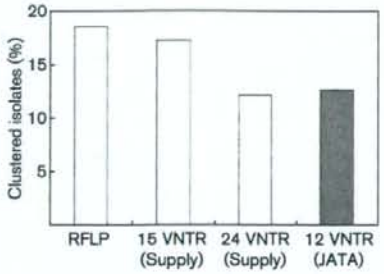

Fig. 1. Comparison of clustering rates in RFLP and VNTR analyses. The percentage of clustered isolates was calculated for each case.

Our data showed that the *h* of VNTR 3336 gave a maximum value in non-Beijing TB analysis (Table 3). The genetic clock of VNTR 3336 in non-Beijing genotypes might be faster than that of Beijing strains. Therefore, the locus of VNTR 3336 in non-Beijing strains might be unstable and that in Beijing strains might be stable. Consequently, the locus of VNTR 3336 might be used in 12-locus VNTR (JATA).

In this study, 325 independent isolates (with no epidemiological link) were collected and analysed. Results show that only four strains formed clusters in both the RFLP (60 strains) and VNTR (41 strains) methods: RKN 246, RKN 275, RKN 283 and RKN 319. The remaining clusters were subdivided using the other method. Cluster 08 of RFLP analysis comprised six strains including the four clustered strains. By 12-locus (JATA) VNTR, cluster 08 was further divided into three groups (Fig. 2). The two remaining strains (RKN 177 and RKN 215) showed independent patterns. Differences of copy number in the two strains were found in two loci. The respective discriminatory powers of RFLP and VNTR analyses were different. The maximum cluster sizes in IS*6110* RFLP and JATA (12) VNTR were, respectively, 8 and 4 (data not shown). The 12-locus (JATA) VNTR had a higher discrimination power than RFLP analysis did.

Analysis of clinical isolates from a suspected outbreak using 12-locus VNTR (JATA)

For examining the effectiveness of the 12-locus VNTR (JATA), 25 clusters (76 strains in total) were analysed. Of them, 17 clusters (54 strains) for which the RFLP patterns were identical in each group had the same VNTR profile (data not shown). Eight initially suspected TB patient clusters (22 strains) were not outbreak related (concurrence of TB): their IS*6110* RFLP showed different patterns. These strains are clearly independent. Therefore, the VNTR profile was also expected to show variant patterns in the respective groups. The VNTR analyses using 12-locus VNTR (JATA) showed that each strain had a different profile in at least 5 loci, except for case 6. Analyses of the other isolates also revealed that the discriminatory power of 12-locus VNTR (JATA) was roughly equivalent to that of IS*6110* RFLP analysis.

In case six, strains S-054 and S-160 differed by only one locus (VNTR 2074). Their RFLP patterns were notably different (data not shown). This concomitant change in two independent markers suggests that these two isolates originated from independent clones. They could still very well represent an outbreak if strains were to have highly similar RFLP (differing by only one or two bands) and have identical VNTR profiles. It is impossible to determine whether or not this was a mass infection case. For that reason, a conclusion should be offered only after considering all results, including contact examination and epidemiological investigation.

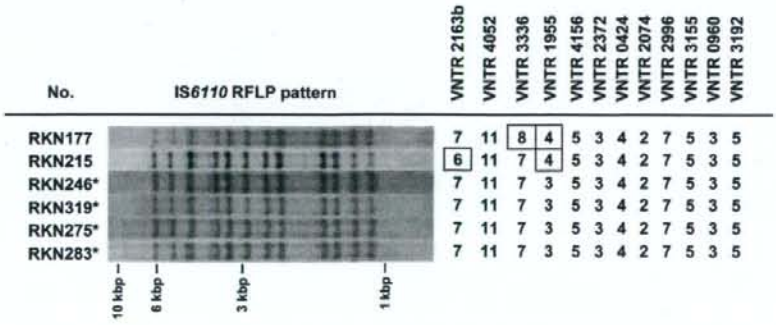

Fig. 2. The subdivision of cluster 08 in IS*6110* RFLP using 12-locus (JATA) VNTR. Cluster 08 in RFLP analysis comprised six strains. The JATA-VNTR divided cluster 08 into three groups. The isolates marked with an asterisk (RKN246, RKN275, RKN283 and RKN319) were clustered using both RFLP and VNTR analyses. Differences in VNTR patterns are boxed.

In VNTR analysis, the frequency of copy number conversion at each locus remains unclear. The copy number was found to be changed at VNTRs 3232 and 3690 (Iwamoto *et al.*, 2007) when the cloned isolate was cultured for 7–23 months. Furthermore, one repeat difference has been reported at MIRU-26 (Savine *et al.*, 2002). In these cases, conversion of the copy number was found at a single locus. Other reports have described that the copy quantities of VNTR changed at multiple loci (Supply *et al.*, 2006; van Deutekom *et al.*, 2005). The frequency of conversion of tandem repeats depended on each locus. Therefore, the accumulation of data related to the stability of each locus is a task of pressing urgency.

The TB isolates without epidemiological links were clearly classified according to differences of VNTR profiles (Oelemann *et al.*, 2007). It is impossible to conclude whether two strains are derived from the same origin, or not, merely by VNTR analysis when a difference of copy number is observed in one or two loci. When more detailed typing is required in a survey IS*6110* RFLP or other loci, including hyper-variable regions in the VNTR, might be used.

Molecular epidemiological analyses are useful both for transmission surveys and population-based retrospective studies. A recent report described that up to 14 % of the strain clusters identified by IS*6110* RFLP analyses might include false clustering (van Deutekom *et al.*, 2005). Apparently, true TB genotyping clusters are obtainable through VNTR analysis because it can analyse *M. tuberculosis* objectively when suitable loci are selected.

For the present PCR platform, analyses of 8 or 12 samples are convenient. Herein, we propose this 12-locus VNTR as a useful means for TB genotyping where Beijing strains are prevalent. This is one report proposing new VNTR loci for Beijing strains. These loci do not represent a final decision. Additional loci can be chosen for TB genotyping in VNTR analysis if further discrimination is necessary. In addition, when the four loci selected here (VNTRs 2074, 2372, 3155 and 3336) are added to 15-locus VNTR (Supply), both genotypes of Beijing and non-Beijing can be analysed.

In this report, we propose new combinations of loci for VNTR analysis for the Beijing family. Furthermore, this study shows that JATA (12) VNTR has higher discriminatory power than IS*6110* RFLP. However, the stability of each locus in VNTR analysis remains unknown. To obtain accumulated data, additional experiments are necessary.

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REFERENCES

Blackwood, K. S., Wolfe, J. N. & Kabani, A. M. (2004). Application of mycobacterial interspersed repetitive unit typing to Manitoba tuberculosis cases: can restriction fragment length polymorphism be forgotten? *J Clin Microbiol* **42**, 5001–5006.

Cave, M. D., Eisenach, K. D., McDermott, P. F., Bates, J. H. & Crawford, J. T. (1991). IS*6110*: conservation of sequence in the *Mycobacterium tuberculosis* complex and its utilization in DNA fingerprinting. *Mol Cell Probes* **5**, 73–80.

CDC (2004). *Guide to the Application of Genotyping to Tuberculosis Prevention and Control. Handbook for TB Controllers, Epidemiologists, Laboratorians, and Other Program Staff — June 2004.* Atlanta, GA; Centers for Disease Control and Prevention. http://www.cdc.gov/tb/genotyping/manual.htm.

Cowan, L. S., Diem, L., Monson, T., Wand, P., Temporado, D., Oemig, T. V. & Crawford, J. T. (2005). Evaluation of a two-step approach for large-scale, prospective genotyping of *Mycobacterium tuberculosis* isolates in the United States. *J Clin Microbiol* **43**, 688–695.

Filliol, I., Driscoll, J. R., Van Soolingen, D., Kreiswirth, B. N., Kremer, K., Valétudie, G., Anh, D. D., Barlow, R., Banerjee, D. & other authors (2002). Global distribution of *Mycobacterium tuberculosis* spoligotypes. *Emerg Infect Dis* **8**, 1347–1349.

Frothingham, R. & Meeker-O'Connell, W. A. (1998). Genetic diversity in the *Mycobacterium tuberculosis* complex based on variable numbers of tandem DNA repeats. *Microbiology* **144**, 1189–1196.

Glynn, J. R., Whiteley, J., Bifani, P. J., Kremer, K. & van Soolingen, D. (2002). Worldwide occurrence of Beijing/W strains of *Mycobacterium tuberculosis*: a systematic review. *Emerg Infect Dis* **8**, 843–849.

Hunter, P. R. & Gaston, M. A. (1988). Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. *J Clin Microbiol* **26**, 2465–2466.

Iwamoto, T., Yoshida, S., Suzuki, K., Tomita, M., Fujiyama, R., Tanaka, N., Kawakami, Y. & Ito, M. (2007). Hypervariable loci that enhance the discriminatory ability of newly proposed 15-loci and 24-loci variable-number tandem repeat typing method on *Mycobacterium tuberculosis* strains predominated by the Beijing family. *FEMS Microbiol Lett* **270**, 67–74.

Kam, K. M., Yip, C. W., Tse, L. W., Wong, K. L., Lam, T. K., Kremer, K., Au, B. K. & van Soolingen, D. (2005). Utility of mycobacterial interspersed repetitive unit typing for differentiating multidrug-resistant *Mycobacterium tuberculosis* isolates of the Beijing family. *J Clin Microbiol* **43**, 306–313.

Kam, K. M., Yip, C. W., Tse, L. W., Leung, K. L., Wong, K. L., Ko, W. M. & Wong, W. S. (2006). Optimization of variable number tandem repeat typing set for differentiating *Mycobacterium tuberculosis* strains in the Beijing family. *FEMS Microbiol Lett* **256**, 258–265.

Kamerbeek, J., Schouls, L., Kolk, A., van Agterveld, M., van Soolingen, D., Kuijper, S., Bunschoten, A., Molhuizen, H., Shaw, R. & other authors (1997). Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. *J Clin Microbiol* **35**, 907–914.

Kremer, K., van Soolingen, D., Frothingham, R., Haas, W. H., Hermans, P. W., Martin, C., Palittapongarnpim, P., Plikaytis, B. B., Riley, L. W. & other authors (1999). Comparison of methods based on different molecular epidemiological markers for typing of *Mycobacterium tuberculosis* complex strains: interlaboratory study of discriminatory power and reproducibility. *J Clin Microbiol* **37**, 2607–2618.

Kremer, K., Glynn, J. R., Lillebaek, T., Niemann, S., Kurepina, N. E., Kreiswirth, B. N., Bifani, P. J. & van Soolingen, D. (2004). Definition

of the Beijing/W lineage of *Mycobacterium tuberculosis* on the basis of genetic markers. *J Clin Microbiol* **42**, 4040–4049.

Kremer, K., Au, B. K., Yip, P. C., Skuce, R., Supply, P., Kam, K. M. & van Soolingen, D. (2005). Use of variable-number tandem-repeat typing to differentiate *Mycobacterium tuberculosis* Beijing family isolates from Hong Kong and comparison with IS*6110* restriction fragment length polymorphism typing and spoligotyping. *J Clin Microbiol* **43**, 314–320.

Mazars, E., Lesjean, S., Banuls, A. L., Gilbert, M., Vincent, V., Gicquel, B., Tibayrenc, M., Locht, C. & Supply, P. (2001). High-resolution minisatellite-based typing as a portable approach to global analysis of *Mycobacterium tuberculosis* molecular epidemiology. *Proc Natl Acad Sci U S A* **98**, 1901–1906.

Mostrom, P., Gordon, M., Sola, C., Ridell, M. & Rastogi, N. (2002). Methods used in the molecular epidemiology of tuberculosis. *Clin Microbiol Infect* **8**, 694–704.

Nikolayevskyy, V., Gopaul, K., Balabanova, Y., Brown, T., Fedorin, I. & Drobniewski, F. (2006). Differentiation of tuberculosis strains in a population with mainly Beijing-family strains. *Emerg Infect Dis* **12**, 1406–1413.

Oelemann, M. C., Diel, R., Vatin, V., Haas, W., Rusch-Gerdes, S., Locht, C., Niemann, S. & Supply, P. (2007). Assessment of an optimized mycobacterial interspersed repetitive- unit-variable-number tandem-repeat typing system combined with spoligotyping for population-based molecular epidemiology studies of tuberculosis. *J Clin Microbiol* **45**, 691–697.

Roring, S., Scott, A., Brittain, D., Walker, I., Hewinson, G., Neill, S. & Skuce, R. (2002). Development of variable-number tandem repeat typing of *Mycobacterium bovis*: comparison of results with those obtained by using existing exact tandem repeats and spoligotyping. *J Clin Microbiol* **40**, 2126–2133.

Savine, E., Warren, R. M., van der Spuy, G. D., Beyers, N., van Helden, P. D., Locht, C. & Supply, P. (2002). Stability of variable-number tandem repeats of mycobacterial interspersed repetitive units from 12 loci in serial isolates of *Mycobacterium tuberculosis*. *J Clin Microbiol* **40**, 4561–4566.

Skuce, R. A., McCorry, T. P., McCarroll, J. F., Roring, S. M., Scott, A. N., Brittain, D., Hughes, S. L., Hewinson, R. G. & Neill, S. D. (2002). Discrimination of *Mycobacterium tuberculosis* complex bacteria using novel VNTR-PCR targets. *Microbiology* **148**, 519–528.

Smittipat, N. & Palittapongarnpim, P. (2000). Identification of possible loci of variable number of tandem repeats in *Mycobacterium tuberculosis*. *Tuber Lung Dis* **80**, 69–74.

Smittipat, N., Billamas, P., Palittapongarnpim, M., Thong-On, A., Temu, M. M., Thanakijcharoen, P., Karnkawinpong, O. & Palittapongarnpim, P. (2005). Polymorphism of variable-number tandem repeats at multiple loci in *Mycobacterium tuberculosis*. *J Clin Microbiol* **43**, 5034–5043.

Sonehara, T., Kawazoe, H., Sakai, T., Ozawa, S., Anazawa, T. & Irie, T. (2006). Ultra-slim laminated capillary array for high-speed DNA separation. *Electrophoresis* **27**, 2910–2916.

Sun, Y. J., Bellamy, R., Lee, A. S., Ng, S. T., Ravindran, S., Wong, S. Y., Locht, C., Supply, P. & Paton, N. I. (2004). Use of mycobacterial interspersed repetitive unit-variable-number tandem repeat typing to examine genetic diversity of *Mycobacterium tuberculosis* in Singapore. *J Clin Microbiol* **42**, 1986–1993.

Supply, P., Mazars, E., Lesjean, S., Vincent, V., Gicquel, B. & Locht, C. (2000). Variable human minisatellite-like regions in the *Mycobacterium tuberculosis* genome. *Mol Microbiol* **36**, 762–771.

Supply, P., Lesjean, S., Savine, E., Kremer, K., van Soolingen, D. & Locht, C. (2001). Automated high-throughput genotyping for study of global epidemiology of *Mycobacterium tuberculosis* based on mycobacterial interspersed repetitive units. *J Clin Microbiol* **39**, 3563–3571.

Supply, P., Allix, C., Lesjean, S., Cardoso-Oelemann, M., Rüsch-Gerdes, S., Willery, E., Savine, E., de Haas, P., van Deutekom, H. & other authors (2006). Proposal for standardization of optimized mycobacterial interspersed repetitive unit-variable-number tandem repeat typing of *Mycobacterium tuberculosis*. *J Clin Microbiol* **44**, 4498–4510.

van Deutekom, H., Supply, P., de Haas, P. E., Willery, E., Hoijng, S. P., Locht, C., Coutinho, R. A. & van Soolingen, D. (2005). Molecular typing of *Mycobacterium tuberculosis* by mycobacterial interspersed repetitive unit-variable-number tandem repeat analysis, a more accurate method for identifying epidemiological links between patients with tuberculosis. *J Clin Microbiol* **43**, 4473–4479.

van Embden, J. D., Cave, M. D., Crawford, J. T., Dale, J. W., Eisenach, K. D., Gicquel, B., Hermans, P., Martin, C., McAdam, R. & other authors (1993). Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: recommendations for a standardized methodology. *J Clin Microbiol* **31**, 406–409.

Wada, T., Maeda, S., Hase, A. & Kobayashi, K. (2007). Evaluation of variable numbers of tandem repeat as molecular epidemiological markers of *Mycobacterium tuberculosis* in Japan. *J Med Microbiol* **56**, 1052–1057.

Yokoyama, E., Kishida, K., Uchimura, M. & Ichinohe, S. (2007). Improved differentiation of *Mycobacterium tuberculosis* strains, including many Beijing genotype strains, using a new combination of variable number of tandem repeats loci. *Infect Genet Evol* **7**, 499–508.

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Histopathological classification of systemic *Mycobacterium avium* complex infections in slaughtered domestic pigs

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Abstract

The aim of this study was to classify the histopathological features of pigs infected with *Mycobacterium avium* complex (MAC). We used slaughtered pig organs systemically infected with MAC. The results showed granulomatous lesions which were observed predominantly in the digestive organs and regional lymph nodes rather than respiratory organs. The histological picture showed a wide range of granulomatous stages from exudative to fibrotic reactions to the MAC infection. Eosinophils and giant cells were characteristically observed in the exudative reactions. The histopathological type in primary focus tended to be maintained in the respective organs. Most strains with the same genotype showed pathogenicity for guinea pigs irrespective of the type of granuloma. Although these findings suggest that different stages of a granulomatous lesion originating from the same causative agent might influence histological patterns, other possibilities such as the hereditary background of the host, or the effects of viral infections should be considered.

Keywords: *Mycobacterium avium* complex; Classification; Histopathological; Granuloma; IS*1245*; Pigs

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Résumé

L'objectif de cette étude est de classifier les caractéristiques histopathologiques des porcs infectés par le complexe *Mycobacterium avium* (MAC). Nous avons examiné des organes de porcs abattus, infectés par le MAC. Les résultats ont montré des lésions granulomateuses observées principalement dans les organes digestifs et les ganglions lymphatiques environnants plus que dans les organes respiratoires. Les schémas histopathologiques ont montré une grande diversité de stades granulomateux, allant de réactions exudatives jusqu'à des réactions fibrotiques. Les eosinophiles et les cellules géantes ont été particulièrement observées dans les réactions exudatives. La plupart des souches de bactéries présentant le même génotype ont montré un pouvoir pathogène chez les cobayes, indépendamment du type granulomateux. Bien que ces résultats suggèrent que différents stades d'une lésion granulomateuse ayant pour origine la même bactérie puissent révéler leurs influences sur les schémas histopathologiques, d'autres possibilités telles que l'hérédité du sujet ou les effets d'une potentielle infection virale doivent également être prises en compte.

Mots clés: Le Complexe *Mycobaterium avium* (MAC); Classification; Histopathologie; Granulome; Séquence d'insertion génomique (IS) *1245*; Porcs

1. Introduction

Mycobacterium avium complex (MAC) infection occurs in immunocompromised hosts or patients with antecedent pulmonary diseases [1]. Recently, the study of pulmonary MAC diseases in patients without a predisposing condition has become very significant for this common clinical problem [2,3]. Such types of MAC diseases cause extensive granuloma formation in the airway and have a progressive course [4]. Therefore, the study of MAC infections is a critical issue.

Some previous studies have reported pathological findings of typical mycobacteriosis, such as leprosy and tuberculosis [5,6]. Ridley subdivided the histopathological picture of leprosy into two main spectrums: (1) tuberculoid granulomas; and (2) lepromatous granulomas [5]. However, controversy exist as to whether or not these classical notions can be applied generally to granulomatous responses to MAC infection in humans. Two spectrums of epithelioid cell granuloma with or without caseation necrosis were observed in human MAC infections [7]. Undifferentiated granuloma was observed in the septic focus with MAC in acquired immunodeficiency syndrome (AIDS) [8–12]. However, whether such histological responses have been maintained among the organs in systemically infected individuals has not been well understood in previous investigations. Since it is very difficult to obtain affected tissues in humans, the evaluation of histopathological findings in MAC patients has not been well defined [4,13].

The domestic pig is an animal susceptible to MAC. In fact, sporadic epidemic outbreaks have occurred throughout the world [14–18]. This has allowed us to engage in relatively extensive histological analyses of the same disease stages due to a large number of pigs being slaughtered during the same growth period in nations

where a modern pig industry is well established [19]. There is the possibility of the pig MAC infection serving an alternative model for MAC infection in human beings.

Some histopathological evaluations of pigs infected with MAC have previously been described [15,20–22]. Histopathological change from a proliferative to an infiltrative character has been observed in lesions of these pigs [20,22]. They demonstrated that dissemination of MAC occurs in domestic pigs. Ellsworth et al. reported that an aborted sow infected with MAC show focal non-encapsulated granulomas in lymph nodes, tonsils, kidney, liver, spleen, lungs as well as the uterine and vaginal walls [23]. However, specific details of the histopathological features have not been demonstrated [15,20]. Moreover, it is not clear whether strains with certain genotypes cause specific histopathological features or whether the features were formed by host reactions.

The aim of the present study was to evaluate the histopathological features of systemically infected domestic pigs. We, then, examined the relationships among the histopathological types, their pathogenesis, and the genotypes of strains.

2. Materials and methods

2.1. Materials for histological examination

We collected organs with granulomatous lesions from pigs ($n = 403{,}792$) slaughtered in Okinawa prefecture between 2002 and 2004. The granulomatous lesions were macroscopically assessed by trained veterinary meat inspectors. The assessment criteria were based on the provisions stipulated in the Standard Procedure Manual of the Japanese National Meat Hygiene Inspection Organization. Thus, the sub-maxillary and mesenteric lymph nodes as well as the liver of the pigs were routinely examined. The sub-maxillary lymph nodes and the mesenteric lymph nodes were incised. The lesions of the lymph nodes were characterized by caseous foci, ranging in size from a pinhead to a broad bean. We distinguished the granulomatous lesions from other similar conditions like nodules caused by toxoplasmosis and milk-spots due to ascarid infections in the liver. We inspected other organs and regional lymph nodes in more detail when we observed caseous lesions in the liver.

We decided to use systemically infected pigs ($n = 276$) as the objective material for this study. A systemically infected pig was defined as a carcass with granulomatous lesions found macroscopically in the sub-maxillary or mesenteric lymph nodes, and any of the visceral organs such as the liver, lung, or spleen, which were routinely examined. All oriented pigs were clinically silent. There was no significant difference in the mean weight between the systemically infected pigs with MAC and the other slaughtered pigs (mean carcass weight was 76.8 kg [$n = 218$ for systemically infected pigs] and 79.5 kg [$n = 21{,}569$ for the other pigs shipped during the fixed period]).

2.2. Histological examination

Tissue ($n = 3312$: 276 pigs $\times$ 12 tissues) from systemically infected pigs was used as material for the histopathological examination. The affected tissues was

macroscopically removed from the organs and fixed immediately in a solution containing 10% neutral buffered formalin fixative. Serial paraffin sections were cut at 3 μ thick on a microtome and thaw-mounted on glass slides. Tissue sections were stained with hematoxylin and eosin (HE). The granulomatous lesions were classified into three categories on the basis of the inflammatory reaction, as well as the spread of the lesion in the tissue with reference to the tuberculoid granuloma stages and classifications described previously [21,24,25] (Fig. 1). (1) Exudative reaction with epithelioid cells (Figs. 1A and 2A). This represents an early stage of the granuloma formation, characterized by clusters of macrophages and lymphocytes. In some cases, activated macrophages are transformed into epithelioid cells. (2) Proliferative reaction with capsulization (Figs. 1B and 2B). Granulomas are formed as circumscribed lesions. These are characterized by central accumulation of epithelioid cells with a surrounding rim consisting of fibroblasts and lymphocytes. The center of the well-developed granuloma transforms itself into caseous necrosis or calcification.

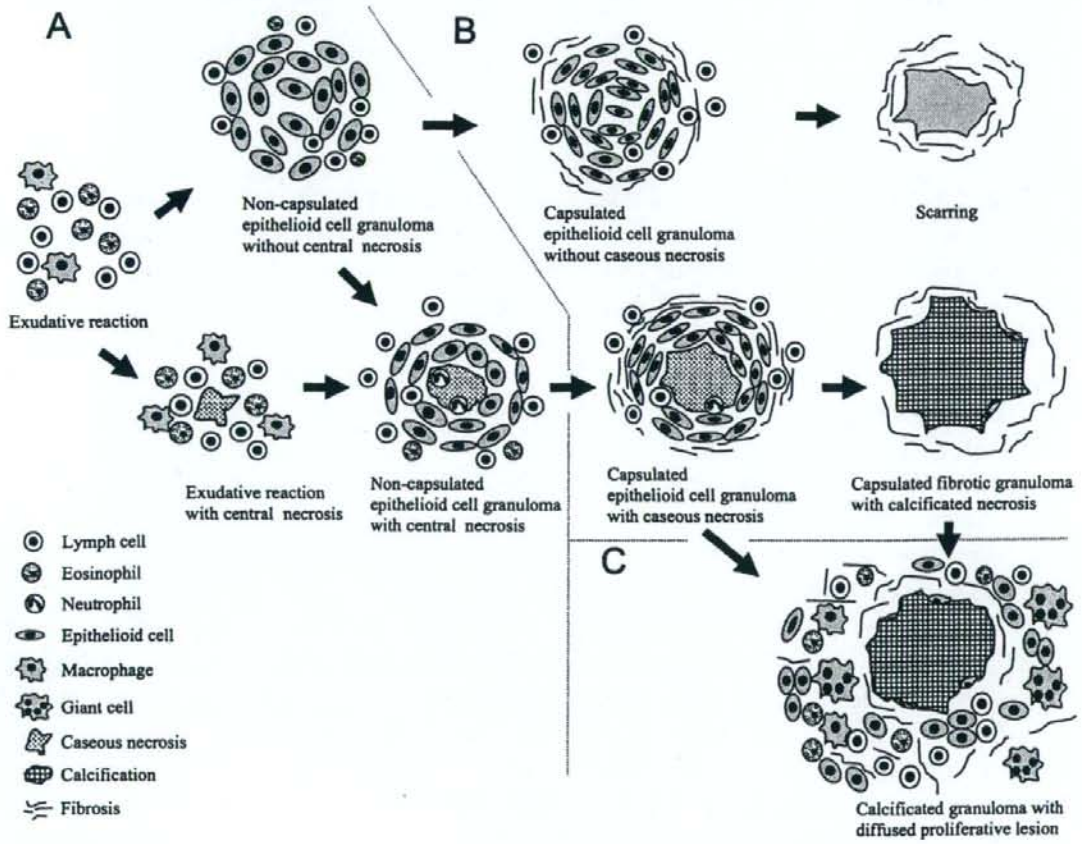

Fig. 1. Schema of pathological changes of *Mycobacterium avium* complex infection in a pig: (A) exudative reaction with epithelioid cells. This represents an early stage of the granuloma formation characterized by clusters of macrophages and lymphocytes. Activated macrophages are transformed into epithelioid cells as granuloma develop. In some cases, the granuloma center causes necrosis without capsulization; (B) proliferative reaction with capsulization. A fibrotic reaction is observed at the rim of the granuloma; and (C) mixed granuloma. In this type of granuloma, an exudative reaction with epithelioid cells is formed around the proliferative reaction with capsulization.

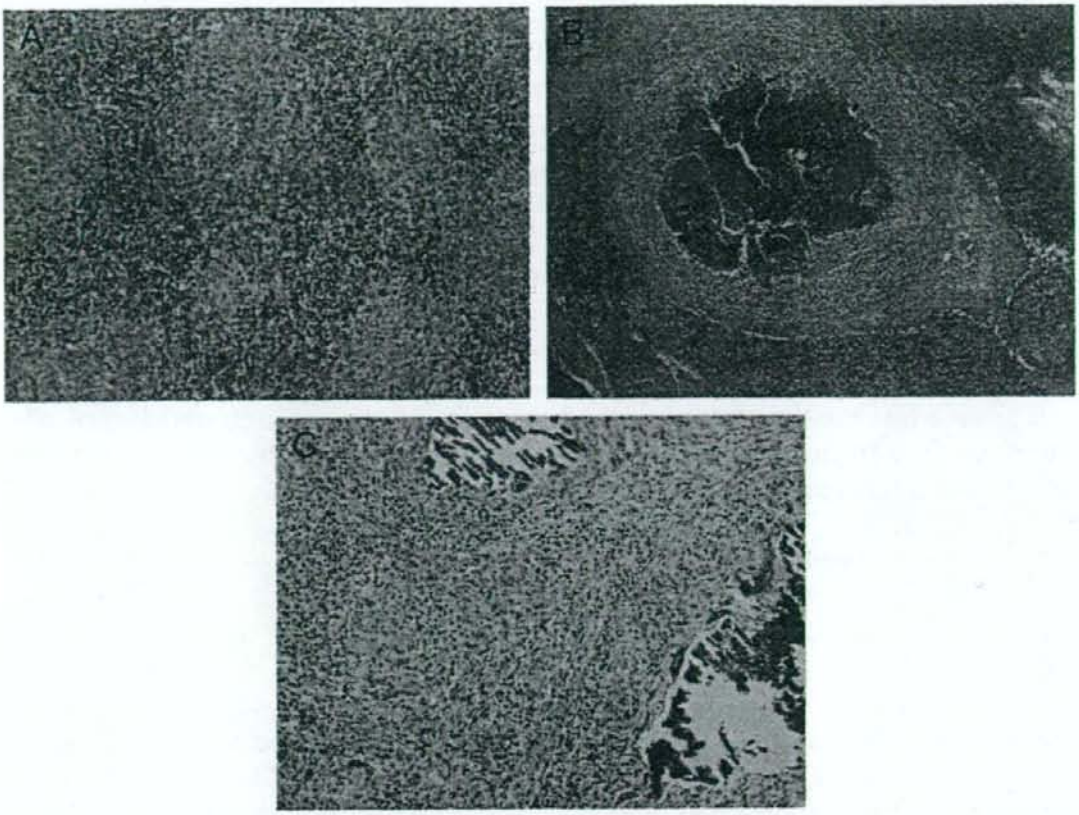

Fig. 2. Histological classification of granulomatous legions in slaughtered pigs: (A) an example of granuloma classified as an exudative reaction with epithelioid cells (mesenteric lymph nodes, HE, × 200). This granuloma has no central necrosis, but infiltration of inflammatory cells (lymphocytes and eosinophils) is observed. Proliferation of macrophages was observed and some of them transformed into epithelioid cells; and (B) an example of granuloma classified as a proliferative reaction with capsulization (mesenteric lymph nodes, HE, × 200). Typical caseous granuloma with central necroses are found surrounded with fibrous capsule. The foci of calcification are also observed in the caseous necroses; and (C) an example of granuloma classified as a mixed granulomas (mesenteric lymph nodes, HE, × 40). Inside the granuloma, extensive proliferations of epithelioid cells, lymphocytes, and fibroblast are observed. Multinucleated giant cells were spreading inside the lesion.

The lesion is distinct from the uninvolved tissues nearby. (3) Mixed granulomas (Figs. 1C and 2C). These are a combination of the exudative reaction with epithelioid cells and the proliferative reaction with capsulization. In some pigs, we found different granuloma stages with in the same tissue section. It was particularly rare to observe a combination of well-developed granuloma and immature granuloma in the same tissue section, and in such cases, we adopted the predominant granuloma pattern for this study.

2.3. Culture examination

After the lesions were collected, the specimens (about 350 mg) were homogenized with ceramic beads in a 1.4 ml carbohydrate solution (TSE kit, Bio-Rad, Paris,

France) using the Multiple-beads Shocker (MB400U, Yasui Kikai, Osaka, Japan), which rapidly cooled them to lower than 5 °C. The homogenized tissues were decontaminated with 2% sodium hydroxide (NaOH) and were centrifuged at 15,000 × *g* for 10 min. The pellets were suspended in 0.1 M phosphate-buffered saline (PBS) (pH 7.4) and inoculated into Ogawa slant medium (Kyokuto Company Limited, Tokyo, Japan) and into the Middlebrook 7H9 medium (Difco, Detroit, MI, USA) with 10% ADC enrichment (Becton, Dickinson and Company, Maryland, USA), followed by incubation for four weeks at 37 °C.

2.4. Identification of isolates

The acid-fast bacilli (AFB) were confirmed using the Fite-Farraco stain method on sliced sections treated according to the methods already mentioned and on a smear of the tissue suspension of an appropriately selected dose.

2.5. Virulence examination

The 11 selected *M. avium*, which were isolated from among the lesions having an exudative reaction with epithelioid cell granulomas ($n = 2$), a proliferative reaction with capsulization ($n = 4$), or the mixed granulomas ($n = 5$), were grown in Middlebrook 7H9 medium to the mid-log phase. The cultured strains were filtered using a 4-μm-pore-size membrane filter (Millipore, Bedford, MA, USA) for dispersal before use. The 11 guinea pigs were infected percutaneously in the lumber portions with an inoculum dose of 10^7 CFU of the strain suspended in 100 μl of PBS. The animal was raised under isolated air condition. The lungs, livers, spleens and kidneys from the guinea pigs were retrieved at seven weeks after infection and prepared for histopathological examination. This experiment was repeated twice using the same strains.

2.6. Isolate genotyping

For MAC DNA typing, restriction fragment length polymorphism (RFLP) was used [26]. Strains were randomly selected from throughout the region during all periods and from all hog farms. The selected 22 isolates from a large collection of the isolated MAC strains were examined by RFLP analysis with insertion sequence IS*1245*, which is representative of a particular MAC strain's mycobacterial pathogenicity [27,28]. Another 30 strains isolated from pigs having limited granulomatous lesions showed the same IS*1245* RFLP pattern in the exploratory experiment. DNA was isolated according to the method described by van Soolingen et al. [26,29]. Approximately 5 μg of purified mycobacterial DNA was digested with restriction endonuclease *Pvu*II (Wako, Osaka, Japan). After separation by electrophoresis in agarose gel, the DNA fragments were exposed to UV in a transilluminator and transferred from the gel to a nylon membrane by vacuum-blot. The DNA was fixed and hybridized with a labeled probe according to the method previously described [29]. The band positions of IS*1245*-containing restriction

fragments were compared with those of a molecular weight marker. The patterns were differentiated according to the number of bands: either "bird type" (three bands) or "non-bird type," (more than three bands) which has been previously described by various authors [30,31].

2.7. Statistical assessment

Elaboration of the data was accomplished using Statview-J5.0 software (Abacus Concept, Inc., Berkeley, CA). Comparison of the groups was done using a paired *t*-test. The χ^2 tests were used to compare categorical variables among groups. $p<0.05$ was considered statistically significant.

3. Results

3.1. Histopathological findings

Granulomatous lesions were found in 986 of the examined 3312 tissues. We classified the mycobacterial lesions into three histopathological categories (Table 1, Fig. 2). Exudative reaction with epithelioid cells was observed in 338 (34.3%) of 986 tissues (Table 1). The appearance of necrosis and fibrosis was observed with a lower prevalence (Table 1, Fig. 2A). The necrosis of this granuloma type was coagulative necrosis. Lymphocytes, eosinophils and giant cells were frequently observed, but neutrophils were rarely observed (Table 1). Most of the giant cells ($n = 135$) were Langhans-type ($n = 50$). Some ($n = 33$) were a foreign-body type with the remaining being of both ($n = 12$). Granulomas that were classified as an exudative reaction with epithelioid cells were predominantly observed in the lung, hepatic and internal iliac lymph nodes (Table 2).

Granulomas classified as a proliferative reaction with capsulization were observed in 500 (50.7%) of 986 tissues from systemically infected slaughtered pigs (Table 1). The appearance of necrosis, calcification and fibrosis was observed with a higher prevalence (Table 1, Fig. 2B). Caseous granulomas were associated with predominant lymphocytes, but also with a few neutrophils and eosinophils (Table 1). Giant cells were relatively rare (Table 1). Langhans-type was observed more than a foreign-body type giant cell (49:5). Granulomas classified as a proliferative reaction with capsulization were predominantly observed in the liver, spleen, mesenteric, pulmonary and superficial lymph nodes (Table 2).

Granulomas classified as a mixed granuloma were observed in 148 (15.0%) of 986 tissues (Table 1). Calcification and fibrosis were observed with a high prevalence, but the fibrosis was formed by irregularly proliferated fibroblasts in diffuse lesions with an accumulation of epithelioid cells (Table 1, Fig. 2C). Lymphocytes, eosinophils, and giant cells were frequently observed in granulomas classified as a mixed granuloma (Table 1). Langhans-type and foreign-body type giant cells were observed (28:22) in about equal measure. Mixed granulomas in each organ were observed with less frequency than the other types of granulomas (Table 2). However, mixed granuloma were observed with comparatively high prevalence in the mesenteric and

Table 1
Difference in stages of progression and cell components for various types of granulomas

Types of granuloma	Total ($n = 986$)	Number (%) having						
		Necrosis	Calcification	Fibrosis	Lymphocytes	Neutrophils	Eosinophils	Giant cells
Exudative reaction with epithelioid cells	338	53 (15.7)	0 (0.0)	41 (12.1)	324 (95.9)	10 (3.0)	170 (50.3)	135 (39.9)
Proliferative reaction with capsulization	500	306 (61.2)	366 (73.2)	489 (97.8)	465 (93.0)	112 (22.4)	115 (23.0)	54 (10.8)
Mixed granuloma	148	66 (44.6)	98 (66.2)	101 (68.2)	148 (100.0)	10 (6.8)	61 (41.2)	85 (57.4)
p-value		<0.0001	<0.0001	<0.0001	NS	<0.0001	<0.0001	<0.0001

NS: not significant ($p<0.05$) for affected tissue obtained from infected pigs ($n = 276$) with MAC.

Table 2
Ratios of various types of granuloma in the organs and lymph nodes of slaughtered pigs

Specimens	Number (%) having:			Total (%)*	*p*-value
	Exudative reaction with epithelioid cells	Proliferative reaction with capsulization	Mixed granuloma		
Organs					
Liver	113 (41.8)	144 (52,7)	16 (5.9)	273 (98.9)	<0.0001
Lung	6 (50.0)	3 (25.0)	3 (25.0)	12 (4.3)	NS
Spleen	10 (46.2)	4 (64.2)	2 (7.7)	16 (5.8)	NS
Kidney	NE	NE	NE	NE	
Lymph nodes					
Mesenteric	28 (10.3)	149 (55.0)	94 (34.7)	271 (98.2)	<0.0001
Hepatic	92 (61.3)	44 (29.3)	14 (9.3)	150 (54.3)	<0.0001
Pulmonary	36 (42.4)	45 (52.9)	4 (4.7)	85 (30.8)	<0.0001
Spleen	3 (75.0)	0 (0.0)	1 (25.0)	4 (1.4)	
Sub-maxillary	31 (26.7)	75 (64.7)	10 (8.6)	116 (42.0)	<0.0001
Superficial cervical	6 (37.5)	9 (56.3)	1 (6.3)	16 (5.8)	NS
Inguinal	9 (24.3)	26 (70.3)	2 (5.4)	37 (13.4)	<0.0001
Internal iliac	2 (66.7)	0 (0.0)	1 (33.3)	3 (1.1)	NS
Sub-iliac	2 (66.7)	1 (33.3)	0 (0.0)	3 (1.1)	NS
Total	338	500	148	986	

NS: not significant ($p<0.05$); and *percentage of total number of examined tissues ($n = 276$) per organs.
NE: not examined.

Table 3
Appearance ratios of acid-fast bacilli among histological types in slaughtered pigs infected with MAC

Organs	Histopathological types			*p*-value
	Exudative reaction with epithelioid cells	Proliferative reaction with capsulization	Mixed granuloma	
Mesenteric lymph nodes	67.9% (19/28)	84.6% (126/149)	78.7% (74/94)	NS
Liver	35.4% (40/113)	34.7% (50/144)	31.3% (5/16)	NS

Figures in parentheses indicate the number of tissues with acid-fast bacilli in relation to the total number for each histological type. NS: not significant ($p<0.05$).

internal iliac lymph nodes (Table 2). In most granulomas with extensive caseous necrosis, a fibrotic reaction replacing the epithelioid cell layer was observed (Fig. 2C).

Eosinophils appeared during any of the granuloma stages, but were frequently observed in granuloma classified as an exudative reaction with epithelioid cells.

There was paucibacillary observation of AFB in all granuloma types (data not shown). The appearance ratios of AFB on mesenteric lymph nodes were 67.9% for the exudative reaction with epithelioid cells, 84.6% for the proliferative reaction with capsulization and 78.7% for the mixed granuloma (Table 3). Histopathological types and bacterial load were not correlated (Table 3).

When the occurrence of AFB was compared according to the criterion of whether or not giant cells exist in the lesions independent of the histopathological type, no significant difference was found in either the mesenteric lymph nodes or the liver (Table 4).

3.2. Distribution of granulomatous lesions across organs

The granulomatous lesions were found almost exclusively in the liver and mesenteric lymph nodes (Table 2). Lesions in sub-iliac and internal-iliac lymph nodes showed the lowest prevalence (Table 2).

Lesions in the liver were concentrated in the portal area with a few exceptions, and they occasionally caused invasive necrosis for the contiguous hepatic lobule (Fig. 5B).

3.3. Comparison of types of granuloma on each organ

When the types of granulomas were compared between mesenteric lymph nodes and liver for systemically infected cases ($n = 209$), the exudative reaction with epithelioid cells and the proliferative reaction with capsulization showed comparatively the same histological types, but differences were observed among the mixed granulomas (Fig. 3). Individuals with mixed granulomas in mesenteric lymph nodes showed different histological types in the liver (Fig. 3).

Furthermore, we examined the histological types for cases having lesions across the mesenteric lymph nodes, liver, and superficial lymph nodes ($n = 46$). An individual with exudative reaction with epithelioid cells in the mesenteric lymph nodes and liver showed a proliferative reaction with capsulization in superficial

Table 4
Appearance ratios of acid-fast bacilli attributable to giant cells in slaughtered pigs infected with MAC

Organs	Existence of giant cell		*p*-Value
	No giant cell	Existence of giant cell	
Mesenteric lymph nodes	86.1% (161/187)	69.0% (58/84)	NS
Liver	34.4% (74/215)	36.2% (21/58)	NS

Figures in parentheses indicate the number of tissues with acid-fast bacilli in relation to the total number with or without giant cells in each organ. NS: not significant ($p > 0.05$).

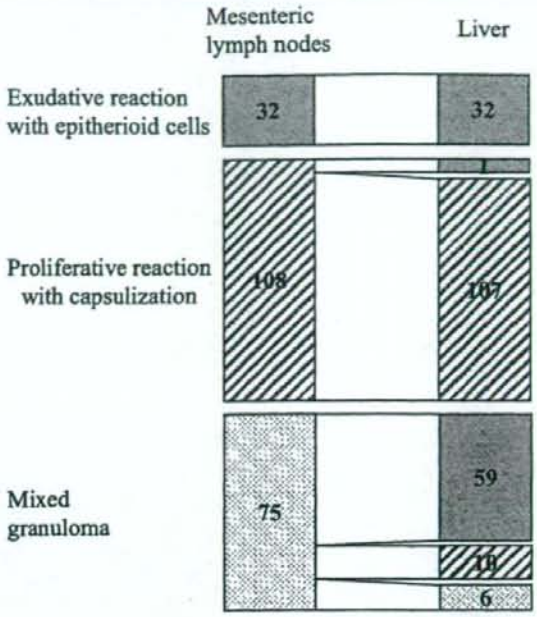

Fig. 3. Comparison of the granuloma types for mesenteric lymph nodes and livers in individuals. Each individual showed a relatively identical granuloma type resulting in the mesenteric lymph nodes as well as in the liver. However, some pigs with mixed granulomas in mesenteric lymph nodes showed different granuloma types in the liver.

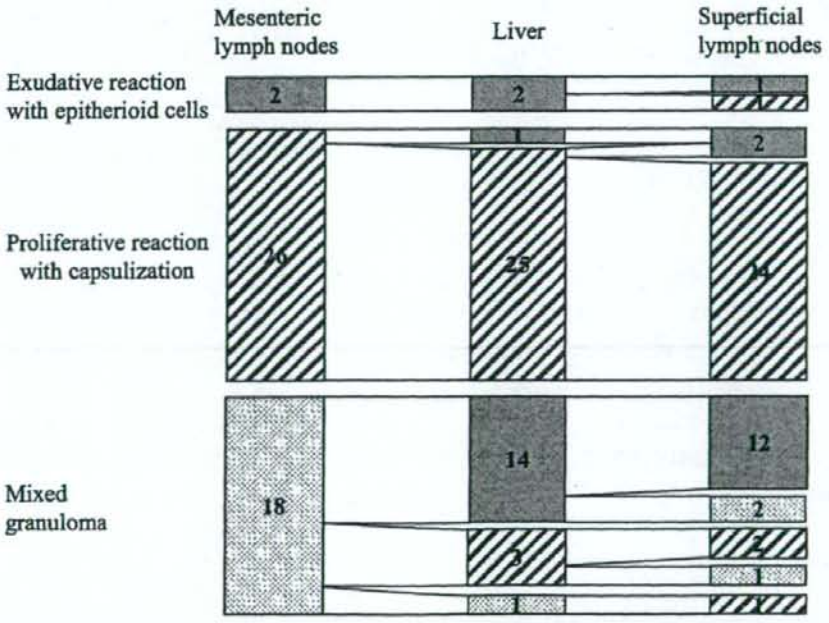

Fig. 4. Comparison of the types of granulomas in the mesenteric lymph nodes, liver, and superficial lymph nodes of individual pigs. Each individual showed relatively identical granuloma types among the various organs. However, some individuals showed different granuloma types among the various organs. There was a relatively higher ratio for individuals with mixed granuloma in the mesenteric lymph nodes.

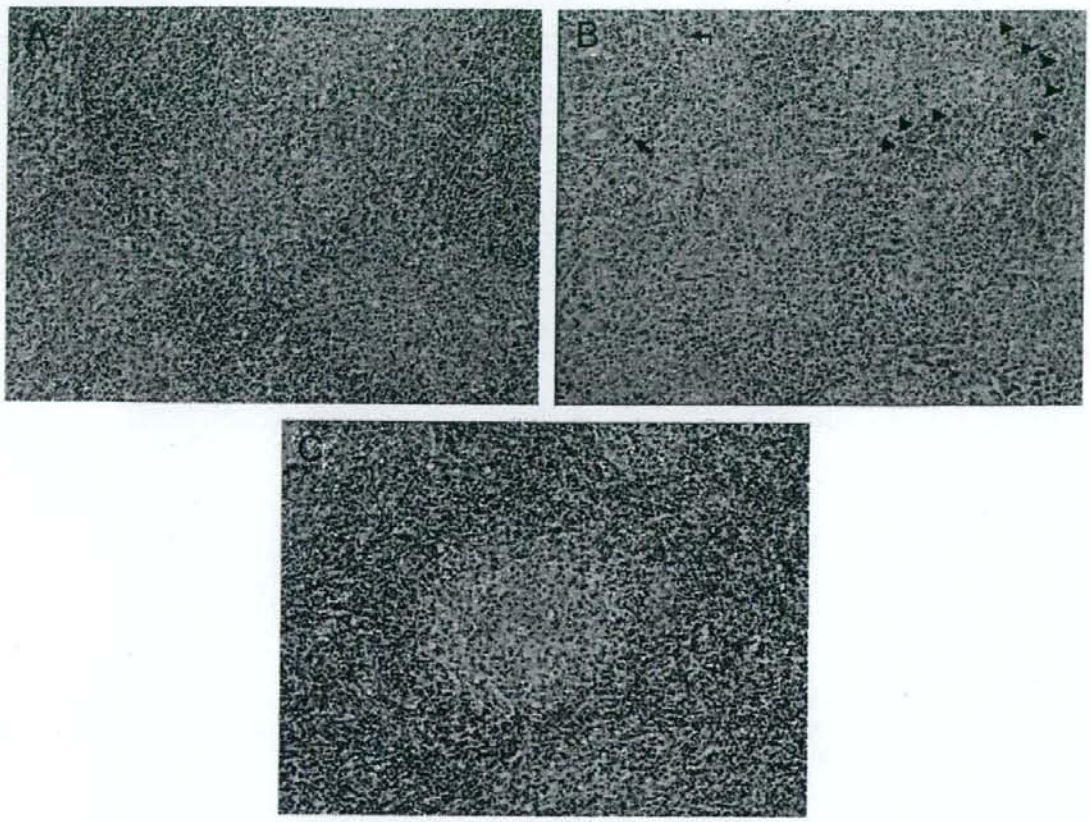

Fig. 5. Exudative reaction with epithelioid cells in a systemically infected pig: (A) a diffuse granulomatous lesion with moderate eosinophils and macrophages observed in the mesenteric lymph node (HE, × 200). A growing epithelioid cell granuloma was observed; (B) an exudative lesion with macrophage, lymphocytes and eosinophils observed in the portal area of the liver (HE, × 200). The lesion invaded the contiguous hepatic lobule, and the remaining hepatic cords were confirmed (arrow heads). Arrows indicate an interlobular bile duct; and (C) a focal accumulation of epithelioid cells with lymphocytes and eosinophils observed in the inguinal lymph node (HE, × 200).

lymph nodes (Fig. 4). Granuloma types of lesions among mesenteric lymph nodes, liver and superficial lymph nodes were almost the same with regard to the proliferative reaction with capsulization (Fig. 4). Individuals with mixed granulomas on mesenteric lymph nodes showed different histological types in the liver and superficial lymph nodes (Fig. 4). Figs. 5–7 show examples of each case that resulted in granuloma across the mesenteric lymph node, liver, and superficial lymph nodes in a systemically infected pig.

3.4. Experimental infection

All infected lesions showed microgranulomas without caseous necrosis in the guinea pigs (Fig. 8A–C). The component cells of the granulomas were epithelioid cells and lymphocytes. There were no significant differences concerning distribution of the lesions in the reproducibility of pig's histological features.

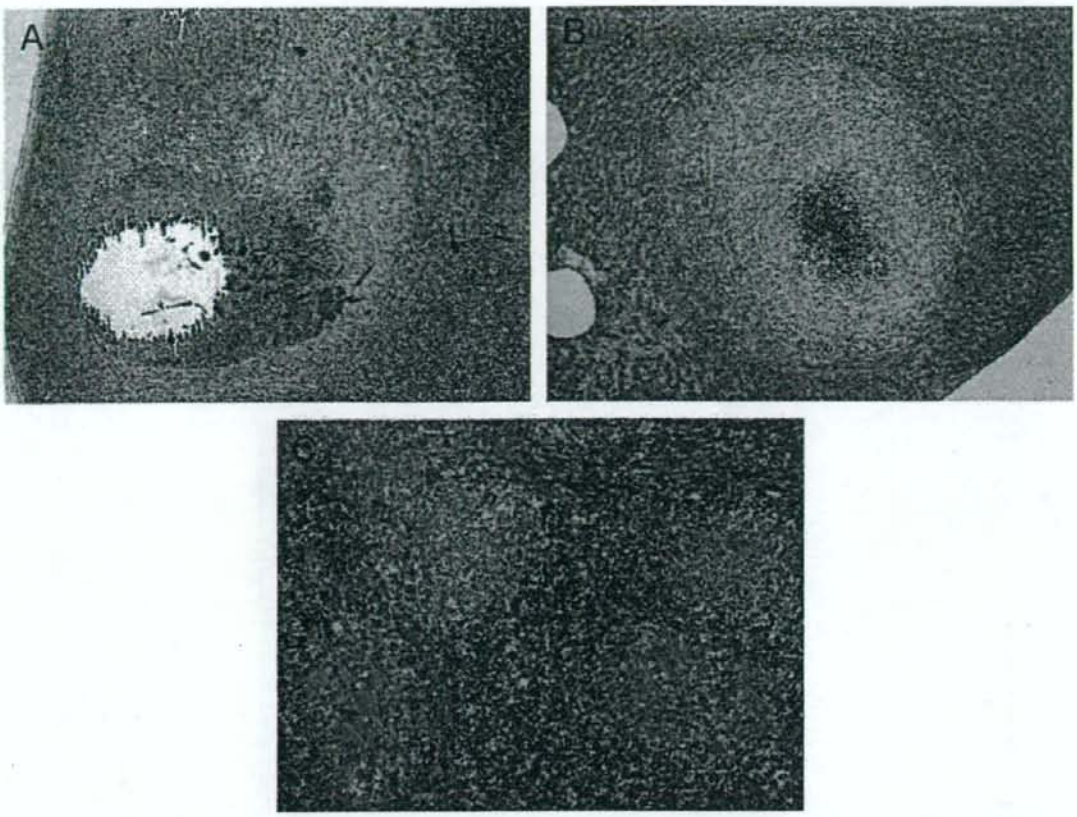

Fig. 6. Proliferative reaction with capsulization in a systemically infected pig: (A) granuloma with caseous necrosis with calcification observed in the mesenteric lymph node (HE, × 100); (B) well-capsulated granulomas with early central caseation observed under hepatic capsule in the liver (HE, × 100); and (C) early active epithelioid cell granuloma observed in the inguinal lymph nodes (HE, × 100).

This experiment was repeated twice using the same strains, and the same results were obtained.

3.5. *Gene analysis of strain with* IS *1245 RFLP*

Isolates were obtained from 33 hog farms on the Okinawa islands. All the isolated strains were also distinguished between *Mycobacterium avium* (*M. avium*) and other sub-species by the multiplex PCR method [32]. All strains possessed IS *1245* in their genes. IS *1245* RFLP analysis was divided into two homogenous types: Type I was characterized with homogenous six bands and Type II was characterized with mostly identical multi-bands >20. However, the "bird-type" with three bands was not observed (Fig. 9). The breakdown by genotype and histological characteristics was as follows: for the strain group with type I, exudative reaction with epithelioid cells ($n = 0$), proliferative reaction with capsulization ($n = 2$), and mixed granulomas ($n = 3$); and for the strain group with type II, and exudative reaction with epithelioid cells ($n = 3$), proliferative reaction with capsulization ($n = 8$), and mixed

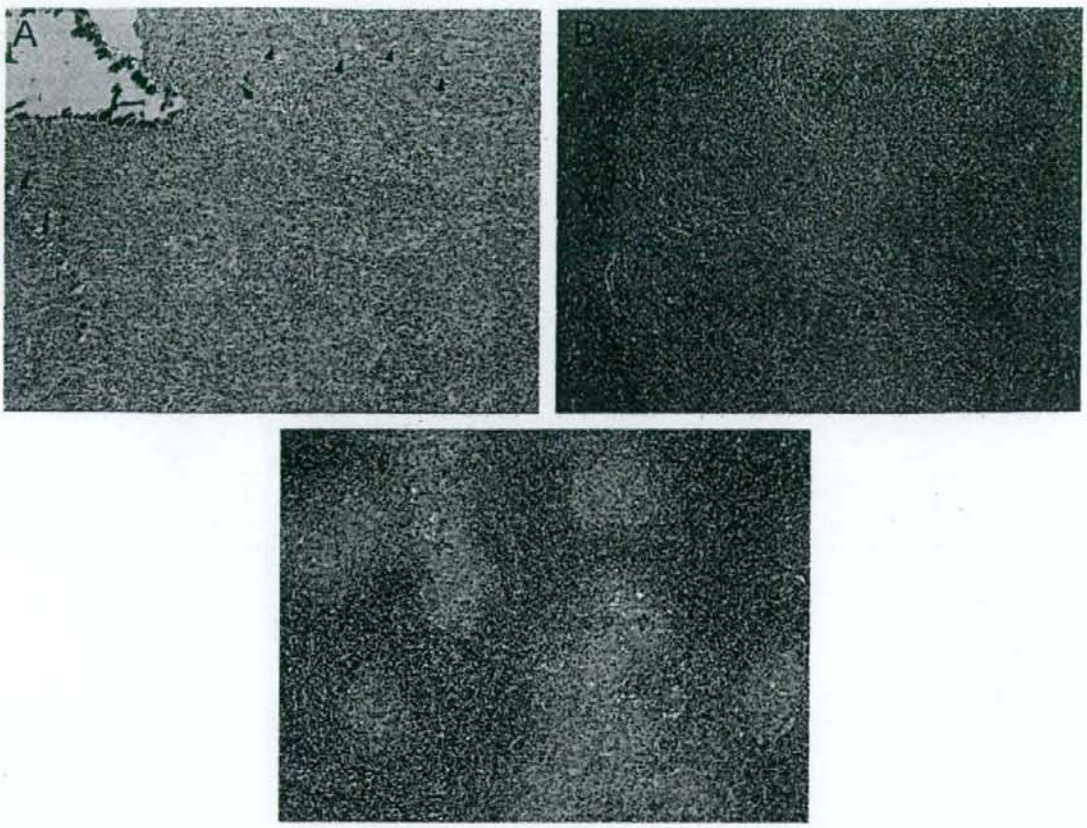

Fig. 7. Mixed granulomas in a systemically infected pig: (A) a mixed granuloma lesion shows a calcified granuloma coexisting with diffuse inflammatory lesions consisting of numerous lymphoid cells and macrophages, Langhans-type giant cells (arrow heads) in mesenteric lymph nodes (HE, × 40); (B) many multifocal epithelioid cell granulomas with severe lymphocyte inflammation observed in the portal area of the liver (HE, × 40); and (C) epithelioid cell granulomas observed focally in the inguinal lymph node (HE, × 40).

granuloma ($n = 8$). A few bands were common between types I (D, E) and II (A, B, C) (Fig. 9A–E).

4. Discussion

The present study indicated that pigs showed a variety of histological pictures from exudative to fibrotic reactions caused by MAC infection. It is common knowledge that in human tuberculosis, epithelioid cell granuloma changes to caseous granuloma with the development of granuloma [24]. Acland et al. examined the sequential morphological changes in orally inoculated pigs and showed that the picture changes from accumulated initial lesion of epithelioid cells to well-capsulated calcificated granuloma [22]. Thus, it may be possible to explain the histological differences of granulomas according to a difference in the phase of infection. Although this might be the main reason that there were a variety of granulomas, it is very difficult to evaluate differences in the phases of infection with spontaneously

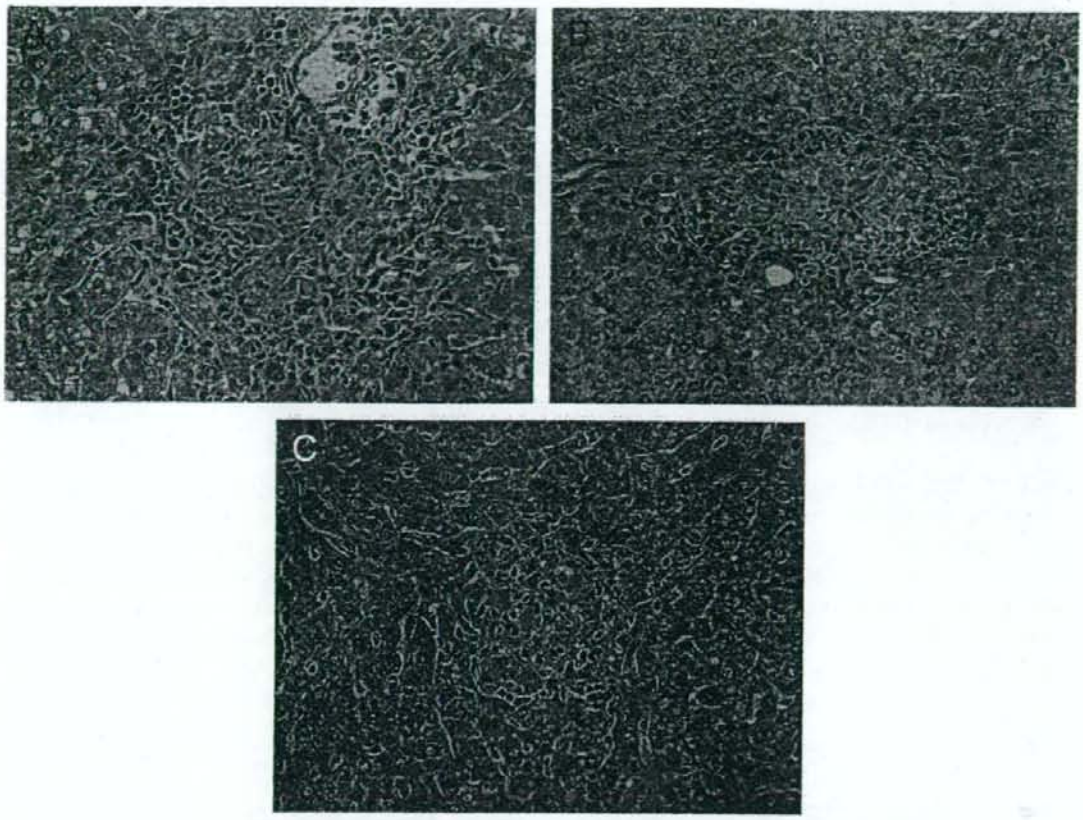

Fig. 8. Histological findings of guinea pigs inoculated with strains that showed different granuloma types in slaughtered pigs. Micro-epithelioid cell granulomas were observed in the livers of all guinea pigs: (A) the liver of a guinea pig inoculated with the strain that showed an exudative reaction with epithelioid cells in pigs (HE, × 200); (B) the liver of guinea pig inoculated with the strain that showed a proliferative reaction with capsulization in pigs (HE, × 200); and (C) the liver of the guinea pig inoculated with the strains that showed the mixed granuloma in pigs (HE, × 200).

infected cases. There are two other possibilities for the cause of the different types of granuloma: bacterial factors and host factors. However, RFLP IS*1245* patterns did not correlate with certain granuloma types in this study. In addition, the most prevalent serotype of the strains isolated from the affected tissue was type 4 (unpublished data). Furthermore, most strains with the same genotype showed pathogenicity for guinea pigs irrespective of the granuloma type. These findings may suggest that bacterial factors did not play a major role in the formation of different types of granuloma. Since we did not assess host immunological status in the present study, this issue needs to be discussed in further immunological research regarding MAC infection in pigs. However, Iwakiri et al. indicated that disseminatedly infected pigs showed a strong reaction to purified protein derived (PPD) from *M. avium* when compared to the lymphoproliferative response between localized infected pigs and disseminatedly infected pigs [33]. Ellsworth et al. also showed the same result in a disseminatedly infected sow [23]. This may suggest that cell-mediated immunity is maintained in systemically infected pigs. The effects of viral infection should also be

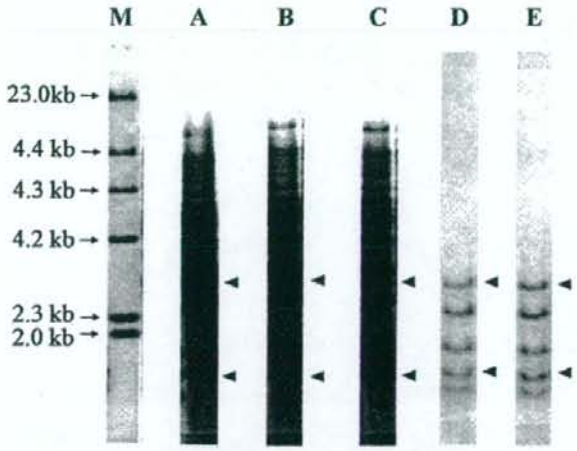

Fig. 9. IS *1245* RFLP type of strains showed different histological types in slaughtered pigs: (A) a genomic pattern in a strain isolated from a proliferative reaction with capsulization; (B) a strain showing an exudative reaction with epithelioid cells showed the same genomic pattern as A; (C) a strain from a mixed granuloma showed the same result; (D) a genomic pattern with six bands was revealed in a strain which showed a proliferative reaction with capsulization; and (E) a strain that appeared as a mixed granuloma showed the same result. *M* is the molecular weight marker. Arrow heads indicate a few common bands that were observed between the group of (A), (B), (C), and the group of (D), (E).

considered because porcine circo virus or porcine reproductive and respiratory syndrome virus affect the immunity of a pig [34–36].

In the present study, we observed the accumulation of eosinophils through a variety of granulomatous lesions. Recent work has suggested a mycobacterial effect of peroxidase in eosinophilial granules [37]. However, it is unknown whether the infiltration is specific to MAC infection because eosinophils are observed more frequently in pigs than in other species. Additionally, infiltrations of eosinophils in granulomatous lesions were not observed in the guinea pig inoculated strains isolated from the pigs. However, Castro et al. showed that heat-killed mycobacteria cause rapid chemotaxis in a large number of eosinophils in mice [38]. Therefore, the role of eosinophils in MAC granuloma should be studied in further research.

It has been reported that giant cells are observed more frequently in granuloma without caseation necrosis than granuloma with caseation necrosis in human MAC disease [39]. This finding is consistent with our present findings that giant cells were observed more frequently in an immature granuloma. The appearance of giant cells was recognized between 6 and 12 weeks after inoculation of *M. avium* in pigs [22], and well-developed granulomas were formed at 12 weeks after oral infection in mesenteric lymph nodes [21]. Because most of the pigs that we used in this study were about 6 months old, these findings may indicate that giant cells are dominant at an earlier granuloma stage.

In the present study, we frequently observed a proliferative reaction with calcification. Some previous researchers have also observed calcificated granuloma in spontaneously affected pigs [20,23]. Experimentally, calcification was shown in more advanced granuloma in pigs scarified at 12 weeks after inoculation [21]. These

findings may suggest that calcification frequently occurs at about 3 months after infection in the pig. In human MAC disease, calcification of caseous necrosis is rare [40] and in a previous review of tuberculosis, the appearance of calcification may require at least one or two years [41]. However, it is very difficult to evaluate the difference in phases and frequency of calcification between pigs and humans.

In pigs, the development of localized lesions in lymph nodes of the digestive tract are usually observed [42]. Windsor et al. showed that lesions commonly appearing in the liver and mesenteric lymph nodes in systemically infected pigs [15]. We observed the same results in the present study and most lesions in the liver were localized in the hepatic portal area. Ellsworth et al. have proved that granulomatous lesions in intestinal organs are caused by oral infection of MAC [43]. Such evidence suggests the gastrointestinal tract as the most likely route of MAC infection, with the respiratory tract as a less frequent pathway in pigs. In addition, although granulomatous lesions are frequently observed in the draining lymph nodes in pigs MAC infection, it is uncommon in human respiratory MAC infection [7,44].

In the present study, a histopathological response was maintained among the organs in systemically infected pigs, but most mixed granuloma showed more immature granuloma in the liver and superficial lymph nodes. Savov and Pavlov showed that the existence of histopathological changes in the lymph nodes had a proliferative to infiltrative character [20]. In addition, thorough histological examination of an aborted sow, they also demonstrated diffuse and focal non-encapsulated granulomas in lymph nodes and parenchymatous organs, and a few encapsulated calcified foci in the endometrium [23]. Farhi et al. have reported histopathological findings in disseminated human cases of immunosuppressed and immunocompetent hosts; and they have demonstrated that tuberculoid granulomas are observed in the liver and spleen, and that a variety of non-necrotizing and necrotizing granulomatous lesions are observed in lymph nodes [11]. These findings support the possibility that the histological futures change across organs in systematically infected host.

Previous studies of systemic mycobacteriosis reported that diffuse lesions are multibacillary, but localized lesions are paucibacillary in an immunocompromised host [1,12]. These findings differ from our present results with domestic pigs in that the exudative reaction with epithelioid cells showed paucibacillary lesions. Nakamura et al. also showed that only a small number of AFB was observed histologically in pigs dosed with MAC [21]. This discrepancy between systemic infection of pigs and systemic infection of humans may be attributed to the immunity of host, and should be clarified it in further investigation.

Our investigation revealed that there are some differences in the histopathological features of pig MAC infection from those of human pulmonary MAC infection [4,7,40]: (1) eosinophils appear with relatively high frequency in immature granuloma; (2) possibility of oral infection; (3) organisms caused dissemination in pigs and show different patterns of granulomatous lesions; and (4) pigs about 6 months old in contrast to that calcification is very rare in human MAC infection.