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REVIEW

Epidemiological situation and control strategies for paratuberculosis in Japan

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Abstract

Paratuberculosis (Ptb), caused by *Mycobacterium avium* subsp. *paratuberculosis* (*Map*), is a chronic and progressive granulomatous enteritis that affects many livestock and wild animals worldwide. The clinical disease is called Johne's disease (JD). In Japan, all dairy cattle (harf million head) are examined for Ptb every five years. About 1000 the officially examined cattle are diagnosed annually as positive for Ptb, but most of these exhibit only minor or no clinical signs and typical lesions in recent years. In contrast to the situation in Japan, the disease prevalence in western countries is very high. We have used ELISA and a culture examination of *Map*, and recently real-time PCR to diagnose this disease. In this review, the author outlines the history of the epidemic and national practical strategies to control paratuberculosis in Japan.

Keywords: Paratuberculosis, Johne's disease, Mycobacterium, bovine, Crohn's disease

Low prevalence of paratuberculosis in Japan

Paratuberculosis (Ptb), caused by *Mycobacterium avium* subsp. *paratuberculosis* (*Map*), is a chronic and progressive granulomatous enteritis (Figs. 1A and B) that affects many livestock and wild animals worldwide[26–28]. The clinical disease is called Johne's disease (JD), however this is used as synonym for paratuberculosis, a *Map* infection. After oral ingestion and invasion into Peryer's patch via M-cells[19], Map infection has very long incubation period (3–6 years) and antibody level is increas in late stage of infection[6].

In Japan, every dairy farm is examined for Ptb every five years in accordance with the Act on Domestic Animal Infectious Diseases Control, after 1998[22]. About 1000 of the half-million head of officially examined cattle are diagnosed as having Ptb annually (the Japanese Animal Health Statistics; MAFF, 2009), but most of these exhibit only minor or no clinical signs and typical lesions. Unlike in Japan, the disease prevalence in western countries is very high. In the United States, for example, over 70% of dairy herds are contaminated[31]. and Ptb causes an estimated annual loss of $220 million to the agricultural economy. The prevalence of Ptb in

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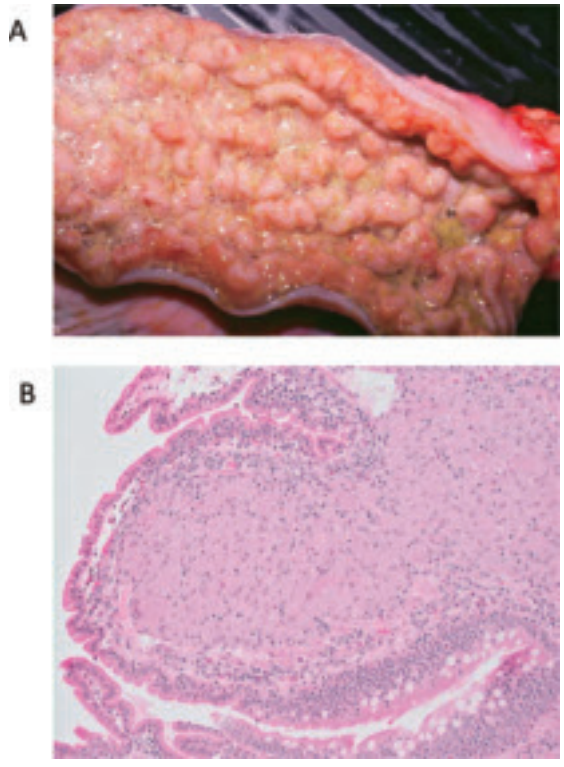


Fig. 1. Pathological findings of paratuberculosis. A: Typical macroscopic finding of bovine paratuberculosis. Swelling of the mucosa is significant, but ulcer or erosion are not observe. B: Typical epithelioid cell granuloma in laminapropria mucosa of ileum. There are few giant cells. (Hematoxylin and Eosin staining).

cattle in Australia, New Zealand, and Europe ranges from 10 to 60%[14]. The low prevalence in Japan is considered to be the result of following active strategies for eradication.

Historical prevalence of Ptb

The first case of bovine paratuberculosis was recorded in Shorthorn breed cattle imported from England in 1930. The first detailed case study was carried out in Holstein breed cattle imported from Wisconsin, USA[32]. In the 1960s, a major outbreak of Ptb occurred in Holstein cattle imported from the USA in Hokkaido[34]. Detailed epidemiological studies suggested that three cattle imported from the USA transmitted Ptb to 35 farms[32]. In the 1980s, major outbreaks of Ptb occurred in the Japanese Black Breed, an important Japanese beef strain, in Hokkaido, Tohoku district, and the Kanto region in Japan. The source of the outbreak was several Aberdeen Angus cattle imported from the USA[33]. Before 1980, outbreaks of Ptb were limited to imported cattle and calves. However, the control was not perfect, and the disease was transmitted to Japanese cattle kept together in the farm and began to spread throughout Japan (Fig. 2). In the 1980s, the author observed many cases of Ptb-infected cattle, including dual infection with tuberculosis, in the Japanese Brown breed in Hokkaido[20,21]. Number of detected cattle as paratuberculosis in Japan is shown in Fig. 3.

Paratuberculosis detection in Animal Quarantine Service

From 1976 to 1997, the Japanese Animal Quarantine Service detected 282 animals infected with paratuberculosis[17]. Fifty-four percent of these were animals imported from the USA. Fig. 4 presents the numbers of imported cattle quarantined from 1975 to 2010. Avarage head of 14 (from 3 to 21) cattle were diagnosed as having Ptb in each year. Despite active examination of imported animals using antibody detection (ELISA), Johnin skin test and bacterial culture (Harold's egg yolk medium), and DNA isolation from fecal samples (Johne Prep or Johne Spin), as well as real-time PCR (SYBR Green), numerous subclinical-stage infected animals passed the quarantine and developed the clinical disease on many Japanese farms. To prevent the import of cattle in the subclinical stage of Ptb, the government should only permit cattle raised on foreign farms having clean conditions analogous to Japanese standard category I. Sweden, one of the most clean country in Ptb, has very good control strategies including animal quarantine and regulation of import of animal[16,29].

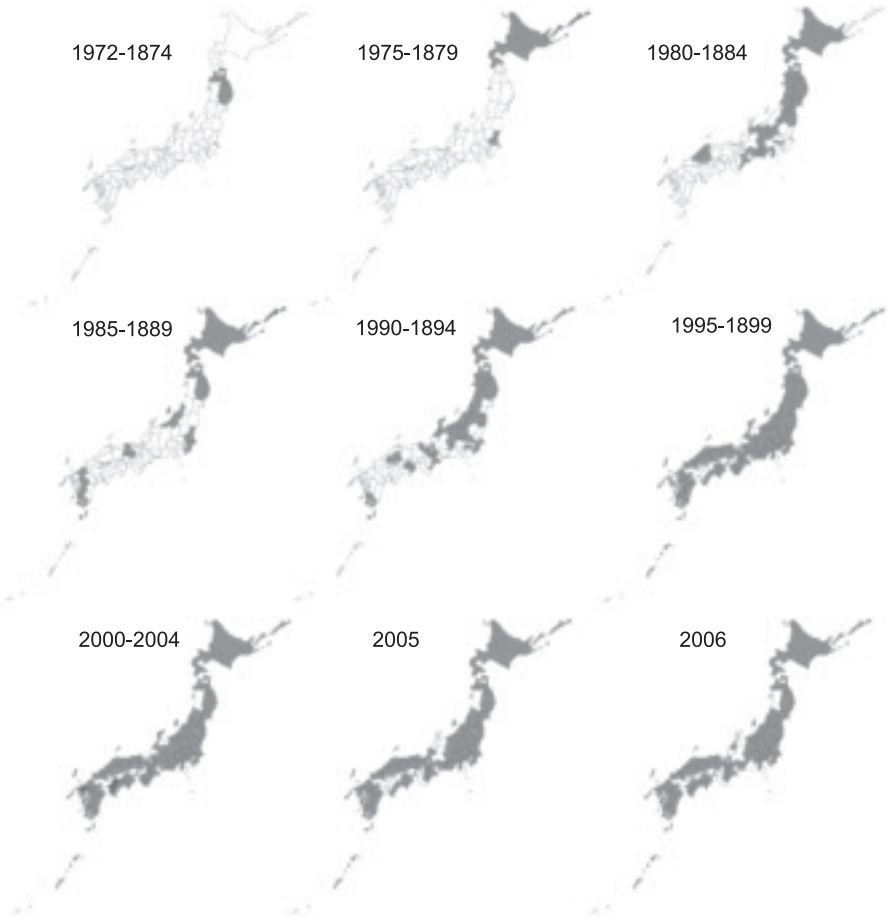


Fig. 2. Spread of bovine paratuberculosis over time in Japan. This figure exhibits onset of paratuberculosis in prefecture of Japan from 1972 to 2006. Gray color indicates onset of paratuberculosis. Chronic spared of the disease is apparent.

Powerful control strategy backed by Japanese law

The current low prevalence of Ptb in Japan strongly suggests that continuous diagnosis with proactive culling of test-positive animals (i.e., a test-and-cull strategy) could be an effective way to eradicate Ptb. Japanese law, specifically the Act on Domestic Animal Infectious Diseases Control, requires that cattle officially diagnosed as having paratuberculosis be killed. The government will compensate farmers for about 80% of the value of the cattle according to the Act. If we had no compensation and test-and-cull were carried out on a voluntary basis, we could not have achieved the current low prevalence of Ptb in Japan.

Guidelines for Control of bovine paratuberculosis

To control Ptb well, the Committee for the Development of Early Diagnosis for Ptb in the Ministry of Agriculture, Forestry and Fisheries (MAFF) of Japan met many times and released a set of guidelines on November 1, 2006. The guidelines were composed of a categorization of farms according to the status of infection and general hygienic guidelines relating to farms and trade.

Dairy farms are categorized based on test results into Category I, clean farms, and Category II, farms under control. A Category I farm is evaluated by a regular ELISA test and bacterial culture. Such a farm has no detectable Ptb-positive cattle. A Category II farm has

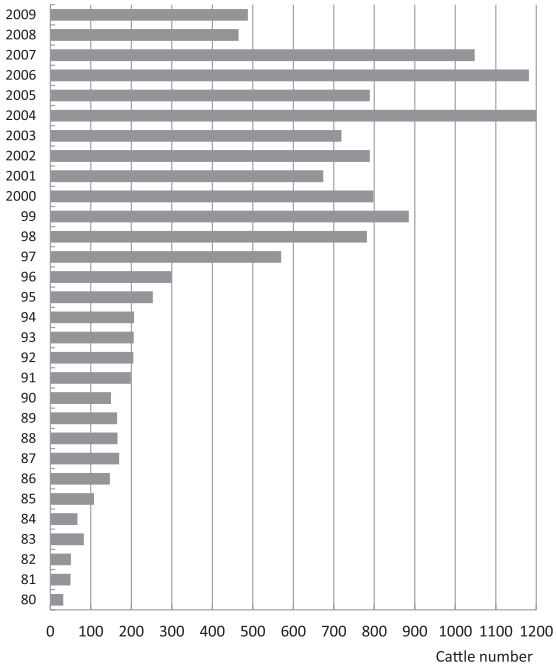


Fig. 3. Number of detected cattle as paratuberculosis in Japan. In this graph, increased number of detected cattle with paratuberculosis in 1997 and 1998 relates introduction of ELISA testing and official surveillance of individual farm with 5 years interval. Acute decrease in 2008 and 2009 was caused by stopping cultural examination.

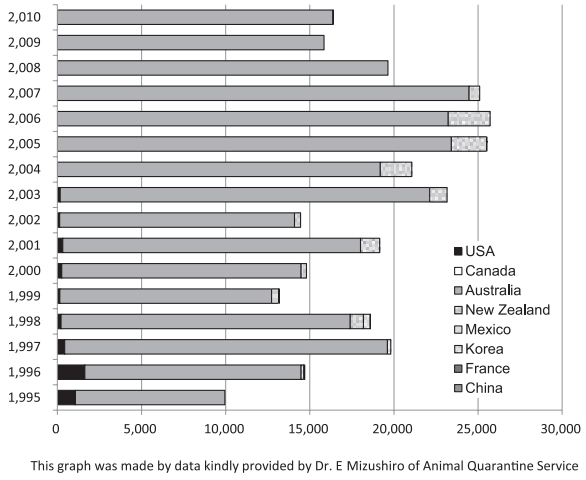


Fig. 4. Number and exported countries of cattle at Animal Quarantine Service. This graph was made by data provided by Dr. Mizushio of Animal Quarantine Service.

Ptb-positive cattle as determined by regular examinations carried out every five years and/or follow-up surveillance and is endeavoring to implement countermeasures. Being a Category II farm does not necessarily indicate the presence of clinical paratuberculosis. Once positive cattle are discovered, follow-up surveillance by ELISA and bacterial culture (PCR) will continue every three months until the result becomes completely negative, at which time it will be reclassified as Category I. However, the prefectural LHSC will continue to carefully monitor the hygienic status of the farm.

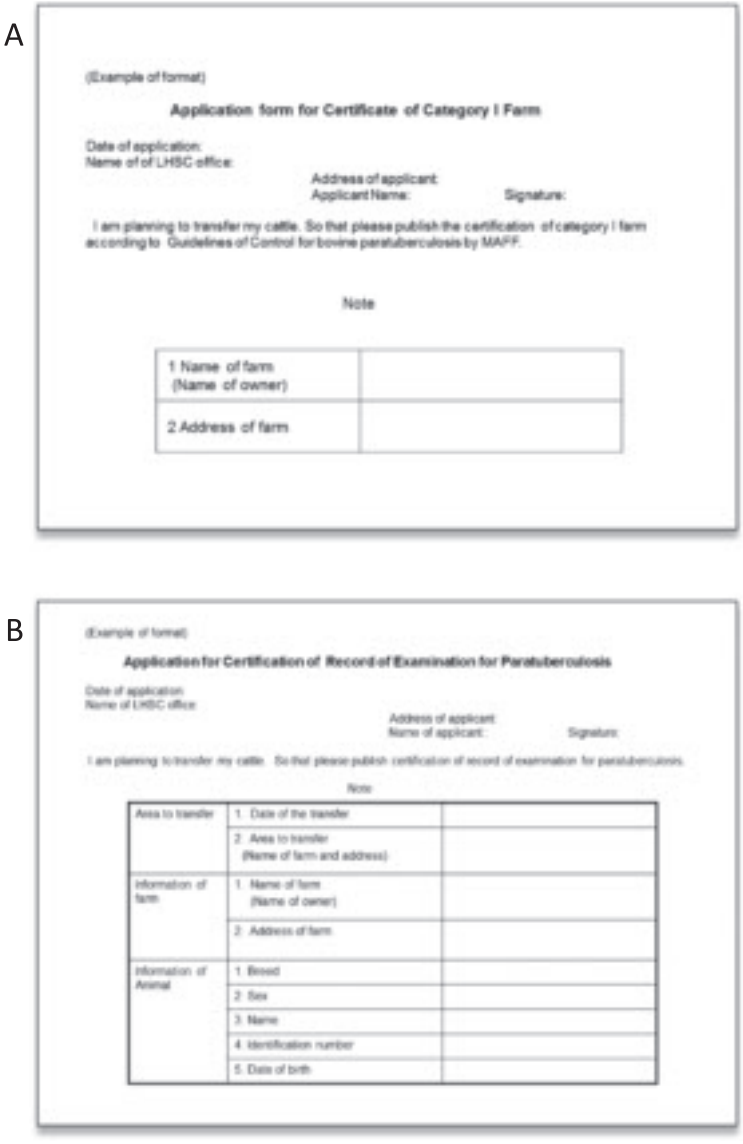
Preventative measures taken by prefectural livestock hygiene service centers

Every prefecture has two to five livestock hygiene service centers (LHSCs), which have a suitable number of veterinary staff for the area. Despite the limited number of cattle, we have a very precise epidemic prevention system, laid out like a spider's web, in Japan. The National Institute of Animal Health trains prefectural veterinary staff members, and they in turn educate farmers about adequate hygienic management for paratuberculosis and if necessary publish certificates confirming the status of the infection. The hygienic management component of LHSC handling includes (1) cleaning the cow house and farm environment, (2) disinfecting the entrance and boots, (3) careful observation and good communications with the veterinarian, (4) maintenance of a clean delivery room, (5) the use of replacement, (6) separation of newborn calves from the mothers as soon as possible, and (7) careful aging of manure. To prevent contaminated calf or cattle, the guidelines provides the following advisories to seller and buyer. (1) They recommend introducing cattle from a Category 1 clean farm by confirmation of the certificate for the category and a re-examination by ELISA at the introduction. (2) If the buyer needs to introduce cattle from a Category II farm, the cattle should have been negative in more than two ELISA tests within a three-month interval during the last six months and negative at least once in *Map* culture. (3) The seller can ask the

LHSC to provide a certificate of the category (Fig. 5A) and an individual record of the history of examinations for Ptb (Fig. 5B). If the buyer needs to introduce cattle less than nine months old, two or more negative culture test results are recommended. The introduced cattle should be kept in a quarantined area until proven negative in two ELISA tests, if possible. The farmer can introduce safer animals with individual (Fig. 6A) and farm certificates (Fig. 6B) indicating paratuberculosis negative.

Regulation of temporary transfer of cattle on a Category 2 farm

When a farmer of a Category II farm would like to enter cattle into a competitive exhibition, the cattle must have been negative in two or more ELISA tests within a three-month interval during the last six months and negative at least once in *Map* culture. Even on a Category I farm, cattle should be confirmed to be ELISA negative prior to temporary transfer to a


A

(Example of format)

Application form for Certificate of Category I Farm

Date of application:
Name of of LHSC office:

Address of applicant:
Applicant Name: Signature:

I am planning to transfer my cattle. So that please publish the certification of category I farm according to Guidelines of Control for bovine paratuberculosis by MAFF.

Note

1 Name of farm (Name of owner)	
2 Address of farm	

B

(Example of format)

Application for Certification of Record of Examination for Paratuberculosis

Date of application:
Name of LHSC office:

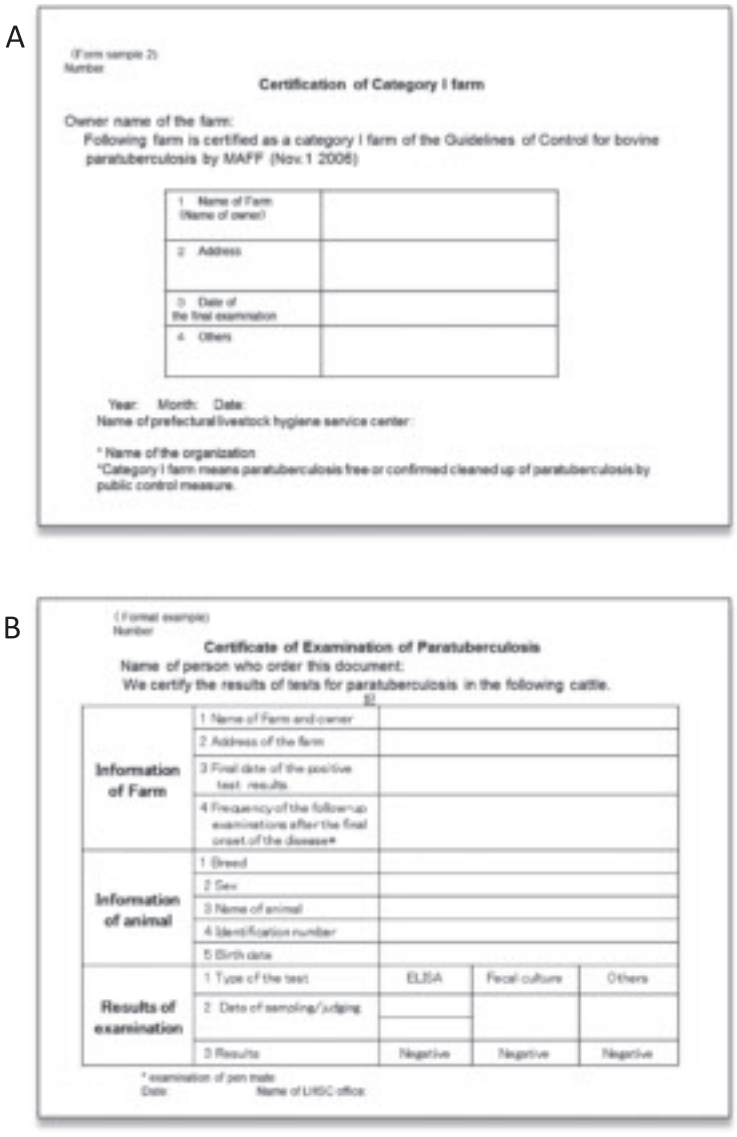
Address of applicant:
Name of applicant: Signature:

I am planning to transfer my cattle. So that please publish certification of record of examination for paratuberculosis.

Note

Area to transfer	1. Date of the transfer	
	2. Area to transfer (Name of farm and address)	
Information of farm	1. Name of farm (Name of owner)	
	2. Address of farm	
Information of Animal	1. Breed	
	2. Sex	
	3. Name	
	4. Identification number	
	5. Date of birth	

Fig. 5. Sample documents of certification of clean revel. A: Translated sample of application form to get category certification. B: Translated sample of application for certification of record of examination for paratuberculosis for individual animal.


A

(Form sample 2)
Number

Certification of Category I farm

Owner name of the farm:
Following farm is certified as a category I farm of the Guidelines of Control for bovine paratuberculosis by MAFF (Nov.1 2006)

1 Name of Farm (Name of owner)	
2 Address	
3 Date of the final examination	
4 Others	

Year: Month: Date:
Name of prefectural livestock hygiene service center:

* Name of the organization
*Category I farm means paratuberculosis free or confirmed cleaned up of paratuberculosis by public control measure.

B

(Format example)
Number

Certificate of Examination of Paratuberculosis

Name of person who order this document:
We certify the results of tests for paratuberculosis in the following cattle.

Information of Farm	1 Name of Farm and owner			
	2 Address of the farm			
	3 Final date of the positive test results			
	4 Frequency of the follow-up examinations after the final onset of the disease*			
Information of animal	1 Breed			
	2 Sex			
	3 Name of animal			
	4 Identification number			
	5 Birth date			
Results of examination	1 Type of the test	ELISA	Fecal culture	Others
	2 Date of sampling/judging			
	3 Results	Negative	Negative	Negative

* examination of pen mate
Date: Name of LHSC office:

Fig. 6. Sample documents of application form to order certification. A: application form to get certification of Category I farm. B: application form to get certification of Category I farm

competitive exhibition, which may present the opportunity for horizontal transmission. Isolation and disinfection should be considered at the competitive exhibition.

LHSC control upon the emergence of Ptb

The prefectural LHSC plays a central role in biosecurity measures affecting farmers and clinical veterinarians. They conduct an inspection upon the emergence of Ptb according to the manual for Ptb examination made by NIAH, isolate infected animals, and issue a binding order to cull positive animals, and subsequently provide epidemiological surveillance, and disinfection of the farm. Upon the emergence of Ptb, all breeding cattle over six months old must be tested by ELISA and fecal cultural and real-time PCR examination. If a clinical Ptb animal exhibits diarrhea, emaciation, and reduced lactation yield, a fecal smear stained with Ziehl-Neelsen staining is examined microscopically. A Johnin skin test is applied if a young calf under

six months old is suspected of being infected. For histopathological examination, they must sample tissues according to the manual. The sampling tissues include, 10, 30, 50 and 100 cm upper ileum from ileocecal junction, jejunum near by mucosal lymphatic tissue, mesenteric lymph noded of ileocecal, ileal jejunual area and supermammary lymph node. Sampling of 10 cm of the cylindrical intestine fixed in 10–20% buffered formalin is recommended. Gentle injection of the fixative into the lumen by syringe is effective to make good tissue sections. A positive result of Ptb diagnosis must be reported to MAFF through the prefectural governor. The same information, with diagnostic samples from the emergence, must be sent to the NIAH.

Recommendation of voluntary culling

The prefectural LHSC conducts testing to prevent dissemination after the emergence and can recommend that the farmer perform voluntary culling if necessary. On a newly discovered positive farm, the remaining pen mates must be tested annually for the next two years. A Category II farm must be tested three times per year by the LHSC. Since current immunological and bacterial examinations cannot diagnose all animals infected with Ptb, cattle having close epidemiological relations to the culled cattle should themselves be culled. This effectively enhances the cleanup of the farm. Cattle shedding a high level of *Map* DNA in the real-time PCR test are also strongly recommended for voluntary culling. This voluntary culling is good for animal hygiene, but means transfer the milk and/or meet from undetected subclunically infected cattle to food chain.

Development of practical control methods and application

ELISA: We used a complement fixation (CF) test before introducing ELISA which is mainly used together with bacterial detection (culture or fecal PCR test) to diagnose paratuberculosis. Research on antibody production in Ptb was reported in the 1970s in Japan[34]. Research on ELISA was started in the 1980s in NIAH[36]. However, since the ELISA method used whole mycobacteria antigen to capture the tested antibody, there were frequent false-positive reactions. A breakthrough technique for avoiding false-positive reactions, *M. phlei*-absorptive-ELISA, was developed by Dr. Y. Yokomizo of NIAH in 1991[35,37]. This ELISA method was introduced as a national standard method after 1997.

This ELISA exhibited a good positive agreement rate in comparative bacterial culture and pathological diagnoses, though the system uses a whole crude *Map* antigen instead of a specific *Map* antigen as the capture antigen[24]. These good results are considered due to a nonspecific reduction of polyclonal antibody level for general mycobacteria antigen, including *Map*, by absorption of test serum with *M. phlei* antigen. Actually, ELISA can detect cattle having a very high anti mycobacteria antibody titer using this absorption approach. Ptb-infected cattle in the advanced stage frequently have the antibody. As a result, the ELISA-positive cattle have significant paratuberculosis, although there are many ELISA-negative infected cattle (Table 1)[13].

Important role of culture examination: Until 2007, about 50% of all Ptb cases detected by national surveillance in Japan were detected by ELISA, and the remaining positive cases were detected by bacterial culture. A few percent were also detected by clinical diagnosis. Animals in

Table 1. Correlation of Map culture and ELISA

Culture	ELISA				Total	
	+		−			
+	91	15%	369	62%	460	77%
−	140	23%	0	0%	140	23%
Total	231	39%	369	62%	600	100%

earlier stages of Ptb shed *Map* to feces, but all cattle have detectable antibodies[25]. The number of ELISA-positive Ptb infected cattle thus began to be reduced, but culture-positive cattle continued to be detected (Table 1)[13].

However, the Ministry of Health, Labour and Welfare (MHLW) of Japan instructed that the use of bacterial culture be terminated after October 2007 because they recognized Ptb as a possible zoonotic infection agent, according to previous papers. Therefore, they provided guidance stating that the milk and meat of cattle diagnosed as having paratuberculosis should not be used for human consumption, and that companies must recall all such products retroactive to the day of sampling. Since it takes more than three months to get the results of a *Map* culture, a "gold standard" Ptb diagnosis could not be applied. Many bottles of milk and a huge number of dairy products thus went to market and were consumed during the culture period. Simply stopping cultural examination suggests overlooking half of the detectable Ptb-infected cattle (estimated at over 500 heads according to previous record) and failing at Ptb control in Japan. The author strongly hopes to resolve the virtual standstill of culture examination by prefectural LHSCs as soon as possible.

Application of real-time PCR: In September 2008, the Ministry of Agriculture, Forestry and Fisheries (MAFF) of Japan introduced a real-time PCR method to detect IS900, a *Map*-specific DNA sequence, as a semi-official diagnostic method.[22] The forward primer is MP10–1: 5'-ATG CGC CAC GAC TTG CAG CCT 3', and the reverse primer is MP11–1: GGC ACG GCT GTT GTA GTC G 3'. A QuantiTect SYBR Green PCR Kit (QUIAGEN, Germany) is recommended as a standard method. Nonetheless, we are still concerned about an increased incidence of Ptb. In diagnosing Ptb by real-time-PCR, we recommend duplicating the extraction from feces and estimating the DNA concentration in comparison with a known concentration in control DNA. The DNA concentration can be measured from 0.001 to 1.000 pg by the recommended protocol, with samples exhibiting over 0.005 pg/well identified as positive. Although the PCR method detects Ptb earlier than the current ELISA method, we should not forget that PCR-positive cattle must have already shed *Map* organisms on the farm in the past.

Voluntary culling

In addition to killing animals diagnosed as positive in official testing on Category II farms, prefectural governments can recommend that farmers voluntarily cull animals with close epidemiological and genetic relations to the positive cattle, and cattle in which real-time PCR detects higher levels of *Map* IS900 DNA. The farmer must apply for voluntary culling to the president of the prefectural funding organization through the director of the prefectural LHSC. The government will compensate farmers for this culling to some extent through the funding organization. This expense compensation is called an "incentive fee for culling." The fee is calculated as the assessed value minus the total sale price including meat, skin, and organ meat. The funding organization pays a handling charge to the person who evaluates the value of the cattle. This is an effective way to realize eradication, but suspected cattle being voluntarily culled, going to slaughter, and finally appearing on the dining table may be a problem from a food safety standpoint[10].

Recent problem of non-specific reaction in ELISA diagnosis

We have encountered at least two types of false-positive reactions in the ELISA test. The first one was caused by unexpected anti-albumin antibody in cattle serum. This auto-antibody was

considered to be generated by a certain type of oil adjuvant vaccine for virus. A commercially available ELISA kit had used bovine albumin to block the reagent of the plate, and the bovine antibody reacted strongly to the blocking materials, exhibiting very strong positive results. This problem was resolved by changing the blocking reagent. The second false-positive reaction seems to be caused by a mycobacteria antibody generated by mycobacterium other than *Map*; this is being evaluated now. The ELISA test is very easy and practical, but should be used as a screening method to find animals sensitized by mycobacteria, while specific diagnosis with "gold standard" methods, such as the isolation of *Map* or PCR detection of specific DNA, should be used for confirmation.

Need for eradication according to many public-health studies

The first report of isolation of *Map* in several Crohn's disease (CD) patients appeared in 1984[7,8,30]. Since then, much suggestive evidence has been accumulated, and interest in the importance of *Map* in food safety has increased[4,5]. There is ongoing concern that *Map* may be an etiological factor of human Crohn's disease[10,12], Due to evidence of *Map* contamination in dairy foods[3,10,11] and beef meat[1,23], the high incidence of bovine Ptb[26]. and the increasing incidence of human CD[9] worldwide, the studies on the comparative pathogenesis of the two diseases must be clarified[2,15]. We recently reported the different pathogenesis of CD and Ptb, and the risk of *Map*-contaminated milk and dairy products in the onset of Crohn's disease[18].

Conclusion

For needs of animal and public health, and development of animal industry, we need to eradicate Ptb by accumulating careful control trials. We eradicated tuberculosis and in Japan and checking Japanese and imported dairy foods for *Map* contamination.

Acknowledgments

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