tissue culture. The differences were proven in lymph nodes of whole GIT and in the mucosae from the jejunum to the rectum between groups with low and high mean number of CFU in faecal culture. The distribution of *M. a. paratuberculosis* also showed that the regions between the middle jejunum and the caecum were adequate for sampling of lymph nodes with low mean number in faecal culture, and that *M. a. paratuberculosis* spread all over mesenteric lymph nodes with increased mean number in faecal culture. In the mucosa, the optimal sampling regions were extended from the regions between the middle jejunum and the distal ileum in low mean number group to the regions between the jejunum and the ileocecal valve in

high mean number group. The lymph nodes and the mucosae belonging to the jejunum and the ileum including the cecal lymph node and the ileocecal valve in a group of $n \geq 10$ CFU showed their superiority over those with the highest positive rate and mean number in a group of $n < 10$ CFU. In shedding animals with high mean number in faecal culture, good detection and high mean number of CFU can be expected in tissue culture of the mesenteric lymph nodes between the duodenum and the caecum, and of the intestinal mucosae between the jejunum and the caecum.

When the lesions were found in the mesenteric lymph nodes and in the intestines, the results were more favourable in tissue culture except in culture of the proximal duodenal mucosa. The optimal sampling sites in animals with lesions were recognised in the jejunum and the ileum including the ileocecal valve and the cecal lymph node, and ranged slightly wider than those in animals without lesions. Most of the optimal sampling sites had superiority over the regions with maximum positive rate and mean number in animals without lesion. Good bacterial growth and detection are presumable in culture of the lymph nodes between the middle jejunum and the caecum and the mucosae between the jejunum and the caecum when the lesions exist in the GIT. But when there were no lesions in the GIT, only the proximal ileal lymph node, the cecal lymph node and the distal jejunal mucosa had the positive rate over 50% and the mean number over 50 CFU. Bacterial proliferation is very poor in culture of the rectal lymph node without lesions in the mesenteric lymph nodes.

The culture method requires a long incubation time (about 2 to 3 months) for the diagnosis of paratuberculosis. PCR techniques have dramatically made significant progress in the past ten years and enabled prompt and accurate identification of *M. a. paratuberculosis* (Svastova et al., 2002). But in the case of the paucibacillary form of paratuberculosis, the low numbers of the microorganisms limit the use of PCR techniques, by which viable and dead bacilli cannot be distinguished. The culture still remains as a gold standard method for the diagnosis of paratuberculosis.

The isolation of the microorganism in the early stage of the disease is essential for the eradication of paratuberculosis. The approach via laparoscopy seems to aid this purpose especially in calves and non-shedders for detecting the causative agent of paratuberculosis directly from tissue samples.

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