國立臺灣大學獸醫專業學院獸醫學系

碩士論文

Graduate Institute of Veterinary Medicine

School of Veterinary Medicine

National Taiwan University

Master Thesis

臺灣牛隻副結核病之傳播風險因子和控制策略

Transmission Risk Factor and Control Strategy of Bovine Paratuberculosis in Taiwan

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中華民國九十九年六月

June, 2010

國立臺灣大學（碩）博士學位論文
口試委員會審定書

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本論文係李文達（R97629018）在國立臺灣大學獸醫學系完成之碩士學位論文，於民國 99 年 6 月 10 日承下列考試委員審查通過及口試及格，特此證明。

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Acknowledgements

Thanks for your reading!

中文摘要

牛隻副結核病的病原是副結核分枝桿菌（*Mycobacterium avium* subsp. *Paratuberculosis*; *MAP*）**，本研究之目標在於探查目前台灣牛隻副結核分枝桿菌感染狀況和傳播風險因子，並發展一套實用的防治計畫。利用血清酵素結合免疫吸附法（ELISA）進行副結核病的監控，並收集牧場環境樣本，用即時定量聚合酶鏈鎖反應檢測** (real time PCR) **和異數重複序列（**VNTRs**）檢測副結核分枝桿菌，並進行親源性的分析。結果顯示副結核病在泌乳牛場的血清盛行率是** 10.1%**，且有** 66.7%**之乳牛場為副結核分枝桿菌陽性場, 肉用牛隻的副結核病血清盛行率是** 2.8%**，而結核菌素皮內測試陽性牛隻的副結核病血清盛行率是** 51.4 %**，且在不同牧場中副結核病感染的狀況和其傳播的風險因子也相當的不同，露天運動場的土壤和糞便是傳播副結核分枝桿菌的媒介。此外本研究法共鑑定出九個亞型的副結核分枝桿菌，並顯示牧場間副結核分枝桿菌傳播的直接關聯性。最後，本研究基於上述結果及其他相關研究，針對台灣副結核病發展出一套控制計畫。**

關鍵字：控制計畫、血清酵素結合免疫吸附法、副結核病、即時定量聚合酶鏈鎖反應檢測、監測、異數重複序列

Abstract

Bovine paratuberculosis is caused by *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*). The aims of this study was to investigate the current *MAP* infection status and *MAP* transmission risk factors in Taiwan; thereby, to develop a practical control program. A nation-wise survey by serum enzyme linked immunosorbent assay (ELISA) was conducted. Environmental samples from dairy herds were collected for detecting *MAP* by real time PCR and for phylogenic analysis by variable number tandem repeats (VNTRs). Results indicated that *MAP* seroprevalence in dairy cow was 10.1%, and 66.7% dairy herds were positive, *MAP* seroprevalence of beef cattle was 2.8%, *MAP* seroprevalence of intradermal tuberculin test (ITT) positive cattle was 51.4%. *MAP* infection status and transmission risk factors are different among different herds. Soil and feces were high risk carries for transmitting *MAP*. Nine substrains of *MAPs* were identified indicating direct link of *MAP* transmission among herds. According to the results above and previous research, a *MAP* control program was developed.

Key words: control program, ELISA, paratuberculosis, real-time PCR, survey, VNTRs

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Chapter 1: Literature Review and Research Direction

Etiology of bovine paratuberculosis

Mycobacterium avium subspecies *paratuberculosis* (*MAP*) is the causative agent of bovine paratuberculosis (bPTB), and it is also called Johne's disease. *MAP* is a slow-growing, acid-fast, and obligate pathogen that can only proliferate in specific hosts, but data suggest that *MAP* survives well in the environment for more than 300 days (Whittington et al., 2004).

Influences of bovine paratuberculosis

The influences of paratuberculosis include:

(1) Animals

Paratuberculosis is a chronic granulomatous enteric infection that most commonly affects cattle, sheep and goat (Hines et al., 2007). According to the clinical signs, potential for shedding *MAP* into feces or milk, the severity of subclinical effects of infection, and the result of diagnostic tests, the infection stages of bPTB may be divided into 4 stages (Whitlock and Buergelt, 1996):

1) Silent stage: this stage generally includes young stock up to 2 year of age; the characters of this stage are no clinical signs, no measurable subclinical effects, and no cost-effective diagnostic tests that can be used.

2) Subclinical stage: Cattle with subclinical infection of *MAP* still don't have clinical signs, but can be detected by fecal culture, direct fecal polymerase chain reaction (PCR) or serological tests (Tiwari et al., 2005). Because of fecal shedding may be intermittent and some animals don't generate antibodies to against *MA*P (Kennedy and Benedictus, 2001), the result of fecal culture, direct fecal PCR or serological examination may be false negative. However, a detectable antibody response is usually after fecal shedding of *MAP* occurs (Lepper et al., 1989).

3) Clinical stage: clinical signs usually appear after 2 to 5 years because of a long incubation period and slow disease course (Kopecky and Larsen, 1975). The main clinical signs of this disease include chronic or intermittent diarrhea, gradual weight loss with a normal or an increased appetite, decreased milk production, and not responding to antibiotic treatment (Whitlock and Buergelt, 1996). Most cattle in clinical stage may be a heavy shedder, and be positive for fecal culture or direct fecal PCR and have enough serum antibody levels which are detectable by serological tests. Most cattle are culled in this stage due to chronic or intermittent diarrhea, decreased milk production, and emaciated (Whitlock and Buergelt, 1996).

4) Advanced clinical stage: clinically affected animals may become increasingly lethargic, weak, and emaciated. Watery diarrhea, hypoproteinemia, and intermandibular edema (bottle jaw) are the characters of this stage (Manning

and Collins, 2001).

(2) Dairy herds and nation

Some reports indicate the economic losses caused by paratuberculosis result from: 1) Decreased milk production: milk production is decreased in enzyme linked immunosorbent assay (ELISA) seropositive cows, when compared with seronegative cows (VanLeeuwen et al., 2001). It's determined that paratuberculosis decrease milk fat and protein yield, and costing almost US$ 205/cow/lactation (Sweeney et al., 1994). On the other side, the similar results of milk production losses associated with subclinical paratuberculosis are determined by fecal culture (Sweeney et al., 1994). Fecal culture positive cattle produce lower milk yield, when compared with fecal culture negative cattle (Wilson et al., 1993). On a whole, paratuberculosis has a considerable negative effect on milk production. 2) Premature culling and reduced slaughter value: The age of cattle culled in tissue culture positive cattle with clinical signs, tissue culture positive cattle without clinical signs, and non-infected cattle are 4.3, 4.9, and 7.7 year, respectively (Buergelt and Duncan, 1978). A study indicated that cattle with positive responses for *MAP* (Fecal culture and ELISA) are seems to be resulting in false positive results on caudal fold tuberculin (CFT) test (Dunn et al., 2005). The cattle affected by *MAP* may interfere with the result of intradermal tuberculin test (ITT), and

cause premature culling. Furthermore, slaughter value may be decreased by paratuberculosis, the reduced slaughter value of culled cattle are ranged from 20% to 30% (Benedictus et al., 1987). In culture based and ELISA based studies, the losses of slaughter value caused by subclinical paratuberculosis are US$ 48/herd and CDN$1330/herd, respectively (Chi et al., 2002a). 3) Reduced fertility: An ELISA based study indicates that the seropositive heifers have a longer open day (approximate 49 days) than seronegative heifers in first lactation (Tiwari et al., 2005). Oppositely, no association was found between subclinical paratuberculosis and fertility in other studies (McNab et al., 1991). Paratuberculosis is thought to be a factor on reduced fertility in some study, but some study indicates that paratuberculosis don't have negative effect on reproduction (McKenna et al., 2006). It's controversial about the association between paratuberculosis and reduced fertility, but a study suggests that paratuberculosis may cause impaired immunity and malnutrition to reduce the fertility in cattle (Johnson-Ifearulundu et al., 2000).

Economic losses associated with paratuberculosis among dairy herds also indicate that paratuberculosis may cause economic impact at herd, regional, and national level. In a United States study, paratuberculosis costs the US dairy industry approximate US$ 250 million/year (Ott et al., 1999). Subsequently, an ELISA based study in the Canadian Maritimes indicate that the annual costs of

paratuberculosis are CD$2472 per herd affected by *MAP*, and annually costs of Canadian dairy industry for paratuberculosis may be estimated at CDN$15 million (Chi et al., 2002a). Nowadays, the sensitivity of diagnostic methods is restricted by the infection stage of cattle, it's difficult to identify the subclinically infected cattle (See paragraph of "Diagnosis of bovine paratuberculosis"). Accordingly, the infected cattle may be misclassified as uninfected cattle by these diagnostic methods, so the estimate of economic losses caused by paratuberculosis is underestimated.

(3) Human

Crohn's disease is a chronic and fatal inflammatory bowel disease in human. *MAP* has been identified and isolated from cases of Crohn's disease (Bull et al., 2003a; Gearry et al., 2005; Ryan et al., 2002; Sechi et al., 2006), but some studies have failed to demonstrate these results (Baksh et al., 2004; Ellingson et al., 2003; Kanazawa et al., 1999; Lozano-Leon et al., 2006). It is controversial about the association between *MAP* and Crohn's disease. However, there is a hypothesis suggest that the Crohn's disease may be caused by a number of different persisting infections, and *MAP* is thought to be one of them (Toracchio et al., 2008). As above, *MAP* is thought to be a particular potential in zoonosis standpoint.

Transmission pathways and risk factors of bovine paratuberculosis

The transmission pathways

(1) Within herd

The main transmission route is the fecal-oral route (Sweeney, 1996). The amount of *MAP* and the age of ingestion are important factors to determine whether the cattle be infected by paratuberculosis. Younger animals are the most susceptible to *MAP* infection (Windsor and Whittington, 2010), and adult animals are not likely to be infected unless they ingest abundance of *MAP* (Rankin, 1962). *MAP* survives well in the environment for more than 300 days, but it doesn't proliferate in the environment (Whittington et al., 2004). Therefore, there is still an indirect transmission rout, such as through fecal contamination of environmental soil and water (Sweeney, 1996). A study confirms that cross species transmission from ovine to cattle may occur sporadically, but the risk of transmission is low (Moloney and Whittington, 2008). Additionally, milk or colostrum may be the transmission route of *MAP* for neonates or calves in 2 ways: 1) milk or colostrum may be contaminated by feces from infected cattle, and the milk or colostrum can act as the mechanical carrier for *MAP* transmission; 2) *MAP* has been isolated from milk and colostrum of infected dams (Streeter et al., 1995), it indicate that *MAP*

may direct transmission through milk and colostrum from infected dams (Sweeney, 1996). Nearly, a study suggest that milk or colostrum may play a minor role in the transmission from dams to neonates and calves, but calves keep with their dams increase the risk of being infected by *MAP* (Nielsen et al., 2008). The results also suggest that the neonates and calves may ingest *MAP* by sucking with contaminated udders from infected dams (Ansari-Lari et al., 2009). Furthermore, Cattles suffer poor nutrition, stress, lactation, parturition, and immunosuppression by other diseases (ex: bovine viral diarrhea) are thought to be the biologically factors to increase the risk of being infected or facilitate the onset of the clinical stage of *MAP* infection (Kennedy and Benedictus, 2001). *MAP* has also been isolated from the reproductive tract of both clinically and subclinically infected cows such that calves born to infected cows could be infected in uterus (Bielanski et al., 2006). Horizontal calf-to-calf transmission may take place by direct contacts or fecal-oral routes (van Roermund et al., 2007).

(2) Among herds

Because subclinical stage of infected cattle is 2 to 5 years and no apparent clinical signs appear, *MAP* is usually introduced into dairy herds through purchase of cattle in this stage (Sweeney, 1996). On the other side,

vehicles or equipments contaminated by *MAP* can act as mechanical carrier for the pathogen as well (Wells and Wagner, 2000).

(3) Zoonosis

The ability of *MAP* to survive commercial pasteurization process is controversial. Some studies reports that *MAP* are inactivated by pasteurization (Keswani and Frank, 1998). Contrarily, *MAP* is found in commercially pasteurized milk purchased from stores in California, Minnesota, and Wisconsin (Ellingson et al., 2005). A study indicates that *MAP* is stronger thermal resistance than other bacteria, resulting in pasteurization of milk and milk products become problematic (Lund et al., 2000). The different pasteurization methods may affect the viability of *MAP* in milk and milk products (Stabel, 2000). As above, milk is thought to a vehicle for transmission of *MAP* from cattle to humans.

Transmission risk factors

The definition of transmission risk factors is the factor that contribute to *MAP* transmission such as the *MAP* positive cattle, *MAP* contaminated environment including soil/water, the trading of infected cattle, mechanical carrier of *MAP*, and management-related risk factors. Management-related risk factors is wildly studied, the

following management-related risk factors have been associated with a lower within-herd prevalence of *MAP* infection: 1) clean and disinfect maternity and calf pens after each use (McKenna et al., 2006), 2) Calve in separate, clean, and dry calving area (Dieguez et al., 2008), 3) Remove calves from their dam immediately after birth (Ansari-Lari et al., 2009), 4) Calf should have a separated area to avoid horizontal calf-to-calf transmission (van Roermund et al., 2007), 5) Calves must be separated from adult cattle for at least the first year of life (Nielsen and Toft, 2007), 6) Avoid vehicular and human traffic from adult cattle areas to young cattle area (Wells and Wagner, 2000), 7) Feed collected colostrum and milk from *MAP* negative cattle with cleaned udders (Dieguez et al., 2008), 8) Feed pasteurized milk or milk replacer to calves (Ansari-Lari et al., 2009), 9) Do not allow shared feed or water between adults and young cattle (Ansari-Lari et al., 2009), 10) Use separate equipment for handling feed and manure (Wells and Wagner, 2000), 11) Feedbunk and water trough should not have risk for fecal contamination (McKenna et al., 2006), 12) do not spread manure on grazing land and fed to cattle of any age group (Obasanjo et al., 1997), 13) cull all cattle with clinical evidence of paratuberculosis as soon as possible (Lu et al., 2008), 14) Colostrum or milk should not be collected or fed to calves or young cattle from *MAP*-positive cattle (ELISA, fecal PCR, or fecal culture) (Dieguez et al., 2008), 15) Maintain a closed herd or purchase replacement cattle only from *MAP* negative herds

or *MAP* free herds (Dieguez et al., 2008).

Diagnosis of bovine paratuberculosis

The main diagnostic methods for bPTB are 1) identification of *MAP* and 2) detection of the specific antibody against *MAP*. Clinically affected cattle are much more likely to be shedding *MAP* or have developed a detectable immune response (Chiodini et al., 1984), it means that when these diagnostic methods are used on cattle of clinical stage, they will have a better sensitivity than when they are utilized on subclinically infected cattle (Whitlock et al., 2000). Because of a long period of silent and subclinical stages, results of false negative are common, particularly in calves, heifers, and even 1st lactation cows (Sweeney, 1996). It is a challenge to interpret and apply the test results from subclinically infected cattle (Dargatz et al., 2001).

1) Identification of *MAP*:

   Theoretically, tissue culture of *MAP* is thought to be the ideal gold standard method (McKenna et al., 2004). Due to the relative high cost and difficulties of sampling for tissue culture, fecal culture is the gold standard in many researches (Collins and Sockett, 1993; Collins et al., 1991; Cox et al., 1991; Dargatz et al., 2001; Milner et al., 1990; Sockett et al., 1992; Sweeney et al., 1995). Fecal culture is the most definitive test to identify both

subclinically and clinically infected cattle (Sweeney et al., 1992), but the availability of fecal culture is greatly limited by the lack of sensitivity and long incubation time (4 to 16 weeks) (Stabel and Whitlock, 2001). Accordingly, molecular analysis of *MAP* nucleic acids using PCR method has been developed for detection of *MAP* present in feces, milk, tissue and environmental samples (Bielanski et al., 2006; Cook and Britt, 2007). The PCR method, targeting *MAP* specific insertion sequence IS900 has been shown to have high sensitivity and specificity for the diagnosis of *MAP* infection in a relatively shorter turn over time, comparing to other conventional diagnostic techniques such as fecal culture (Stabel and Bannantine, 2005). Furthermore, real time PCR enables both detection and accurate quantification of a specific target sequence of *MAP* (Cook and Britt, 2007; Eishi et al., 2002; Schonenbrucher et al., 2008). A study indicate that 10 or fewer *MAP* in milk and feces are consistently detected by using real time PCR, it's very useful and cost-effective for the diagnosis of clinical and subclinical paratuberculosis (Kudahl et al., 2004).

2) Detection of the specific antibody against *MAP*:

Serum ELISA is the most widely used technique for the diagnosis of bPTB because of its low cost and easy automation (Dieguez et al., 2009).

Regardless of which ELISA is applied, sensitivity is best for detection of heavy fecal shedders (Collins et al., 2005). A study indicate that the sensitivity of a commercial ELISA kit is 87% when applied to cows of clinical stage or advanced clinical stage, but only 15% when applied to subclinically infected cattle (Sweeney et al., 1995). The specificity is increased by a procedure use a serum absorption step to remove antibodies that cross react with Mycobacterium phlei (Yokomizo et al., 1985) and the specificity of ELISA kits in detecting fecal culture-positive cattle are > 94% (Collins et al., 2005). ELISA is generally used as a herd level diagnostic method to identify infected herds (Dieguez et al., 2009). Furthermore, some studies suggest that environmental real time PCR for *MAP* may accurately predicts herd status, and the potential advantages to this method include relative lower cost than ELISA, ease for sampling and specificity that is near 100% (Berghaus et al., 2006; Cook and Britt, 2007; Raizman et al., 2004). Within infected herds, fecal real time PCR is the best opinion to identify cattle shedding *MAP*.

Molecular epidemiology of bovine paratuberculosis

MAP strain identification by molecular analysis is a useful tool in epidemiological investigations for a better understanding of the origin of an infection, identification of

risk factors influencing transmission, characterization of the pathogenesis, and evaluation of regional control strategy which can lead to more rational control measures (Motiwala et al., 2006). Different control strategies may be proposed depending on whether a new *MAP* infection is introduced from another herd or is resulting from cattle ingest *MAP* from contaminated environment (Motiwala et al., 2006). There are many different molecular subtyping techniques for *MAP* include multiplex PCR for fingerprint analysis of the IS900 integration loci (MPIL), IS900, IS1311, and ISI245-restriction fragment length polymorphism (RFLP), and amplified fragment length polymerphism (AFLP) (Pavlik et al., 2000; Pavlik et al., 1999). Unfortunately, these techniques are limited for *MAP* subtyping because of the poor discriminatory power and low throughput. Repetitive elements in *MAP* genome are frequently used as markers for the differentiation and subtyping. The short sequence repeats (SSR) method showed a high discriminatory power for the differentiation and subtyping of *MAP*. However, the high cost and time of sequencing may limit the usefulness of SSR (El-Sayed et al., 2009). The variable number tandem repeats (VNTRs) is an alternative genotyping method for *MAP* (Bull et al., 2003b). DNA sequences of VNTRs show allelic hypervariability related to the number of repeats and inter-allelic sequence variability and are called variable number of tandem repeats (VNTRs) (Li et al., 2005). Phylogenetic analysis of VNTRs is a PCR-based strain

typing method to detect the variations in the number of tandem repeated sequences distributed across several loci of the *MAP* genome (Motiwala et al., 2006). The PCR products are analyzed by gel electrophoresis to detect tandem repeats (TRs) with different sizes at the different loci. Each strain of *MAPs* is designated a code for different sizes of tandem repeats at different loci. Two *MAPs* with different codes may represent that they are different strains of *MAPs*. The discriminatory power is calculated by the discriminatory index, and the discriminatory index of greater than 0.90 is deemed confident on epidemiologic study (Hunter and Fraser, 1989).

Control of bovine paratuberculosis

Control of paratuberculosis is a colossal challenge, because of vaccination against *MAP* in cattle (Kalis et al., 2001), and the cost of treating infected cattle are not cost-effective (Brumbaugh et al., 2000; Brumbaugh et al., 2004).

The ubiquitous nature of *MAP*, the long incubation period, most cases are subclinical, and diagnostic methods are insufficient sensitivity for identifying subclinically infected cattle allows the infection spread within and among herds. Effective control strategy based on closuring the transmission routes is the only way to eradicate paratuberculosis (Kudahl et al., 2008). However, the process of eradication is slow; it's needed the livestock owners keep the control strategy going for years.

Furthermore, effective closure of transmission routes depends on intensive laboratory procedures (Dorshorst et al., 2006; Groenendaal et al., 2002; Kudahl et al., 2007), a successful control strategy depends on whether the livestock owners understand the fundamental cognizance of bPTB (Kudahl et al., 2008; Wells and Wagner, 2000). Risk-based control strategies allows the livestock owners to optimize management, thereby saving time and money (Kudahl et al., 2008). The control strategies of bPTB including herd risk assessment, test and cull strategies, and certification and monitoring program are introduced as following (Lu et al., 2008; Raizman et al., 2006; Weber et al., 2004).

(1) Herd risk assessment:

The herd management risk assessment (RA) is endorsed by the US Animal Health Association's National Johne's Working Group. The RA is performed by a certified veterinarian to evaluate the past history of bPTB and identify the *MAP* transmission pathways on the herd. The keystone of this control program is "calves", because the young cattle are at highest risk for infection and transmission to other cattle in the future (Windsor and Whittington, 2010). The RA evaluates five areas and sources of replacements, and each area and sources of replacements are scored based on the potential risk of the *MAP* transmission within or between herds, assuming the herd is

infected. Each area is evaluated and assigned a score-based upon different criteria, with a maximum possible score of 216 points from the following: calving area (the maximum score of this area is 80 points), preweaned heifer area (the maximum score of this area is 60 points), postweaned heifer area (the maximum score of this area is 35 points), bred heifer area (the maximum score of this area is 35 points), and cow area (the maximum score of this area is 16 points). The higher the score is, the higher the risk of *MAP* transmission. A total score higher than 80 points is considered as ‘‘high risk’’, between 50 and 79 points as ‘‘moderate risk’’, 30–49 points as ‘‘low risk’’, and lower than 30 points is considered as ‘‘very low risk’’ (Raizman et al., 2006). As a part of the bPTB control program, herd testing is initially performed using serum antibody tests as a baseline to estimate within herd prevalence, and to identify test positive cattle at highest risk of being a source of *MAP* transmission. Livestock owners are benefited by testing a number of cattle per year free of laboratory charge. Each subsequent year, a RA is performed to evaluate the progress of the management plan and identify areas where further improvement is required. In order to participate in the control program and benefit from free testing, herds are encouraged to show progress in herd practices change (decreasing RA score).

(2) test and cull strategies:

To control the spread of *MAP*, test-based culling strategy is recommended. Current diagnostic tests, such as fecal culture test, fecal PCR test, and ELISA have high sensitivities for detecting infectious cattle shedding high levels *MAP*, but relatively low sensitivities for detecting infectious cattle shedding low levels *MAP* (Collins et al., 2006; Collins et al., 2005; Stabel and Bannantine, 2005; Tiwari et al., 2006). A general practice on many dairy farms is to immediately cull animals shedding high levels of *MAP*, as they are considered to be a greater risk for spreading *MAP* (McKenna et al., 2006). For cattle shedding low levels of *MAP*, culling low shedding cattle may be more costly to the herd than the infections they cause (Dorshorst et al., 2006). A study indicate culling of only high shedding animals is effective in controlling *MAP* transmission within herds with good management, but for herds with poor management, in addition to immediate culling of high shedding animals, culling of low shedding animals (based on fecal culture), is necessary (Lu et al., 2008). The study also suggests that if only high shedding cattle are culled, faster *MAP* diagnostic methods (such as fecal PCR and ELISA) should be applied with high testing frequency (6 months interval), particularly on herds with poor management. In general, culling of infectious cattle with longer

testing intervals is not effective in controlling *MAP* (Lu et al., 2008). However, other study suggest that ELISA positive cattle don't have be culled until their daily milk yield below 10 kg (Kudahl et al., 2008).

(3) certification and monitoring program

The purchase of *MAP* infected cattle is the main cause of the introduction of the pathogen in a *MAP* free herd (Sweeney, 1996). Certification and monitoring programs have been implemented to determine and certify the health status of herds in several countries, such as in The Netherlands, the USA and Australia. In these countries, programs are formalized to classify herds based on the likelihood of being *MAP* infected (Collins, 2003). This certification and monitoring programs limits the spread of paratuberculosis by creating a *MAP* free source of replacement. Several levels of herd status based on successive herd tests are defined, given a lower risk to introduce the infection into herds. The likelihood of becoming *MAP* free increases with the number of negative results to herd tests. Certification and monitoring programs are different through the testing methods (serology or fecal culture), the time interval between successive herd tests, the selection of cattle within herds to be tested, and imposed rules for the purchase of animals and through actions taken after positive test results (Kovich et al., 2006; Weber et al.,

2004). In most certification and monitoring programs, all or a sample of the animals in each herd are tested by ELISA (generally ≧ 3 years of age) or fecal culture (individual or pooled; ≧ 2 years of age) (Weber et al., 2004). Both tests have a poor sensitivity, resulting in a large number of undetected but infected cattle. Hence, undetected herds can persist in the certified group of herds. These herds may sell infected cattle to truly *MAP*-free herds in the same or lower level of certification. In order to reduce the risk of introducing an infection in a *MAP*-free herd, purchasing cattle from herds at a lower level of certification is not allowed (Ezanno et al., 2005). A study indicted the designation '*MAP*-free' should be changed into '*MAP* low-risk' (Weber et al., 2004). The cost-effective certification and monitoring scheme may be reported, and as following: (1) certification of '*MAP* low-risk' herds after four herd examinations at 2 year intervals consisting of pooled fecal culture of all cattle ≥ 2 years of age, and (2) monitoring of '*MAP* low-risk' herds by pooled fecal culture of all cattle ≥ 1year of age at 2 year intervals (Kalis et al., 2004; Weber et al., 2004).

Bovine paratuberculosis in Taiwan

The average annual milk production (6000 kg/per cattle, Council of Agriculture,

Executive Yuan, R.O.C) is lower in Taiwan than in United States (9333 kg/per dairy cattle, National Agricultural Statistics Service, Department of Agriculture, U.S.). There is still some room to improve milk production in Taiwan. A survey conducted in 1987 shows that 4% of cattle were serologically positive for paratuberculosis in Taiwan (Lu et al., 1987), and clinical cases of paratuberculosis are found through the recent years, but no related control strategy has been implemented. Additionally, a human inflammatory bowel disease is now believed to be related to the infection of *MAP*. There is a custom for Taiwanese countrymen to eat viscera of cattle (from culled dairy cattle and beef cattle), and *MAP* may transmit to human in Taiwan. Intradermal tuberculin tests (ITT) in cattle are frequently interfered by paratuberculosis and present as false positive results, which is problematic for the control of bovine tuberculosis. The current infection status of bPTB in Taiwanese dairy herds and beef cattle are unknown. It's still unknown the policy for the control of bovine tuberculosis, ITT, is interfered by paratuberculosis or not. Furthermore, the transmission risk factors of paratuberculosis in Taiwan are not definite, and relation between each factor that contributes to *MAP* infections is thought to be complex and very markedly different among different herds. In order to gain a better understanding of the *MAP* infection in Taiwan and to develop a practical control strategy, it's necessary to investigate the associated transmission risk factors in each dairy herd. Furthermore, a successful

control strategy depends on whether the livestock owners understand the fundamental cognizance of bPTB (Kudahl et al., 2008; Wells and Wagner, 2000). It is necessary to know "what information about bPTB should be propagandized to livestock owners and to enhance the cooperation of livestock owners". Risk-based control program are the most cost-effective strategy to control bPTB in dairy herds (Kudahl et al., 2008). The "*MAP* low-risk" certification and monitoring program limits the spread of paratuberculosis between herds by creating a *MAP* free source of additions and replacements (Kovich et al., 2006). The risk-based control program, certification program, and monitoring program are different through the testing methods (serology or fecal culture), the time interval between successive herd tests, the selection of cattle within herds to be tested, and imposed rules for the purchase of animals and through actions taken after positive test results. In order to develop a practical control strategy in Taiwan, it is necessary to understand 1) what is the most cost-effective diagnostic method to identify the *MAP* infection status of dairy herd, 2) what is the most cost-effective diagnostic method to identify the subclinical infected cattle, and 3) what is the optimal method for certification and monitoring scheme of "*MAP* low-risk" herd. A test-based control strategy on its own is inadequate, because current diagnostic tests, such as fecal culture, fecal real time PCR, and ELISA have highest sensitivities for detecting infectious animals shedding high levels of *MAP*, but relatively low test

sensitivities for detecting infectious animals shedding low levels of *MAP* (Alinovi et al., 2009; Collins et al., 2006; Collins et al., 2005; Tiwari et al., 2006). Therefore, preventive management practices must be the starting point in a control program. Prevention should be directed in particular at protecting the most susceptible cattle (young cattle), reducing contamination of the environment, and avoiding the spread of the bacterium between herds (Chi et al., 2002b; Muskens et al., 2003). It is necessary to understand "what are the preventive management practices to control bPTB".

Chapter II: Specific Aim and Hypothesis

Specific aim

The unsolved problems about bPTB in Taiwan are 1) the current infection status of bPTB in dairy herds are unknown, 2) the current infection status of bPTB in beef cattle are unknown, 3) it's still unknown the policy for the control of bovine tuberculosis, ITT, is interfered by paratuberculosis or not, 4) the transmission risk factors related to *MAP* infection of dairy herd in Taiwan is also unknown, and 5) no related control strategy has been implemented. As above, the aims of this study are:

1) To know the current infection status of paratuberculosis in dairy herds.
2) To know the current infection status of beef cattle.
3) To investigate whether the bPTB interfere the result of ITT or not.
4) To investigate the *MAP* transmission risk factors within and among dairy herds.
5) To develop a practical control strategy for bPTB in Taiwan.

Hypothesis

1) Comparing with the results of a survey conducted in 1987, the seroprevalence of paratuberculosis in Taiwan may increase through these two decades.
2) The beef cattle are affected by *MAP*, and it may be a possible pathway to

transmitting *MAP* from meat production to human.

3) The result of ITT is interfered by bPTB.

4) The transmission risk factor contributes to *MAP* infections is thought to be complex and very markedly different among different herds. The transmission risk factors focused in this study are *MAP* positive cattle, *MAP* contaminated environment including soil and water, and the exogenous transmission pathway including trading of cattle and mechanical carrier of *MAP*.

5) Evidence-based decision making is a useful method to understand 1) What information about bPTB should be propagandized to livestock owners, 2) what is the most cost-effective diagnostic method to identify the *MAP* infection status of dairy herd, 3) what are the preventive management practices to control bPTB, 4) what is the most cost-effective diagnostic method to identify the subclinical infected cattle", and 5) what is the optimal method for certification and monitoring scheme of "*MAP* low-risk" herd. Based on the above results and evidence-based decision making, the practical control strategy for bPTB is developed.

Chapter III: Material and methods

Bovine paratuberculosis surveillance

Collection of bovine serum samples

A survey was proposed for bPTB specific antibody in approximately 2,600 cattle nationwide, including 2000 serums of dairy cattle were randomly collected by the animal disease control center as part of an annual investigation of bovine infectious diseases, 500 serums of beef cattle at slaughter facilities, and 100 serums of ITT positive cattle.

Detect the MAP specific antibody for bovine serum samples

All serum samples were tested for *MAP* specific antibody by using commercialized ELISA kit (Institut Pourquier). The ELISA was conducted according to the manufacturer's instructions. The S/P Ratio lower than 55% was defined as negative for *MAP* specific antibody, and S/P Ratio equal to / higher than 55% was defined as positive for *MAP* specific antibody. Comparing to fecal culture, the sensitivities of this commercialized ELISA kit were 1.44%, 6.99%, 19.12%, 55.56%, and 81.71% for cattle with fecal shedding score 0, 0.1 to 1.1, 1.1 to 2.0, 2.1 to 3.0, and 3.1 to 4.0, respectively; the specificity of this commercialized ELISA kit was 100% (Collins et al., 2005).

Descriptive statistical analysis

Because of the low sensitivity of the serological test for *MAP*, an estimate of the true individual-level prevalence (adjusted for imperfect sensitivity and specificity) was calculated. A herd was defined as *MAP*-positive herd, if at least one positive serum sample was detected.

Spatial Statistical analysis

The geocodes of all herds were displayed on a map of Taiwan, using the Arc View GIS 9.3 (ESRI, Redlands, CA, USA). The herd location data were converted into continuous surfaces by using the kernel-density function, expressing the case occurrence. The kernel density was estimated by using a bandwidth of 5 km and a cell size of 200 m.

Transmission Risk Factor Analysis for Bovine Paratuberculosis in Taiwan

Collection of environmental samples for transmission risk factor analysis

To investigate the transmission risk factors in dairy herds, feces, soil and water samples of dairy herds were collected from these locations (Berghaus et al., 2006; Lombard et al., 2006; Raizman et al., 2004):

1) Feeding troughs or area/ Alleyways/ Return alley of milking parlor: taking 10 g samples of soil at approximately 10 m intervals while walking along its length, and grab samples were collected in marked sterile 50 mL centrifuge tubes.

2) Sick cow pen/ Dairy cow pen/ Dry cow pen/ Pregnant cow pen/ Calving area/ Calf pen/ Pasture/ Outdoor sport field: one 10 g sample of soil was collected for every 10 m^2 of surface area, and the grab samples were collected in marked sterile 50 mL centrifuge tubes.

3) Watering troughs/ Wastewater gutter/ Lagoons: samples were collected at 3 ~ 4 locations along their perimeter where was possible to safely approach the edge. Water or manure samples were collected by immersing a sterile 50 mL centrifuge tube up to 10 cm beneath the surface.

4) Feed equipment: 10 g soil/feces samples were collected at 1 ~ 2 locations from feed equipment (If it's possible), and the samples were collected in marked sterile 50 mL centrifuge tubes.

Pretreatment of environmental samples were described as following:

(1) All samples were shipped and stored under refrigeration (4°C) within 2 days (Khare et al., 2008).

(2) Soil and fecal samples: 1 g soil or fecal sample was put into 1.5 mL microcentrifuge tube, and stored at -70 °C.

(3) Water samples: water samples (30 ~ 40 mL) were centrifuged at 3000 ×g for 30 min. The supernatant was discarded (5 mL supernatant was leaved), and the pellet of each tube was resuspended in the supernatant. 1.3 mL water sample was put into 1.5 mL microcentrifuge tube, and stored at -70 °C.

Real time PCR of environmental samples

1. Primers design of for IS900 real time PCR

Specific primer of real time PCR for the IS900 sequence was designed by using the Primer 3 on-line tool (Rozen and Skaletsky, 2000). The primer sequences were as follows: MAPF, AAT GAC GGT TAC GGA GGT GGT TGT G; and MAPR, AAT CGC TGC GCG TCG TCG TTA ATA.

2. Preparation of plasmid for positive control

Plasmid for real time PCR were prepared by cloning the real time PCR

product of IS900 into a pCR2.1 cloning vector (Invitrogen) according to the manufacturer's recommendations. Plasmids containing the inserts of IS900 were purified by using QIAprep Spin Miniprep Kit (Qiagen), eluted into Tris-EDTA buffer and sequenced to confirm that the sequence was accurate. The exact concentration at A260 was determined and subsequently the exact plasmid copy number was calculated.

3. DNA extraction from soil, fecal and water samples

All extractions were performed by using FastDNA® Spin Kit for soil (Qbiogene). All steps were conducted according to the manufacturer's instructions, except 1) 350 mg soil or feces samples and 350 µL water samples, 2) the homogenized step for 45 seconds at a speed of 6.0 m/s twice rather than 40 seconds at a speed of 6.0 m/s once, 3) the final DNA elution was 200 µL. Dilutions of the DNA (1:10) were made and 4µL of the diluted DNA were used as template for real time PCR analysis (Cook and Britt, 2007).

4. Real time PCR assay

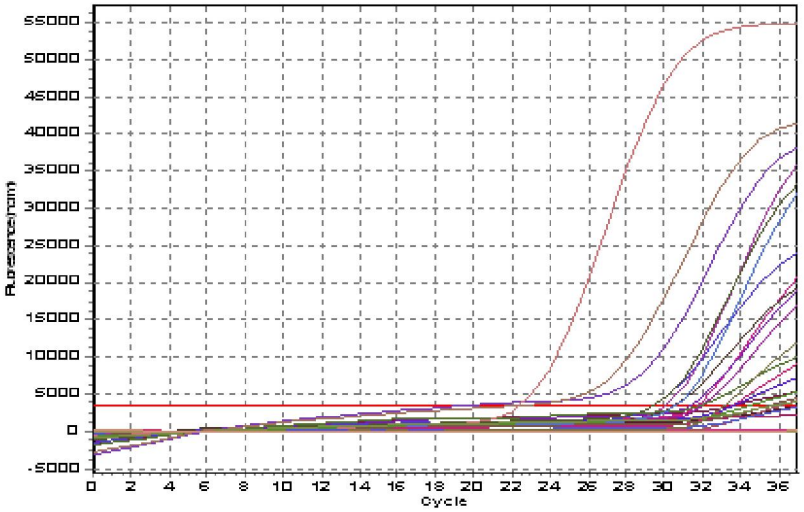
Samples were examined by real time PCR to detect the specific sequence IS900 of *MAP*. Real time PCR was conducted on the Mastercycler ep *realplex*[4]

(eppendorf). The reaction mixture was contained 10μL of 2X SYBR® Advantage® qPCR Premix (Clontech), 0.2 μM of the primers MAPF and MAPR, 4 μL of the DNA template, and added double distilled water to 20 μL in total volume. Positive controls added 1×10^1 and 1×10^2 copies of IS900 plasmids instead of the DNA template, and negative control added double distilled water instead of the DNA template. Positive and negative controls were included for each PCR process. The program of real time PCR was as following conditions: PCR initial: 95 ℃ for 2 min, followed by 37 cycles of subsequent denature at 95℃ for 5 s, annealing at 66℃ for 34 s, and a single fluorescence acquisition was performed at the end of each cycle. After 37 cycles, melting curve analysis was performed. Samples were heated to 95°C, then immediately cooled to 66℃ and held for 1 min. Samples were then heated to 95℃ at a rate of 0.2°C with continuous fluorescence monitoring. Then samples were cooled to 4 °C. A sample was considered positive if it showed an increase in fluorescence during amplification and the corresponding melting curve was observed at 86 to 88 °C (Figure 1).

5. IS900 real time PCR data analysis

If there was ≧1 environmental sample in the herd showed positive result for IS900 real time PCR, the herd was defined as *MAP* positive herd.

Sample	Ct SYBR	Tm x (°C)
UL1-A1	35.67	
UL1-A2	32.35	87.5
UL1-A3		
UL1-A4	33.18	87.0
UL1-A5	31.36	86.8
UL1-A6	31.67	86.7
UL1-A7	30.82	86.4
UL1-A8	33.02	86.0
UL1-A9	30.11	87.2
UL1-B1		
UL1-B2		
UL1-B3		
UL2-A1		
UL2-A2	29.75	86.6
UL2-A3	16.36	86.2
UL2-A4	28.99	85.8
UL2-A5	28.99	87.3
UL2-A6	34.70	87.3
UL2-A7	35.39	
UL2-B1		
UL2-B2		
UL2-B3		
UL2-B4		
UL3-A1	36.10	
UL3-A2	31.02	87.1
UL3-A3	32.92	87.3
UL3-A4	18.00	87.2
UL3-A5	22.09	87.1
UL3-B1		
UL3-B2		
UL3-B3		
P-1	30.27	86.0
P-2	33.73	87.5
P-3	34.63	87.5
N	-	



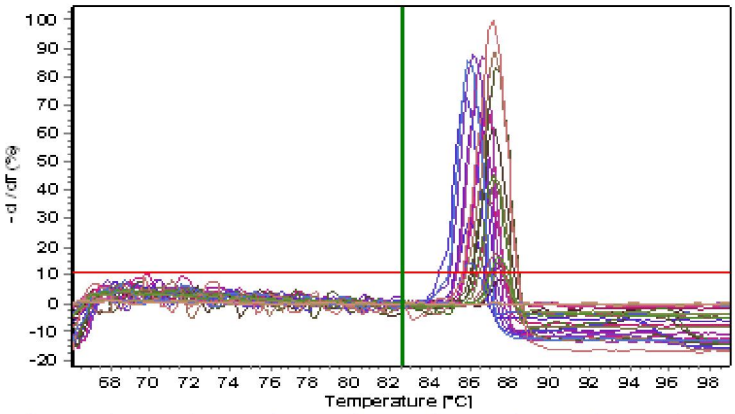


Figure 1. The example for the result of real time PCR: UL1-A1 to UL3-B3 were field soil environmental samples; P-1 was positive control contained 1×10^2 copies of IS900 plasmids; P-2 was positive control contained 1×10^1 copies of IS900 plasmids; P-3 was positive control contained 1 copies of IS900 plasmids; N was negative control containing double distilled water. The samples of UL1-A1, UL1-A2 to UL1-A9, UL-A2 to UL1-A6 and UL3-A2 to UL3-A5 showed an increase in fluorescence during amplification and the corresponding melting curve was observed at 86~88 °C. The results indicated UL1-A1, UL1-A2 to UL1-A9, UL-A2 to UL1-A6 and UL3-A2 to UL3-A5 were positive for IS900 real time PCR.

Molecular epidemiology of bovine paratuberculosis

1. Resource of samples

The phylogenetic analysis by nested PCR of VNTRs was performed on IS900 real time PCR positive environmental samples.

2. Nested PCR of VNTRs

Nested PCR was conducted on PCT-200 thermal cycler (Bio-Rad) with ten pair of primers including five pair of outer primers and five pair of inner primers (List in Table 1). The outer primer of VNTRs were designed by using the Primer 3 on-line tool (Rozen and Skaletsky, 2000), and the inner primers was described in other studies (El-Sayed et al., 2009; Overduin et al., 2004; Thibault et al., 2007).

The reaction mixtures of first round containing 10 µL of 5X Green GoTaq® Flexi buffer (Promega), 1.5 mM of $MgCl_2$ (Promega), 0.25 mM of mixed deoxynucleoside triphosphates (Invitrogen), 0.2 mM of each forward primer and revere primer, 5 µL of DNA template, 1.25 U of Go Taq® Flexi DNA polymerase (Promega), and added double distilled water to 50 µL in total volume. The program of first stage was as following: initial denature at 95°C for 2 min, followed by 40 cycles of subsequent denature at 95°C for 1 minutes, annealing at 63°C (X3) for 1 minute, extension at 72°C for 1 minute, and additional extension at 72°C for 5

minutes for ending. The annealing temperature for TR25, TR292, 1067 and 9425 was 65°C.

The reaction mixtures of second round containing 10 μL of 5X Green GoTaq® Flexi buffer (Promega), 1.5 mM of $MgCl_2$ (Promega), 0.25 mM of mixed deoxynucleoside triphosphates (Invitrogen), 0.2 mM of each forward primer and revere primer, 1 μL of DNA template (from PCR product of first round), 1.25 U of Go Taq® Flexi DNA polymerase (Promega), and added double distilled water to 50 μL in total volume. The program of second round was as following: initial denature at 95°C for 2 min, followed by 40 cycles of subsequent denature at 95°C for 1 minutes, annealing at 65°C (X3 and 9425) for 1 minute, extension at 72°C for 1 minute, and additional extension at 72°C for 5 minutes for ending. The annealing temperature for TR25 was 57°C, that for TR292 was 51°C, and that for 1067 was 59°C. The PCR products were analyzed by gel electrophoresis to detect tandem repeats (TRs) with different sizes at the different loci. Each strain of *MAPs* was designated a code for different sizes of tandem repeats at different loci. Two *MAPs* with different codes may represent that they were different strains of *MAPs* (Figure 2).

Table 1. PCR primer profiles and amplicon sizes of VNTRs

Target	Primer	Sequence	Flanking	Period size
X3	OX3F	AATCCGAGCAGCCGCAGGGT	210	53X
	OX3R	ACATCCCGGGGAGCATCAGGTC		
	X3F	GCCCAACCGTTCCCAACGAGAG	102	
	X3R	AGCCCTCCTTACGGAGCAGGAA		
TR25	O25F	TTCGCGTTGCTCGTCGTCCA	266	58X
	O25R	TTGTTGCCGCCGTTGACCGA		
	TR25F	GTCAAGGGATCGGCGAGG	191	
	TR25R	TGGACTTGAGCACGGTCAT		
TR292	O292F	TCCAGCCAGAAGTGCCGGGT	332	53X
	O292R	TGCTGCAGCGCTGTTTCGC		
	TR292F	CTTGAGCAGCTCGTAAAGCGT	137	
	TR292R	GCTGTATGAGGAAGTCTATTCATGG		
1067	O1067F	AGCGCCTACCTGTACAGCCG	368	53X
	O1067R	GATCGTCTTGGCGCGCAGGT		
	1067F	CGCCGCCCGCCGAAAAAG	266	
	1067R	CGGGACATCACAAATACAGAAGAA		
9425	O9425F	CGGAAAGCCGGGACGACTGA	222	57X
	O9425R	ACGCTGCTGGGCCGGTTCTA		
	9425F	AGGTGGCCAGGCGGGTCATCTTCC	135	
	9425R	CAGAAGGCGACGGAGGACTACATC		

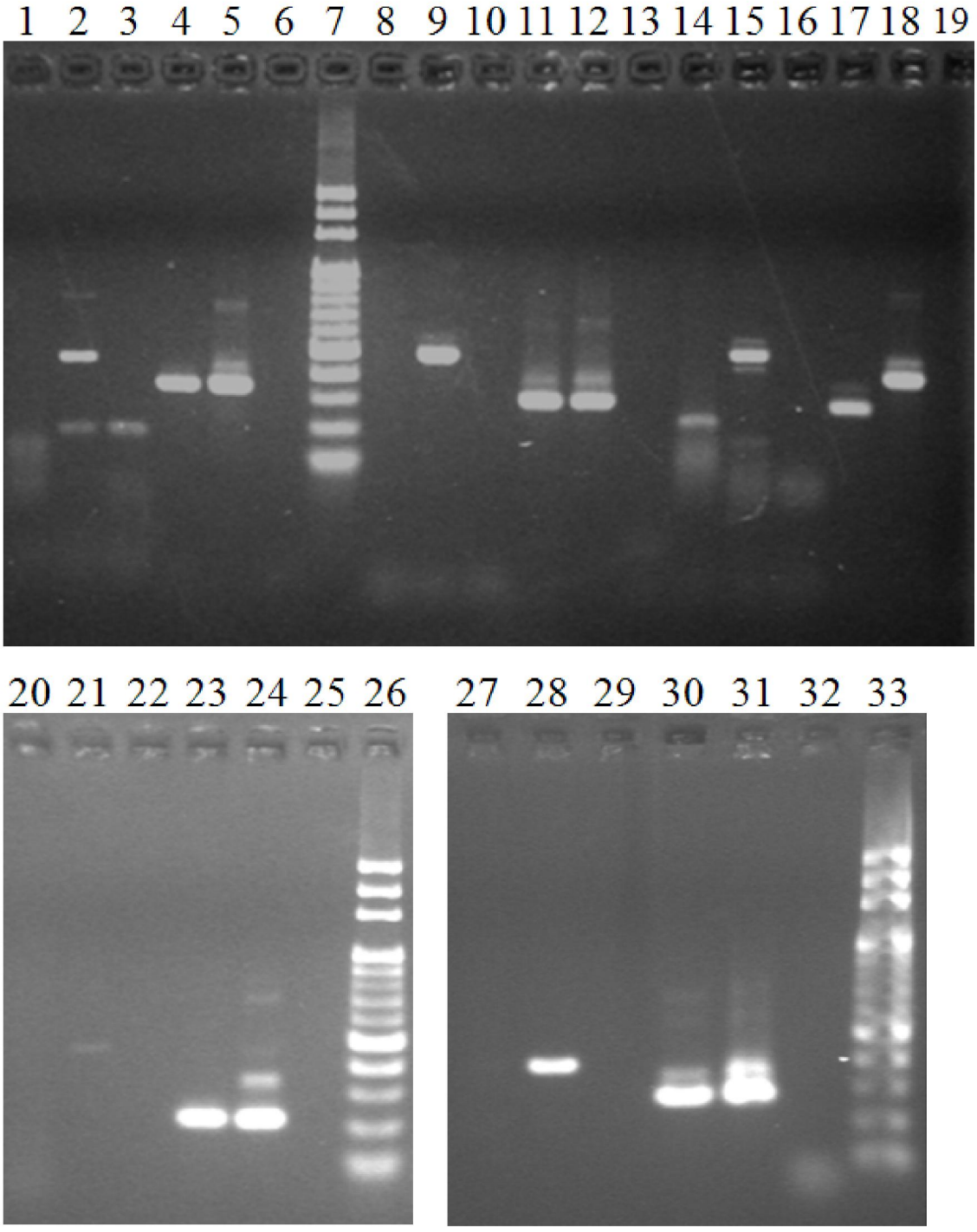


Figure 2. The example for the result of VNTRs: the primers of lane 1 to 3, lane 4 to 6, lane 8 to 10, lane 11 to 13, lane 14 to 16, lane 17 to 19, lane 20 to 22, lane 23 to 25, lane 27 to 29, lane 30 to 32 were O25 (forward and reverse), TR25 (forward and reverse), O292 (forward and reverse), TR292 (forward and reverse), O1067 (forward

and reverse), 1067 (forward and reverse), OX3 (forward and reverse), X3 (forward and reverse), O9425 (forward and reverse), and 9425(forward and reverse), respectively. The sample of lane 1, 8, 14, 20, and 27 was soil sample which was positive for IS900 real time PCR (sample A); the sample of lane 2, 9, 15, 21, and 28 was the other soil sample which was positive for IS900 real time PCR (sample B). The samples of lane 4, 5, 11, 12, 17, 18, 23, 24, 30, and 31 were PCR product of lane 1, 2, 8, 9, 14, 15, 20, 21, 27, and 28, respectively. Lane 3, 6, 10, 13, 16, 19, 22, 25, 29, and 32 were negative control. Lane 7, 26, and 33 were 100 bp leader marker. The production sizes of lane 1, 2, 4, 5, 7, 8, 9, 11, 12, 14, 15, 17, 18, 20, 21, 23, 24, 26, 27, 28, 30, 31 were none, 450 base pair (bp), 350 bp, 350 bp, none, 500 bp, 300 bp, 300 bp, 475bp, none, 250 bp, 350 bp, none, none, 220 bp, 220 bp, none, 300 bp, 250 bp, 250 bp, respectively. The results indicated the VNTRs code (X3-TR25-TR292-1067-9425) of sample A was 23302, and Sample B was 23322. The *MAPs* in these two samples were different substrains.

3. VNTRs data analyses

The allelic diversity (h) at a locus was calculated as $h = 1- \Sigma \chi_i^2 [n/(n-1)]$, and χ_i was the frequency of the *i*th allele at the locus, and n the number of isolates (Mazars et al., 2001). The discriminatory index (DI) was used as a numerical index for the discriminatory power of typing method (Hunter and Gaston, 1988). The DI was calculated using the following formula:

$$DI = 1 - \left[\frac{1}{N(N-1)} \sum_{j=1}^{s} n_j(n_j - 1) \right]$$

and *N* was the total number of samples in the typing method, *s* was the total number of distinct patterns discriminated by the typing method, and nj was the number of substrains belonging to the jth pattern. The *MAP* genetic relatedness was analyzed by the eBURST (http://eburst.mlst.net/) programs (Feil et al., 2004).

Evidence-based control strategy of bovine paratuberculosis in Taiwan

Evidence-based decision making applied the available evidence gained from the scientific method (Croft et al., 2001). The systematic review of published research studies was a major method. Literature and information related to the bPTB risk factor, bPTB control program, bPTB certification, and bPTB test parameters were identified from Pubmed. Unsolved problems addressed by the systematic review on control strategy of bPTB are 1) “What information about bPTB should be propagandized to livestock owners?”, 2) “what is the most cost-effective diagnostic method to identify the *MAP* infection status of dairy herd?”, 3) “what is the preventive management practices to control bPTB?”, 4) what is the most cost-effective diagnostic method to identify the subclinical infected cattle, and 5) “what is the optimal method for certification and monitoring scheme of “*MAP* low-risk” herd?”. For the initial identification of primary studies the following search terms combined with “paratuberculosis”, “control”, “program”, “certification”, “test”, “status”, “PCR”, “ELISA”, “risk factor”, “environment”, “management”. The following criteria were used to enable primary identification of the relevant literatures: 1) in English language and 2) address at least one of the unsolved problems to deem the papers relevant or irrelevant. These literatures and information were reviewed to facilitate developing bPTB control strategy in Taiwan. In combination with the results of bPTB surveillance,

bPTB transmission risk factors, and the reviewed literatures, the bPTB control strategy was developed.

Chapter III: Results

Bovine paratuberculosis surveillance

Collection of bovine serum samples

Totally, 1) 2170 bovine serum samples from 2170 dairy cattle in 63 dairy herds were collected from seven counties (Taichung, Yunlin, Changhua, Nantou, Tainan, Pingtung, and Taitung) and Kaohsiung city in Taiwan in 2008. The number of sampled dairy herds in each country and the sample number of each herd were listed in table 2; 2) 542 bovine serum samples from 542 beef cattle (including 424 male cattle, 86 female cattle, and 32 sex-unknown cattle) were collected from Yahsen slaughterhouse (table 3); 3) 148 bovine serum samples from 148 ITT positive dairy cattle including 63 young cattle (< 1 years old) and 85 adult cattle (> 1 years old) were collected (table 4).

MAP seroprevalence of dairy herds in Taiwan

The individual dairy cattle seroprevalence of paratuberculosis was 10.1%. The herd-level prevalence in Taiwan was 66.7% (42/63). The seroprevalences of individual dairy herds were ranged from 0% to 60% in dairy herds, and the seroprevalences of half *MAP* positive herds were higher than 10% (high prevalence), and the others were equal to /lower than 10% (low prevalence) (figure 3). The seroprevalences of bPTB in Taichung, Yunlin, Changhua, Nantou, Tainan, Kaohsiung city, Pingtung, and Taitung

were 8.0%, 8.9%, 4.7%, 0.0%, 23%, 12.5%, 11.4%, and 19.2%, respectively (figure 4). The herd-level prevalences of bPTB in Taichung, Yunlin, Changhua, Nantou, Tainan, Kaohsiung city, Pingtung, and Taitung were 50.0%, 100.0%, 78.6%, 0.0%, 100.0%, 100.0%, 50.0%, and 100.0%, respectively (figure 5).

MAP seroprevalence of beef cattle in Taiwan

The *MAP* seroprevalence of beef cattle was 2.8%. The seroprevalences of male cattle, female cattle, and sex-unknown cattle were 0.2%, 12.8%, and 10.3%, respectively (table 3).

MAP seroprevalence of ITT positive cattle in Taiwan

The *MAP* seroprevalence of ITT positive cattle was 51.4%. The *MAP* seroprevalence of young cattle and adult cattle were 27% and 69.4% (table 4).

Bovine paratuberculosis spatial analysis of dairy herds in Taiwan

The geographic location information of dairy herds in Pingtung were obtained, and the kernel density map of positive herds (t = 5 km, cell size 100 m) showed in figure 6. The different densities of positive herds per square kilometer (sqkm) were indicated by the colors (from white to dark blue, see the upper right of figure 6). The

different herd status of *MAP* specific antibody were indicated by different colors, such as red spots meant positive herds, green spots meant negative herds, and yellow spots meant untested herds. The “K” meant Kaohsiung city and “P” meant Pingtung.

Table 2. The sample sizes and seroprevalences of each dairy herds and each country/city

	Total samples	Positive samples	Positive rate (%)
Taichung (Tc)	**351**	**28**	**8.00%**
Tc-A	45	0	0.00%
Tc-B	36	0	0.00%
Tc-C	29	0	0.00%
Tc-D	30	10	33.30%
Tc-E	30	3	10.00%
Tc-F	35	0	0.00%
Tc-G	30	1	3.30%
Tc-H	36	13	36.10%
Tc-I	45	0	0.00%
Tc-J	35	1	2.90%
Yunlin (Ul)	**180**	**16**	**8.90%**
Ul-1	35	3	8.60%
Ul-A	45	5	11.10%
Ul-B	35	1	2.90%
Ul-C	30	3	10.00%
Ul-D	35	4	11.40%
Changhua (Ch)	**618**	**31**	**5.00%**
Ch-A	46	6	13.00%
Ch-B	46	2	4.30%
Ch-C	46	12	26.10%
Ch-D	48	1	2.10%
Ch-E	37	2	5.40%
Ch-F	48	1	2.10%
Ch-G	37	2	5.40%
Ch-H	48	1	2.10%
Ch-I	37	2	5.40%
Ch-J	47	0	0.00%
Ch-K	37	1	2.70%
Ch-L	48	0	0.00%
Ch-M	47	1	2.10%
Ch-N	46	0	0.00%
Nantou (Nt)	**30**	**0**	**0.00%**
Nt-A	30	0	0.00%
Kaohsiung city (Kc)	**24**	**3**	**12.50%**
Kc-1	24	3	12.50%

	Total samples	Positive samples	Positive rate (%)
Tainan (Tn)	**209**	**48**	**23.00%**
Tn-1	45	27	60.00%
Tn-A	55	10	18.20%
Tn-B	55	7	12.70%
Tn-C	54	4	7.40%
Pingtung (Pt)	**518**	**59**	**11.40%**
Pt-A	5	0	0.00%
Pt-B	10	0	0.00%
Pt-C	28	0	0.00%
Pt-D	30	0	0.00%
Pt-E	40	6	15.00%
Pt-F	45	3	6.70%
Pt-G	26	2	7.70%
Pt-H	20	1	5.00%
Pt-I	20	9	45.00%
Pt-J	3	0	0.00%
Pt-K	36	10	27.80%
Pt-L	20	6	30.00%
Pt-M	30	10	33.30%
Pt-N	30	0	0.00%
Pt-O	10	0	0.00%
Pt-P	35	0	0.00%
Pt-Q	6	0	0.00%
Pt-R	35	3	8.60%
Pt-S	35	0	0.00%
Pt-T	24	7	29.20%
Pt-U	30	2	6.70%
Taitung (Tt)	**240**	**50**	**20.80%**
Tt-A	30	8	26.70%
Tt-B	45	9	20.00%
Tt-C	35	6	17.10%
Tt-D	35	3	8.60%
Tt-E	30	1	3.30%
Tt-F	30	3	10.00%
Tt-G	35	20	57.10%
Total	**2170**	**235**	**10.80%**

Table 3. The *MAP* seroprevalence of beef cattle

	Total sample	Positive samples	Positive rate (%)
Male cattle	424	1	0.2%
Female cattle	86	11	12.8%
Sex-unknown cattle	32	3	10.3%
Total	542	15	2.8%

Table 4. The *MAP* seroprevalence of ITT positive cattle

	Total sample	Positive samples	Positive rate (%)
Young cattle	63	18	28.6%
Adult cattle	85	60	70.6%
Total	148	78	52.7%

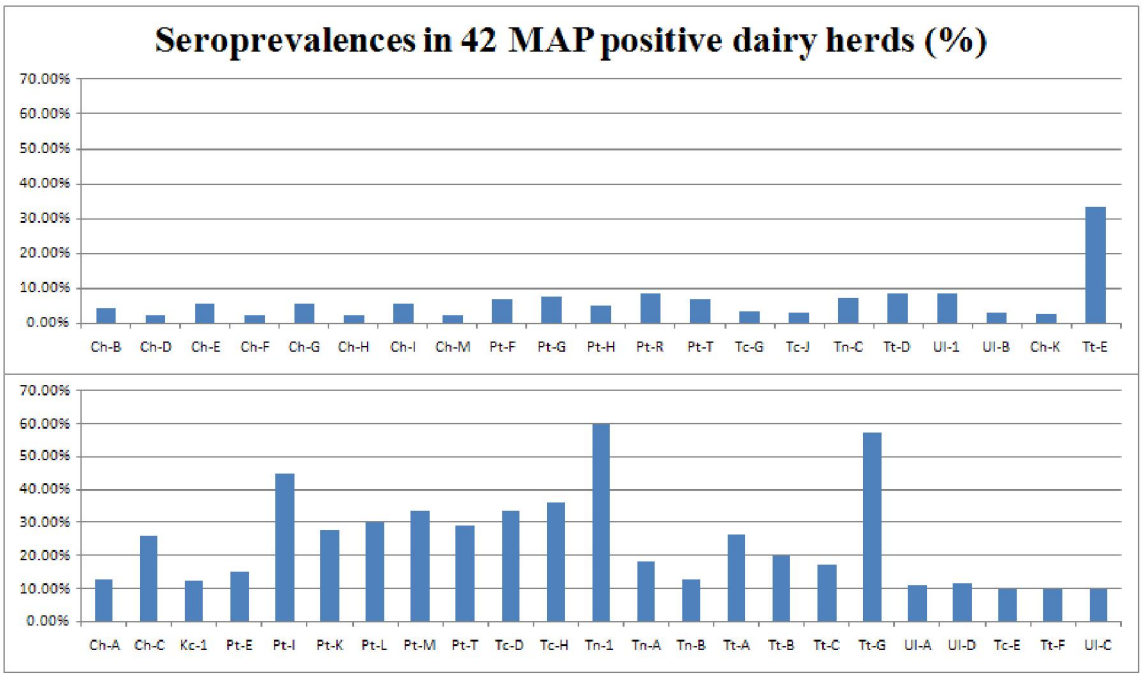


Figure 3. The seroprevalences of 42 *MAP* positive dairy herds: it were ranged from 0% to 60% in dairy herds, and the seroprevalences of half herds were higher than 10% (high prevalence), and the others were equal to /lower than 10% (low prevalence).

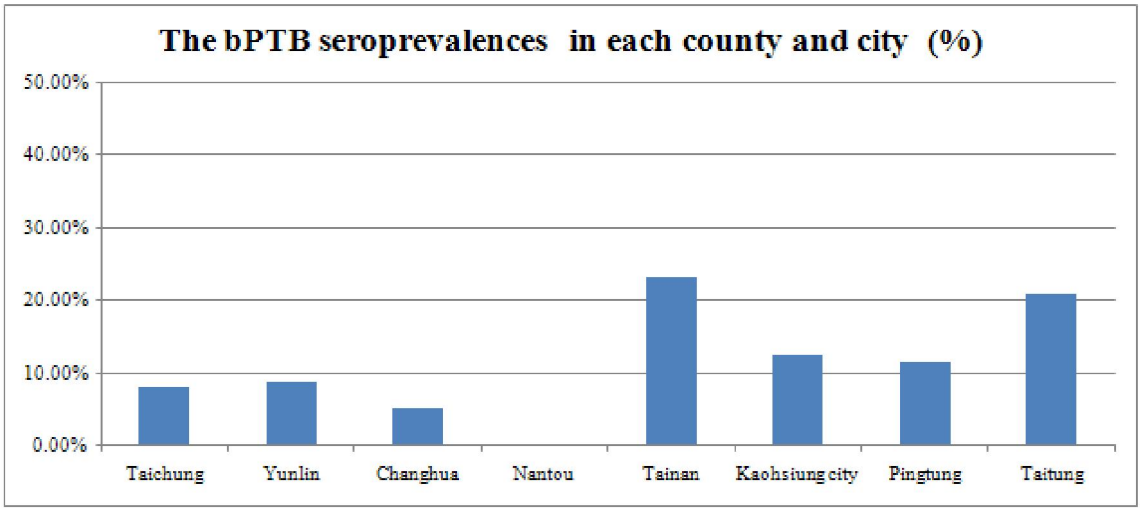


Figure 4. The *MAP* seroprevalences of each country and city: the seroprevalences of bPTB in Taichung, Yunlin, Changhua, Nantou, Tainan, Kaohsiung city, Pingtung, and Taitung were 8.0%, 8.9%, 4.7%, 0%, 23%, 12.5%, 11.4%, and 19.2%, respectively.

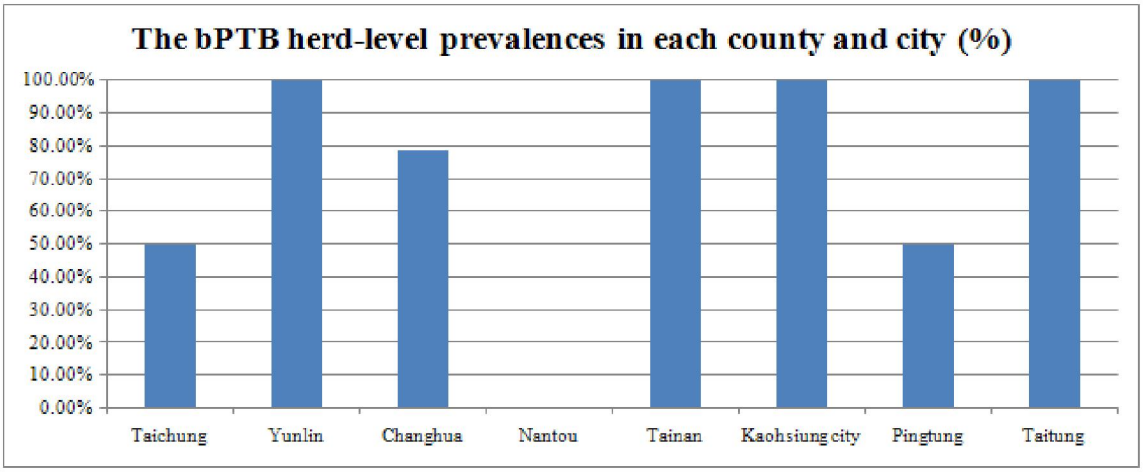


Figure 5. The herd-level *MAP* prevalences of each country and city: the herd-level *MAP* prevalences of bPTB in Taichung, Yunlin, Changhua, Nantou, Tainan, Kaohsiung city, Pingtung, and Taitung were 50.0%, 100.0%, 78.6%, 0.0%, 100.0%, 100.0%, 50.0%, and 100.0%, respectively.

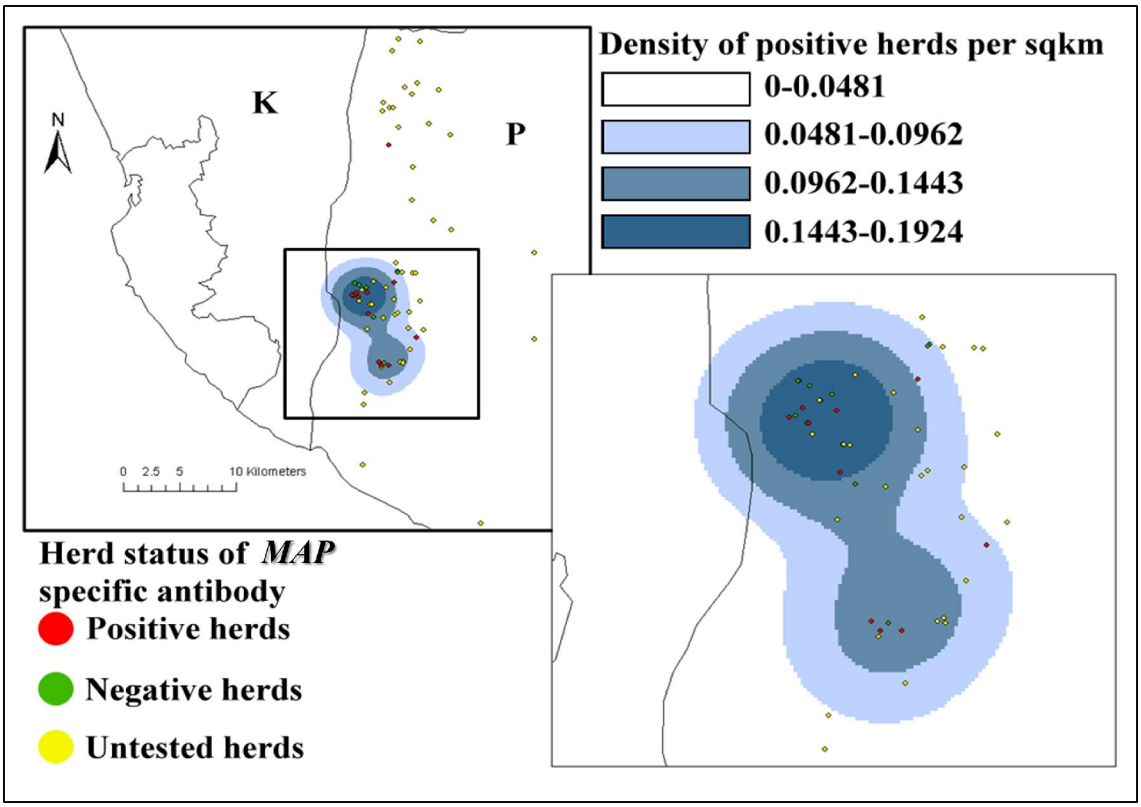


Figure 6. Serological bPTB herd infection status and kernel estimate of positive density infected herds. The different densities of positive herds per square kilometer (sqkm) were indicated by the colors (from white to dark blue, see the upper right of fiqure 3). The different herd status of *MAP* specific antibody were indicated by different colors, such as red spots meant positive herds, green spots meant negative herds, and yellow spots meant untested herds. The “K” meant Kaohsiung city and “P” meant Pingtung.

Transmission Risk Factor Analysis for Bovine Paratuberculosis

Environmental samples for IS900 real time PCR

Totally, 130 environmental samples including 89 soil/feces samples and 41 water samples were collected from 8 dairy herds (table 5). The location of each sample in herd Tt1, Tt2, Tt3, Tn1, Ul1, Ul2, and Ul3 were indicated in sampling maps of each herd (figure 7 to 13) and table 5, but the sampling map of herd Tc1 didn't be obtained.

IS900 Real time PCR of environmental samples

Environmental samples of 8 dairy herds from Taichung (Tc1), Yunlin (Ul1, Ul2 and Ul3), Tainan (Tn1) and Taitung (Tt1, Tt2 and Tt3) were examined by IS900 real time PCR. At least one environmental sample in herd Tt1, Tt2, Tn1, Ul1, Ul2 and Ul3 was positive for IS900 real time PCR, but all environmental samples in herd Tc1 and Tt3 are negative. 9 soil samples and 1 water sample from dairy cow pen, 9 soil samples from outdoor sport field, 1 soil sample from pregnant cow pen, 4 soil samples from heifer pen, and 1 soil sample from calf pen are positive for *MAP*. The prevalence of soil and feces samples from *MAP*-positive herds in outdoor sport field, dairy cow pen, pregnant cow pen, heifer pen and calf pen was 82% (9/11), 41% (9/22), 100% (1/1), 67% (4/6), 14% (1/7), respectively. The prevalence of water samples from *MAP*-positive herds in dairy cow pen was 7% (1/14). The positive rates of *MAP*

positive herds were ranging from 0 to 58% and the positive rates of Tt1, Tt2, Tn1, Ul1, Ul2 and Ul3 were 11%, 13%, 26%, 58%, 45% and 50%, respectively (table 5).

Environmental samples for molecular epidemiology

Totally, 25 environmental samples were positive for IS900 real time PCR, and further investigation for the phylogenetic analysis were performed by VNTRs.

Molecular epidemiology of bovine paratuberculosis by VNTRs

Twenty five *MAP* positive environmental samples were performed on VNTR typing using five polymorphic loci (X3, TR25, TR292, 1067 and 9425). Totally, 9 substrains (From A to I) of *MAPs* were identified among these *MAP* positive environmental samples based on polymorphism of alleles (table 6).

The discriminatory power differed for the individual locus, which displayed variable allele diversity (h) ranging from 0.00 to 0.52. VNTR allelic diversity in each locus was listed in table 7. Among the five VNTR loci, 1067 showed the highest discriminatory power that differentiated three allelic types and the allele diversity (h) was 0.52. The allele diversity (h) of X3, TR25 and TR292 were 0.04, 0.32 and 0.47, respectively. Differentiated allelic types of X3, TR25 and TR292 were 2, 2, and 3, respectively. There was no allele diversity (h = 0.00) for 9425 representing

monomorphic allele among these twenty five *MAP* positive environmental samples. The total discriminatory power of VNTRs calculated by discriminatory index was 0.84. The phylogenetic tree based on VNTRs genotypes among 25 *MAP* positive environmental samples were draw by the eBURST programs (figure 14), and the locations of each substrain *MAP* were indicated in the sampling map of each herd (figure 7 to 13). One sample in herd Ul3, two samples in herd Tt1 and two samples in herd Ul2 were *MAP* substrain A. One sample in herd Ul3, three samples in herd Ul1, and four samples in herd Tn1 were *MAP* substrain B. One sample in herd Tn1 was *MAP* substrain C. Two samples in herd Tt2 was *MAP* substrain D. One sample in herd Ul3, two samples in herd Ul1, and two samples in herd Ul2 were *MAP* substrain E. One sample in herd Ul2 was *MAP* substrain F. One sample in herd Ul1 was *MAP* substrain G. One sample in herd Ul1 was *MAP* substrain H. *MAP* substrain A, B, C, D, E, F, G, and I were classified in group 1, but *MAP* substrain H was not in the group. The VNTRs codes of *MAP* strain K-10 (Li et al., 2005) (GenBank accession number NC_002944) was identified as 23322 (Pattern B).

Table 5. Results of real time PCR for environmental samples

Soil and feces samples																	
Location\Herd	Tt1		Tt2		Tc1*		Tt3*		Tn1		UL1		Ul2		Ul3		Positive
	Total	Positive	Total	Positive	Total	Positive	Total	Positive	Total	Positive	Total	Positive	Total	Positive	Total	Positive	rate**
Outdoor sport field	3	2	2	1	0	0	10	0	0	0	2	2	2	2	2	2	82% (9/11)
Dairy cow pen	3	0	3	1	2	0	4	0	8	3	2	1	3	2	3	2	41% (9/22)
Pregnant cow pen	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	100% (1/1)
Dry cow pen	0	0	0	0	0	0	9	0	1	0	0	0	0	0	0	0	0 (0/1)
Heifer pen	2	0	0	0	2	0	0	0	2	0	4	4	0	0	0	0	67% (4/6)
Calf pen	2	0	2	0	0	0	5	0	0	0	1	0	2	1	0	0	14% (1/7)
Pasture	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0 (0/2)
Lagoon	1	0	2	0	0	0	1	0	0	0	0	0	0	0	0	0	0 (0/3)
Water samples																	
Location\Herd	Tt1		Tt2		Tc1*		Tt3*		Tn1		UL1		Ul2		Ul3		Positive
	Total	Positive	Total	Positive	Total	Positive	Total	Positive	Total	Positive	Total	Positive	Total	Positive	Total	Positive	rate**
Outdoor sport field	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0% (0/1)
Dairy cow pen	2	0	2	0	2	0	4	0	4	1	1	0	2	0	3	0	7% (1/14)
Pregnant cow pen	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0 (0/1)
Dry cow pen	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0 (0/1)
Heifer pen	3	0	0	0	2	0	0	0	0	0	2	0	2	0	0	0	0 (0/7)
Calf pen	1	0	2	0	0	0	1	0	1	0	0	0	0	0	0	0	0 (0/4)
Pasture	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0 (0/0)
Lagoon	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0 (0/0)
Total positive rate	11% (2/18)		13% (2/15)		0% (0/8)		0% (0/37)		26% (5/19)		58% (7/12)		45% (5/11)		50% (4/8)		19% (25/131)

*: *MAP*-negative herds

**: The rate was calculated from *MAP*-positive herds

Table 6. Data of VNTRs of *MAP* positive environmental samples

Herd	Location	Sample type	X3	TR25	TR292	1067	9425	Pattern
Tt1	Outdoor sport field	Soil and feces	2	3	3	0	2	A
	Outdoor sport field	Soil and feces	2	3	3	0	2	A
Tt2	Outdoor sport field	Soil and feces	2	0	1	2	2	D
	Dairy cow pen	Soil and feces	2	0	1	2	2	D
Tn1	Dairy cow pen	Soil and feces	2	3	3	2	2	B
	Dairy cow pen	Soil and feces	2	3	3	2	2	B
	Dairy cow pen	Soil and feces	2	3	3	2	2	B
	Pregnant cow pen	Soil and feces	2	3	3	2	2	B
	Dairy cow pen	Water	2	3	3	1	2	C
Ul1	Outdoor sport field	Soil and feces	2	0	2	0	2	E
	Outdoor sport field	Soil and feces	2	3	3	2	2	E
	Dairy cow pen	Soil and feces	2	3	3	2	2	B
	Heifer pen	Soil and feces	2	3	3	2	2	B
	Heifer pen	Soil and feces	2	0	3	0	2	B
	Heifer pen	Soil and feces	4	0	6	0	2	H
	Heifer pen	Soil and feces	2	0	3	0	2	G
Ul2	Outdoor sport field	Soil and feces	2	3	3	0	2	A
	Outdoor sport field	Soil and feces	2	3	3	0	2	E
	Dairy cow pen	Soil and feces	2	0	3	0	2	E
	Dairy cow pen	Soil and feces	2	0	3	0	2	F
	Calf pen	Soil and feces	2	0	3	2	2	A
Ul3	Outdoor sport field	Soil and feces	2	0	3	0	2	E
	Outdoor sport field	Soil and feces	2	0	2	2	2	A
	Dairy cow pen	Soil and feces	2	3	3	0	2	B
	Dairy cow pen	Soil and feces	2	3	3	2	2	I

Table 7. VNTR allelic diversity in each locus

	No. of distinct alleles in each locus				
No. of copy	X3	TR25	TR292	1067	9425
0	0	11	0	12	0
1	0	0	2	1	0
2	24	0	2	12	25
3	0	14	20	0	0
4	1	0	0	0	0
5	0	0	0	0	0
6	0	0	1	0	0
Allele diversity (h)	0.04	0.47	0.32	0.52	0.00

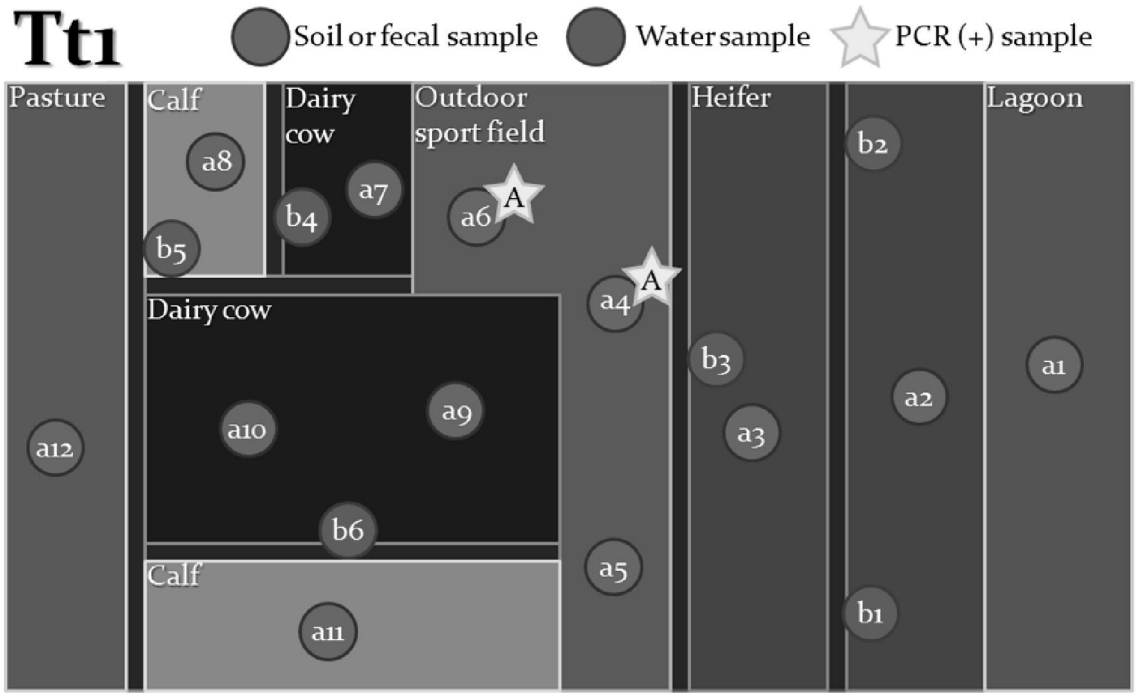


Figure 7. Sampling map of herd Tt1: red spot indicated a soil/feces sample, and blue spot indicated a water sample. A red or blue spot was asterisked meant the sample was positive for IS900 real time PCR The code in asterisk was the *MAP* substrain identified by VNTRs. Totally, 18 environmental samples (12 soil/feces samples and 6 water samples) including 3 soil/feces samples of outdoor sport field, 3 soil/feces samples and 2 water samples of dairy cow pen, 2 soil/feces samples and 3 water samples of heifer pen, 2 soil/feces samples and 1 water sample of calf pen, and 1 soil/feces sample of lagoon and pasture were collected from herd Tt1. The sample a4 and a6 located at outdoor sport field were positive for IS900 real time PCR, and the *MAP* substrains of these 2 sample identified by VNTR were substrain A.

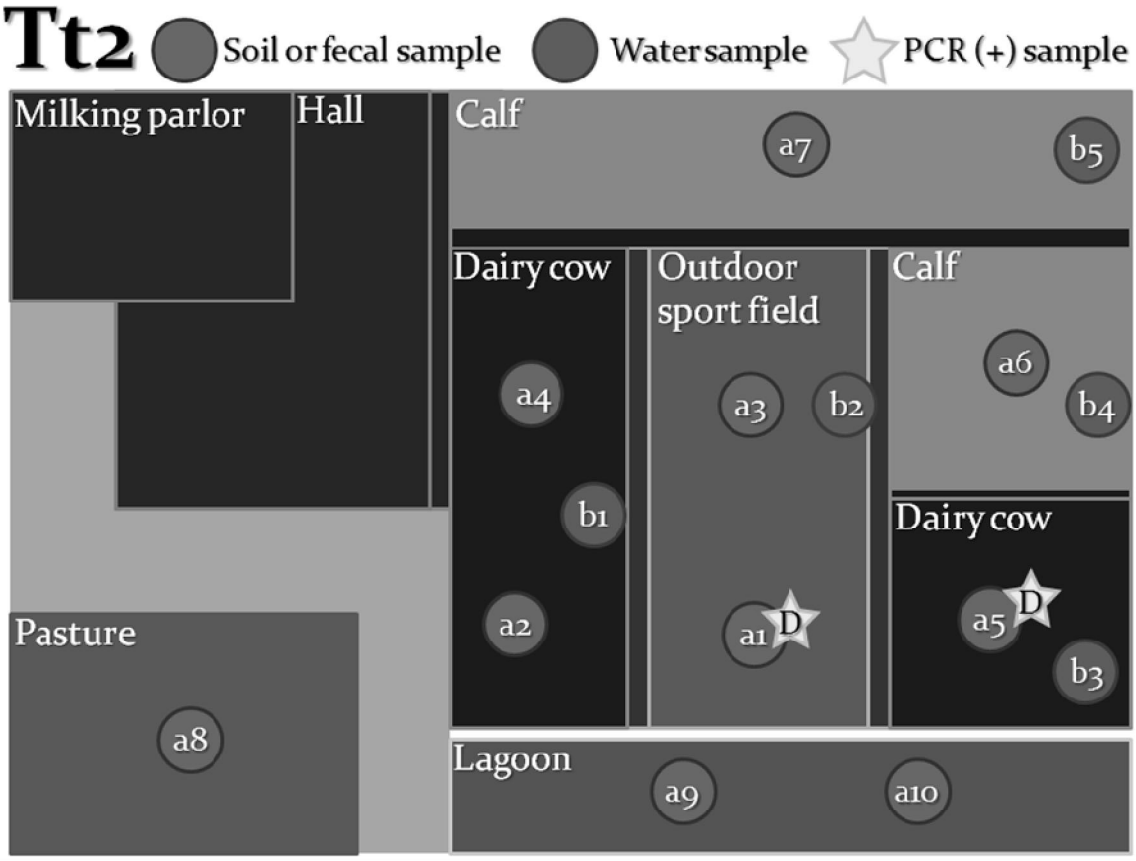


Figure 8. Sampling map of herd Tt2: red spot indicated a soil/feces sample, and blue spot indicated a water sample. A red or blue spot was asterisked meant the sample was positive for IS900 real time PCR The code in asterisk was the *MAP* substrain identified by VNTRs. Totally, 15 environmental samples (10 soil/feces samples and 5 water samples) including 2 soil/feces samples and 1 water sample of outdoor sport field, 3 soil/feces samples and 2 water samples of dairy cow pen, 2 soil/feces samples and 1 water sample of calf pen, 2 soil/feces sample of lagoon, and 1 soil/feces sample of pasture were collected from herd Tt2. The sample a1 located at outdoor sport field and a5 located at dairy cow pen were positive for IS900 real time PCR, and the *MAP* substrains of these 2 sample identified by VNTR were substrain D.

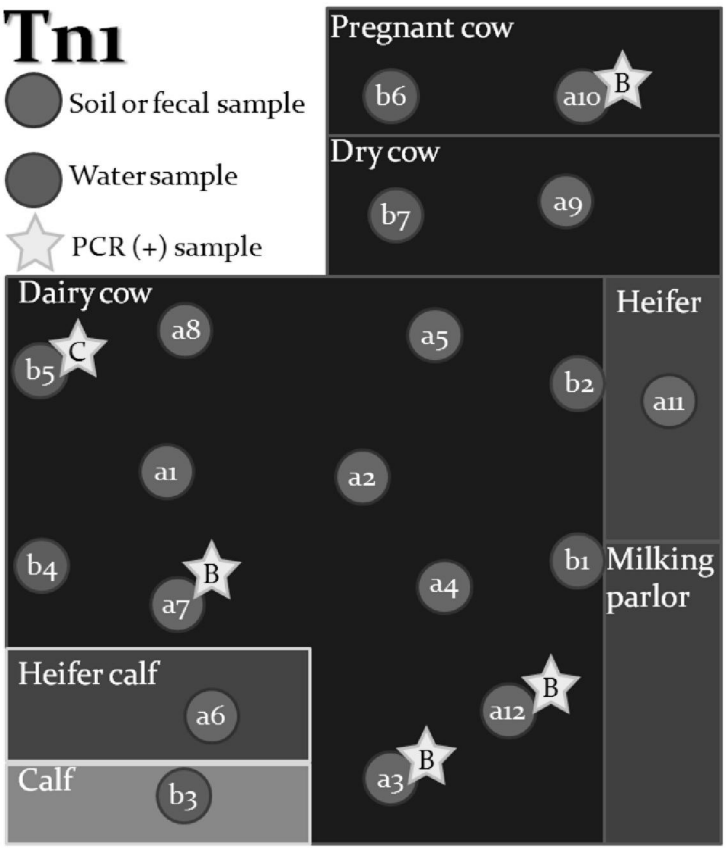


Figure 9. Sampling map of herd Tn1: red spot indicated a soil/feces sample, and blue spot indicated a water sample. A red or blue spot was asterisked meant the sample was positive for IS900 real time PCR The code in asterisk was the *MAP* substrain identified by VNTRs. Totally, 19 environmental samples (12 soil/feces samples and 7 water samples) including 8 soil/feces samples and 4 water sample of dairy cow pen, 1 soil/feces samples and 1 water sample of dry cow pen, 1 soil/feces samples and 1 water sample of pregnant cow pen, 2 soil/feces samples and of heifer pen, and 1 water sample of calf pen were collected from herd Tn1. The samples (a3, a7, a12, and b5) located at dairy cow pen and a10 located at pregnant cow pen were positive for IS900 real time PCR. The *MAP* substrains identified by VNTR of these 4 soil/feces samples (a3, a7, a10, and a12) were substrain B, and the water sample (b5) was . substrain C.

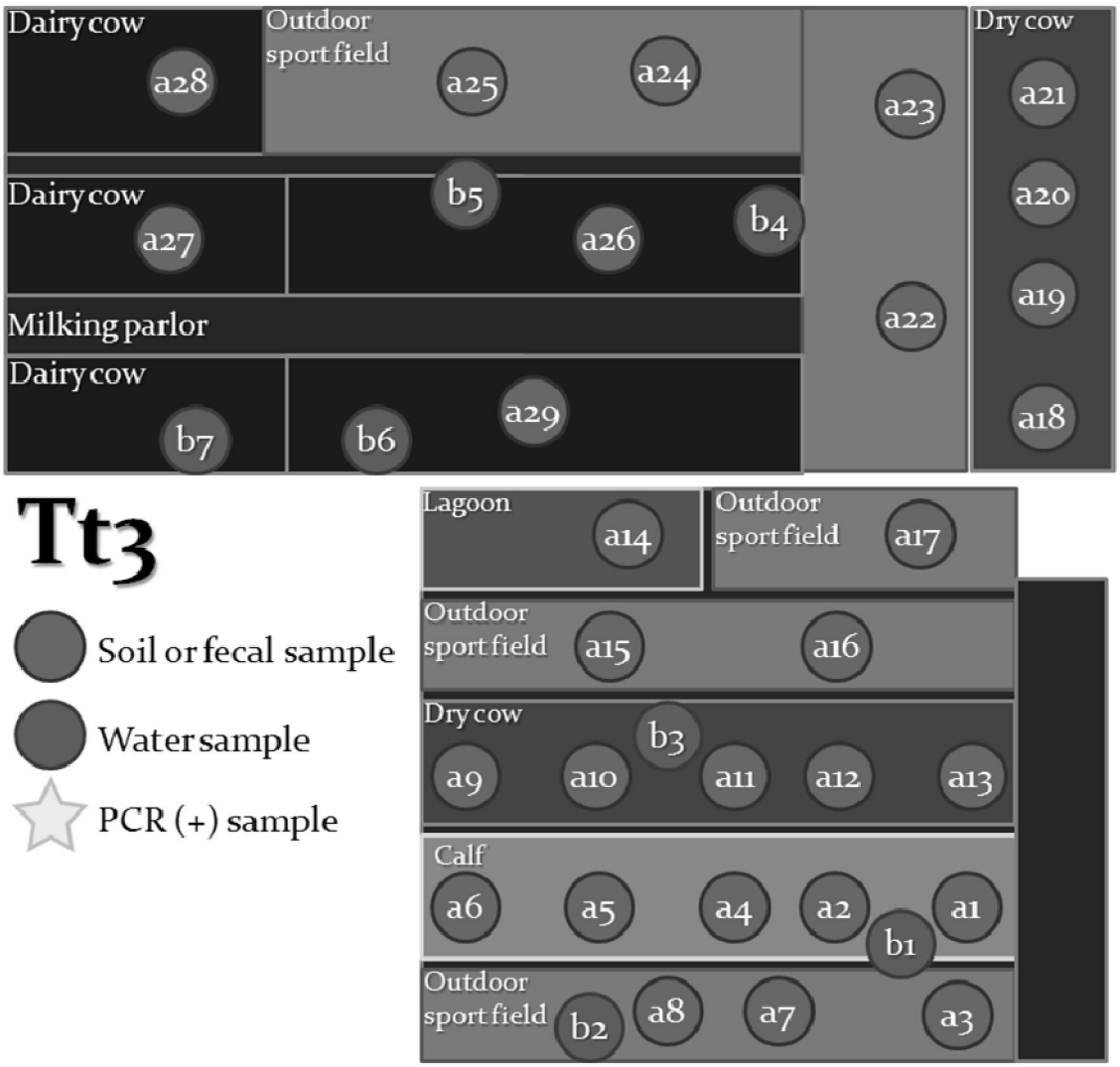


Figure 10. Sampling map of herd Tt3: red spot indicated a soil/feces sample, and blue spot indicated a water sample. A red or blue spot was asterisked meant the sample was positive for IS900 real time PCR The code in asterisk was the *MAP* substrain identified by VNTRs. Totally, 35 environmental samples (28 soil/feces samples and 7 water samples) including 10 soil/feces samples and 1 water sample of outdoor sport field, 4 soil/feces samples and 4 water samples of dairy cow pen, 9 soil/feces samples and 1 water samples of dry cow pen, 5 soil/feces samples and 1 water samples of calf pen, 2 soil/feces samples and 1 water sample of calf pen, and 1 soil/feces sample of lagoon, were collected from herd Tt3.

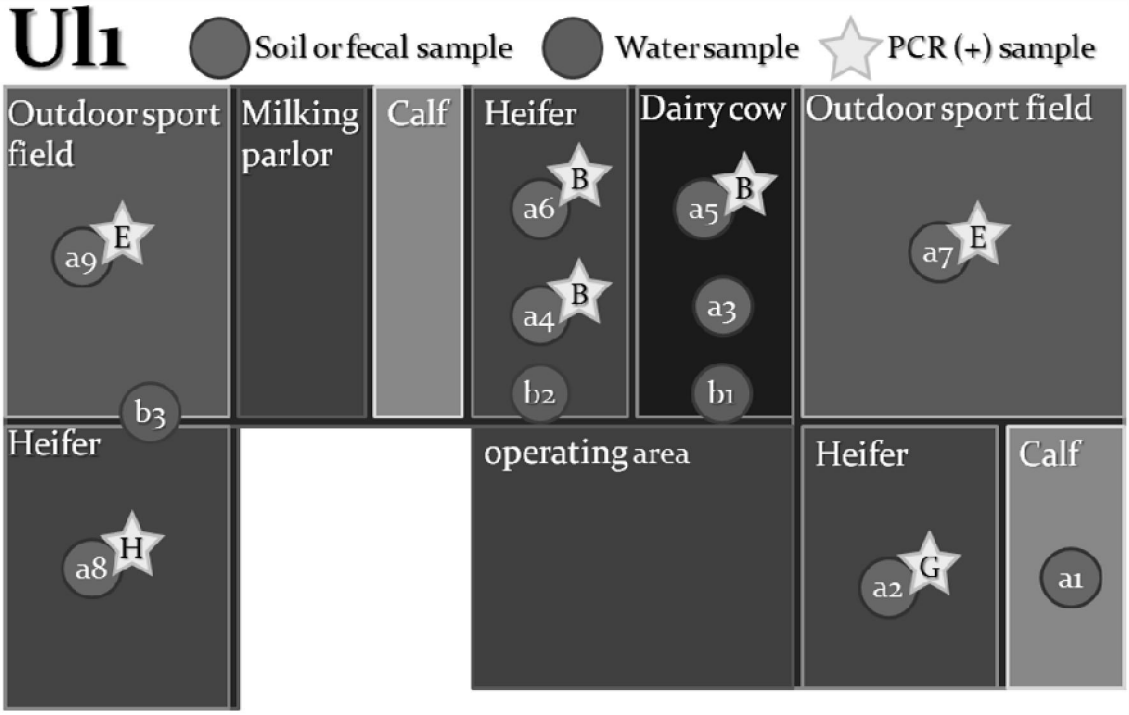


Figure 11. Sampling map of herd Ul1: red spot indicated a soil/feces sample, and blue spot indicated a water sample. A red or blue spot was asterisked meant the sample was positive for IS900 real time PCR The code in asterisk was the *MAP* substrain identified by VNTRs. Totally, 12 environmental samples (9 soil/feces samples and 3 water samples) including 2 soil/feces samples of outdoor sport field, 2 soil/feces samples and 1 water samples of dairy cow pen, 4 soil/feces samples and 2 water samples of heifer pen, and 1 soil/feces samples of calf pen were collected from herd Ul1. The samples located at outdoor sport field (a7 and a9), dairy cow pen (a5) and heifer pen (a2, a4, a6, and a8) were positive for IS900 real time PCR. The *MAP* substrains identified by VNTR of a2 (heifer pen), a4 (heifer pen), a5 (dairy cow pen), a6 (heifer pen), a7 (outdoor sport field), a8 (heifer pen), and a9 (outdoor sport field) were substrain G, B, B, B, E, H, and E, respectively.

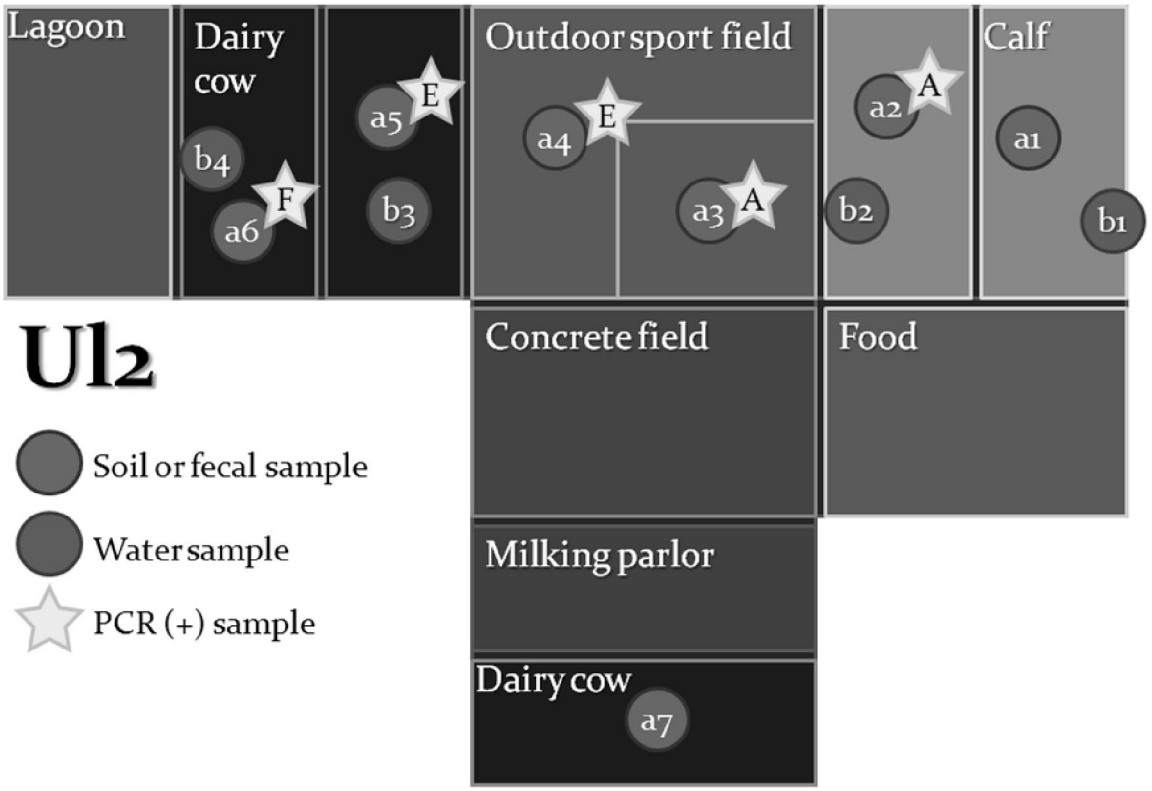


Figure12. Sampling map of herd Ul2: red spot indicated a soil/feces sample, and blue spot indicated a water sample. A red or blue spot was asterisked meant the sample was positive for IS900 real time PCR The code in asterisk was the *MAP* substrain identified by VNTRs. Totally, 11 environmental samples (7 soil/feces samples and 4 water samples) including 2 soil/feces samples of outdoor sport field, 3 soil/feces samples and 2 water samples of dairy cow pen, 2 soil/feces samples and 2 water samples of calf pen were collected from herd Ul2. The samples located at outdoor sport field (a3 and a4), calf pen (a2) and dairy cow pen (a5 and a6) were positive for IS900 real time PCR. The *MAP* substrains identified by VNTR of a2 (calf pen), a3 (outdoor sport field), a4 (outdoor sport field), a5 (dairy cow pen), and a6 (dairy cow pen) were substrain A, A, E, E, and F, respectively.

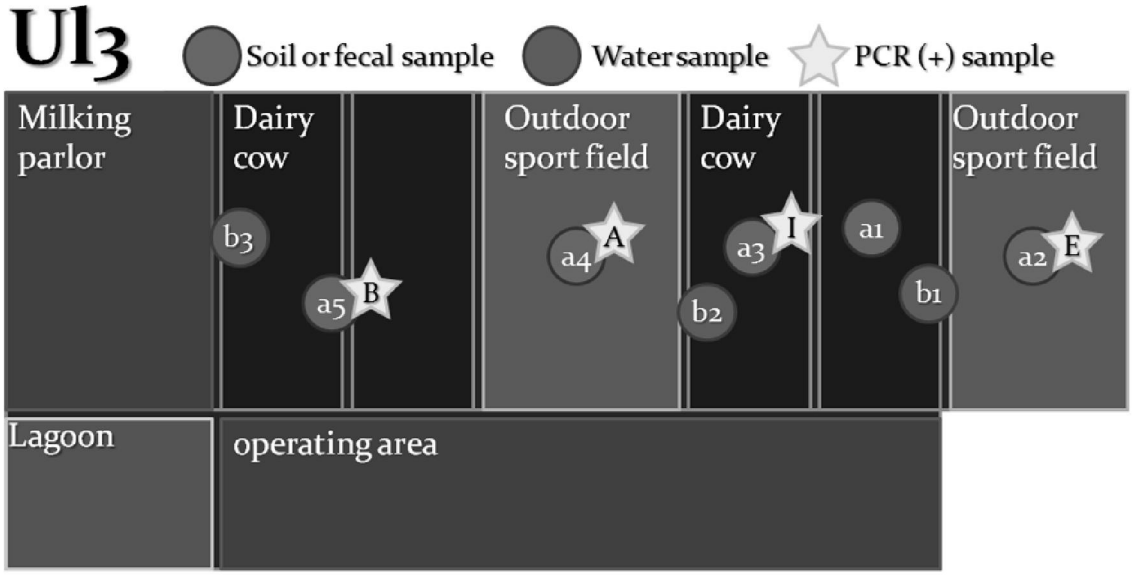


Figure 13. Sampling map of herd Ul3: red spot indicated a soil/feces sample, and blue spot indicated a water sample. A red or blue spot was asterisked meant the sample was positive for IS900 real time PCR The code in asterisk was the *MAP* substrain identified by VNTRs. Totally, 8 environmental samples (5 soil/feces samples and 3 water samples) including 2 soil/feces samples of outdoor sport field, and 3 soil/feces samples and 3 water samples of dairy cow pen were collected from herd Ul3. The samples located at outdoor sport field (a2 and a4), and dairy cow pen (a3 and a5) were positive for IS900 real time PCR. The *MAP* substrains identified by VNTR of a2 (outdoor sport field), a3 (dairy cow pen), a4 (outdoor sport field), and a5 (dairy cow pen) were substrain E, I, A, and B, respectively.

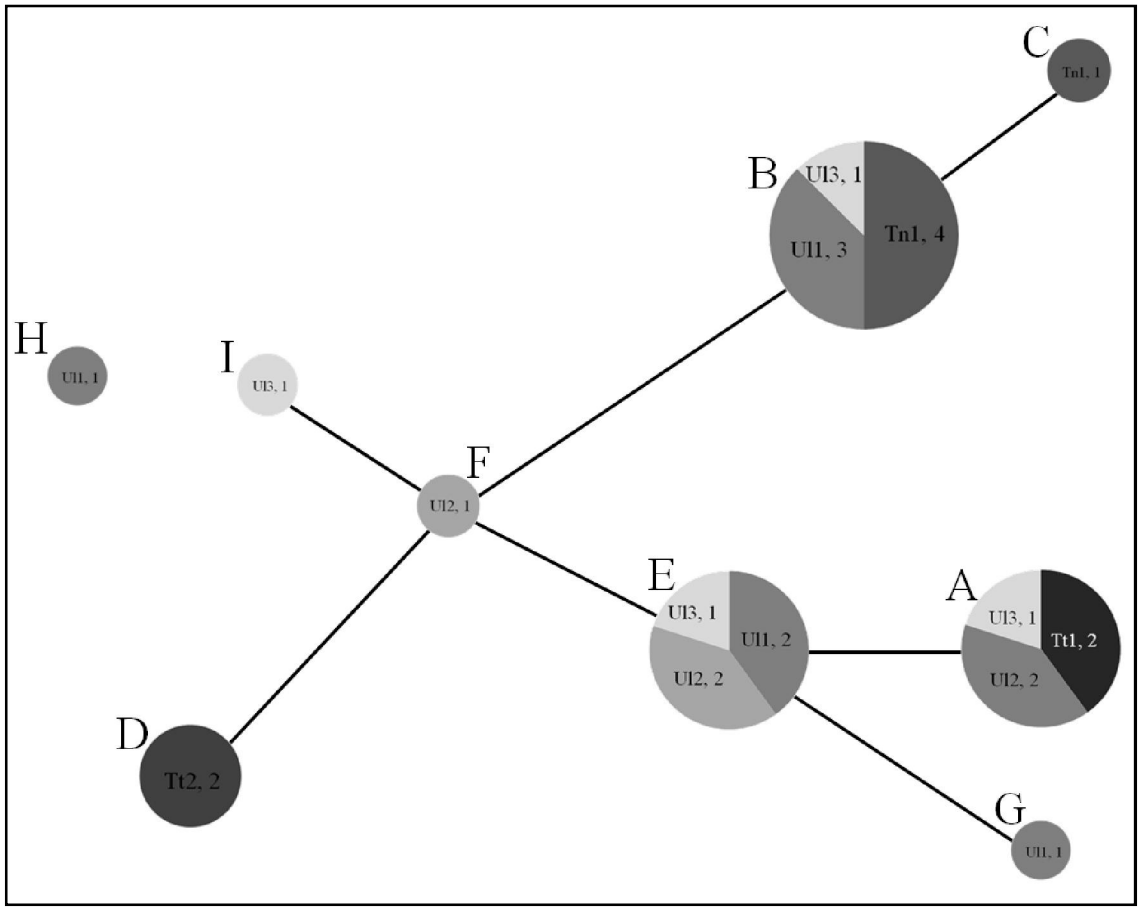


Figure 14. Phylogenetic tree based on VNTRs genotypes among 25 *MAP* positive environmental samples: The phylogenetic tree was draw by the eBURST programs. Each pie chart means the different *MAP* substrain, and each color and the ratio of each color in each pie chart means the number of the *MAP* substrain in the herd. (e.g. herd code, number of substrain). One sample in herd Ul3, two samples in herd Tt1 and two samples in herd Ul2 were *MAP* substrain A. One sample in herd Ul3, three samples in herd Ul1, and four samples in herd Tn1 were *MAP* substrain B. One sample in herd Tn1 was *MAP* substrain C. Two samples in herd Tt2 was *MAP* substrain D. One sample in herd Ul3, two samples in herd Ul1, and two samples in herd Ul2 were *MAP* substrain E. One sample in herd Ul2 was *MAP* substrain F. One sample in herd Ul1

was *MAP* substrain G. One sample in herd Ul1 was *MAP* substrain H. The *MAP* substrain A, B, C, D, E, F, G, and I were classified in group 1, but *MAP* substrain H was not in the group.

Evidence-based control strategy of bovine paratuberculosis in Taiwan

The initial search revealed 2443 literatures. After exclusion, 67 literatures were deemed relevant and 22 literatures used in this study.

The information about bPTB should be propagandized to livestock owners

There were some relative information for the livestock owners including: 1) "younger cattle are most susceptible to *MAP* infection" (Windsor and Whittington, 2010); 2) "clinical signs usually appear after 2 to 5 years, so *MAP* is usually introduced into dairy herds through purchase of infected but clinically normal cattle" (Sweeney, 1996); 3) "subclinically infected cattle may reduced milk production, premature culling, reduced slaughter value, and reduced fertility rates" (McKenna et al., 2006); 4) "The estimative annually cost of bPTB in US dairy is US$200 to US$250" (Ott et al., 1999), 5) "the main transmission routes are direct fecal-oral route or ingesting *MAP* from milk, colostrums, fecal-contaminated feed, soil, and water" (Sweeney, 1996; Whittington et al., 2004); 6) "calves born to infected cattle have a higher risk of being infected than calves born to uninfected cattle" (Bielanski et al., 2006); 6) "it must be managed as a herd-level disease, not individual cattle disease. This disease should be control to improve the productivity and profitability of the herd" (Harris and Barletta, 2001); 7) "paratuberculosis can be controlled. Diagnostic procedures are available to

identify transmission risk factors and to prevent new infections" (McKenna et al., 2006); 8) "the rate of infection and impact of this disease will increase with time unless effective control strategy is implemented" (Kudahl et al., 2008); 9) "it takes time to control bovine paratuberculosis in a herd, and reducing the prevalence within a herd may requires 3 to 7 years (Kudahl et al., 2008), it is needed the livestock owners' cooperation to keep the control strategy going for years", and 10) "the value of "*MAP* low-risk" certification program is enhancing economic value of certified herds and support the certified herds as a source for replacement cattle of low risk for *MAP* infection" (Kovich et al., 2006).

The most cost-effective diagnostic method to identify the MAP infection status of dairy herd

A study indicated ELISA alone was a sensitive and low-cost testing method to indentify the status of dairy herd, and the sensitivity to identify *MAP* positive herd (HSe) was 54 to 82% (when sample was large than 30 cattle or whole herd). In addition, culture of environmental samples was the most cost-effective method for herds. It yielded 99% HSe for herds with 16% within herd prevalence, but was not sufficient to achieve 95% HSe in low prevalence herds (5%) (Tavornpanich et al., 2008). Furthermore, detection of *MAP* by real time PCR was the best methods for

detecting *MAP* in soil and manure extracts, because real time PCR was high sensitivity and specificity for the detection of *MAP* in a relatively shorter turn over time, comparing to other conventional diagnostic techniques such as fecal culture, the methods was greatly limited by the lack of sensitivity, specificity and/or turn over time (Cook and Britt, 2007). The serum ELISA and real time PCR of environment were most effective methods to identify the *MAP* status of herds.

The preventive management practices to control bPTB

There were some preventive management practices of control bPTB including: 1) "clean and disinfect maternity and calf pens after each use" (McKenna et al., 2006), 2) "calve in separate, clean, and dry calving area" (Dieguez et al., 2008), 3) "remove calves from their dam immediately after birth" (Ansari-Lari et al., 2009), 4) "calf should have a separated area to avoid horizontal calf-to-calf transmission" (van Roermund et al., 2007), 5) "calves must be separated from adult cattle for at least the first year of life" (Nielsen and Toft, 2007), 6) "avoid vehicular and human traffic from adult cattle areas to young cattle area" (Wells and Wagner, 2000), 7) "feed collected colostrum and milk from cattle with cleaned udders" (Dieguez et al., 2008), 8) "feed pasteurized milk or milk replacer to calves" (Ansari-Lari et al., 2009), 9) "do not allow shared feed or water between adults and young cattle" (Ansari-Lari et al., 2009), 10)

"use separate equipment for handling feed and manure" (Wells and Wagner, 2000), 11) "feedbunk and water trough should not have risk for fecal contamination" (McKenna et al., 2006), 12) "do not spread manure on grazing land and fed to cattle of any age group" (Obasanjo et al., 1997), 13) "cull all cattle with clinical evidence of paratuberculosis as soon as possible" (Lu et al., 2008), and 14) "maintain a closed herd or purchase replacement cattle only from *MAP* negative herds or "*MAP* low-risk" herds" (Dieguez et al., 2008).

The most cost-effective diagnostic method to identify the subclinical infected cattle

MAP infected cattle were a major transmission risk factor, and the subclinical infected cattle was difficult to be identified. A study indicated the combined use of ELISA and direct fecal PCR had the potential to increase the overall sensitivity for the diagnosis of *MAP* infection (Pinedo et al., 2008). Direct fecal real time PCR was greater sensitivity than the ELISA and faster than fecal culture. The greater sensitivity also allowed the testing of pooled fecal samples, making whole herd and environmental sampling more economical (Clark et al., 2008). In order to identify the *MAP* infected cattle within herd, the combined use of ELISA and direct fecal real time PCR were an economical and effective opinion.

The optimal method for certification and monitoring scheme of "MAP low-risk" herd

A study conclude that the optimal certification and monitoring scheme for "*MAP* low-risk" herds were 1) certification of "*MAP* low-risk" herds after four herd examinations at 2 year intervals consisting of pooled fecal culture of all cattle ≥ 2 years of age, and 2) monitoring of "*MAP* low-risk" herds by pooled fecal culture of all cattle ≥ 1 year of age at 2 year intervals (Weber et al., 2004). In addition, direct fecal real time PCR was greater sensitivity than the ELISA and faster than fecal culture. The greater sensitivity also allowed the testing of pooled fecal samples, making whole herd and environmental sampling more economical (Clark et al., 2008). In conclusion, the optimal certification and monitoring scheme for "*MAP* low-risk" herds were 1) certification of "*MAP* low-risk" herds after four herd examinations at 2 year intervals consisting of pooled fecal IS900 real time PCR of all cattle ≧ 2 years of age, and 2) monitoring of "*MAP* low-risk" herds by pooled fecal IS900 real time PCR of all cattle ≧ 1 year of age at 2 year intervals

Chapter V: Discussion

Bovine paratuberculosis surveillance

The MAP seroprevalence of paratuberculosis is 10.1%

The *MAP* seroprevalence in this study, comparing with the result reported in 1987, the seroprevalence of bPTB in Taiwan has risen considerably in these twenty years (Lu et al., 1987). When the within herd prevalence of bPTB is lower than 5% (Kudahl et al., 2008), the economic impact of bPTB may be insusceptible for livestock owners. Nowadays, the seroprevalence of bPTB in Taiwan is about 10%, the economic effect of paratuberculosis for dairy herds in Taiwan is thought to be a major impact.

The herd-level MAP prevalence is 66.7%

The result also indicates that 67 % of the evaluated dairy herds have been affected by *MAP*. Because of the lower sensitivity of ELISA, the herd-level prevalence may be underestimated. It also suggest the 21 *MAP* negative herds may be not *MAP*-free herds, further investigating the infection statuses of these herds are necessary to evaluated the risk of *MAP* exist in these herds.

The prevalence of individual herd are ranged from 0% to 60%

Among these 42 *MAP*-positive herds, the seroprevalence among these herds is

varied considerably, ranging from 1 percent to 60 percent. The seroprevalences of half *MAP* positive herds are equal to / higher than 10% (high prevalence), and the others are lower than 10% (low prevalence). The results indicate that seroprevalences in positive herds are markedly different among different herds and the transmission risk factors that contributed to *MAP* infections may be complex and different among different herds. In order to develop a risk-based control strategy for each herd with different infection status, it's necessary to further investigate the transmission risk factors in each herd.

The seroprevalence and herd-level prevalence of bPTB in each country and city

The results indicate each country and city sampled in this study is affected by *MAP* except Nanton (Figure 4 and 5). It is necessary further investigating the *MAP* infection status of herds in these *MAP* affected countries and cities to effectively closure the transmission pathway between *MAP* positive herds and *MAP* negative herds. The seroprevalences in Tainan, Kaohsiung city, Pingtung, and Taitung are higher than 10% (the average *MAP* seroprevalence), it indicates the economic loss in these countries and city is more serious than other countries. The herd-prevalences are higher than 50% in Taichung, Changhua, and Pingtung, and are 100% in Yunlin, Tainan, Kaohsiung city, and Taitung. In addition, there is only one herd sampled in Nanton, so

the *MAP* infection status of Nanton is still unclear. Purchasing *MAP* infected but clinical normal cattle is a major pathway for transmitting *MAP* among herds (Sweeney, 1996). It indicates the trading of cattle in each country and city is necessary to be further observant. The "*MAP* low-risk" certification and monitoring program must be developed to provide a *MAP* low-risk source of additions and replacements.

The MAP seroprevalence of beef cattle is 2.8%

The sources of these cattle are mixture of beef cattle and culled cattle. The result indicates the cattle in slaughterhouse are affected by *MAP,* some studies indicate beef can be contaminated with *MAP* via dissemination of the pathogen in the tissues of infected cattle (Eltholth et al., 2009). This data suggests that the likelihood of meat products being contaminated with *MAP* on retail meats should not be ignored. Furthermore, the seroprevalence of male cattle is lower than female cattle. It is resulting from that the long disease course, and the male cattle are slaughtered at two to three years of age (as beef cattle). Currently data indicates the culled cattle with older age plays an important role in *MAP* transmission. Further investigation of the *MAP* of retail meats is necessary.

The MAP seroprevalence of ITT positive cattle

A study indicated that cattle with positive responses for *MAP* (Fecal culture and ELISA) are seems to be resulting in false positive results on caudal fold tuberculin (CFT) test (Dunn et al., 2005). The same test is implemented for identifying tuberculosis (TB) infected cattle in Taiwan. The result in this study indicates the PTB plays an important role on interfering in the results of intradermal tuberculin test (ITT). Furthermore, the difference of the PTB ELISA positive rates between adult and young cattle may suggests the results of ITT on adult cattle are more odds to be interfered with PTB. It causes premature culling and makes Taiwan can't be classified into TB-free areas to cause serious economic loss in dairy herds, reduce the value of dairy industry, and hamper the international trade of Taiwan. As above, it is necessary to modify the current policy about TB control. For dairy herds, it's suggested that the strategic deployment of ancillary in vitro tests alongside the ITT has enhanced the detection of TB-infected cattle and reduced the number of cattle culled as false positives of ITT. For national level, the definition of TB-positive cattle and herds must be re-defined. It's suggested that the ITT-positive cattle with culture positive or PCR positive is defined as TB-positive cattle, and the herd would be defined as TB-positive herds, if $\geqq 1$ TB-positive cattle in the herd (Department of Health and Ageing, Australia).

The kernel density map of MAP positive dairy herds

In kernel density map of *MAP* positive herds, the hot spot is located at the west area of Pingtung. Dairy herds in hot spot are thought to be face with more risk of *MAP* infection, because the odds of introducing *MAP* (mechanical carriers or purchasing infected cattle) are increased. This viewpoint may be confirmed by molecular epidemiology in this area, an method to analysis the *MAP* transmission pattern, such as VNTRs. Furthermore, the results are useful for developing a regional level control strategy.

Transmission Risk Factor Analysis for Bovine Paratuberculosis

The prevalence of soil and feces samples from MAP-positive herds

The results indicate soils and feces in outdoor sport field are the most representative area to represent the *MAP* status of dairy herds and high risk for transmitting *MAP* from environment to cattle, because 1) cattle is mixing in outdoor sport field, it offers a possibility to transmit *MAP* from environment to cattle; 2) the outdoor sport field is covered by soil and pad in Taiwan. It hampered the disinfection and cleaning of this area, and *MAP* may survival in this substance for more than 300 days. Cattle pens are concrete field and may be washed 2 to 3 times a day, so it may not be an index for us to understanding the *MAP* status in dairy herds. The IS900 real time PCR result of cattle pens demonstrate that the positive rates of these area are affected by the number of infectious cattle and the grade of disinfection or cleaning in these area. The results suggest that 1) it is necessary to investigate the infectious cattle in these areas by direct fecal real time PCR; and 2) enforcing the disinfection and cleaning in these areas to closure the possible transmission pathway of *MAP* from environment to cattle.

The results of lagoon and pasture are all negative for IS900 real time PCR, but it's not indicated these areas are not important for *MAP* transmission. Comparing with the study reported by Raizman et al, the lagoon is the targeted common and contaminated

areas in the environment (Raizman et al., 2004). The reason of the difference between this study and the study reported by Raizman et al may be that *MAP* in this area is diluted by other substance such as water, feed, or feces and urine of *MAP* negative cattle. Pasture is a similar area with outdoor sport field, but the density of cattle and stay time of cattle are relative low.

The prevalence of water samples from MAP-positive herds

Compare the result of soil and feces samples with water sample, it indicate that the water is relative lower risk for transmitting *MAP*. This results may result from the water trough is flowing and elevated. Because of the number of infectious cattle, herd hygiene level, and endemic type of management or environment, the infection and contamination of *MAP* in different herds or different time may be different.

Nine substrains (From A to I) of MAPs were identified

The results of VNTRs indicated that there are direct link of *MAP* transmission among 1) herd Tt1, Ul2 and Ul3; 2) herd Ul1, Ul2 and Ul3; and 3) herd Tn1, Ul2 and Ul3. The results of VNTRs provide evidence about exogenous transmission, and *MAP* transmission of regional level. It's necessary to closure the exogenous transmission pathway, such as purchasing cattle from *MAP* negative herds and reducing the risk to

contact other cattle or herds with unknown *MAP* status. On the other hand, the exogenous transmission pathway for herd Tt2 may not exist, it indicate the point to control paratuberculosis for herd Tt2 is to closure the transmission pathway within herd.

The Phylogenetic tree of 9 MAP substrains

The substrain B is same as *MAP* K-10, a bovine clinical isolate was isolated from a dairy herd in Wisconsin at the U.S. The substrain A to G and I are classified into same group, it indicate there may be some relationship of *MAP* infection between Taiwan and the U.S. The results also indicate the original source of substrain H is different with other substrains, it means substrain H is from different country or the Taiwanese aboriginal strain. The further investigating of genetic relatedness by VNTRs is necessary to provide more evidence about the original resource of *MAP* infection.

The distributions of MAP substrains in each herd

The different *MAP* substrains in each herd are a method to investigate the *MAP* transmission pathway among herds. Besides, the distributions of different *MAP* substrains in each herd are a method to investigate the *MAP* transmission pathway within herd. In the herd, the same substrain between two or more areas indicates there

is a transmission pathway among these areas. In this situation, blocking this transmission pathway is necessary and essential to control bPTB in the herd. In herd Ul1, there are possible transmission pathways between dairy cow pen and heifer pen, and between outdoor sport field and heifer pen (figure 11). In herd Ul2, there are possible transmission pathways between dairy cow pen and outdoor sport field, and between outdoor sport field and calf pen (figure 12). Further investigating the VNTRs of *MAP* infected cattle (shedders) in the herd is necessary to find out the detail transmission pathway, and to block the transmission pathway. A study indicates polymorphism of VNTRs may cause variations on the gene expression and this could be important in the understanding of differences in the pathogenic effect (Romano et al., 2005). Therefore, it is suspected the different *MAP* substrains may have the different resistances and features of the *MAP*, and causes the different distributions in herds.

Evidence-based control strategy of bovine paratuberculosis in Taiwan

Based on the results of above chapters and reviewed literatures, a bPTB control program is developed. The goal of this bPTB control program is aimed to prevent 1) transmission of *MAP* infection from infected cattle to other cattle, 2) contamination of the environment with *MAP*, and 3) introduction of *MAP* infected cattle from infected herds to non-infected herds. A successful control strategy depends on whether the owners understand the fundamental cognizance of bPTB, so the first point of this bPTB control is propagandizing the related information to the livestock owner. Second, the status of each herd will be identified to implement different control strategies. Third, the "*MAP* low-risk" certification and monitoring program is developed to provide a source of additions and replacements for dairy herds. The flowchart of this bPTB control program is indicated in figure 15, and the specific descriptions in this bPTB control program are as following paragraphs

Livestock owner education and propaganda of bPTB

Some relative information for the livestock owners including 1) younger cattle are most susceptible to *MAP* infection (Windsor and Whittington, 2010); 2) clinical signs usually appear after 2 to 5 years, so *MAP* is usually introduced into dairy herds through purchase of infected but clinically normal cattle (Sweeney, 1996); 3)

subclinically infected cattle may reduced milk production, premature culling, reduced slaughter value, and reduced fertility rates (McKenna et al., 2006); 4) The estimative annually cost of bPTB in US dairy is US$200 to US$250 (Ott et al., 1999), 5) the main transmission routes are direct fecal-oral route or ingesting *MAP* from milk, colostrums, fecal-contaminated feed, soil, and water (Sweeney, 1996; Whittington et al., 2004); 6) calves born to infected cattle have a higher risk of being infected than calves born to uninfected cattle (Bielanski et al., 2006); 6) It must be managed as a herd-level disease, not individual cattle disease. This disease should be control to improve the productivity and profitability of the herd (Harris and Barletta, 2001); 7) paratuberculosis can be controlled. Diagnostic procedures are available to identify transmission risk factors and to prevent new infections (McKenna et al., 2006); 8) the rate of infection and impact of this disease will increase with time unless effective control strategy is implemented (Kudahl et al., 2008); 9) it takes time to control bovine paratuberculosis in a herd, and reducing the prevalence within a herd may requires 3 to 7 years (Kudahl et al., 2008), it is needed the livestock owners' cooperation to keep the control strategy going for years, and 10) The value of "*MAP* low-risk" certification program was enhancing economic value of certified herds and support the certified herds as a source for replacement cattle of low risk for *MAP* infection (Kovich et al., 2006) are

propagandized by fly sheet (Figure 16) and periodical conference to enhance the cooperation of livestock owners. Thereby, it could attract the interest of livestock owner to participate in this control program. All dairy herds participate in this program are voluntary, and in order to reduce the risk of introducing *MAP* in these herds, cattle might be added to these herds only if the cattle are from a herd with level 1 to 4 of "*MAP* low-risk" certification.

Identify the MAP status of voluntary dairy herds

The status of voluntary dairy herd is classified into *MAP* positive herds and *MAP* negative herds by the serum ELISA, pooled fecal IS900 real time PCR and environmental IS900 real time PCR:

(1) Serum ELISA: serum of all cattle over 2 years of age are collected and tested by commercial serum ELISA kit (Institut Pourquier). The detailed protocol is indicted in appendix 2. A herd is defined as *MAP* positive herd, if at least one positive serum sample is detected.

(2) Pooled fecal IS900 real time PCR: feces of all cattle over 2 years of age are collected. Five individual fecal samples of 2 g are pooled to make a composite 10 g sample, and then 350 μg from the pooled sample are used for DNA extraction and IS900 real time PCR (the detailed protocol is indicted in

appendix 2). A positive result of pooled fecal IS900 real time PCR is confirmed by individual fecal IS900 real time PCR of the feces from previously positive pooled sample. If there are ≧1 fecal sample in the herd showed positive result for IS900 real time PCR, the herd is defined as *MAP* positive herd.

(3) Environmental IS900 real time PCR:≧10 soil/feces samples are collected. The samples are used for DNA extraction and tested by IS900 real-time PCR (the detailed protocol is indicted in appendix 2). If there are ≧1 environmental sample in the herd showed positive result for IS900 real time PCR, the herd is defined as *MAP* positive herd. Furthermore, the results of environmental IS900 real time PCR are a convictive evidence for the livestock owner improving the management practices and the cleaning of herd.

The control and test strategy for MAP positive herds

There are some preventive management practices of control bPTB including 1) clean and disinfect maternity and calf pens after each use (McKenna et al., 2006), 2) calve in separate, clean, and dry calving area (Dieguez et al., 2008), 3) remove calves from their dam immediately after birth (Ansari-Lari et al., 2009), 4) calf should have a separated area to avoid horizontal calf-to-calf transmission (van Roermund et al., 2007), 5) calves must be separated from adult cattle for at least

the first year of life (Nielsen and Toft, 2007), 6) avoid vehicular and human traffic from adult cattle areas to young cattle area (Wells and Wagner, 2000), 7) feed collected colostrum and milk from cattle with cleaned udders (Dieguez et al., 2008), 8) feed pasteurized milk or milk replacer to calves (Ansari-Lari et al., 2009), 9) do not allow shared feed or water between adults and young cattle (Ansari-Lari et al., 2009), 10) use separate equipment for handling feed and manure (Wells and Wagner, 2000), 11) feedbunk and water trough should not have risk for fecal contamination (McKenna et al., 2006), 12) do not spread manure on grazing land and fed to cattle of any age group (Obasanjo et al., 1997), 13) cull all cattle with clinical evidence of paratuberculosis as soon as possible (Lu et al., 2008), and 14) maintain a closed herd or purchase replacement cattle only from *MAP* negative herds or "*MAP* low-risk" herds (Dieguez et al., 2008) are implemented to reduce the risk of *MAP* transmission. Furthermore, In order to effectively prevent transmission of *MAP* infection, a test strategy for investigating the infectious cattle in *MAP* positive herds is proposed:

1) Serum ELISA and: these tests would be performed to effectively identify infectious cattle. Serum of all cattle over 2 years of age are collected and tested by commercial serum ELISA kit (Institut Pourquier). The detailed protocol is indicted in appendix 2.

2) Pooled fecal IS900 real time PCR: feces of all cattle over 2 years of age are collected. Five individual fecal samples of 2 g are pooled to make a composite 10 g sample, and then 350 mg from the pooled sample are used for DNA extraction and IS900 real time PCR (the detailed protocol is indicted in appendix 2). A positive result of pooled fecal IS900 real time PCR is confirmed by individual fecal IS900 real time PCR of the feces from previously positive pooled sample.
3) Environmental IS900 real time PCR is performed to effectively identify infectious environment, and to evaluate the progress of the improvement of management practices. The detailed protocol is indicted in appendix 2.

Test positive cattle are highest risk to be a source for transmitting *MAP*, and the related management practices are as following: 1) test positive cattle are isolated to close transmission pathway, and would be culled if their daily milk yield below 10 kg, 2) All calves from test positive cattle are fattened and slaughtered because they had a high risk of being infected with *MAP*, and test positive cattle are not inseminated, and 3) Feed collected colostrum and milk from *MAP* negative cattle with cleaned udders. Test positive environmental areas indicated that it's necessary to enforce the disinfection and cleaning in these areas for closuring the possible transmission pathway of *MAP* from environment to cattle, and to avoid the

susceptible cattle (i.e. young cattle) contacting the contaminated environment. These tests would be annually performed to optimize management practices and closure the *MAP* transmission pathway until the herd is negative for these tests, and then the herds with negative result of the tests would participate the "*MAP* low-risk" certification and monitoring program.

"MAP low-risk" certification and monitoring program

The *MAP* negative herds would participate in "*MAP* low-risk" certification program. The certification of "*MAP* low-risk" herds including four levels is conducted after four herd examinations at 2 year intervals consisting of pooled fecal IS900 real time PCR of all cattle ≥ 2 years of age (the detailed protocol is indicted in appendix 2). With each subsequent annual pooled fecal IS900 real time PCR is negative, the herds are awarded a higher certification level (from 1 to 4), reflecting the increased confidence that the herd is truly low-risk of the *MAP* infection (table 8). To reduce the risk of introducing *MAP* in "*MAP* low-risk" herds, cattle might be added to these herds only if the cattle are from another "*MAP* low-risk" herd. In addition, cattle might be added to herds that are in the process of "*MAP* low-risk" certification only if they are from a herd with an equal or higher level of "*MAP* low-risk" certification. Furthermore, the herds in level 4 would enter

into “*MAP* low-risk” monitoring program. The monitoring of “*MAP* low-risk” herds is performed by pooled fecal IS900 real time PCR of all cattle ≥ 1 year of age at 2 year intervals (the detailed protocol is indicted in appendix 2). A positive result of pooled fecal IS900 real time PCR is confirmed by individual fecal IS900 real time PCR of the feces from previously positive pooled sample. If all individual fecal samples of a previously positive pool are negative, then this pool is regarded as negative. If an individual fecal IS900 real time PCR is positive, the herd is expelled from the “*MAP* low-risk” certification and monitoring program. To reduce the risk of introducing *MAP* in “*MAP* low-risk” herds, cattle might be added to these herds only if the cattle are from another “*MAP* low-risk” herd. In addition, cattle might be added to herds that are in the process of “*MAP* low-risk” certification only if they are from a herd with an equal or higher level of “*MAP* low-risk” certification. To reduce the risk of introducing *MAP* in “*MAP* low-risk” herds, cattle might be added to these herds only if the cattle are from another “*MAP* low-risk” herd.

Table 8. "*MAP* low-risk" certification program

Year	Level	Component
1	1	All tests of voluntary dairy herd are negative.
2	1	None
3	2	The herd has met the requirements for level 1. All cattle ≧ 2 years of age are tested by pooled fecal IS900 real time PCR, and all cattle are negative. The level 2 test must be complete between 22 to 26 months after level 1 test.
4	2	None
5	3	The herd has met the requirements for level 2. All cattle ≧ 2 years of age are tested by pooled fecal IS900 real time PCR, and all cattle are negative. The level 3 test must be complete between 22 to 26 months after level 2 test.
6	3	None
7	4	The herd has met the requirements for level 3. All cattle ≧ 2 years of age are tested by pooled fecal IS900 real time PCR, and all cattle are negative. The level 4 test must be complete between 22 to 26 months after level 3 test.

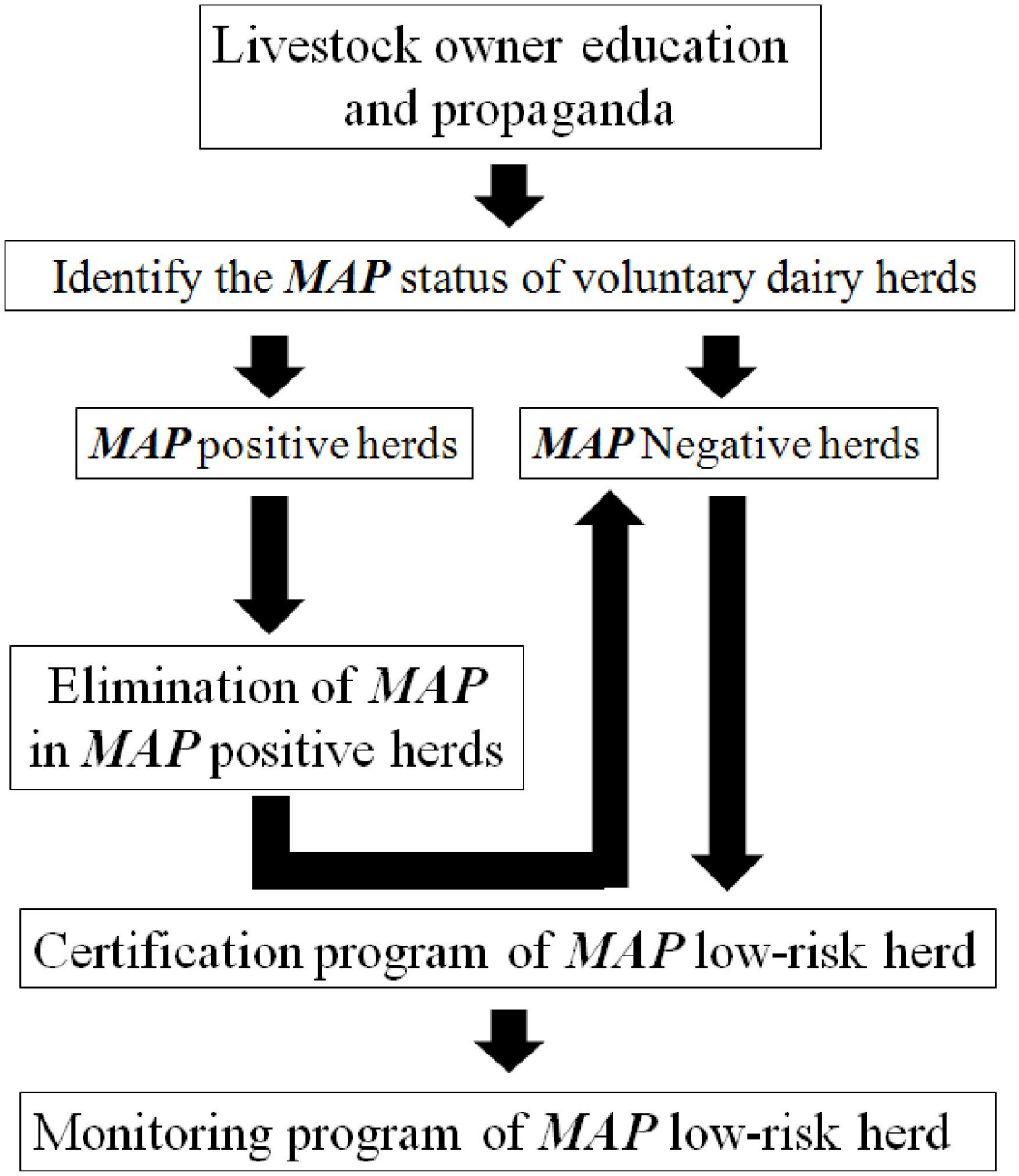


Figure 15. Flow chart of the bPTB control program.

Figure 16. Fly sheet for propaganda of bPTB.

Chapter VI: Conclusion

The *MAP* seroprevalence of dairy herds was 10.1%, and 66.7% dairy herds were *MAP* positive herd. The results indicated the *MAP* infection status and transmission risk factors are different among different herds. The *MAP* seroprevalence of beef cattle was 2.8%, and suggested that the likelihood of meat products being contaminated with *MAP* on retail meats should not be ignored. The *MAP* seroprevalence of intradermal tuberculin test (ITT) positive cattle was 51.4%, and indicated the PTB plays an important role on interfering in the results of ITT. In kernel density map of *MAP* positive herds, the hot spot was located at the west area of Pingtung. Soil and feces were high risk carries for transmitting *MAP*, but water was relative lower risk for transmitting *MAP*. Nine substrains of *MAPs* were identified, and indicate there were direct link of *MAP* transmission among herds. According to the results of this study and related researches of this disease, a bPTB control program was developed.

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Appendix I

Transmission Risk Factor and Control Strategy of Bovine Paratuberculosis in Taiwan

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Appendix I

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Abstract Bovine paratuberculosis is caused by *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*). The aims of this study was to investigate the current *MAP* infection status and *MAP* transmission risk factors in Taiwan; thereby, to develop a practical control program. A nation-wise survey by serum enzyme linked immunosorbent assay (ELISA) was conducted. Environmental samples from dairy herds were collected for detecting *MAP* by real time PCR and for phylogenic analysis by variable number tandem repeats (VNTRs). Results indicated that *MAP* seroprevalence in dairy cow was 10.1%, and 66.7% dairy herds were positive, *MAP* seroprevalence of beef cattle was 2.8%, *MAP* seroprevalence of intradermal tuberculin test (ITT) positive cattle was 51.4%. *MAP* infection status and transmission risk factors are different among different herds. Soil and feces were high risk carries for transmitting *MAP*. Nine substrains of *MAPs* were identified indicating direct link of *MAP* transmission among herds. According to the results above and previous research, a *MAP* control program was developed.

Key words: paratuberculosis, real time PCR, spatial analysis, survey, VNTRs.

Appendix I

Introduction

Mycobacterium avium subsp. *paratuberculosis* (*MAP*) is the causative agent of paratuberculosis (Johne's disease). Outbreaks of Johne's disease often result in severe economic loss to dairy industry [15]. *MAP* is usually introduced into dairy herds through purchase of infected but clinically normal cattle. Contaminated vehicles or equipments can act as mechanical carrier for the pathogen as well. Infectious cattle may shed *MAP* into their feces, milk and colostrum. The main transmission route within a herd is the direct fecal-oral route from ingesting fecal-contaminated feed, soil, and water [22]. Younger cattle are most susceptible to *MAP* infection, and may ingest *MAP* through feeding milk and colostrums [29]. Transplacental infection also takes place in infected cattle [3]. Enzyme-linked immunosorbent assay (ELISA) for detecting the specific antibodies against *MAP* in blood is widely used. Real-time PCR detecting *MAPs* in feces, milk, and environmental samples has been developed [5]. Phylogenetic analysis of variable number of tandem repeats (VNTRs) is a PCR-based strain typing method for tracing back the origin of *MAP* infections, for identifying the risk factors influencing *MAP* transmission, and for developing control strategies to block the routes of *MAP* transmission within and among dairy herds [24]. In Taiwan, a survey conducted in 1987 shows that 4% of cattle were serologically positive for paratuberculosis [1], but no related control strategy has been implemented. Vaccination against *MAP* in cattle and the cost of treating infected cattle are not cost-effective. Risk-based control strategy is recommended for the control of *MAP* in dairy herds [13]. The aims of this study are: 1) to know the current infection status of paratuberculosis in dairy herds and beef cattle, 2) to investigate whether the bPTB interfere the result of ITT or not, 3) to investigate the *MAP* transmission risk factors within and among dairy herds, and 4) to develop a practical control strategy for bPTB in Taiwan.

Material and methods

Bovine Paratuberculosis Surveillance A survey was proposed for bPTB specific antibody in approximately 2,600 cattle nationwide, including 2000 serums of dairy cattle were randomly collected by the animal disease control center as part of an annual investigation of bovine infectious diseases, 500 serums of beef cattle at slaughter facilities, and 100 serums of ITT positive cattle. Furthermore, the spatial point pattern analysis was performed to analysis the survey of dairy herds. The geocodes of all herds were displayed on a map of Taiwan, using the Arc View GIS 9.3 (ESRI, Redlands, CA, USA). The herd location data were converted into continuous surfaces by using the kernel-density function, expressing the case occurrence. The kernel density was estimated by using a bandwidth of 5 km and a cell size of 200 m.

Transmission Risk Factor Analysis To investigate the transmission risk factors in dairy herds, feces, milk, colostrums, and environmental soil/water samples from watering/feeding troughs, alleyways, pens, outdoor sport field, grazing land, wastewater gutter and lagoons were collected for *MAP* detection by real-time PCR and for *MAP* strain typing by phylogenetic analysis of VNTRs. All extractions were performed by using FastDNA® Spin Kit for soil (Qbiogene). All steps were

conducted according to the manufacturer's instructions, except 1) 350 mg soil or feces samples and 350 μL water samples, 2) the homogenized step for 45 seconds at a speed of 6.0 m/s twice rather than 40 seconds at a speed of 6.0 m/s once, 3) the final DNA elution was 200 μL. Dilutions of the DNA (1:10) were made and 4 μL of the diluted DNA were used as template for real time PCR analysis. Real-time PCR to detect the specific sequence IS900 of *MAP* was conducted on the Mastercycler ep realplex^4 (eppendorf®) with primer sequences were as follows: MAPF, AAT GAC GGT TAC GGA GGT GGT TGT G; and MAPR, AAT CGC TGC GCG TCG TCG TTA ATA. Phylogenetic analysis was conducted on PCT-200 thermal cycler (Bio-Rad) with ten pair primers were listed in table 1. The outer primers of VNTRs were designed by using the Primer 3 on-line tool, and the inner primers were described by other studies [8,19,24]. The PCR products were analyzed by gel electrophoresis to detect tandem repeats (TRs) with different sizes at the different loci. Each strain of *MAPs* was designated a code for different sizes of tandem repeats at different loci. The discriminatory index (DI) was used as a numerical index for the discriminatory power of typing method [11]. The *MAP* genetic relatedness was analyzed by the eBURST (http://eburst.mlst.net/) programs.

Evidence-based Control Strategy of Bovine Paratuberculosis in Taiwan Evidence-based decision making applied the available evidence gained from the scientific method. The systematic review of published research studies was a major method. Literature and information related to the bPTB risk factor, bPTB control program, bPTB certification, and bPTB test parameters were identified from Pubmed. Unsolved problems addressed by the systematic review on control strategy of bPTB were 1) "What information about bPTB should be propagandized to livestock owners?", 2) "what is the most cost-effective diagnostic method to identify the *MAP* infection status of dairy herd?", 3) "what is the preventive management practices to control bPTB?", 4) what is the most cost-effective diagnostic method to identify the subclinical infected cattle, and 5) "what is the optimal method for certification and monitoring scheme of "*MAP* low-risk" herd?". For the initial identification of primary studies the following search terms combined with "paratuberculosis", "control", "program", "certification", "test", "status", "PCR", "ELISA", "risk factor", "environment", "management". The following criteria were used to enable primary identification of the relevant literatures: 1) in English language and 2) address at least one of the unsolved problems to deem the papers relevant or irrelevant. These literatures and information were reviewed to facilitate developing bPTB control strategy in Taiwan. In combination with the results of bPTB surveillance, bPTB transmission risk factors, and the reviewed literatures, the bPTB control strategy was developed.

Results

Bovine Paratuberculosis Surveillance Totally, 1) 2170 bovine serum samples from 2170 dairy cattle in 63 dairy herds were collected from seven counties (Taichung, Yunlin, Changhua, Nantou, Tainan, Pingtung, and Taitung) and Kaohsiung city in Taiwan in 2008. The number of sampled dairy herds in each country and the sample number of each herd were listed in table 2; 2) 542

bovine serum samples from 542 beef cattle (including 424 male cattle, 86 female cattle, and 32 sex-unknown cattle) were collected from Yahsen slaughterhouse; 3) 148 bovine serum samples from 148 ITT positive dairy cattle including 63 young cattle (< 1 years old) and 85 adult cattle (> 1 years old) were collected. The individual dairy cattle seroprevalence of paratuberculosis was 10.1%. The herd-level prevalence in Taiwan was 66.7% (42/63). The seroprevalences of individual dairy herds were ranged from 0% to 60% in dairy herds, and the seroprevalences of half *MAP* positive herds were higher than 10% (high prevalence), and the others were equal to /lower than 10% (low prevalence) (figure 1). The seroprevalences of bPTB in Taichung, Yunlin, Changhua, Nantou, Tainan, Kaohsiung city, Pingtung, and Taitung were 8.0%, 8.9%, 4.7%, 0.0%, 23%, 12.5%, 11.4%, and 19.2%, respectively. The herd-level prevalences of bPTB in Taichung, Yunlin, Changhua, Nantou, Tainan, Kaohsiung city, Pingtung, and Taitung were 50.0%, 100.0%, 78.6%, 0.0%, 100.0%, 100.0%, 50.0%, and 100.0%, respectively. The *MAP* seroprevalence of beef cattle was 2.8%, and the seroprevalences of male cattle, female cattle, and sex-unknown cattle were 0.2%, 12.8%, and 10.3%, respectively. The *MAP* seroprevalence of ITT positive cattle was 51.4%, and the *MAP* seroprevalence of young cattle and adult cattle were 27% and 69.4%. The geographic location information of dairy herds in Pingtung were obtained, and the kernel density map of positive herds (t = 5 km, cell size 100 m) showed in figure 6. The different densities of positive herds per square kilometer (sqkm) were indicated by the colors. The different herd status of *MAP* specific antibody were indicated by different colors, such as red spots meant positive herds, green spots meant negative herds, and yellow spots meant untested herds. The “K” meant Kaohsiung city and “P” meant Pingtung (figure 2).

Transmission Risk Factor Analysis for Bovine Paratuberculosis Totally, 130 environmental samples including 89 soil/feces samples and 41 water samples were collected from 8 dairy herds (table 3). Environmental samples of 8 dairy herds from Taichung (Tc1), Yunlin (Ul1, Ul2 and Ul3), Tainan (Tn1) and Taitung (Tt1, Tt2 and Tt3) were examined by IS900 real time PCR. At least one environmental sample in herd Tt1, Tt2, Tn1, Ul1, Ul2 and Ul3 was positive for IS900 real time PCR, but all environmental samples in herd Tc1 and Tt3 are negative. 9 soil samples and 1 water sample from dairy cow pen, 9 soil samples from outdoor sport field, 1 soil sample from pregnant cow pen, 4 soil samples from heifer pen, and 1 soil sample from calf pen are positive for *MAP*. The prevalence of soil and feces samples from *MAP*-positive herds in outdoor sport field, dairy cow pen, pregnant cow pen, heifer pen and calf pen was 82% (9/11), 41% (9/22), 100% (1/1), 67% (4/6), 14% (1/7), respectively. The prevalence of water samples from *MAP*-positive herds in dairy cow pen was 7% (1/14). Totally, 25 environmental samples were positive for IS900 real time PCR, and further investigation for the phylogenetic analysis were performed by VNTRs. Twenty five *MAP* positive environmental samples were performed on VNTR typing using five polymorphic loci (X3, TR25, TR292, 1067 and 9425). Totally, 9 substrains (From A to I) of *MAPs* were identified among these *MAP* positive environmental samples based on polymorphism of alleles (table 4). VNTR allelic diversity in each locus was listed in table 5. The phylogenetic tree based on VNTRs

genotypes among 25 *MAP* positive environmental samples were draw by the eBURST programs (figure 3).

Evidence-based control strategy of bovine paratuberculosis in Taiwan *The information about bPTB should be propagandized to livestock owners*: there were some relative information for the livestock owners including: 1) "younger cattle are most susceptible to *MAP* infection" [30]; 2) "clinical signs usually appear after 2 to 5 years, so *MAP* is usually introduced into dairy herds through purchase of infected but clinically normal cattle" [22]; 3) "subclinically infected cattle may reduced milk production, premature culling, reduced slaughter value, and reduced fertility rates" [15]; 4) "The estimative annually cost of bPTB in US dairy is US$200 to US$250" [18], 5) "the main transmission routes are direct fecal-oral route or ingesting *MAP* from milk, colostrums, fecal-contaminated feed, soil, and water" [22,28]; 6) "calves born to infected cattle have a higher risk of being infected than calves born to uninfected cattle" [3]; 6) "it must be managed as a herd-level disease, not individual cattle disease. This disease should be control to improve the productivity and profitability of the herd" [10]; 7) "paratuberculosis can be controlled. Diagnostic procedures are available to identify transmission risk factors and to prevent new infections" [15]; 8) "the rate of infection and impact of this disease will increase with time unless effective control strategy is implemented" [13]; 9) "it takes time to control bovine paratuberculosis in a herd, and reducing the prevalence within a herd may requires 3 to 7 years [13], it is needed the livestock owners' cooperation to keep the control strategy going for years", and 10) "the value of "*MAP* low-risk" certification program is enhancing economic value of certified herds and support the certified herds as a source for replacement cattle of low risk for *MAP* infection" [12]. *The most cost-effective diagnostic method to identify the MAP infection status of dairy herd*: the serum ELISA and real time PCR of environment were most effective methods to identify the *MAP* status of herds [5,23]. *The preventive management practices to control bPTB*: there were some preventive management practices of control bPTB including: 1) "clean and disinfect maternity and calf pens after each use" [15], 2) "calve in separate, clean, and dry calving area" [6], 3) "remove calves from their dam immediately after birth" [2], 4) "calf should have a separated area to avoid horizontal calf-to-calf transmission" [25], 5) "calves must be separated from adult cattle for at least the first year of life" [16], 6) "avoid vehicular and human traffic from adult cattle areas to young cattle area" [27], 7) "feed collected colostrum and milk from cattle with cleaned udders" [6], 8) "feed pasteurized milk or milk replacer to calves" [2], 9) "do not allow shared feed or water between adults and young cattle" [2], 10) "use separate equipment for handling feed and manure" [27], 11) "feedbunk and water trough should not have risk for fecal contamination" [15], 12) "do not spread manure on grazing land and fed to cattle of any age group" [17], 13) "cull all cattle with clinical evidence of paratuberculosis as soon as possible" [14], and 14) "maintain a closed herd or purchase replacement cattle only from *MAP* negative herds or "*MAP* low-risk" herds" [6]. *The most cost-effective diagnostic method to identify the subclinical infected cattle*: a study indicated the combined use of ELISA and direct fecal PCR had the potential to increase the overall sensitivity for

the diagnosis of *MAP* infection [20]. Direct fecal real time PCR was greater sensitivity than the ELISA and faster than fecal culture. The greater sensitivity also allowed the testing of pooled fecal samples, making whole herd and environmental sampling more economical [4]. In order to identify the *MAP* infected cattle within herd, the combined use of ELISA and direct fecal real time PCR were an economical and effective opinion. *The optimal method for certification and monitoring scheme of "MAP low-risk" herd*: a study concludes that the optimal certification and monitoring scheme for "*MAP* low-risk" herds [26]. In addition, the pooled fecal culture was replaced by pooled fecal IS900 real time PCR [4].

Discussion

Bovine Paratuberculosis Surveillance The *MAP* seroprevalence in this study, comparing with the result reported in 1987, the seroprevalence of bPTB in Taiwan has risen considerably in these twenty years [1]. When the within herd prevalence of bPTB is lower than 5% [13], the economic impact of bPTB may be insusceptible for livestock owners. Nowadays, the seroprevalence of bPTB in Taiwan is about 10%, the economic effect of paratuberculosis for dairy herds in Taiwan is thought to be a major impact. The result also indicates that 67 % of the evaluated dairy herds have been affected by *MAP*. Because of the lower sensitivity of ELISA, the herd-level prevalence may be underestimated. It also suggest the 21 *MAP* negative herds may be not *MAP*-free herds, further investigating the infection statuses of these herds are necessary to evaluated the risk of *MAP* exist in these herds. Among these 42 *MAP*-positive herds, the seroprevalence among these herds is varied considerably, ranging from 1 percent to 60 percent. The seroprevalences of half *MAP* positive herds are equal to / higher than 10% (high prevalence), and the others are lower than 10% (low prevalence). The results indicate that seroprevalences in positive herds are markedly different among different herds and the transmission risk factors that contributed to *MAP* infections may be complex and different among different herds. In order to develop a risk-based control strategy for each herd with different infection status, it's necessary to further investigate the transmission risk factors in each herd. The results indicate each country and city sampled in this study is affected by *MAP* except Nanton (Figure 4 and 5). It is necessary further investigating the *MAP* infection status of herds in these *MAP* affected countries and cities to effectively closure the transmission pathway between *MAP* positive herds and *MAP* negative herds. The seroprevalences in Tainan, Kaohsiung city, Pingtung, and Taitung are higher than 10% (the average *MAP* seroprevalence), it indicates the economic loss in these countries and city is more serious than other countries. The herd-prevalences are higher than 50% in Taichung, Changhua, and Pingtung, and are 100% in Yunlin, Tainan, Kaohsiung city, and Taitung. In addition, there is only one herd sampled in Nanton, so the *MAP* infection status of Nanton is still unclear. Purchasing *MAP* infected but clinical normal cattle is a major pathway for transmitting *MAP* among herds [22]. It indicates the trading of cattle in each country and city is necessary to be further observant. The "*MAP* low-risk" certification and monitoring program must be developed to provide a *MAP* low-risk source of additions and

replacements. The sources of these cattle are mixture of beef cattle and culled cattle. The result indicates the cattle in slaughterhouse are affected by *MAP*, some studies indicate beef can be contaminated with *MAP* via dissemination of the pathogen in the tissues of infected cattle [9]. This data suggests that the likelihood of meat products being contaminated with *MAP* on retail meats should not be ignored. Furthermore, the seroprevalence of male cattle is lower than female cattle. It is resulting from that the long disease course, and the male cattle are slaughtered at two to three years of age (as beef cattle). Currently data indicates the culled cattle with older age plays an important role in *MAP* transmission. Further investigation of the *MAP* of retail meats is necessary. A study indicated that cattle with positive responses for *MAP* (Fecal culture and ELISA) are seems to be resulting in false positive results on caudal fold tuberculin (CFT) test [7]. The same test is implemented for identifying tuberculosis (TB) infected cattle in Taiwan. The result in this study indicates the bPTB plays an important role on interfering in the results of intradermal tuberculin test (ITT). Furthermore, the difference of the bPTB ELISA positive rates between adult and young cattle may suggests the results of ITT on adult cattle are more odds to be interfered with PTB. It causes premature culling and makes Taiwan can't be classified into TB-free areas to cause serious economic loss in dairy herds, reduce the value of dairy industry, and hamper the international trade of Taiwan. As above, it is necessary to modify the current policy about TB control. For dairy herds, it's suggested that the strategic deployment of ancillary in vitro tests alongside the ITT has enhanced the detection of TB-infected cattle and reduced the number of cattle culled as false positives of ITT. For national level, the definition of TB-positive cattle and herds must be re-defined. It's suggested that the ITT-positive cattle with culture positive or PCR positive is defined as TB-positive cattle, and the herd would be defined as TB-positive herds, if $\geqq 1$ TB-positive cattle in the herd (Department of Health and Ageing, Australia). In kernel density map of *MAP* positive herds, the hot spot is located at the west area of Pingtung. Dairy herds in hot spot are thought to be face with more risk of *MAP* infection, because the odds of introducing *MAP* (mechanical carriers or purchasing infected cattle) are increased. This viewpoint may be confirmed by molecular epidemiology in this area, an method to analysis the *MAP* transmission pattern, such as VNTRs. Furthermore, the results are useful for developing a regional level control strategy.

Transmission Risk Factor Analysis for Bovine Paratuberculosis The results indicate soils and feces in outdoor sport field are the most representative area to represent the *MAP* status of dairy herds and high risk for transmitting *MAP* from environment to cattle, because 1) cattle is mixing in outdoor sport field, it offers a possibility to transmit *MAP* from environment to cattle; 2) the outdoor sport field is covered by soil and pad in Taiwan. It hampered the disinfection and cleaning of this area, and *MAP* may survival in this substance for more than 300 days. Cattle pens are concrete field and may be washed 2 to 3 times a day, so it may not be an index for us to understanding the *MAP* status in dairy herds. The IS900 real time PCR result of cattle pens demonstrate that the positive rates of these area are affected by the number of infectious cattle and the grade of disinfection or cleaning in these area. The results suggest that 1) it is necessary to

investigate the infectious cattle in these areas by direct fecal real time PCR; and 2) enforcing the disinfection and cleaning in these areas to closure the possible transmission pathway of *MAP* from environment to cattle. The results of lagoon and pasture are all negative for IS900 real time PCR, but it's not indicated these areas are not important for *MAP* transmission. Comparing with the study reported by Raizman et al, the lagoon is the targeted common and contaminated areas in the environment [21]. The reason of the difference between this study and the study reported by Raizman et al may be that *MAP* in this area is diluted by other substance such as water, feed, or feces and urine of *MAP* negative cattle. Pasture is a similar area with outdoor sport field, but the density of cattle and stay time of cattle are relative low. Compare the result of soil and feces samples with water sample, it indicate that the water is relative lower risk for transmitting *MAP*. This results may result from the water trough is flowing and elevated. Because of the number of infectious cattle, herd hygiene level, and endemic type of management or environment, the infection and contamination of *MAP* in different herds or different time may be different. The results of VNTRs indicated that there are direct link of *MAP* transmission among 1) herd Tt1, Ul2 and Ul3; 2) herd Ul1, Ul2 and Ul3; and 3) herd Tn1, Ul2 and Ul3. The results of VNTRs provide evidence about exogenous transmission, and *MAP* transmission of regional level. It's necessary to closure the exogenous transmission pathway, such as purchasing cattle from *MAP* negative herds and reducing the risk to contact other cattle or herds with unknown *MAP* status. On the other hand, the exogenous transmission pathway for herd Tt2 may not exist, it indicate the point to control paratuberculosis for herd Tt2 is to closure the transmission pathway within herd. The substrain B is same as *MAP* K-10, a bovine clinical isolate was isolated from a dairy herd in Wisconsin at the U.S. The substrain A to G and I are classified into same group, it indicate there may be some relationship of *MAP* infection between Taiwan and the U.S. The results also indicate the original source of substrain H is different with other substrain, it means substrain H is from different country or the Taiwanese aboriginal strain. The further investigating of genetic relatedness by VNTRs is necessary to provide more evidence about the original resource of *MAP* infection.

Evidence-based control strategy of bovine paratuberculosis in Taiwan Based on the results of above chapters and reviewed literatures, a bPTB control program is developed. The goal of this bPTB control program is aimed to prevent 1) transmission of *MAP* infection from infected cattle to other cattle, 2) contamination of the environment with *MAP*, and 3) introduction of *MAP* infected cattle from infected herds to non-infected herds. A successful control strategy depends on whether the owners understand the fundamental cognizance of bPTB, so the first point of this bPTB control is propagandizing the related information to the livestock owner. Second, the status of each herd will be identified to implement different control strategies. Third, the "*MAP* low-risk" certification and monitoring program is developed to provide a source of additions and replacements for dairy herds. The flowchart of this bPTB control program is indicated in figure 15, and the specific descriptions in this bPTB control program are including: 1) *Livestock owner education and propaganda of bPTB*: the information is propagandized by fly sheet and periodical conference to

enhance the cooperation of livestock owners. Thereby, it could attract the interest of livestock owner to participate in this control program. All dairy herds participate in this program are voluntary, and in order to reduce the risk of introducing *MAP* in these herds, cattle might be added to these herds only if the cattle are from a herd with level 1 to 4 of "*MAP* low-risk" certification, *2) Identify the MAP status of voluntary dairy herds*: all cattle over 2 years of age are tested by serum ELISA kit (Institut Pourquier) and pooled fecal IS900 real time PCR [23], and ≧10 soil/feces environmental samples are tested by environmental IS900 real time PCR. If there is a positive result, the herd is classified into *MAP* positive herd, 3) *The control and test strategy for MAP positive herds*: preventive management practices is implemented to control bPTB. All cattle over 2 years of age are tested by serum ELISA kit (Institut Pourquier) and pooled fecal IS900 real time PCR, and ≧10 soil/feces environmental samples are tested by environmental IS900 real time PCR for investigating the transmission risk factors in *MAP* positive herds. Test positive cattle are highest risk to be a source for transmitting *MAP*, and the related management practices are as following: 1) test positive cattle are isolated to close transmission pathway, and would be culled if their daily milk yield below 10 kg, 2) All calves from test positive cattle are fattened and slaughtered because they had a high risk of being infected with *MAP*, and test positive cattle are not inseminated, and 3) Feed collected colostrum and milk from *MAP* negative cattle with cleaned udders. Test positive environmental areas indicated that it's necessary to enforce the disinfection and cleaning in these areas for closuring the possible transmission pathway of *MAP* from environment to cattle, and to avoid the susceptible cattle (i.e. young cattle) contacting the contaminated environment. These tests would be annually performed to optimize management practices and closure the *MAP* transmission pathway until the herd is negative for these tests, and then the herds with negative result of the tests would participate in the "*MAP* low-risk" certification and monitoring program. *"MAP low-risk" certification and monitoring program*: the certification of "*MAP* low-risk" herds including four levels is conducted after four herd examinations at 2 year intervals consisting of pooled fecal IS900 real time PCR of all cattle ≥ 2 years of age. With each subsequent annual pooled fecal IS900 real time PCR is negative, the herds are awarded a higher certification level (from 1 to 4), reflecting the increased confidence that the herd is truly low-risk of the *MAP* infection. To reduce the risk of introducing *MAP* in "*MAP* low-risk" herds, cattle might be added to these herds only if the cattle are from another "*MAP* low-risk" herd. In addition, cattle might be added to herds that are in the process of "*MAP* low-risk" certification only if they are from a herd with an equal or higher level of "*MAP* low-risk" certification. Furthermore, the herds in level 4 would enter into "*MAP* low-risk" monitoring program. The monitoring of "*MAP* low-risk" herds is performed by pooled fecal IS900 real time PCR of all cattle ≥ 1 year of age at 2 year intervals. A positive result of pooled fecal IS900 real time PCR is confirmed by individual fecal IS900 real time PCR of the feces from previously positive pooled sample. If all individual fecal samples of a previously positive pool are negative, then this pool is regarded as negative. If an individual fecal IS900 real time PCR is positive, the herd is expelled from the "*MAP* low-risk" certification and monitoring program. To reduce the risk

of introducing *MAP* in "*MAP* low-risk" herds, cattle might be added to these herds only if the cattle are from another "*MAP* low-risk" herd. In addition, cattle might be added to herds that are in the process of "*MAP* low-risk" certification only if they are from a herd with an equal or higher level of "*MAP* low-risk" certification. To reduce the risk of introducing *MAP* in "*MAP* low-risk" herds, cattle might be added to these herds only if the cattle are from another "*MAP* low-risk" herd.

Conclusion

The *MAP* seroprevalence of dairy herds was 10.1%, and 66.7% dairy herds were *MAP* positive herd. The results indicated the *MAP* infection status and transmission risk factors are different among different herds. The *MAP* seroprevalence of beef cattle was 2.8%, and suggested that the likelihood of meat products being contaminated with *MAP* on retail meats should not be ignored. The *MAP* seroprevalence of intradermal tuberculin test (ITT) positive cattle was 51.4%, and indicated the PTB plays an important role on interfering in the results of ITT. In kernel density map of *MAP* positive herds, the hot spot was located at the west area of Pingtung. Soil and feces were high risk carries for transmitting *MAP*, but water was relative lower risk for transmitting *MAP*. Nine substrains of *MAPs* were identified, and indicate there were direct link of *MAP* transmission among herds. According to the results of this study and related researches of this disease, a bPTB control program was developed.

Acknowledgement

This work was supported by Toxicology, pathology, and immunology laboratory, Graduate Institute of Veterinary Medicine, School of Veterinary Medicine National Taiwan University.

Appendix I

Table 1. PCR primer profiles and amplicon sizes of VNTRs

Target	Primer	Sequence	Flanking	Period size
X3	OX3F	AATCCGAGCAGCCGCAGGGT	210	53X
	OX3R	ACATCCCGGGGAGCATCAGGTC		
	X3F	GCCCAACCGTTCCCAACGAGAG	102	
	X3R	AGCCCTCCTTACGGAGCAGGAA		
TR25	O25F	TTCGCGTTGCTCGTCGTCCA	266	58X
	O25R	TTGTTGCCGCCGTTGACCGA		
	TR25F	GTCAAGGGATCGGCGAGG	191	
	TR25R	TGGACTTGAGCACGGTCAT		
TR292	O292F	TCCAGCCAGAAGTGCCGGGT	332	53X
	O292R	TGCTGCAGCGCTGTTTCGC		
	TR292F	CTTGAGCAGCTCGTAAAGCGT	137	
	TR292R	GCTGTATGAGGAAGTCTATTCATGG		
1067	O1067F	AGCGCCTACCTGTACAGCCG	368	53X
	O1067R	GATCGTCTTGGCGCGCAGGT		
	1067F	CGCCGCCCGCCGAAAAAG	266	
	1067R	CGGGACATCACAAATACAGAAGAA		
9425	O9425F	CGGAAAGCCGGGACGACTGA	222	57X
	O9425R	ACGCTGCTGGGCCGGTTCTA		
	9425F	AGGTGGCCAGGCGGGTCATCTTCC	135	
	9425R	CAGAAGGCGACGGAGGACTACATC		

Table 2. The sample sizes and seroprevalences of each dairy herds and each country/city

	Total samples	Positive samples	Positive rate (%)		Total samples	Positive samples	Positive rate (%)
Taichung (Tc)	**351**	**28**	**8.00%**	**Tainan (Tn)**	**209**	**48**	**23.00%**
Tc-A	45	0	0.00%	Tn-1	45	27	60.00%
Tc-B	36	0	0.00%	Tn-A	55	10	18.20%
Tc-C	29	0	0.00%	Tn-B	55	7	12.70%
Tc-D	30	10	33.30%	Tn-C	54	4	7.40%
Tc-E	30	3	10.00%	**Pingtung (Pt)**	**518**	**59**	**11.40%**
Tc-F	35	0	0.00%	Pt-A	5	0	0.00%
Tc-G	30	1	3.30%	Pt-B	10	0	0.00%
Tc-H	36	13	36.10%	Pt-C	28	0	0.00%
Tc-I	45	0	0.00%	Pt-D	30	0	0.00%
Tc-J	35	1	2.90%	Pt-E	40	6	15.00%
Yunlin (Ul)	**180**	**16**	**8.90%**	Pt-F	45	3	6.70%
Ul-1	35	3	8.60%	Pt-G	26	2	7.70%
Ul-A	45	5	11.10%	Pt-H	20	1	5.00%
Ul-B	35	1	2.90%	Pt-I	20	9	45.00%
Ul-C	30	3	10.00%	Pt-J	3	0	0.00%
Ul-D	35	4	11.40%	Pt-K	36	10	27.80%
Changhua (Ch)	**618**	**31**	**5.00%**	Pt-L	20	6	30.00%
Ch-A	46	6	13.00%	Pt-M	30	10	33.30%
Ch-B	46	2	4.30%	Pt-N	30	0	0.00%
Ch-C	46	12	26.10%	Pt-O	10	0	0.00%
Ch-D	48	1	2.10%	Pt-P	35	0	0.00%
Ch-E	37	2	5.40%	Pt-Q	6	0	0.00%
Ch-F	48	1	2.10%	Pt-R	35	3	8.60%
Ch-G	37	2	5.40%	Pt-S	35	0	0.00%
Ch-H	48	1	2.10%	Pt-T	24	7	29.20%
Ch-I	37	2	5.40%	Pt-U	30	2	6.70%
Ch-J	47	0	0.00%	**Taitung (Tt)**	**240**	**50**	**20.80%**
Ch-K	37	1	2.70%	Tt-A	30	8	26.70%
Ch-L	48	0	0.00%	Tt-B	45	9	20.00%
Ch-M	47	1	2.10%	Tt-C	35	6	17.10%
Ch-N	46	0	0.00%	Tt-D	35	3	8.60%
Nantou (Nt)	**30**	**0**	**0.00%**	Tt-E	30	1	3.30%
Nt-A	30	0	0.00%	Tt-F	30	3	10.00%
Kaohsiung city (Kc)	**24**	**3**	**12.50%**	Tt-G	35	20	57.10%
Kc-1	24	3	12.50%	**Total**	**2170**	**235**	**10.80%**

Table 3. Results of real time PCR for environmental samples

Soil and feces samples																	
Location\Herd	Tt1		Tt2		Tc1*		Tt3*		Tn1		UL1		Ul2		Ul3		Positive rate**
	Total	Positive	Total	Positive	Total	Positive	Total	Positive	Total	Positive	Total	Positive	Total	Positive	Total	Positive	
Outdoor sport field	3	2	2	1	0	0	10	0	0	0	2	2	2	2	2	2	82% (9/11)
Dairy cow pen	3	0	3	1	2	0	4	0	8	3	2	1	3	2	3	2	41% (9/22)
Pregnant cow pen	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	100% (1/1)
Dry cow pen	0	0	0	0	0	0	9	0	1	0	0	0	0	0	0	0	0 (0/1)
Heifer pen	2	0	0	0	2	0	0	0	2	0	4	4	0	0	0	0	67% (4/6)
Calf pen	2	0	2	0	0	0	5	0	0	0	1	0	2	1	0	0	14% (1/7)
Pasture	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0 (0/2)
Lagoon	1	0	2	0	0	0	1	0	0	0	0	0	0	0	0	0	0 (0/3)
Water samples																	
Location\Herd	Tt1		Tt2		Tc1*		Tt3*		Tn1		UL1		Ul2		Ul3		Positive rate**
	Total	Positive	Total	Positive	Total	Positive	Total	Positive	Total	Positive	Total	Positive	Total	Positive	Total	Positive	
Outdoor sport field	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0% (0/1)
Dairy cow pen	2	0	2	0	2	0	4	0	4	1	1	0	2	0	3	0	7% (1/14)
Pregnant cow pen	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0 (0/1)
Dry cow pen	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0 (0/1)
Heifer pen	3	0	0	0	2	0	0	0	0	0	2	0	2	0	0	0	0 (0/7)
Calf pen	1	0	2	0	0	0	1	0	1	0	0	0	0	0	0	0	0 (0/4)
Pasture	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0 (0/0)
Lagoon	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0 (0/0)
Total positive rate	11% (2/18)		13% (2/15)		0% (0/8)		0% (0/37)		26% (5/19)		58% (7/12)		45% (5/11)		50% (4/8)		19% (25/131)

*: *MAP*-negative herds

**: The rate was calculated from *MAP*-positive herds

Appendix I

Table 4. Data of VNTRs of *MAP* positive environmental samples

Herd	Location	Sample type	X3	TR25	TR292	1067	9425	Pattern
Tt1	Outdoor sport field	Soil and feces	2	3	3	0	2	A
	Outdoor sport field	Soil and feces	2	3	3	0	2	A
Tt2	Outdoor sport field	Soil and feces	2	0	1	2	2	D
	Dairy cow pen	Soil and feces	2	0	1	2	2	D
Tn1	Dairy cow pen	Soil and feces	2	3	3	2	2	B
	Dairy cow pen	Soil and feces	2	3	3	2	2	B
	Dairy cow pen	Soil and feces	2	3	3	2	2	B
	Pregnant cow pen	Soil and feces	2	3	3	2	2	B
	Dairy cow pen	Water	2	3	3	1	2	C
Ul1	Outdoor sport field	Soil and feces	2	0	2	0	2	E
	Outdoor sport field	Soil and feces	2	3	3	2	2	E
	Dairy cow pen	Soil and feces	2	3	3	2	2	B
	Heifer pen	Soil and feces	2	3	3	2	2	B
	Heifer pen	Soil and feces	2	0	3	0	2	B
	Heifer pen	Soil and feces	4	0	6	0	2	H
	Heifer pen	Soil and feces	2	0	3	0	2	G
Ul2	Outdoor sport field	Soil and feces	2	3	3	0	2	A
	Outdoor sport field	Soil and feces	2	3	3	0	2	E
	Dairy cow pen	Soil and feces	2	0	3	0	2	E
	Dairy cow pen	Soil and feces	2	0	3	0	2	F
	Calf pen	Soil and feces	2	0	3	2	2	A
Ul3	Outdoor sport field	Soil and feces	2	0	3	0	2	E
	Outdoor sport field	Soil and feces	2	0	2	2	2	A
	Dairy cow pen	Soil and feces	2	3	3	0	2	B
	Dairy cow pen	Soil and feces	2	3	3	2	2	I

Appendix I

Table 5. VNTR allelic diversity in each locus

	No. of distinct alleles in each locus				
No. of copy	X3	TR25	TR292	1067	9425
0	0	11	0	12	0
1	0	0	2	1	0
2	24	0	2	12	25
3	0	14	20	0	0
4	1	0	0	0	0
5	0	0	0	0	0
6	0	0	1	0	0
Allele diversity (h)	0.04	0.47	0.32	0.52	0.00

Appendix I

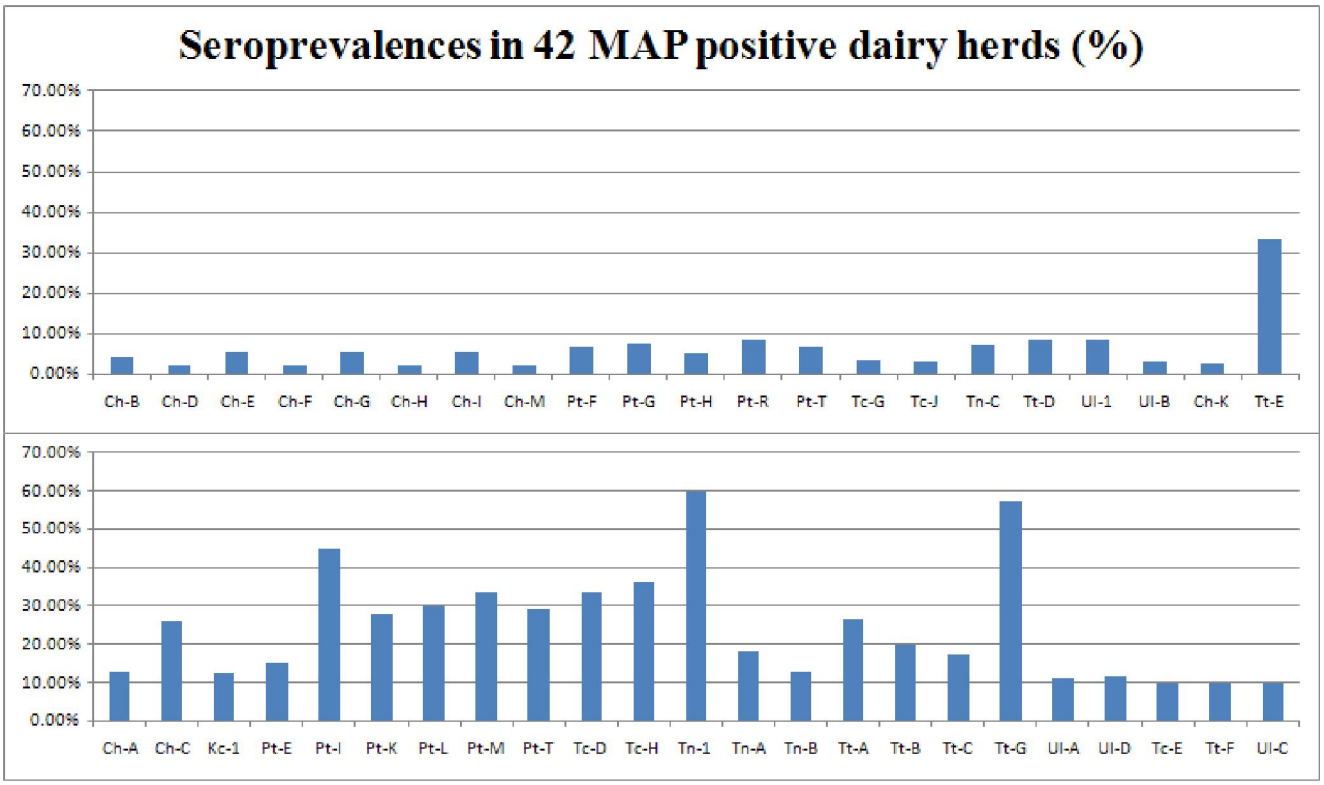

Figure 1. The seroprevalences of 42 *MAP* positive dairy herds: it were ranged from 0% to 60% in dairy herds, and the seroprevalences of half herds were higher than 10% (high prevalence), and the others were equal to /lower than 10% (low prevalence).

Appendix I

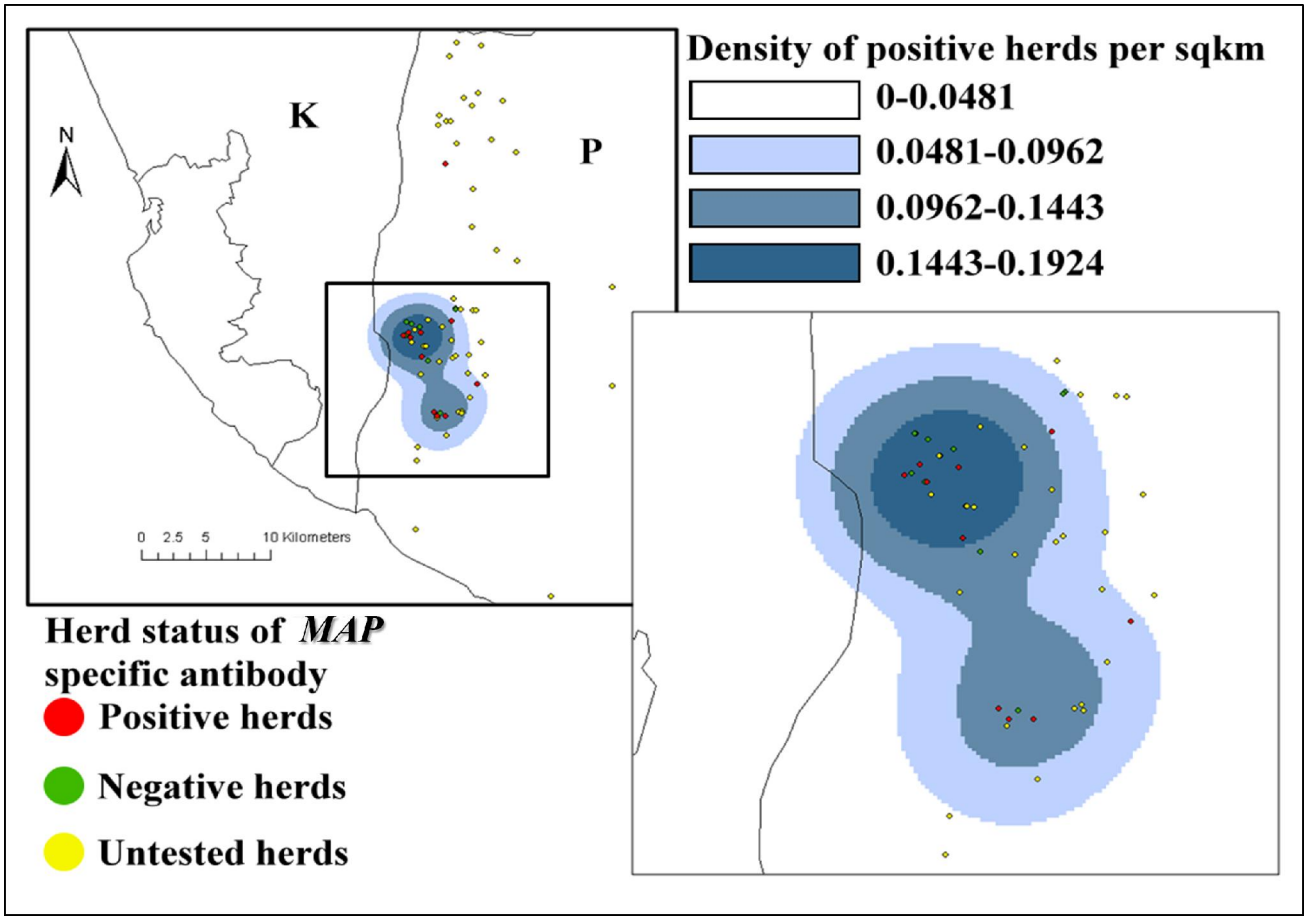

Figure 2. Serological bPTB herd infection status and kernel estimate of positive density infected herds. The different densities of positive herds per square kilometer (sqkm) were indicated by the colors (from white to dark blue, see the upper right of figure 3). The different herd status of *MAP* specific antibody were indicated by different colors, such as red spots meant positive herds, green spots meant negative herds, and yellow spots meant untested herds. The "K" meant Kaohsiung city and "P" meant Pingtung.

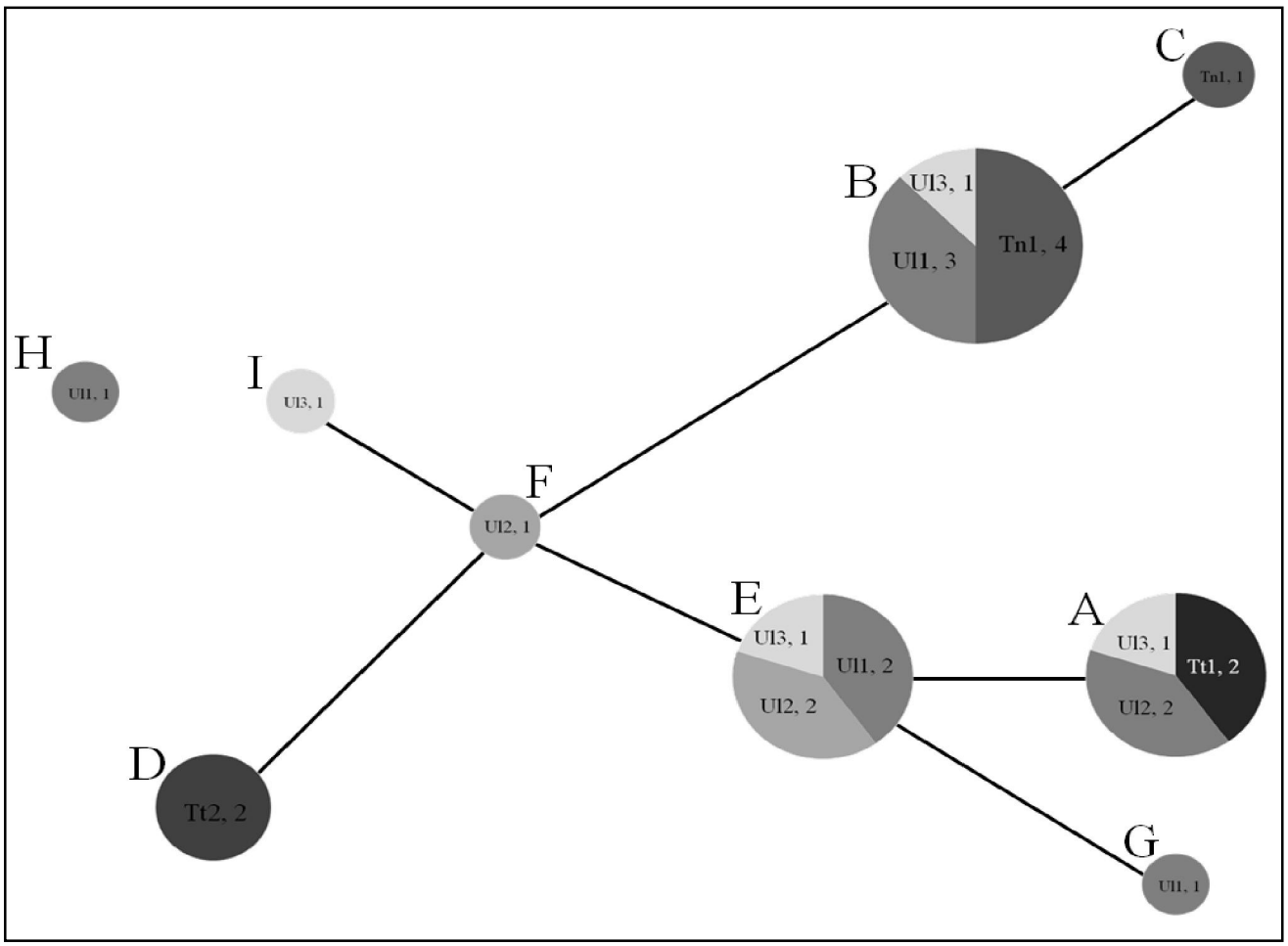

Figure 3. Phylogenetic tree based on VNTRs genotypes among 25 *MAP* positive environmental samples: The phylogenetic tree was draw by the eBURST programs. Each pie chart means the different *MAP* substrain, and each color and the ratio of each color in each pie chart means the number of the *MAP* substrain in the herd. (e.g. herd code, number of substrain). One sample in herd Ul3, two samples in herd Tt1 and two samples in herd Ul2 were *MAP* substrain A. One sample in herd Ul3, three samples in herd Ul1, and four samples in herd Tn1 were *MAP* substrain B. One sample in herd Tn1 was *MAP* substrain C. Two samples in herd Tt2 was *MAP* substrain D. One sample in herd Ul3, two samples in herd Ul1, and two samples in herd Ul2 were *MAP* substrain E. One sample in herd Ul2 was *MAP* substrain F. One sample in herd Ul1 was *MAP* substrain G. One sample in herd Ul1 was *MAP* substrain H. The *MAP* substrain A, B, C, D, E, F, G, and I were classified in group 1, but *MAP* substrain H was not in the group.

Appendix I

Reference

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Appendix I

台灣牛隻副結核病 (Bovine Paratuberculosis) 之傳播風險因子和控制策略

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摘要　牛隻副結核病的病原是副結核分枝桿菌（*Mycobacterium avium* subsp. *Paratuberculosis*; *MAP*），本研究之目標在於探查目前台灣牛隻副結核分枝桿菌感染狀況和傳播風險因子，並發展一套實用的防治計畫。利用血清酵素結合免疫吸附法（ELISA）進行副結核病的監控，並收集牧場環境樣本, 用即時定量聚合酶鏈鎖反應檢測 (real time PCR) 和異數重複序列（VNTRs）檢測副結核分枝桿菌，並進行親源性的分析。結果顯示副結核病在泌乳牛場的血清盛行率是 10.1%, 且有 66.7%之乳牛場為副結核分枝桿菌陽性場, 肉用牛隻的副結核病血清盛行率是 2.8%，而結核菌素皮內測試陽性牛隻的副結核病血清盛行率是 51.4 %, 且在不同牧場中副結核病感染的狀況和其傳播的風險因子也相當的不同, 露天運動場的土壤和糞便是傳播副結核分枝桿菌的媒介。此外本研究法共鑑定出九個亞型的副結核分枝桿菌，並顯示牧場間副結核分枝桿菌傳播的直接關聯性。最後，本研究基於上述結果及其他相關研究，針對台灣副結核病發展出一套控制計畫。

關鍵字：控制計畫、血清酵素結合免疫吸附法、副結核病、即時定量聚合酶鏈鎖反應檢測、監測、異數重複序列

Appendix II

Standard of procedure for diagnosis of bovine paratuberculosis

Material

- 0.5, 1.5 and 2.0 mL microcentrifuge tube (Axygen, QSP®)
- 15, 50 mL centrifuge tubes (Gene Direx®)
- 5 c.c. Syringe and 18# needle (Terumo®)
- Cold-bag & ice-blocks
- Wooden tongue depressor (東軒)
- Gloves (Anderson®)
- Protective clothing (召寶)
- Rain boot
- Pourquier® Elisa Paratuberculosis Antibody Screening (Pourquier®)
- FastDNA® SPIN Kit for Soil (MP Biomedicals)
- SYBR® Advantage® qPCR Premix (Clontech®)
- 99.9% Ethanol (Sigma®)
- Primers for real time PCR**:**

Primers	Forward/reverse sequences (5’ → 3’)
MAPF	AATGACGGTTACGGAGGTGGTTGTG
MAPR	AATCGCTGCGCGTCGTCGTTAATA

- Agarose (invitrogen)
- 100 bp leader marker (One-star biotechnology Co., Ltd)
- 5X TBE buffer (One-star biotechnology Co., Ltd)

Appendix II

- Ethidium bromide (Sigma®)
- Tube rack
- Pipetman (Eppendorf®, Rainin® and Gibson®)
- Tips (QSP®)

Equitment

- Dry-bath (Thermolyne® Type 16500)
- Vortexer (Scientific Industries SI-0236)
- Electronic Balance (Excell® BH-1500)
- Refrigerater (RevcoULT-1386-3 and Whirlpool)
- Mastercycler ep *realplex*^{4} (eppendorf®)
- Microcentrifuge (Beckman microfuge® 18)
- SpectraMax M5 (Molecular devices)

Methods

Sample collection

Procedure for serum sample collection

Blood is collected by tail vein venepuncture into anticoagulant-free tubes.

Procedure for fecal sample collection (Berghaus et al., 2006; Raizman et al., 2004):

Approximately 30 g of fecal samples are obtained from pen floor with a wooden tongue

depressor, and the sample is collected in marked sterile 50 mL centrifuge tubes. A new wooden tongue depressor is used for each fecal sample.

- **Procedure for environmental sample collection** (Berghaus et al., 2006; Lombard et al., 2006; Raizman et al., 2004)**:**

Composite manure or effluent samples are collected from farms in these locations:

1. Feeding troughs or area/Alleyways/Return alley of milking parlor:

   Taking 10 g samples of soil at approximately 10 m intervals while walking along its length, and grab samples are collected in marked sterile 50 mL centrifuge tubes.

2. Sick cow pen/Calving area/Dry cow pen/Preweaned calves pen/Postweaned calves pen/Outdoor sport field/Grazing or hay land:

   One 10 g sample of soil is collected for every 10 m^2 of surface area, and the grab samples are collected in marked sterile 50 mL centrifuge tubes.

3. Watering troughs/Wastewater gutter/Lagoons:

   Samples are collected at 3 to 4 locations along their perimeter where is possible to safely approach the edge. Water or manure samples are collected by immersing a sterile 50 mL centrifuge tube up to 10 cm beneath the surface.

4. Feed equipment

   10 g soil and feces samples are collected at 1 to 2 locations from feed equipment (If it's possible), and the samples are collected in marked sterile 50 mL centrifuge tubes.

- **Shipping of samples** (Khare et al., 2008)**:**

Samples are shipped under refrigeration (4℃) within 1 week.

- **Pretreatment and storage of samples**

1. Serum samples

   Serum is separated by centrifugation at 1000 xg for 10 min. 1 mL serum sample is put into 1.5 mL microcentrifuge tube, and stored at -20 °C.

2. Soil and fecal samples

   1.3 g soil or fecal sample is put into 1.5 mL microcentrifuge tube, and stored at -70 °C.

3. Pooled fecal samples

   5 individual fecal samples of 2 g are pooled to make a composite 10 g sample, and 1.3 g pooled fecal sample is put into 1.5 mL microcentrifuge tube, and stored at -70 °C.

4. Water samples

   Water samples (30 ~ 40 mL) are centrifuged at 3000 xg for 30 min. The supernatant is discarded (5 mL supernatant is leaved), and the pellet of each tube is resuspended in the supernatant. 1.3 mL water sample is put into 1.5 mL microcentrifuge tube, and stored at -70 °C.

5. Milk sample

   Milk samples (10 to 15 mL) are centrifuged at 3000 xg for 30 min and the supernatant is discarded. The pellet of each tube is resuspended in a small portion of the supernatant, and transfer into 1.5 mL microcentrifuge tube. The samples are

centrifuged at 14,000 xg for 10 min and the remainder of the supernatant discarded, and the pellet of each tube is resuspended in 1.3 mL DDW. The samples are put into 1.5 mL microcentrifuge tube, and stored at -70 ℃.

ㄧ ELISA test for *Map* Antibody (Pourquier® Elisa Paratuberculosis Antibody Screening) (Collins et al., 2005):

1. Thaw serum sample at room temperature or in 37°C water bath.
2. Dilute test serum and controls (1:20) with dilution buffer.
3. Incubate diluented sample for 15 minutes to 2 hours at 21°C (±5°C).
4. Dilute the wash concentrate (1:20) with double-distilled water.
5. Dispense 100 µL of Diluted sample into wells A1 ~ L5 (Total 93 wells).
6. Dispense 100 µL of diluted positive control into wells L6 and L7.
7. Dispense 100 µL of diluted negative control into well L8.
8. Incubate for 45 minutes ± 3 minute at 21°C (±5°C).
9. Dilute the conjugate to 1:100 with the “Dilution buffer 1”
10. Aspirate the liquid content of all wells into an appropriate waste reservoir.
11. Wash each well 3 washes with 300 µL of Wash solution.【Avoid plate drying.】
12. Dispense 100 µL of Conjugate into each well incubate for 30 min at 21°C (±5°C).
13. Repeat steps 9 and 10.
14. Dispense 100 µL of Revelation solution into each well.
15. Incubate for 10 minutes at 21°C (±5°C).

16. Dispense 100 μL of Stop solution into each well.

17. Blank the reader on air.

18. Measure and record the absorbance at 450 nm.

19. Calculate the results:

    A. OD_{nc} = Negative control

    B. OD_{pc} = Positive control

    C. S/P Ratio = 【Sample－OD_{nc}】／【OD_{pc}－OD_{nc}】

    D. S/P Ratio ≦ 45% (Negative)

    E. S/P Ratio ≧ 55% (Positive)

    F. The positive control had a minimal OD_{450} value of 0.350.

    G. The ratio between the mean OD_{450} value of positive control and OD_{450} value of the negative control is≧3.

l DNA extraction & real time PCR assay for *MAP*

n DNA extraction from soil and fecal samples (FastDNA® SPIN Kit for Soil) (Cook and Britt, 2007; Layton et al., 2006):

1. Add up to 350 mg of soil or fecal samples to Lysing Matrix E tubes.

2. Add 978 μL Sodium Phosphate Buffer to sample in Lysing Matrix E tube.

3. Add 122 μL MT Buffer.

4. Homogenize in the FastPrep® Instrument for 45 seconds at a speed setting of 6.0.

5. Put the Lysing Matrix E tubes on ice for 5 min and repeat step7.
6. Centrifuge at 14,000 xg for 15 minutes to pellet debris.
7. Transfer supernatant to a clean 2.0 mL microcentrifuge tube. Add 250 µL PPS (Protein Precipitation Solution) and mix by shaking the tube by hand 10 times.
8. Centrifuge at 14,000 xg for 5 minutes to pellet precipitate. Transfer supernatant to a clean 5 mL tube.
9. Resuspend Binding Matrix suspension and add 1.0 mL to supernatant in 5 mL tube.
10. Place on rotator or invert by hand for 2 minutes to allow binding of DNA. Place tube in a rack for 3 minutes to allow settling of silica matrix.
11. Remove and discard 500 µL of supernatant being careful to avoid settled Binding Matrix.
12. Resuspend Binding Matrix in the remaining amount of supernatant. Transfer approximately 600 µL of the mixture to a SPIN™ Filter and centrifuge at 14,000 xg for 1 minute. Empty the catch tube and add the remaining mixture to the SPIN™ Filter and centrifuge as before. Empty the catch tube again.
13. Add 500 µL prepared SEWS-M and gently resuspend the pellet using the force of the liquid from the pipet tip.
14. Centrifuge at 14,000 xg for 1 minute. Empty the catch tube and replace.
15. Repeat step 12 to 13 twice.
16. Without any addition of liquid, centrifuge a second time at 14,000 x g for 2 minutes to "dry" the matrix of residual wash solution. Discard the catch tube and replace with a new,

clean catch tube.

17. Air dry the SPIN™ Filter for 5 minutes at room temperature.

18. Incubation for 5 minutes at 55˚C in a heat block.

19. Gently resuspend Binding Matrix (above the SPIN filter) in 200 µL of DES.

20. Centrifuge at 14,000 xg for 1 min to bring eluted DNA into the clean catch tube. Discard the SPIN filter.

21. DNA elution is 10-fold diluted by DDW. (15 µL DNA elution + 135 µL DDW)

22. 10-fold diluted DNA elution is now ready for real-time PCR.

23. Store at -20°C for extended periods or 4°C until use.

- **DNA extraction from water** (FastDNA® SPIN Kit for Soil) (Donaghy et al., 2008; Pinedo et al., 2008; Rodriguez-Lazaro et al., 2005; Sweeney et al., 2006):

1. Add up to 350 µL of water samples to Lysing Matrix E tubes.

2. Add 978 µL Sodium Phosphate Buffer to sample in Lysing Matrix E tube.

3. Add 122 µL MT Buffer.

4. Homogenize in the FastPrep® Instrument for 45 seconds at a speed setting of 6.0.

5. Put the Lysing Matrix E tubes on ice for 5 min and repeat step7.

6. Centrifuge at 14,000 xg for 15 minutes to pellet debris.

7. Transfer supernatant to a clean 2.0 mL microcentrifuge tube. Add 250 µL PPS (Protein Precipitation Solution) and mix by shaking the tube by hand 10 times.

8. Centrifuge at 14,000 xg for 5 minutes to pellet precipitate. Transfer supernatant to a clean 5

mL tube.

9. Resuspend Binding Matrix suspension and add 1.0 mL to supernatant in 5 mL tube.
10. Place on rotator or invert by hand for 2 minutes to allow binding of DNA. Place tube in a rack for 3 minutes to allow settling of silica matrix.
11. Remove and discard 500 µL of supernatant being careful to avoid settled Binding Matrix.
12. Resuspend Binding Matrix in the remaining amount of supernatant. Transfer approximately 600 µL of the mixture to a SPIN™ Filter and centrifuge at 14,000 xg for 1 minute. Empty the catch tube and add the remaining mixture to the SPIN™ Filter and centrifuge as before. Empty the catch tube again.
13. Add 500 µL prepared SEWS-M and gently resuspend the pellet using the force of the liquid from the pipet tip.
14. Centrifuge at 14,000 xg for 1 minute. Empty the catch tube and replace.
15. Repeat step 12 to 13 twice.
16. Without any addition of liquid, centrifuge a second time at 14,000 x g for 2 minutes to "dry" the matrix of residual wash solution. Discard the catch tube and replace with a new, clean catch tube.
17. Air dry the SPIN™ Filter for 5 minutes at room temperature.
18. Incubation for 5 minutes at 55˚C in a heat block.
19. Gently resuspend Binding Matrix (above the SPIN filter) in 200 µL of DES.
20. Store at -20°C for extended periods or 4°C until use.

Appendix II

- **Real time PCR assay** (SYBR® Advantage® qPCR Premix, Clontech®)**:**

1. Thaw SYBR® Advantage® qPCR Premix, template DNA, primers, and water on ice.

   Gently mix the individual solutions.

2. Prepare a reaction mix as describe below:

Component	Volume for reaction mix	Final Concentration
SYBR® Advantage® qPCR Premix(2X)	10 µL	1x
Primer A	0.4 µL	0.2 µM/reaction
Primer B	0.4 µL	0.2 µM/reaction
RNase-free water	5.2 µL	
Template DNA	4 µL	< 100 ng/reaction
Total reaction volume	20 µL	

3. Mix the reaction mix thoroughly, and dispense into PCR tubes.

4. Add template DNA to the individual PCR tubes containing the reaction mix.

   Note: Negative and positive control are included for each PCR process.

5. Program your real-time cycler according to the program as describe below:

Step		Time	Temperature
PCR initial		30s	95°C
37 cycles	Denaturation	10s	95°C
	Annealing*	30s	66°C

*A single fluorescence acquisition during the extension cycle.

6. Place the PCR tubes or plates in the real-time cycler, and start the cycling program.

- **Analysis** (Chen et al., 2008; Clark et al., 2008)

1. Melting curve analysis:

   Samples are heated to 95°C, then immediately cooled to 66°C and held for 1 min.

   Samples are then heated to 95°C at a rate of 0.2°C with continuous fluorescence

   monitoring. Then Samples are cooled to 4 °C. A sample is considered positive if it showed

an increase in fluorescence during amplification and the corresponding melting curve is observed at 86~88 °C.

2. Agarose gel electrophoresis :

   After PCR, 6 μL of the PCR product is mixed with a dye mixture and electrophoresed in 0.5 % TBE buffer through a 2 % agarose gel. Bands of the appropriate size are identified by comparison with a 100 bp DNA ladder maker. A sample is considered positive if a signal band corresponding to 100 bp is visualized under UV light.

3. DNA sequencing

Reference

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