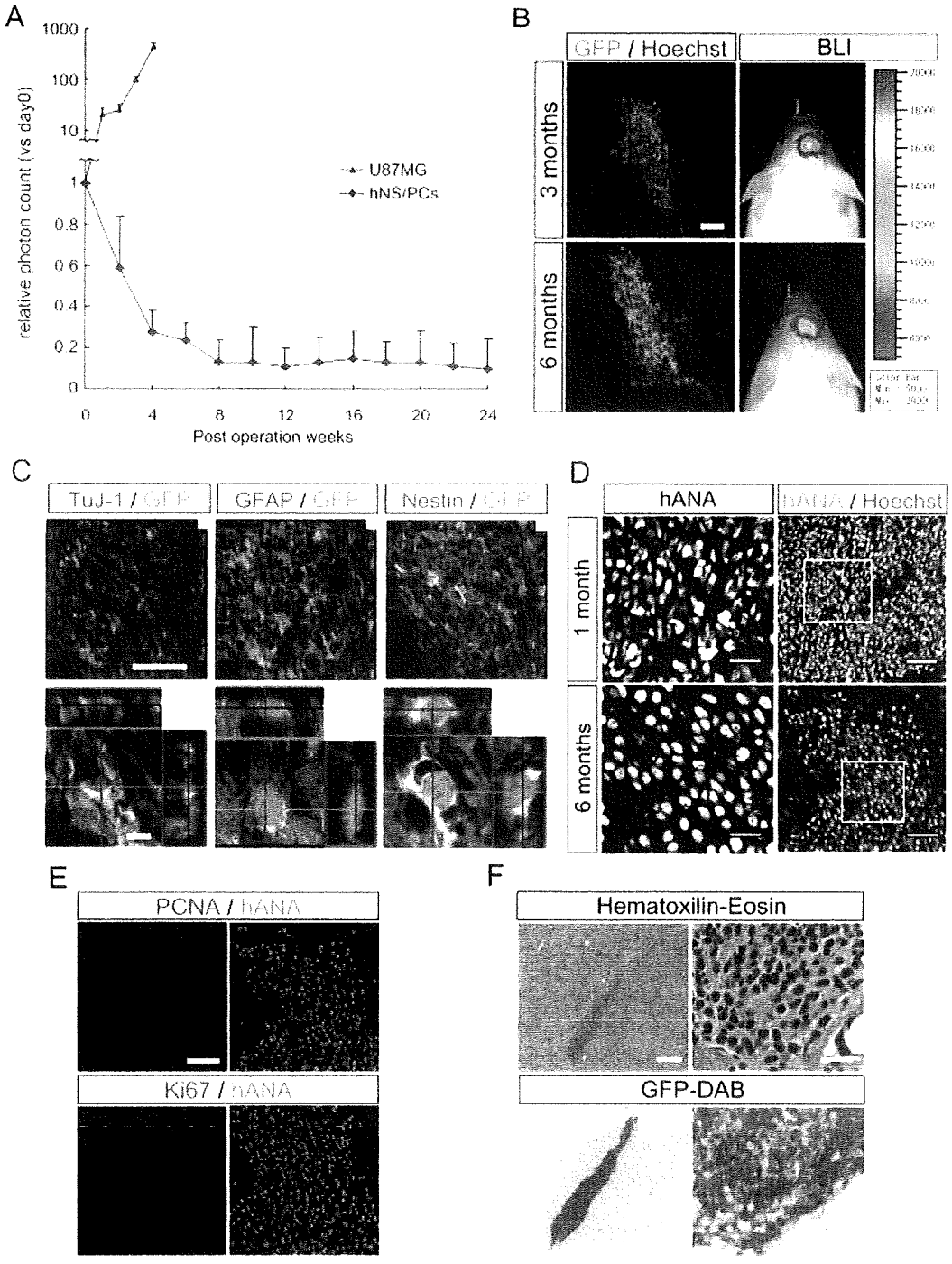

Fig. 5. hNS/PCs did not exhibit tumorigenesis for 6 months. A: Long-term observation of grafted hNS/PCs-250 by BLI for 6 months. The photon count of hNS/PCs decreased for 8 weeks after transplantation but was stable thereafter. The hNS/PCs did not show any evidence of tumorigenicity during the 6-month observation period, unlike the control U87MG cells. Data are presented as the mean 6 SEM. B: Immunohistochemical analysis of Venus-expressing grafted hNS/PCs 3 and 6 months after transplantation showed consistent correlation of the Venus-expressing area with the photon counts detected by BLI. C: Immunohistochemical analysis of grafted hNS/PCs 6 months after transplantation. hNS/PCs resided at the site of injection and differentiated into TuJ-1-positive neurons and GFAP-positive astrocytes. However, Nestin-positive neural progenitors were also observed, even 6 months after transplantation. D: hANA and Hoechst nuclear staining of the grafted area 1 month and 6 months after the transplantation. Grafted cells showed larger nuclei and lower cell densities in the long-term analysis than in the short-term analysis. E: Immunohistochemical analysis of proliferation markers PCNA and Ki67. Neither PCNA- nor Ki67-positive cells were observed. F: Hematoxylin-eosin and GFP-DAB staining indicated no malignant invasive features in the transplanted cells. Scale bars 5 100 1m in B; low-magnification image 50 1m, high-magnification image 5 1m in C; low-magnification image 50 1m, high-magnification image 20 1m in D; 50 1m in E; low-magnification image 200 1m, high-magnification image 20 1m in F.

these findings indicate that the grafted hNS/PCs could survive and differentiate properly in the brain of NOD/SCID mice without any tumor formation, at least for 6 months after transplantation.

DISCUSSION

Human Fetal Neurospheres Exhibited Altered Proliferation and Differentiation Properties In Vitro and In Vivo, Depending on the Culture Period

Because large numbers of human fetus-derived cells are difficult to obtain unless they are expanded in vitro, it is essential to evaluate the differentiation and proliferation properties as well as tumorigenic potential of in-vitro-expanded hNS/PCs when considering their clinical use in cell replacement therapies. In the present study, we clearly showed the likely senescence of hNS/PCs maintained for more than 500 DIV, from their differentiation and proliferation properties. More importantly, we also showed the lack of tumorigenicity of hNS/PCs-250 over the long term in vivo. This is the first report in which the in vivo tumorigenicity of transplanted in-vitro-maintained hNS/PCs was evaluated by long-term monitoring with BLI.

The in vitro ATP assay (Fig. 1A) and in vivo BLI study (Fig. 3A) showed that hNS/PCs-250 had significantly higher growth and survival rates than did hNS/PCs-500 both in vitro and in vivo. This difference between hNS/PCs-250 and -500 in vitro was attributable to a reduced proportion of dividing cells in the hNS/PCs-500, shown by positive staining for Ki67 or PCNA, or in the cell cycle of S/G2/M, but not to the proportion of annexin V-positive apoptotic cells. In addition, the proportion of $CD133^{+}$ undifferentiated hNS/PCs was lower in the older hNS/PCs-500 than in the younger hNS/PCs-250. Thus, hNS/PCs seem to lose their ability to self-renew and acquire the properties of committed progenitors or postmitotic cells during their long-term culture in vitro.

Interestingly, this difference in self-renewability between hNS/PCs-250 and -500 seemed to be correlated with an alteration in their differentiation potentials. hNS/PCs-250 exhibited more neurogenic and fewer gliogenic properties than hNS/PCs-500 (Fig. 2A,B). Given that the $CD24^{+}$ cells are proposed to be associated with the population of committed neuronal progenitors and neurons (Calaora et al., 1996; Shewan et al., 1996; Doetsch et al., 1999; Murayama et al., 2002; Nieoullon et al., 2005; Pruszak et al., 2007), the significantly higher proportion of $CD24^{+}$ cells in hNS/PCs-250 than in hNS/PCs-500 (Fig. 2C,D) also supports the idea that the hNS/PCs-250 contain more neurogenic progenitors than do the hNS/PCs-500, which contain more gliogenic progenitors. Taken together, these findings suggest that human fetal neurospheres lose multipotent and self-renewable hNS/PCs, which are replaced by progenitors committed to become glial cells, after long-term maintenance in vitro.

Long-Term Observation by BLI Revealed hNS/PCs To Be Nontumorigenic up to 6 Months After Their Transplantation Into NOD/SCID Striatum

Although some reports have examined the safety of grafted cells, including their long-term potential for tumorigenicity, by histological analyses, this type of analysis does not allow the dynamics of donor cells to be observed over time in the same live animal. In the present study, we monitored the survival of hNS/PCs grafted into the NOD/SCID striatum by BLI. Because we do not have to sacrifice the animals at each time point for histological analysis, we can repeatedly examine the same grafted animal and sequentially evaluate the in vivo tumorigenicity of the donor cells. This monitoring system allows a more accurate analysis than the conventional intermittent histological method.

The findings that the photon counts of engrafted hNS/PCs-250 decreased to 12.8% of the initial count within 8 weeks after transplantation and that their signals were maintained thereafter for up to 6 months without any tumorigenic proliferation, unlike the U87MG glioblastoma cell line, suggested that hNS/PCs-250 are not tumorigenic. These results were confirmed by histological analyses. The Venus-positive area 6 months after transplantation was no greater than the area 3 months after the surgery. No Ki67- or PCNA-positive proliferative cells were observed 6 months after the transplantation. HE staining indicated a pathology that lacked any malignant invasive behavior. Taken together, these results strongly indicate that the hNS/PCs-250 were negative for tumorigenicity. Surprisingly, the Venus-positive grafted cells were still positive for Nestin even 6 months after the transplantation, but they were negative for the proliferation markers Ki67 and PCNA (Fig. 5E). Moreover, the density of the grafted cells was much lower in animals 6 months after grafting than 1 month after grafting (Fig. 5D). Although we cannot clarify the properties of these Nestin-positive but proliferation marker-negative cells, they might reside in the grafted sites as dormant neural stem cells. Therefore, further evaluation of the safety of these donor cells, such as observation periods much longer than 6 months, is warranted.

In conclusion, we showed that the maintenance and safety of transplanted hNS/PCs could be assessed by monitoring the dynamics of these cells in vivo using BLI. Our present study provides a reliable system for evaluating the tumorigenicity of hNS/PCs in vivo and addresses several issues that are prerequisites for the clinical application of hNS/PCs, including defining their properties in vitro and in vivo and their tumorigenicity when transplanted after long-term maintenance in culture. Taken together with previous reports, our data indicate that the prospect for the future application of hNS/PCs to cell replacement therapies for neurological disorders is very promising.

ACKNOWLEDGMENTS

We are grateful to Dr. S. Mitani for the anti-GFP antibody; Drs. T. Nomura and K. Tamaoki for the NOG mice; Dr. M. Yamasaki for the human neural

stem/progenitor cells; Dr. H. Miyoshi for the lenti-virus vector; Dr. Y. Toyama for continuous encouragement; Mrs. Y. Yamashita, Ms. A. Yokokawa, and Mrs. T. Harada for technical assistance; and all the members of Dr. Okano's laboratory for encouragement and kind support.

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CIMID-713; No of Pages 21

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Comparative Immunology, Microbiology and Infectious Diseases xxx (2009) xxx–xxx

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Descriptive analysis of the prevalence and the molecular epidemiology of *Mycobacterium avium* complex-infected pigs that were slaughtered on the main island of Okinawa

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Accepted 17 March 2009

Abstract

Recent genetic studies have revealed that several epidemiological factors affect *Mycobacterium avium* complex (MAC) infection in pig populations. However, mechanisms underlying the spread of MAC infection among hog farms have not been clarified. In consideration of this situation, we cross-sectionally investigated the mechanisms underlying the spread of MAC on the island of Okinawa. Pigs slaughtered (n = 706,763) and 331 hog farms on Okinawa were surveyed during the years 2002–2004. Two outbreaks of MAC infection were occurred in several farms during survey period. Bacteria were isolated from randomly selected pigs and genotype of isolates was determined by using genetic finger printing methods with the insertion sequence (IS) *1245* restriction fragment length polymorphism (RFLP). Most isolates had large numbers of IS*1245* copies, while strains with low copy numbers of IS*1245* and isolates without IS*1245* were seen in few farms. MACs strains were repeatedly isolated from pigs of the affected farms during the survey period. Those farms with an identical pig rearing systems showed synchronic changes in the prevalence of MAC infection. An industrial farm without an outbreak had an independent pig flow, but maintained distinct MAC strains. Multivariate analysis did not reveal independent factors for the prevalence of the MAC infection. These findings suggest that there were three clusters distinguished genetically in the main

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0147-9571/$ – see front matter
doi:10.1016/j.cimid.2009.03.002

island of Okinawa, which were potentially spread by common pig flow. However, the outbreaks occurred because of unspecified conditions on each farm environment.

Keywords: *Mycobacterium avium* complex; Pigs; Epidemiology; Outbreak; Environment

Résumé

De récentes études en génétique ont dévoilé que plusieurs facteurs épidémiologiques ont des répercussions néfastes sur le complexe *Mycobacterium avium* (MAC) des porcs, ce qui les rend malades. Cependant, les mécanismes sous-jacents à la diffusion de l'infection du MAC dans les élevages de porcs n'ont pas été clairement identifiés. Par conséquent, nous avons examiné les mécanismes latents à la propagation du MAC sur l'île d'Okinawa en découpant la population totale des porcs en groupes d'animaux présentant les mêmes caractéristiques. Notre étude recense donc 706 763 bêtes réparties dans 331 fermes. Sur toute la période de nos travaux, le taux moyen des infections du MAC atteind au maximum 9,2% des animaux. Les fermes présentant des systèmes d'élevages identiques ont montré des modifications simultanées dans la fréquence des infections, et les organismes aux mêmes caractéristiques génétiques ont été séparés des porcs de ces fermes.

Une ferme d'élevage en batterie dont les animaux proviennent d'un marché indépendant ne présente aucun pic d'infection, et garde des distinctions génétiques au niveau du MAC. Ces conclusions suggèrent qu'il y avait trois groupes distingués génétiquement dans l'île principale d'Okinawa qui s'est été étendu potentiellement par courant du cochon commun. Cependant, les premières manifestations se sont produites à cause de conditions non spécifiées sur chaque environnement de ferme.

Mots clés : le complexe *Mycobacterium avium* ; Porcs ; Épidémiologiques ; Pic d'infection ; Environnement

1. Introduction

Granulomatous lesions are frequently observed during edibility inspection of slaughtered pigs. Although *Mycobacterium* (M) *bovis*, *M. avium* complex (MAC), *M. fortuitum*, *M. chelonae* and *Rhodococcus equi* have been reported as causative pathogens [1–6], the frequency of identification is higher in MAC [2,7,8]. MAC, which is a complex of *Mycobacterium* (M) *avium* and *M. intracellulare*, is an ubiquitous organism and zoonotic pathogen; *M. avium* can be further subdivided into subspecies corresponding to the serotyping divides, with 28 serovars and with restriction fragment length polymorphism (RFLP) typing with the insertion sequences (IS) *900* [9] */901* [10] / IS*1245* [11] as probes. These subspecies include *M. avium* subsp. *paratuberculosis* (IS*900*+/IS*901*−/IS*1245*−), *M. avium* subsp. *avium* {serotypes 1–3, IS*900*−/IS*901*+/IS*1245*+ (low copy umber)}, *M. avium* subsp. *silvaticum* (IS900−/IS901+/IS*1245*+) and *M. avium* subsp. *hominissuis* {serotypes 4–6, 8–11 and 21, IS900−/IS*901*−/IS*1245*+ (usually high copy number)} [12,13]. *M. intracellulare* mainly causes respiratory infections in humans, and results in the formation of granulomatous lesions in the lungs and the bronchial system [14]. *M. avium* subsp. *paratuberculosis* is the etiological agent of a type of severe enteritis in ruminants known as Johne's disease [15]. *M. avium* subsp. *avium*

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originates from avian species [12] and is particularly virulent in poultry [16]. *M. avium* subsp. *silvaticum* is isolated from parenchymatous organs of wood pigeons with a tuberculous pathology [17]. *M. avium.* subsp. *hominissius* is the most prevalent opportunistic pathogen for pigs and humans [5,18,19].

M. avium generally causes localized lymphadenitis to a pig. In contrast, *M. avium* forms progressive destructive lesions in the lungs of human immunocompetent hosts, or forms disseminated lesions in human immunocompromised hosts. Some researchers have reported about the homology of the strains isolated from humans and pigs [19,20–23]. They showed highly polymorphic multi-band profiles of human isolates that share a high degree similarity with a significant proportion of isolates from pigs using genetic finger printing methods by IS*1245* RFLP. The isolates from human patients were distributed randomly among the clusters of porcine isolates [19]. Tirkkonen et al. reported tat IS*1245* RFLP patterns of 38% of the porcine and human *M. avium* subsp. *hominissuis* isolates showed the similarity over 90% [22]. These finding indicates that pigs may be an important vehicle for *M. avium*. For this reason, eco-epidemiological researches about *M. avium* are important from the point of public health.

In the pig population, sporadic outbreaks of mycobacteriosis have been reported from the industrialized countries [2,5,24–26]. Previous studies have been show that infected pigs, small free-living wild animals, non-vertebrates, and especially farm-type environments are the main sources of the causal agents of mycobacterial infections for pigs [27–32]. Birds are the most popular infectious source of *M. avium* subsp. *avium* [32], while environment is that of *M. avium* subsp. *hominissuis* [12,24,32,33]. Recently, *M. avium* with the identical genotype were isolated from infected pigs as well as the sawdust used as bedding material [5]. In addition, Gardner and Hird have shown that infrequent exposure to a common environmental source is the most important factor in the spread of these organisms [26]. However, the propagation mechanism among farms has not been fully explained in these studies.The current study was undertaken to determine a mechanism for the propagation of MAC infection among hog farms on the Okinawa main island using descriptive epidemiology and genotyping of isolated MACs. In addition, we considered the epidemic factors of MAC infections in the pig population.

2. Material and methods

2.1. Epidemiological background

The main island of Okinawa is located in Okinawa prefecture, which is approximately 500 km from the main islands of Japan. There are two pig markets in Okinawa prefecture and they are supplied piglets from breeding farms which are approved by an official organization. The consistent managed farms purchase piglets for breeding from the pig markets. Fattening farms also purchase piglets from the pig markets. Cross-fertilization of pigs is usually performed on each farm. The actual number of hog farms varied from 343 to 407, and the number of evaluated hog farms varied from 278 to 331 during the surveillance period (Table 1). There are some reasons for this discrepancy: (1) there are uncounted farms include breeding or growing farms that do not send pigs to the slaughter houses; (2)

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Table 1
Epidemiological analysis and prevalence fluctuation in slaughtered pigs during outbreaks.

	Baseline (9 months) Apr.–Dec. 2002	First outbreak (11 months) Jan.–Nov. 2003	Second outbreak (6 months) Dec. 2003–May 2004
Total number of hog farms in Okinawa[a]	407	382	343
Number of hog farms evaluated (% evaluated)	278 (68.3)	312 (81.7)	331 (96.5)
Number of infected hog farm (% affected)[b]	130 (31.9)	179 (46.9)	129 (37.6)
Total number of pigs slaughtered in Okinawa	295,614	323,399	189,604
Total number of pigs evaluated (% evaluated)[c]	292,878 (99.1)	271,307 (83.9)	142,578 (75.2)
Total number of MAC infection (%)[d]	906 (0.3[e])	5,445 (2.0[e])	7,015 (4.9[e])
Local infection (%)	879 (0.3[e])	5,213 (1.9[e])	6,672 (4.7[e])
Systemic infection (%)	27 (0.01[e])	232 (0.09[e])	343 (0.24[e])

[a] The total number of hog farm includes the number of farms that did not ship pigs during the each of the periods.

[b] Hog farms those having many depositary hog farms were considered to be one hog farm.

[c] Pigs slaughtered at isolated slaughtered houses could not be examined except for those located on the Okinawa main island.

[d] Infected pigs were classified into two categories. If a gross lesion was observed only in the mesenteric or submaxillary lymph nodes, it was categorized as having a local infection; and if the gross lesion was observed in the visceral organs and/or dressed in carcass lymph nodes, it was categorized as having a systemic infection.

[e] The percent figures were calculated using the total numbers of pigs evaluated.

some industrial farms being followed by several depositary hog farms are considered to be identical. The hog farms were divided into four groups (A, B, C and D) according to the criterion of whether there was a common pig rearing system or not. Each group is organized by different companies and the major hog farms in the Okinawa islands. Farms within each group share piglets for fattening/breeding and feedstuffs, but each farm within the group is independent. Groups A–C represent large hog farms managed by different organizations in Okinawa prefecture. They employed strict hygiene controls using an all-in/all-out batch system for each grower unit and followed the multi-site pig production system. This latter system refers to the rearing of various age groups of swine at different locations [34]. Basically, the fattening was performed on a concrete floor without bedding material like sawdust. The pigs are bred in an opening pig pen. Fattening pigs are not allowed to graze. Group A represented the largest share of pork meat production in Okinawa prefecture and are divided into three large-scale hog farms (farms AI, AII and AIII) that use independent accounting systems. The AI functions as one of the reproduction centers in Okinawa prefecture and performed the central role in the breeding supply of candidate pigs to the piggeries belonging to Group A.

We investigated 706,763 pigs between April 2002 and May 2004. This accounts for 87.4% of the total number of pigs (n = 808,617) slaughtered in the Okinawa prefecture during this period. Approximately 100 pigs each year were imported from outside the Okinawa prefecture before 2002, which declined to 37 in 2003, and 35 in 2004. All slaughtered pigs were raised on islands in Okinawa. The number of pigs sent from Group A was 165,250 (23.4% of the total number of pigs investigated), of which Farm AI 32,072 (4.5%), Farm AII 49,472 (7.0%) and Farm AIII sent 83,706 (11.8%) respectively. Farms in

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Groups B and C are typical organizationally managed hog farms in the Okinawa prefecture. Group B farms typically provide over 25% and Group C farms over 14% of pigs slaughtered. In our study, we included 238,703 (33.8%) from farms in Group B and 58,144 (8.2%) from farms in Group C. We included a total of 244,666 (34.6%) slaughtered pigs that were sent from privately managed hog farms (Group D) in this study. We counted the prevalence of infection based on macroscopic observation of tuberculoid lesions in the organs of slaughtered pigs. Then, the prevalence per hog farms was calculated based on slaughter registers.

2.2. Macroscopic examination

Macroscopic examination was performed by trained veterinary meat inspectors. Assessment criteria of the lesions were based on the provisions stipulated in the Standard Procedure Manual of the Japanese National Meat Hygiene Inspection Organization. The sub-maxillary lymph nodes and the mesenteric lymph nodes were incised. Lesions of the lymph nodes are characterized by caseous foci, which range in size from a pinhead to a broad bean. We distinguished granulomatous lesions by MAC infections from other similar lesions such as nodules caused by *R. equi*, *Salmonella*, toxoplasmosis and ascarid infections. We also inspected other organs and the regional lymph nodes in more detail when caseous lesions were found in the liver.

The pigs were divided into locally infected and systemically infected groups according to the spread of granulomatous lesions throughout the body; local infection was defined as a carcass with granulomatous lesions macroscopically found only in the sub-maxillary or mesenteric lymph nodes, while systemic infection was defined as the presence of additional granulomatous lesions in visceral organs.

2.3. Histopathological examination

Histopathological studies were performed using tissues, which were taken from all of the systemically infected pigs. The affected tissues were macroscopically removed from the organs and fixed immediately in a solution containing 10% neutral buffered formalin fixative overnight at room temperature. Serial paraffin sections were cut at 3 μm thick using a microtome and thaw-mounted on glass slides. Tissue sections were stained with hematoxylin and eosin (HE). We distinguished the granulomatous lesions histologically from other similar conditions such as other granulomatous diseases.

2.4. Culture methods

Isolation of bacteria was performed using tissues taken from all of the systemically infected pigs. The specimens including lesions (about 350 mg) were collected and homogenized with ceramic beads in 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 below 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

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Ogawa slant medium (Kyokuto Company Limited, Tokyo, Japan) and into Middlebrook 7H9 medium (Difco, Detroit, MI, USA) with 10% ADC enrichment (Becton, Dickinson and Company, Maryland, USA), followed by incubation for 4 weeks at 37 °C. A polyclonal colony on the Ogawa slant medium was observed occasionally, and included *M. avium* and other non-virulent mycobacteria. Therefore, we repeatedly isolated monoclonal colonies. Accordingly, in this investigation, we collected all strains that were isolated without fungal or bacterial contamination.

2.5. Identification of isolates

The acid-fast bacilli on the sliced sections or the smear of tissue suspension were confirmed by the method of Fite-Farraco stain. All of the isolated strains were also distinguished between *M. avium* and other subspecies by the multiplex PCR method [35] and DNA–DNA hybridization with Amplicor Mycobacterium (Roche Diagnostics K.K., Switzerland).

2.6. Analysis of epidemic factors

The following five independent variables were included in the analysis of variables affecting the prevalence of mycobacterial infection on hog farms: farm size, density of farms, production district, production use and sanitary management. Farm size was classified into three categories based on the total number of pigs sent to the slaughterhouses during the survey: small (<623), medium (623–2340) and large (>2341). Farm density was classified into two categories: hog complexes where several farms were gathered in one place, and scattered farms, where several farms lie scattered within the area. Production district was classified into four categories by administration division: north, central, south and the isolated islands divisional. Also, the precise location of hog farms or piggeries was investigated based on the information obtained from the local authorities. Production use was classified into two categories: pigs bred for edible meat, and pigs bred for breeding. As a result, this classification ultimately classified the pigs in terms of their age (about 6 months or over 1 year). Sanitary management was classified into two categories: all-in/all-out category, in which the pigs are sent as a unit, and the picked-up category, in which the pigs are not sent as a unit. This classification reflects whether or not a farm performs strict hygiene management, for example, multi-site systems including farrowing accommodations, dry sow stalls, trough feeding, routine cleaning and disinfection of pen, disinfectant foot-baths and arrangement of exclusive employees at each piggery.

2.7. Genotyping of isolates

To analyze transmission routes among pigs in the two outbreaks of MAC infection observed in this study, genotype of the isolates was determined. The 93 strains for genotyping (including 4 at baseline, 67 at the first outbreak, and 22 strains from the second outbreak) were obtained from 32 hog farms. The isolates were examined by the IS*1245* RFLP method proposed by van Soolingen et al. [36] because this will allow future

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comparison of results obtained in different laboratories and the stability of this insertion element was confirmed in several laboratories [37,38]. DNA was isolated according to the method described by van Soolingen et al. [36]. Approximately 5 μg of purified mycobacterial DNA was digested with restriction endonuclease *Pvu* II. After separation by electrophoresis in agarose gel (I.D.$_{NA}$ Agarose, Main, Camblex Bio Science Rockland, Inc.), DNA fragments were exposed to UV in a transilluminator and transferred from the gel to a nylon membrane by vacuum blotting. DNA was fixed and hybridized with a labeled probe according to a previously described method [36]. The band positions for the IS*1245*-containing restriction fragments were compared with those of a molecular weight marker. The RFLP profiles show approximately a maximal of 27 copies of IS*1245* [11] and were classified to the bird-type group, which has a profile that comprises three stable IS*1245* bands, a low copy number group, a high copy number group, and a no copy number group, as described previously [39,40]. We repeated the RFLP analysis, but the results were consistent.

2.8. Analysis of MAC serotype

Pig isolates ($n = 40$) with the IS*1245* and human clinical isolates ($n = 17$) as reference strains, were assayed for serotyping. Human clinical isolates were obtained from patients with pulmonary MAC disease, who had been admitted to the Okinawa National Hospital or University of the Ryukyus Hospital between 2000 and 2005. These isolates were identified as *M. avium* by biochemical analyses and DNA probes (AccuProbe Culture Identification test, Gene Probe). The strains obtained from patients ($n = 17$) and pigs ($n = 40$) were classified into 28 serovars on the basis of the antigenic glycopeptidolipids (GPLs) present on the cell surface. The serovars of the strains were identified using liquid chromatography/mass spectrometry [41]. In some cases, we found a lack of GPL or apolar GPL rather than serospecific GPL. Apolar GPL did not have serospecific oligosaccharides in the GPL. These analyses were done at the Toneyama Institute for Tuberculosis Research at the Osaka City University Medical School.

2.9. Statistical assessment

Data were analyzed using SPSS (SPSS Japan Inc., Tokyo). Comparison of the groups was done with the paired *t*-test. χ^2 tests were used to compare categorical variables between groups. One-way ANOVA was applied for univariate analysis and logistic multiple regression was used for multiple variables to identify the specific epidemic factors. $P < 0.001$ was considered to be statistically significant.

3. Results

3.1. Epidemiological background

Between 1997 and 2003, the prevalence of MAC was broadly stable at less than 0.3% (Fig. 1), although there was a small peak in April 1999 reaching 1% (Fig. 1). However, the

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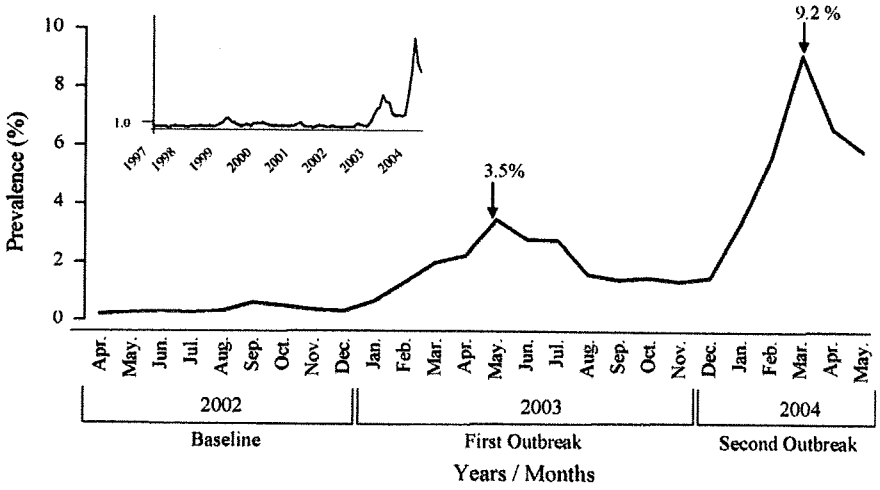

Fig. 1. Prevalence of slaughtered pigs with mycobacterial lesions in Okinawa prefecture during 2002–2004. Two peaks of prevalence were observed in the early part of the 2003 and 2004 summers.

prevalence rate started to increase in January 2003, and peaked in May 2003 (Fig. 1). Since then, the prevalence rate decreased to less than 2% by August 2003. The prevalence rate increased again starting in December 2003 (Fig. 1). Based on these findings, the interval was divided into three periods; the baseline (dormant occurrence period), the first outbreak and the second outbreak (Fig. 1, Table 1). Table 1 shows the actual number of hog farms evaluated, which were farms that sent raised pigs during the survey period, and the number of affected hog farms, which were those that sent pigs infected with MAC. In addition, this table shows the number of evaluated pigs, which were the pigs that were slaughtered during the survey, and the prevalence of MAC infection among those pigs. Of the 292,878 pigs evaluated during the baseline period, mycobacterial lesions were detected in 906 (0.3%) (Table 1). A significantly increased prevalence of locally infected or systemically infected pigs was observed during the following outbreak stages compared with the baseline period (Table 1). Farm AII shipped the largest number of infected pigs during the first outbreak (Fig. 2). In the second outbreak, Group C was primarily responsible for the increased prevalence (Fig. 2).

3.2. *Identification of species*

Most of the affected pigs with MAC were clinically silent. The total number of samples isolated with MAC from systemically infected pigs was 94. Of these, 93 strains of the positive cultures were identified as *M. avium* and the remaining mycobacterium was identified as *M. neoaurum*.

3.3. *Pig flow, genotypes and prevalence fluctuation*

To evaluate the mechanisms of propagation of MAC infection among hog farms, we selected 32 hog farms, as shown in Fig. 3. Most hog farms purchased piglets for breeding

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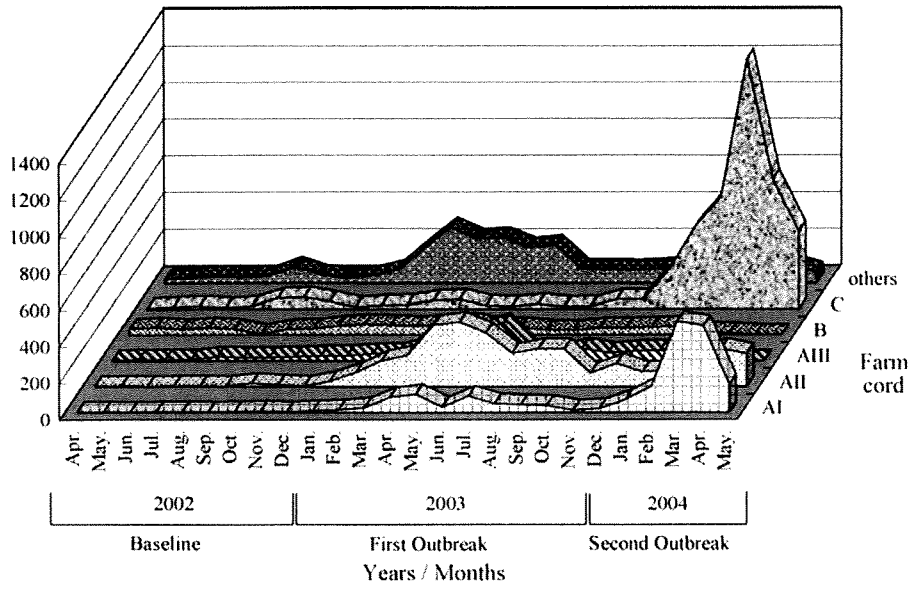

Fig. 2. Change in infected pigs at all of the main hog farms. Farm name codes are correlating to the codes shown in subsequent Fig. 3. Area charts illustrate the infection head-count every hog farm changing with progress in time.

from pig markets located within Okinawa prefecture. These piglets were raised in breeding houses on the respective farms. However, the hog farms belonging to Group B did not purchase piglets from the market, but introduced all piglets from their own breeding house (Fig. 3).

Based on the IS*1245* RFLP analysis, all of isolates were classified into three groups according to the copy number of IS*1245* (low, high or no copy number group; hereafter referred to as Type I and Type II and Type III, respectively) (Fig. 4). The Type II was dominant in the pigs sent from the various hog farms; however, MACs with the Type I genotype had six identical bands and were the minority strain in Group A and among privately managed hog farms (Fig. 3). Infected pigs shipped from the hog farms belonging to Group B had MACs of Type III genotype. Strains with these genotypes were isolated repeatedly in the identical fattening herd (data not shown). On some hog farms, strains of an identical genotype were isolated from both sows raised in a breeding house and piglets nursed in the breeding house. Each strain isolated in the first outbreak and second outbreak showed similar genotypes (Fig. 4). Bird-type strains were not isolated thorough entire investigation period.

We then evaluated the prevalence of MAC infection during each period according to this pig rearing systems, as shown in Fig. 3. Each of the deposit farms belonging to Farm AII that received piglets from a mother farm showed a high prevalence of MAC infection during the second outbreak period, but Farm AI showed a gradual increase in prevalence during the three periods (Fig. 3). Different patterns of prevalence were seen for the privately managed hog farms (Fig. 3). In Group C, the rate of prevalence (the average value for each deposit farm) was very high during the second outbreak period (Fig. 3). All deposit farms belonging to Group B had an extremely low rate of prevalence during all periods, and there were no systemically infected pigs (Fig. 3).

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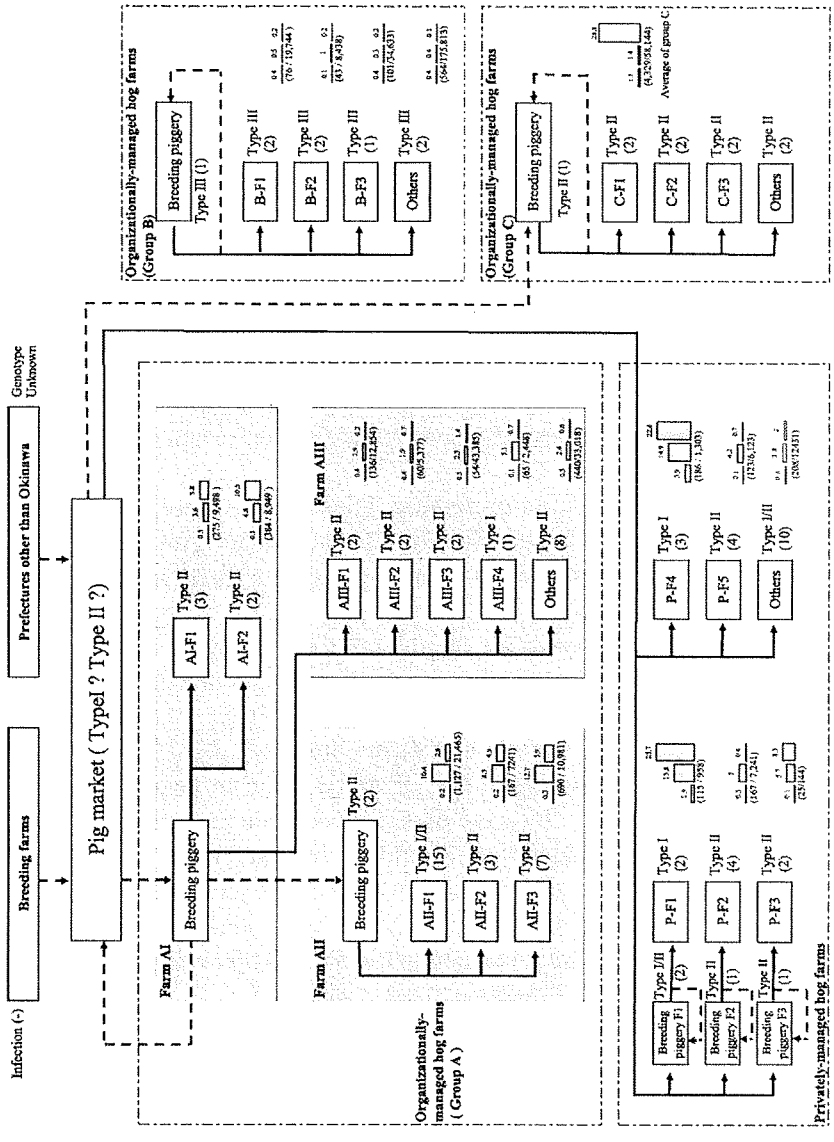

Fig. 3. Flow chart of the pig market in Okinawa prefecture, IS*1245* RFLP types and the fluctuation of prevalence for each hog farm. We selected 32 hog farms to evaluate prevalence and to perform genotyping, since it was possible to analyze the transmission route of pigs in these 32 hog farms. We described only 20 fatting farms and as "others" including remain farms. Strains having Type II IS*1245* RFLP genotype are dominant among these hog farms, but strains having Type I genotype are observed only on certain hog farms. Piggeries belonging to Group B which are organizationally managed had only Type III. Bar graphs indicate the fluctuation in prevalence at the piggeries during the investigated periods: the baseline period (left side bar), the first outbreak period (middle bar), and the second outbreak period (right side bar). "F" of each code represents fattening pigs. A dashed line shows the flow of pigs for breeding. A solid line shows the flow of pigs for pork meat.

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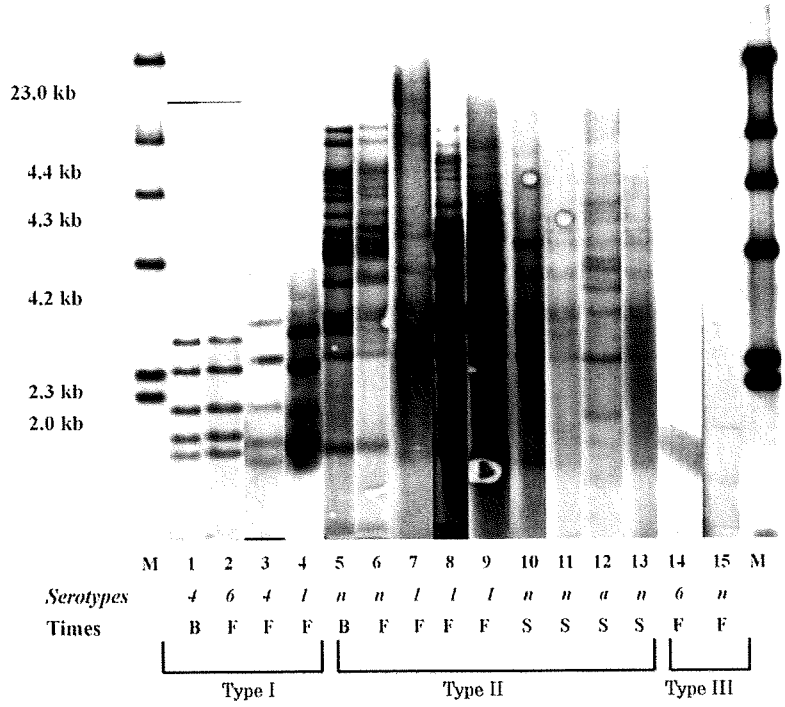

Fig. 4. Examples of IS*1245* RFLP typing of the *Mycobacterium avium* strains ($n = 93$) isolated from affected pigs. Types were distinctly classified into three clusters: one cluster, Type I having identical six bands, and another cluster, Type II having multiple bands where most are identical. Type III was negative for IS*1245*. Farm name codes correlate to the codes shown in Fig. 3. 1: Privately managed farm, 2: Privately managed farm, 3: Farm AII, 4: Farm AIII, 5: Privately managed farm, 6–7: Farm AI, 8–9: Farm AII, 10–11: Farm AII, 12–13: Group C, 14: Group B-Others, 15: Group B-F1, n: no typing, a: apolar type, M: molecular weight markers, B: baseline, F: first outbreak, S: second outbreak.

3.4. Regional aggregation of MAC infection

We investigated the status of regional aggregation in the rural area of Okinawa prefecture. Fig. 5 represents a typical area with a high density of hog farms. This is located in a remote area away from other piggeries. Several hog farms belonging to the two organizationally managed groups are located within 1000 m of this area. A piggery, which belonged to Farm AII, exhibited an outbreak, while the B farming family maintained a low infection rate throughout the investigation period. Most of these hog farms belonging to AII did not use sawdust or other bedding materials; therefore, the pigs were raised on a slatted floor. All hog farms belonging to Group A showed a high prevalence of MAC infection during the first outbreak period (Fig. 5). Hog farms belonging to Group B showed an extremely low prevalence of MAC infection throughout all periods (Fig. 5). Similar results were obtained from analysis of hog farm complexes (data not shown).

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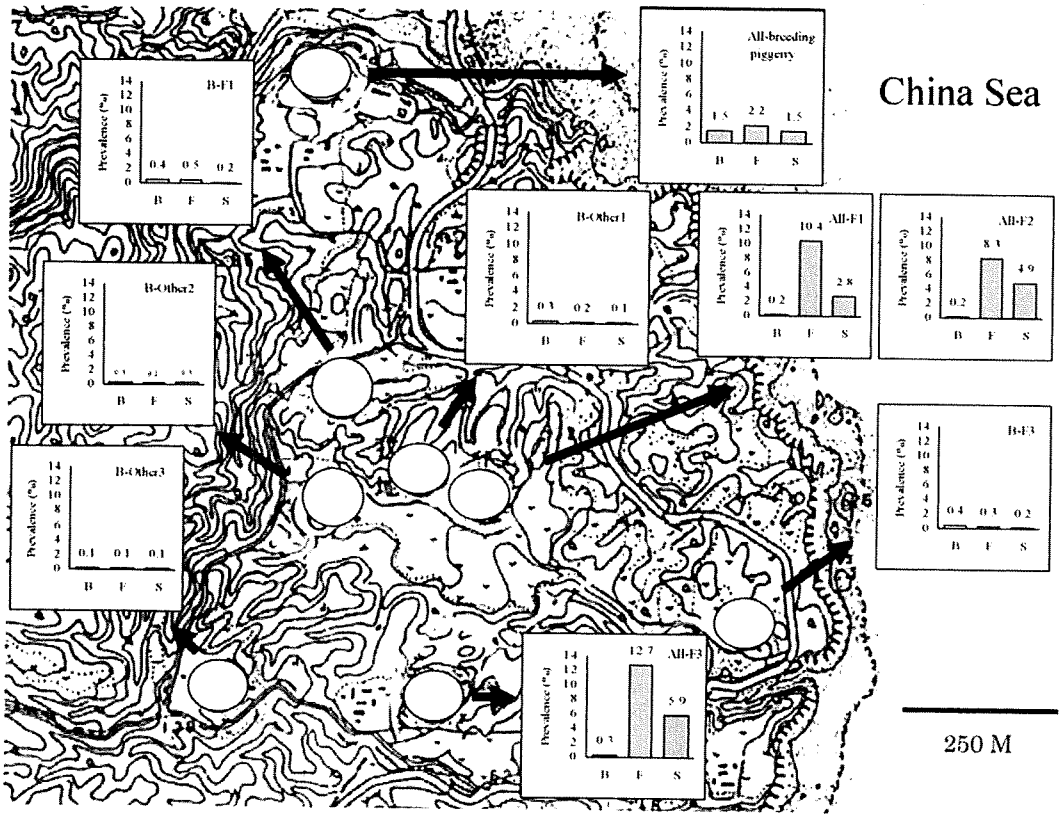

Fig. 5. Location and prevalence for each piggery in the rural area of Okinawa prefecture. The codes are piggery names correlating to the codes shown in Fig. 2. Bar graphs indicate the change in prevalence of MAC infection in piggeries. B: baseline period, F: first outbreak period, S: second outbreak period.

3.5. Epidemic factors of hog farms

We classified the hog farms into two categories; those with or without MAC infected pigs at the baseline period. The fluctuation in prevalence at each hog farm was successively followed. The prevalence of MAC-affected hog farms at the baseline period was significantly higher during the following outbreak periods than those of non-affected farms at the baseline period (Table 2). Using the univariate analysis, we found significant differences in terms of farm size, production district and application purpose (Table 3). However, there was no significant difference in each factor using the multiple logistic regression model (Table 4).

3.6. Serotyping results

The strain obtained from patients ($n = 5$) and pigs ($n = 33$) could be classified into the classical 28 serovars of *M. avium* and other strains were classified into new serotypes, apolar GPL type, and no GPL type (Table 5). In strains obtained from humans, the apolar

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Table 2
Prognostic tree of hog farms classified by the existence of mycobacterial infection.

Baseline Apr.–Dec. 2002		First outbreak Jan.–Nov. 2003		*P*-values	Second outbreak Dec. 2003–May 2004		*P*-values
Total	155		155			155	
Infection (−)	61 (39.4%)	(−)	37 (60.7%)	<0.001	(−)	32 (86.5%)	<0.001
					(+)	5 (13.5%)	
		(+)	24 (39.3%)		(−)	13 (54.2%)	NS
					(+)	11 (45.8%)	
Infection (+)	94 (60.6%)	(−)	1 (1.1%)	<0.001	(−)	1 (100.0%)	NS
					(+)	0 (0.00%)	
		(+)	93 (98.9%)		(−)	17 (18.3%)	<0.001
					(+)	76 (81.7%)	

Statistical analysis was performed between the number of infection-negative and infection-positive samples. Apr: April, Dec: December, Jan: January, Nov: November, NS: not significant. The number of hog farms evaluated ($n = 155$) is the number of farms that shipped raised pigs throughout each period.

Table 3
Analysis of valuables those affect the prevalence of mycobacterial infection in hog farms.

Independent valuables		With or without infection per hog farms		*P*-values[a]
		−	+	
		$n = 61$	$n = 94$	
Farm size	Small (<623)	43 (70.5%)	8 (8.5%)	<0.001
	Middle (624–2,340)	14 (23.0%)	38 (40.4%)	
	Large (2,341<)	4 (6.6%)	48 (51.1%)	
Density of farm	Hog complex	4 (6.6%)	20 (21.3%)	NS
	Scattering farm	57 (93.4%)	74 (78.7%)	
Production district	North	15 (24.6%)	42 (44.7%)	<0.001
	Central	8 (13.1%)	19 (20.2%)	
	South	32 (52.5%)	33 (35.1%)	
	Divisional isolated islands	6 (9.8%)	0 (0.0%)	
Productive use (age)	Fattening (6 month)	38 (62.3%)	91 (96.8%)	<0.001
	Breeding (>1 year)	23 (37.7%)	3 (3.2%)	
Sanitary management	All-in all-out	38 (62.3%)	54 (57.4%)	NS
	Pick up	23 (37.7%)	40 (42.6%)	

[a] To evaluate the differences among multi-groups, one-way ANOVA was used.

GPL was predominantly (10/17: 58.8%). In strains obtained from pigs, the predominant serotypes were Type 1 (15/40: 37.5%) and Type 4 (11/40: 27.5%) (Table 5). Although there were significant overlaps, it was difficult to demonstrate any similarities related to both strains.

In IS*1245* RFLP analysis, most of the isolates with serotype 4 were of genotype Type I, while some isolates with serotype 1 were of genotype Type II (Fig. 4).

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Table 4
Multiple logistic regression analyses for mycobacterial infection of hog farms.

Risk factors	*P*-values	Odd ratios	95%CI
Farm scale	0.008	0.164	0.043–0.619
Density of farm	0.465	0.593	0.146–2.408
Production district	0.220	0.465	1.368–6.472
Productive use (age)	0.313	0.416	0.075–2.290
Sanitary management	0.145	2.311	0.750–7.120

Table 5
Serotyping of strains isolated from humans and pigs infected with *Mycobacterium avium*.

Serotype	Human ($n = 17$)	Pig ($n = 40$)
Type 1	2	15
Type 2	0	6
Type 4	2	11
Type 6	1	1
New serotype	1	1
Apolar GPL	10	2
No GPL	1	4

GPL: glycopeptidolipids.

4. Discussion

The transport of infected animals has long been considered to be the critical factor in the spread of livestock diseases [42]. In the case of bovine tuberculosis the number of cattle imported from an infected area is associated with a high prevalence of the infection [43]. In the present study, a genetically identical bacteria were isolated from the individuals in the farms with the same pig flow. These findings indicate that the pig flow facilitated dissemination of the organism among the hog farms. This hypothesis is supported by the finding that hog farms belonging to the same group showed a synchronous increase in the prevalence of MAC infection. To better clarify the mechanism of transmission, we need to collect evidence that the piglets introduced from the pig market were infected with a genetically identical strain.

As described in the introduction section, the predominant causative strains of granulomatous lesions of pig lymph nodes are *M. avium* subsp. *hominissuis* (serotypes 4–6, 8–1, and 21; genotype IS*1245*+, and IS*901*−) [5,19,22]. In certain regions, strains of *M. avium* subsp. *avium* (serotypes 1–3; genotype IS*1245*+, and IS*901*+) are also causative agents for pigs [44]. *M. avium* subsp. *avium* is the cause of avian tuberculosis and is known to be a contagious disease among birds [16]. Previous studies have suggested that infected free-living birds were the most common source of *M. avium* [45,46]. Therefore, exposure to birds has been proposed to be a source of infection for pigs [32,47,48]. It has been reported that the isolates *M. avium* subsp. *avium* that originate from birds have profiles comprising three stable IS*1245* bands. In contrast, *M. avium* subsp. *hominissuis* show highly polymorphic multi-band profiles [23,36]. In the present study, we identified 15 isolates with serotype 1, but a three-banded bird-type profile was not shown by genotyping.

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In addition, most of the isolates with serotype 1 showed multiple-band profiles and most of the isolates with serotype 4 showed six copies of IS*1245*. Furthermore, various numbers of tandem repeat analysis revealed that isolates of serotype 1 were clustered in a group and the isolates with six copies of IS*1245* were clustered in a group but these clusters differed from a cluster of bird-type strains with IS*901* (these results could not be presented owing to competing interests). However, we cannot exclude the possibility of any existence of *M. avium* subsp. *avium* originating from avians. A recent study in the Czech Republic revealed that some birds are infected with *M. avium hominissuis* [16]. In Sweden, more than half of pig isolates had bird-type profiles [48]. This suggests that the influence of birds must differ depending on the area.

The importance of a free-living non-vertebrate or protozoa as the causal agent of the MAC infection has been reported [29–31,49,50]. Fischer et al. studied the role of syrphid flies for mycobacteriosis of pigs, and concluded that the larvae spread the mycobacteria throughout the pig herds and the surrounding environment [49]. In the present study, since regional aggregation was not recognized through the analysis of MAC infections in the rural area, the role of a vector for propagation of MAC between hog farms was seen as negative on the Okinawa Archipelago. Further investigations are needed to investigate the participation of insects in propagation of MAC infection on the islands within the Okinawa Archipelago.

It has been reported that IS*1245* RFLP patterns can be divided into more than 20 clusters [40] or 10 clusters [19]. A classification of IS*1245* RFLP into three types (Type I–III) seems to have less discriminatory power for the molecular epidemiology of *M. avium* infection in pigs. To answer this question, we considered that these results were obtained because certain strains were conserved in pig populations because the islands of Okinawa are relatively isolated with limited introduction of pigs from outside the prefecture, particularly during the investigated period. Furthermore, all of slaughtered pigs were raised in Okinawa. Moreover, the initiator farm could not be determined because the numbers of isolates collected from each farm were limited in this study. Therefore, we believe that IS*1245* RFLP is useful for the discrimination of the isolates obtained from the islands of Okinawa. However, IS*1245* RFLP may have limitations for analyzing strains with low copy numbers or for resolving extended epidemiologic relationships, as described by Pestel-Caron and Arbeit [37]. Alternatively, Dvorska et al. also proposed a standardized IS*901* RFLP method for typing *M. avium* subsp. *avium* and *M. avium* subsp. *silvaticum* isolates [51]. Previous report demonstrated that the copy number of IS*901* was always greater than that of IS*1245* found in the bird isolates [10,13,39]. Consequently, other detailed genotyping methods such as *Pvu*II-*Pst*I-IS*901*, as proposed by Dvorska et al. [51] or RFLP with new probe of IS*1311*/IS*1245*, as proposed by Johansen et al. [19] will be required in future studies.

In the present investigation, the first outbreak and second outbreak happened in different farms (AII and Group C), while another group of farms (Group B) appeared to be spared any outbreaks. Therefore, the question remains whether there is any relationship between these two outbreaks. We have demonstrated that the strains obtained during both outbreaks were genetically closely related although we could not determine the precise relationship between the two outbreaks in Farm AII and Group C (Fig. 4). Therefore, we considered that the two outbreaks were related to each other. In addition, in this study, we could not

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describe whether Group B was protected from these outbreaks because the number of isolates from Group B was limited and the isolated strains did not possess IS*1245*. However, Group B might be isolated from these outbreaks because Group B did not have a common pig flow with Group A and C.

It has been reported that strict sanitary management, such as the all-in/all-out batch system within each grower unit, is effective in preventing hoof-and-mouth disease and other infectious diseases [42,52]. In the present study, an outbreak was observed in both the hog farms, which used pens continually without floor cleaning, and the hog farms, which used pens non-continually, with floor cleaning after shipping the pigs, based on strict sanitary management system {for example Farms AII and P-F2 (Fig. 3)}. It has been reported that *M. avium* is excreted continuously in feces from infected pigs [53,54]. This indicates the possibility that pig-to-pig transmission can occur by fecal–oral infection, if there is even one infected pig in a herd. Experimentally, the contact infection can be generated within the pig population, but the length of the contact time seems to affect the outcome [53,55–57]. In the present study, *M. avium* with an identical genotype was isolated from pigs belong to the same batch (Type III or II). This indicates that contact infection took place in nature.

In this study, many systematically infected pigs were observed during the epidemic. The role of systemic infection for propagation of MAC in the pig population is not well known. If the amount of MAC excreted from the body of systemically infected pigs is greater than that from locally infected pigs, their role as the source of infection may be important. In patients with AIDS, the amount of acid-fast bacilli can be observed in the affected tissue and in stool samples [58,59]. They may not be an infectious source even though their amount of excretion is significant, since human-to-human transmission has not been proven in patients with AIDS or immunocompetent patients [14]. A small amount of acid-fast bacilli was observed at any stage of granulomatous lesion samples taken from infected pigs in a previous histological study [60]. This indicates that systemically infected pigs are unlikely to be a major infectious source in the population and the increased number of systemically infected individual is a result of the epidemic.

A previous study suggested that the mother sow is the infectious source [61,62]. In the present study, the identical genotype (Type II) was shown in isolates from the sows and the fattening pigs on the same hog farm. These findings suggest the possibility that the piglets were infected through contact with the mother pigs or by the environment of the farrowing house in which they were raised.

Recent studies have used molecular epidemiological methods to determine whether the used bedding materials or feed provided the source of the outbreak [5,24,63]. However, our study did not identify any common factor, such as bedding materials. Matlova et al. reported that an isolate from non-used sawdust has a different IS*1245* RFLP profile compared with an isolate from used-sawdust and affected pigs [5]. In their later study, they reported that the isolates from affected pigs are virulent, but the strains obtained from environmental samples are not virulent for pullets [63]. These findings indicate that fresh sawdust cannot become the epidemic factor, and that infrequent exposure to another common environmental source is the important factor [26]. In addition, since MAC is a ubiquitous organism and can be easily aerosolized [64–66], the eradication of MAC from the environment might be difficult, even if a very strict sanitary management system is introduced.

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In the present study, IS*1245*-negative *M. avium* strains were observed only in Group C, which had its own pig flow and the total prevalence in Group C was lower than that of other Groups throughout the study period (Fig. 3). Komijn et al. observed that only 1 out of 91 MACs isolated from pigs lacked IS*1245* DNA [20]. In addition, a *M. avium* with IS*1245* was commonly isolated (84% of 577 tested) from pig lymph nodes in the Czech Republic [63]. A Japanese genetic study reported that *M. avium* was isolated from pigs at a high rate, and a few other mycobacteria without IS*1245* were also isolated, although it does not confirm a holding ratio of IS*1245* [35]. Thus *M. avium* with IS*1245* is the main pathogen in pigs and the strain without IS*1245* may be maintained under the environment of certain hog farms with original pig flow. However, further studies need to clarify the genetic characteristics of these strains in the Okinawa islands.

Some epidemiological studies in Japan have reported the seasonality of MAC infection (personal communication). A remarkable increase in MAC infection was observed in the summer and fall months in Arizona [24]. In addition, it is known that MAC shows increased tolerance in high temperatures and seems to prefer warm properties [67–69]. Furthermore, Horsburgh et al. showed in prospective cohorts that the occurrence of human MAC disease is more common in tropical than in temperate climates [70]. However, in the present study, a significant difference between prevalence of MAC infection and weather factor was not observed.

We have presented the results of a comparison of MAC serotypes that were isolated from humans and those from pigs, which were infected with *M. avium* during the same interval. The interpretation of similarities in both groups was difficult. In addition, our collaborators are currently using variable numbers of tandem repeats (VNTR) to analyze the genotype of the *M. avium* strains isolated from humans and pigs, including these strains from Okinawa (under submission). The preliminary results indicate that it is difficult to definitively confirm the similarity of both strains. In our previous report, however, we demonstrated the pathogenicity of *M. avium* strains isolated from pigs in experiments using guinea pigs [60]. At present, because we cannot ignore the potential infectiveness and pathogenicity in humans of MAC obtained from pigs, the significance of MAC infection should be considered not only as a zoonotic issue but also a public health issue.

The strength of this study is the extensive investigation of a large number of pigs slaughtered on the islands of Okinawa. However, since this epidemiological study was a cross-sectional study and descriptive analysis, we are unable to clarify the cause of the outbreaks. A prospective study should be performed to precisely evaluate the prevalence and to evaluate the cause of outbreaks.

In conclusion, our present study demonstrated that there were three clusters of infection that could be distinguished genetically in the main island of Okinawa. The stains were potentially spread by a common pig flow, but outbreaks occurred because of unspecified conditions on each farm environment.

Role of the funding source

We declare that there are no study sponsors involved in the study design, or in the collection, analysis and interpretation of data.

Please cite this article in press as: Hibiya K, et al. Descriptive analysis of the prevalence and the molecular epidemiology of *Mycobacterium avium* complex-infected pigs that were slaughtered