オーペリのメイドの秘密

タイトル
エピデミオロジーの状況とパラチーピーレリアの制御戦略に対する日本の

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Epidemiological situation and control strategies for paratuberculosis in Japan

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Abstract
Paratuberculosis (Ptbc), 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 (half million head) are examined for Ptbc every five years. About 1000 the officially examined cattle are diagnosed annually as positive for Ptbc, 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 (Ptbc), 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. 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, Map infection has very long incubation period (3–6 years) and antibody level is increases in late stage of infection.

In Japan, every dairy farm is examined for Ptbc every five years in accordance with the Act on Domestic Animal Infectious Diseases Control, after 1998. About 1000 of the half-million head of officially examined cattle are diagnosed as having Ptbc 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 and Ptbc causes an estimated annual loss of $220 million to the agricultural economy. The prevalence of Ptbc in

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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. 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. 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. Fifty-four percent of 
these were animals imported from the USA. 
Fig. 4 presents the numbers of imported cattle 
quarantined from 1975 to 2010. Average 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.
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...
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 I 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

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.
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

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.
Status of paratuberculosis in Japan

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

**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
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 jejunal area and super-mammary 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 subclinically 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. Research on ELISA was started in the 1980s in NIAH. 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. 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. 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).

**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>
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<tr>
<th>Culture</th>
<th>ELISA</th>
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<tr>
<td>+</td>
<td>91</td>
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<tr>
<td></td>
<td>369</td>
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<td>-</td>
<td>140</td>
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<td>0</td>
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<tr>
<td>Total</td>
<td>231</td>
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<td></td>
<td>369</td>
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**Table 1. Correlation of *Map* culture and ELISA**
earlier stages of Ptbb sheds Map to feces, but all cattle have detectable antibodies\textsuperscript{(25)}. The number of ELISA-positive Ptbb infected cattle thus began to be reduced, but culture-positive cattle continued to be detected (Table 1)\textsuperscript{(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 Ptbb 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” Ptbb 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 Ptbb-infected cattle (estimated at over 500 heads according to previous record) and failing at Ptbb 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\textsuperscript{(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 Ptbb. In diagnosing Ptbb 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 Ptbb 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\textsuperscript{(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 19847,8,30. Since then, much suggestive evidence has been accumulated, and interest in the importance of Map in food safety has increased4,5. There is ongoing concern that Map may be an etiological factor of human Crohn’s disease10,12. Due to evidence of Map contamination in dairy foods3,10,11 and beef meat12,30, the high incidence of bovine Ptb26, and the increasing incidence of human CD9 worldwide, the studies on the comparative pathogenesis of the two diseases must be clarified2,15. 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 disease18.

**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.

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