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Mycolactone-producing *Mycobacterium marinum* infection in captive Hong Kong warty newts and pathological evidence of impaired host immune function

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ABSTRACT: A mass mortality event of captive Hong Kong warty newts *Paramesotriton hongkongensis* with non-granulomatous necrotic lesions occurred in Taipei Zoo, Taiwan, in 2014. Clinically, the sick newts were lethargic and often covered with water mold *Saprolegnia* sp. on the skin of the body trunk or extremities. Predominant pathological findings were multifocal non-granulomatous necrotic lesions in the liver, spleen, and kidneys and severe skin infection with *Saprolegnia* sp., with deep invasion and involvement of underlying muscles. The possibility of ranavirus infection was ruled out by negative PCR results. Unexpectedly, abundant intralesional acid-fast positive bacilli were found in the necrotic lesions of the liver, spleen, and kidney in all 14 sick newts. PCR targeting the *hsp65*, *ITS* region, and partial *16S rRNA* genes was performed, and the sequence identity from amplified amplicons of *hsp65* and partial *16S rRNA* genes was 100% identical to that of the corresponding gene fragment of *Mycobacterium marinum*. Further molecular investigations demonstrated that the current *M. marinum* was a mycolactone-producing mycobacterium with the presence of *esxA*/*esxB* genes. Mycolactone is a plasmid-encoded, immunosuppressive, and cytotoxic toxin. The possible immunosuppression phenomenon characterized by systemic non-granulomatous necrotic lesions caused by *M. marinum* and the unusual deep invasive infection caused by water mold might be associated with the immunosuppressive effect of mycolactone. Therefore, it should be noted that non-granulomatous necrotic lesions in amphibians can be caused not only by ranavirus infection but also by mycobacteriosis.

KEY WORDS: Hong Kong warty newt · *Mycobacterium marinum* · Mycolactone · *Saprolegnia* sp.

INTRODUCTION

Infectious diseases such as ranavirus infection and chytridiomycosis have recently been suggested to play a major role in the global decline of amphibian populations (Gray et al. 2009, Miller et al. 2011, Van Rooij et al. 2015). The diseases are of particular concern in captive amphibians, as increasing numbers of amphibians are being bred for zoo exhibits, the pet industry, and endangered species repopulation (Densmore & Green 2007, Miller et al. 2011). Ranaviruses have been reported to cause several mass mortality events in wild and captive amphibians in different countries (Gray et al. 2009, Miller et

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al. 2011). At least 72 amphibian species in 14 families are known to have been infected by ranaviruses, and host susceptibility varies among species and developmental stages (Miller et al. 2011). The histopathological lesions caused by ranaviruses are multifocal hemorrhagic and necrotic foci in multiple internal organs, and the renal tubular epithelium is also considered as one of the primary targets (Robert et al. 2005, Gray et al. 2009, Miller et al. 2011). *Batrachochytrium dendrobatidis* is the causative agent of chytridiomycosis, a potentially fatal epidermal infection in amphibians (Van Rooij et al. 2015). Recently, a new species of *Batrachochytrium* sp., named *B. salamandrivorans*, was discovered (Martel et al. 2014). This species, which most likely originated in Asia, is highly pathogenic to salamanders (Martel et al. 2014).

Infectious diseases in amphibians such as mycobacteriosis, do not only affect ectothermic vertebrates, but can also cause disease in mammals, including humans (Densmore & Green 2007). Therefore, the zoonotic potential of some infectious diseases in amphibians and in exotic companion animals should not be overlooked. The lesions caused by mycobacteria are granulomas in the skin and multiple internal organs, with varying numbers of acid-fast positive bacilli (Green et al. 2000, Chai et al. 2006, Ferreira et al. 2006, Sánchez-Morgado et al. 2009, Fremont-Rahl et al. 2011, Haridy et al. 2014). A group of mycobacteria called mycolactone-producing mycobacteria (MPM), harboring a mycolactone-producing plasmid, is able to generate an immunosuppressive and cytotoxic toxin (mycolactone), which is considered an important virulence factor in MPM infection (Hong et al. 2008, Pidot et al. 2008). Although only one species of MPM, named *Mycobacterium ulcerans*, is reported to cause a disease called Buruli ulcer in humans (Pidot et al. 2010), other species of MPM, including *M. liflandii*, *M. marinum*, and *M. pseudoshottsii*, have been reported to cause disease in amphibians and fish (Mve-Obiang et al. 2005, Rhodes et al. 2005, Ranger et al. 2006, Hong et al. 2008).

From November 2014 to January 2015, a mass mortality event among captive Hong Kong warty newts *Paramesotriton hongkongensis* with non-granulomatous necrotic lesions occurred in the Taipei Zoo, Taiwan. This study reports on the pathological and molecular investigations of 14 newts conducted using histopathological examination, histochemical staining, and PCR to investigate possible ranaviruses and mycolactone-producing mycobacteria infection in these cases. The possible mechanisms of the unusual non-granulomatous necrotic lesions are also discussed.

MATERIALS AND METHODS

Sample collection

All 14 Hong Kong warty newts were captive in a semi-aquatic 20-gallon tank in an isolated building of the Taipei Zoo, Taiwan, for several years. During this time, the conditions of the captive environment and animals were stable. In November 2014, the thermostat and water circulation systems in this building malfunctioned for one day because of a power outage. A few days later, lethargy and water mold (*Saprolegnia* sp.) covering the skin of the body trunk or extremities were observed in some of the animals. Although a bath treatment with chlorine dioxide (0.5 ppm overnight once) for water mold disease was performed, and newts with more severe water mold were further treated with Dessamor (Aquarium Münster) according to the manufacturer's instructions, the clinical signs of these newts did not improve and they subsequently died between November 2014 and January 2015, and eventually the entire group died off. Necropsy and fresh liver tissue collection were performed in 4 individuals, and representative tissue samples were collected and fixed in 10% neutral buffered formalin. For the other 10 individuals that did not have fresh liver preserved, their bodies were opened, examined grossly, and directly preserved in 10% neutral buffered formalin. The samples from these 14 individuals were submitted to the Graduate Institute of Molecular and Comparative Pathobiology, School of Veterinary Medicine, National Taiwan University, for further examination.

Histopathology

For histopathological examination, formalin-fixed and paraffin-embedded (FFPE) tissue blocks (n = 14) were prepared, sectioned at 4 µm, and stained with hematoxylin and eosin (H&E), acid-fast (Ziehl–Nielsen's method), and Gomori methenamine silver (GMS).

DNA extractions

Fresh liver tissue samples from 4 necropsied individuals were used for DNA extraction by the FastDNA™ Spin Kit (MP Biomedical) according to the manufacturer's instructions. These DNA extractions were then used as PCR templates. DNA was not

extracted from the 10 individuals directly preserved in 10% neutral buffered formalin.

PCR

To detect ranavirus DNA, primer sets targeting the DNA polymerase, major capsid protein (MCP), and neurofilament triplet H1-like (NF-H1) protein genes of ranavirus were used in PCR reactions following procedures used in previous studies (Sun et al. 2006, Holopainen et al. 2009). DNA from a tissue known to be infected by ranavirus was used as the positive control, and reaction mixtures without the DNA template were used as the negative control.

Mycobacterial species identification by PCR and DNA sequencing was performed with primer sets targeting the heat-shock protein 65 gene (*hsp65*), the internal transcribed spacer (*ITS*) region, and partial 16S rRNA gene (Hughes et al. 2000, Kim et al. 2005, Mokaddas & Ahmad 2007) (Table 1). The obtained amplicons were directly sequenced and compared with sequences available in GenBank using the BLAST server from the National Center for Biotechnology Information.

To detect the mycolactone-producing plasmid in the DNA isolated from the fresh liver samples, 3 primer sets targeting different gene regions of mycolactone-producing plasmid, namely, putative mycolactone polyketide synthase A (*mlsA*) gene (GenBank accession no. DQ508261), putative type I polyketide synthase loading module (*mlsB*) gene (GenBank accession no. DQ508260), and putative 3-oxoacyl-acyl-carrier-protein synthase (*fabH*) gene (GenBank accession no. DQ508259), were designed by the Primer-BLAST software (Mve-Obiang et al. 2005, Ranger et al. 2006). To detect the gene associated with highly antigenic proteins (ESAT-6 and CFP-10), PCR targeting both *esxA* and *esxB* genes was performed (Mve-Obiang et al. 2005).

The PCR reaction was conducted with the OnePCR mixture (GeneDireX), and consisted of 25 µl of 2× OnePCR mixture, 1.0 µl of 10 µM of each primer, and 1.0 µg of the DNA template for a total reaction volume of 50 µl. All PCR reactions were performed as follows: initial denaturation (94°C for 5 min), followed by 35 cycles of denaturation (94°C for 30 s), annealing (different temperatures with different primer sets for 1 min) and extension (72°C for 2 min), and final extension (72°C for 5 min) (Hughes et al. 2000, Kim et al. 2005, Mve-Obiang et al. 2005, Mokaddas & Ahmad 2007, Holopainen et al. 2009). Amplified DNA was fractionated by electrophoresis on 1.5% agarose gels and visualized by ethidium bromide staining. The sequences and annealing temperatures of these primer sets and their expected sizes are summarized in Table 1.

Table 1. PCR primers used in the present study

Target gene	Expected size (bp)	Primer sequence (5′ to 3′)	Annealing temp. (°C)	Source
16s rRNA	550	AGA GTT TGA TCC TGG CTC AG GCG ACA AAC CAC CTA CGA G	60	Hughes et al. (2000)
ITS	350–450	GAT TGG GAC GAA GTC GTA ACA AG AGC CTC CCA CGT CCT TCA TCG GCT	55	Mokaddas & Ahmad (2007)
hsp65	644	ACC AAC GAT GGT GTG TCC AT CTT GTC GAA CCG CAT ACC CT	62	Kim et al. (2005)
exsA	183	GAC AGA ACA GCA GTG GAA TTT CG CTT CTG CTG CAC ACC CTG GTA	60	Mve-Obiang et al. (2005)
esxB	227	TTT TGA AGA ACG ATG CCG CTA C TGA CGG ATG TTC GTC GAA ATC	60	Mokaddas & Ahmad (2007)
mlsA	183	GTC CCG ACG TCT ACG GAT TC TCT CGG CCA AAG CGA TGT AG	60	Present study
mlsB	204	CAG CCA ACT GCG CTA CTA CA GAC CAC ACT GAT CCC GTC TC	60	Present study
fabH	194	CCC CGT ATG TCA CGT CCA AA CTG ACG ACC AAC GGC TGA TA	60	Present study
DNA polymerase	560	GTG TAY CAG TGG TTT TGC GAC TCG TCT CCG GGY CTG TCT TT	50	Holopainen et al. (2009)
NF-H1	639	CCA AAG ACC AAA GAC CAG GTT GGT CTT TGG TCT CGC TC	55	Holopainen et al. (2009)
MCP	531	GAC TTG GCC ACT TAT GAC GTC TCT GGA GAA GAA GAA	58	Mao et al. (1999)

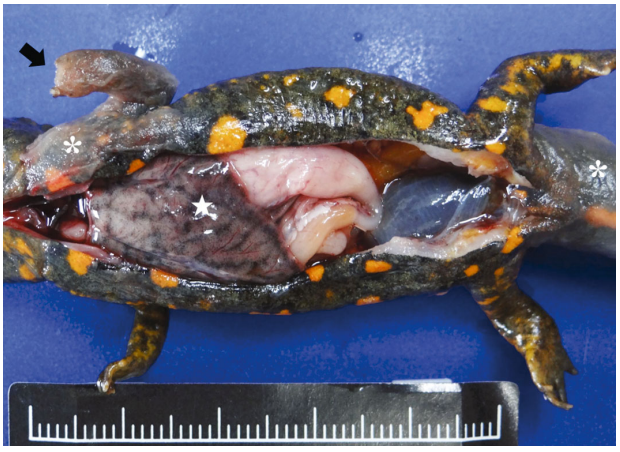

Fig. 1. Gross findings, Newt no. 8 (see Table 2). The body trunk is multifocally covered by a layer of a cotton-like white substance (❀), and one of the extremities is ulcerated (black arrow). The liver has a grey and dark red mottled appearance (☆)

RESULTS

Pathology

Grossly, the body trunks of the newts were multifocally covered by a layer of a cotton-like white substance, and some of their extremities were swollen and/or ulcerated. The liver had a grey and dark red mottled appearance, but no remarkable abnormities were found in other internal organs (Fig. 1). Microscopically, multifocal necrotic lesions composed of pyknotic, karyorrhectic, or karyolytic cells with none to minimal inflammatory cell infiltration were noted in the liver (n = 14), spleen (n = 9), and kidney (n = 5). The size and number of melanomacrophage centers were variably increased in the livers of these individuals (Fig. 2A). Although the necrotic foci were randomly distributed in the hepatic parenchyma, some of them showed scattered melanin pigments and melanomacrophages (Fig. 2B). The necrotic foci in the spleen were similar to those in the liver, and a small number of melanomacrophages were noted around the lesions or the adjacent blood vessels (Fig. 3A,B). The necrotic lesions found in the kidneys were located at renal tubules and glomerular tufts, and extended into the peripheral renal interstitium (Fig. 4A,B). Under acid-fast stain, abundant acid-fast bacilli were noted within/around all necrotic lesions, and the acid-fast bacilli displayed an intracellular distribution (Figs. 2C, 3C, 4C).

The epidermis was multifocally necrotic with an accumulation of proteinaceous exudate and infiltrated by numerous irregular, 5 to 15 µm in width,

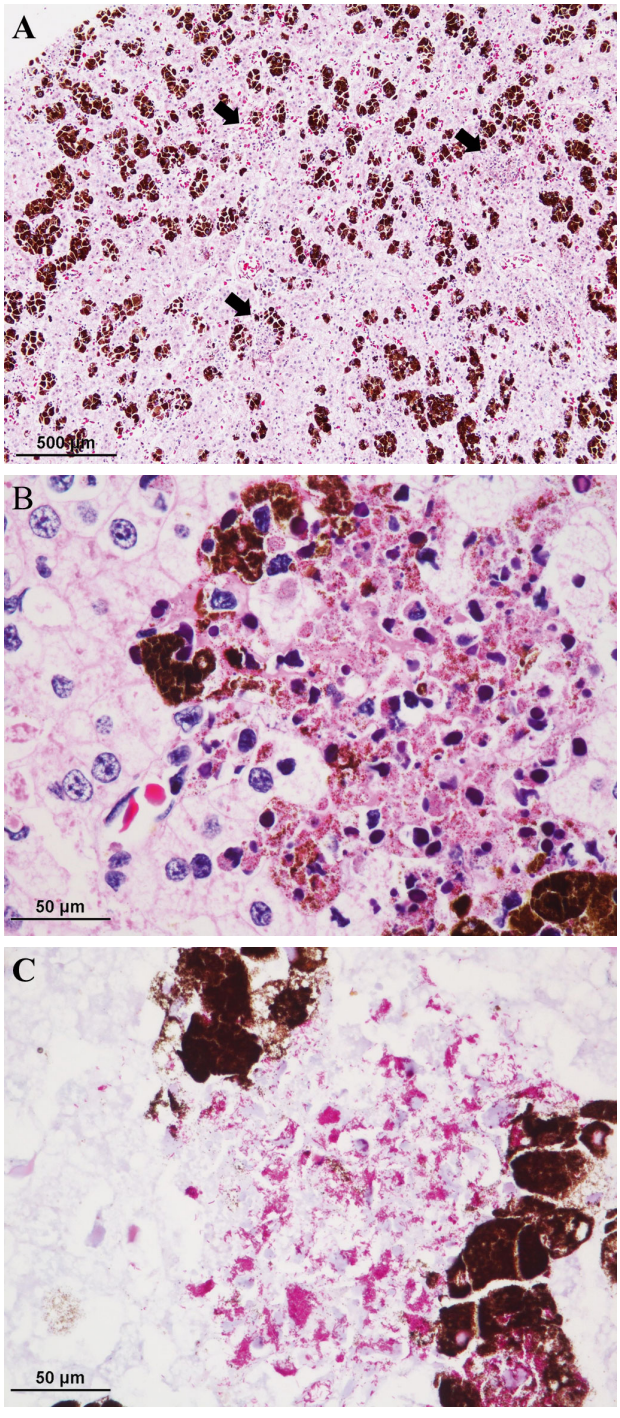

Fig. 2. Liver, Newt no. 10 (see Table 2). Histopathology of hepatic lesions. (A) Multifocal, prominent, and enlarged melanomacrophage centers are noted with multifocal necrotic lesions (black arrows) in the liver parenchyma (H&E stain). (B) The necrotic lesions are composed of pyknotic, karyorrhectic, and/or karyolytic cells with a granular eosinophilic cytoplasm, and there is accumulation of eosinophilic substance with none to minimal inflammatory cell infiltration (H&E stain). (C) Abundant acid-fast bacilli are noted within/around the necrotic lesions, and the acid-fast bacilli display intracellular distribution (acid-fast stain)

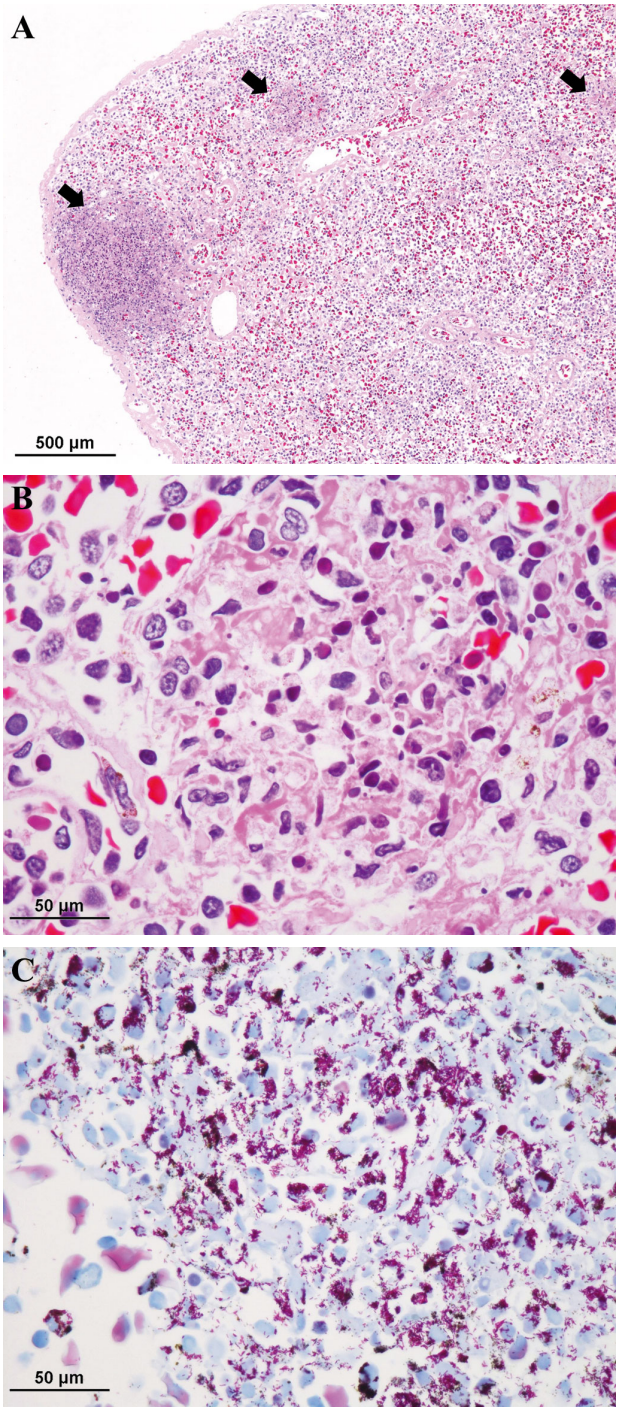

Fig. 3. Spleen, Newt no. 14 (see Table 2). Histopathology of splenic lesions. (A) There are multifocal necrotic lesions (black arrows) in the spleen parenchyma (H&E stain). (B) The necrotic lesions are composed of pyknotic, karyorrhectic, or karyolytic cells with a granular eosinophilic cytoplasm, and there is accumulation of eosinophilic substance with no to minimal inflammatory cell infiltration (H&E stain). (C) Abundant acid-fast bacilli are noted within/around the necrotic lesions, and the acid-fast bacilli display an intracellular distribution (acid-fast stain)

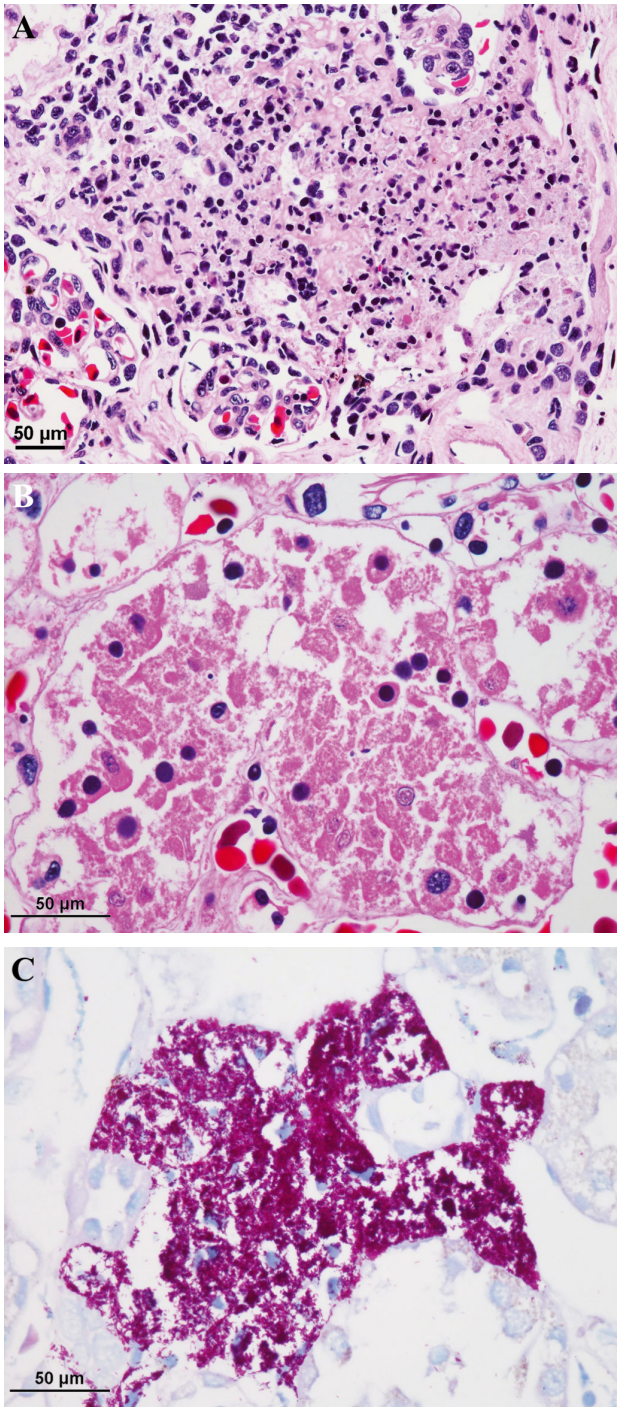

Fig. 4. Kidney, Newt no. 4 (see Table 2). Histopathology of renal lesions. (A) The necrotic lesions in the kidney are located at renal tubules and glomerular tufts, extending into the peripheral renal interstitium with minimal inflammatory cell infiltration (H&E stain). (B) Some necrotic lesions are only confined within a single renal tubule without inflammatory response (H&E stain). (C) Abundant acid-fast bacilli are noted within/around the necrotic lesions, and the acid-fast bacilli display intracellular distribution (acid-fast stain)

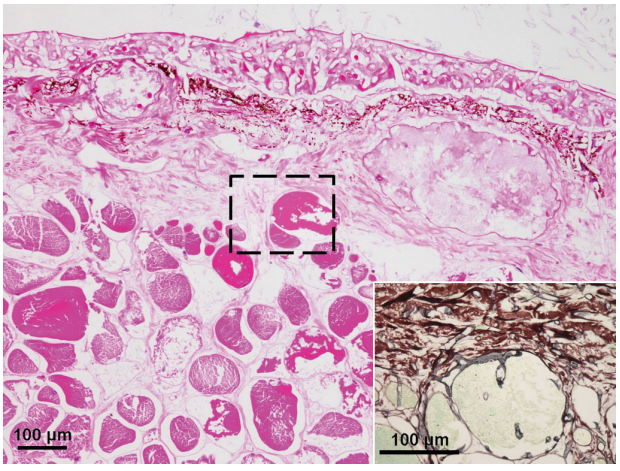

Fig. 5. Skin, Newt no. 9 (see Table 2). Histopathology of skin lesions. The epidermis is multifocally necrotic and infiltrated by numerous fungal hyphae. The hyphae further penetrate into the underlying dermis and muscles, causing varying degrees of myofiber degeneration to necrosis (H&E stain). Inset: Higher magnification of area outlined by black dashed line illustrates that the fungal hyphae are irregular shaped, 5 to 15 µm in width, acute-angle branching, and non-septate, morphologically consistent with *Saprolegnia* sp. (GMS stain)

acute-angle branching and non-septate hyphae (n = 10), morphologically consistent with *Saprolegnia* sp. These hyphae infiltrated into the adjacent dermis, and the blood vessels and serous/mucous glands in the dermis were variably dilated (Fig. 5). Furthermore, these hyphae also penetrated into the underlying muscles and caused varying degrees of myofiber degeneration to necrosis, featured by the loss of cross striations, hypereosinophilic sarcoplasm, vacuolation and fragmentation (Fig. 5, inset).

PCR

Using primers specific for ranaviruses including DNA polymerase, MCP, and NF-H1 protein genes, no specific signals were detected (data not shown).

Using primers specific for mycobacterial species identification, including *hsp65* gene, *ITS* region, and partial *16S rRNA* genes, PCR reactions were performed with DNA templates from 4 fresh liver samples, and the PCR products were directly sequenced. The DNA sequence from amplified amplicons of *hsp65* gene and the partial *16S rRNA* genes were 100% identical to that of the *Mycobacterium marinum* strain *cyprinum* CC240299 (GenBank accession no. AF456239) (Fig. 6). The sequence identity was slightly less similar to those of *M. liflandii*, *M. pseudoshottsii*, *M. shottsii*, and *M. ulcerans*. The DNA sequence from amplified amplicons of *ITS* region was 99% identical to those of *M. liflandii*, *M. marinum*, and *M. ulcerans*. The results of the PCR reactions targeting mycolactone-related genes, including *mlsA*, *mlsB*, and *fabH*, and the genes encoding highly antigenic proteins, including *esxA* and *esxB*, were all positive in the 4 fresh liver samples (Fig. 7).

Results of histopathological examination and PCR for ranavirus and mycobacteria are shown in Table 2.

DISCUSSION

Non-granulomatous mycobacteriosis in 14 Hong Kong warty newts *Paramesotriton hongkongensis* characterized by multifocal necrotic lesions with none to minimal inflammatory cell infiltration in various internal organs is reported in the present study. Initially, ranavirus infection was highly suspected but subsequently excluded by negative PCR results. Unexpectedly, abundant intralesional acid-fast positive bacilli were found in the necrotic lesions of the liver, spleen, and kidneys in all affected newts. Further molecular tests to identify mycobacteria species were performed, and the results indicated that the intralesional acid-fast positive bacilli were *Mycobacterium marinum*. Therefore, mycobacteriosis caused by *M. marinum* is likely the final diagnosis in these 14 newts. The present study addresses attention to the unusual lesions caused by mycobacterial infection and its potential zoonotic risk to public health.

Several possibilities regarding the lack of granulomatous lesions in these newts are worthy of discussion. *M. ulcerans*, harboring a mycolactone-producing plasmid and producing mycolactone, has been reported to cause non-granulomatous necrotic cutaneous lesions in humans (Buruli ulcer) (Ranger et al. 2006, Hong et al. 2008, Pidot et al. 2008). It is known that the non-granulomatous necrotic skin lesions caused by *M. ulcerans* are associated with the presence of mycolactone and caused by a lack of highly antigenic proteins (ESAT-6 and CFP-10), the deletions of *esxA* and *esxB* genes (Mve-Obiang et al. 2005, Ranger et al. 2006). At present, a mycolactone-producing capability has also been found in *M. liflandii*, *M. marinum*, and *M. pseudoshottsii* (Mve-Obiang et al. 2005, Ranger et al. 2006, Pidot et al. 2008). The *M. marinum* detected in the present study is considered to harbor mycolactone-producing plasmid because of the positive PCR results for *mlsA*, *mlsB*, and *fabH* genes, the component genes of mycolactone. However, *esxA* and *esxB* genes were

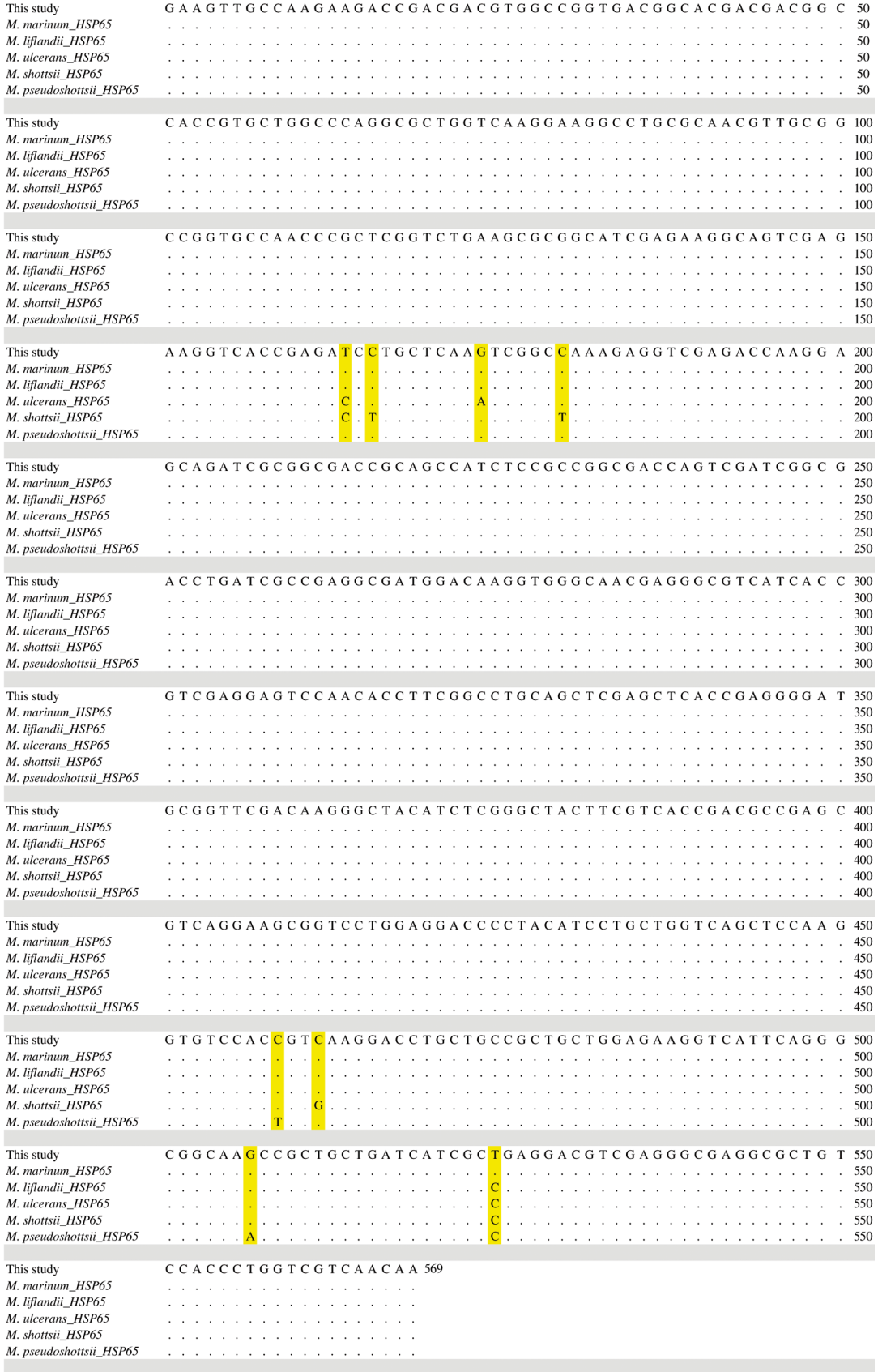

Fig. 6. Multiple alignments of (A) *hsp65* and (B) *16s rRNA* DNA sequences in *Mycobacterium* species. Areas of DNA sequence variation are highlighted in yellow (continued on next page)

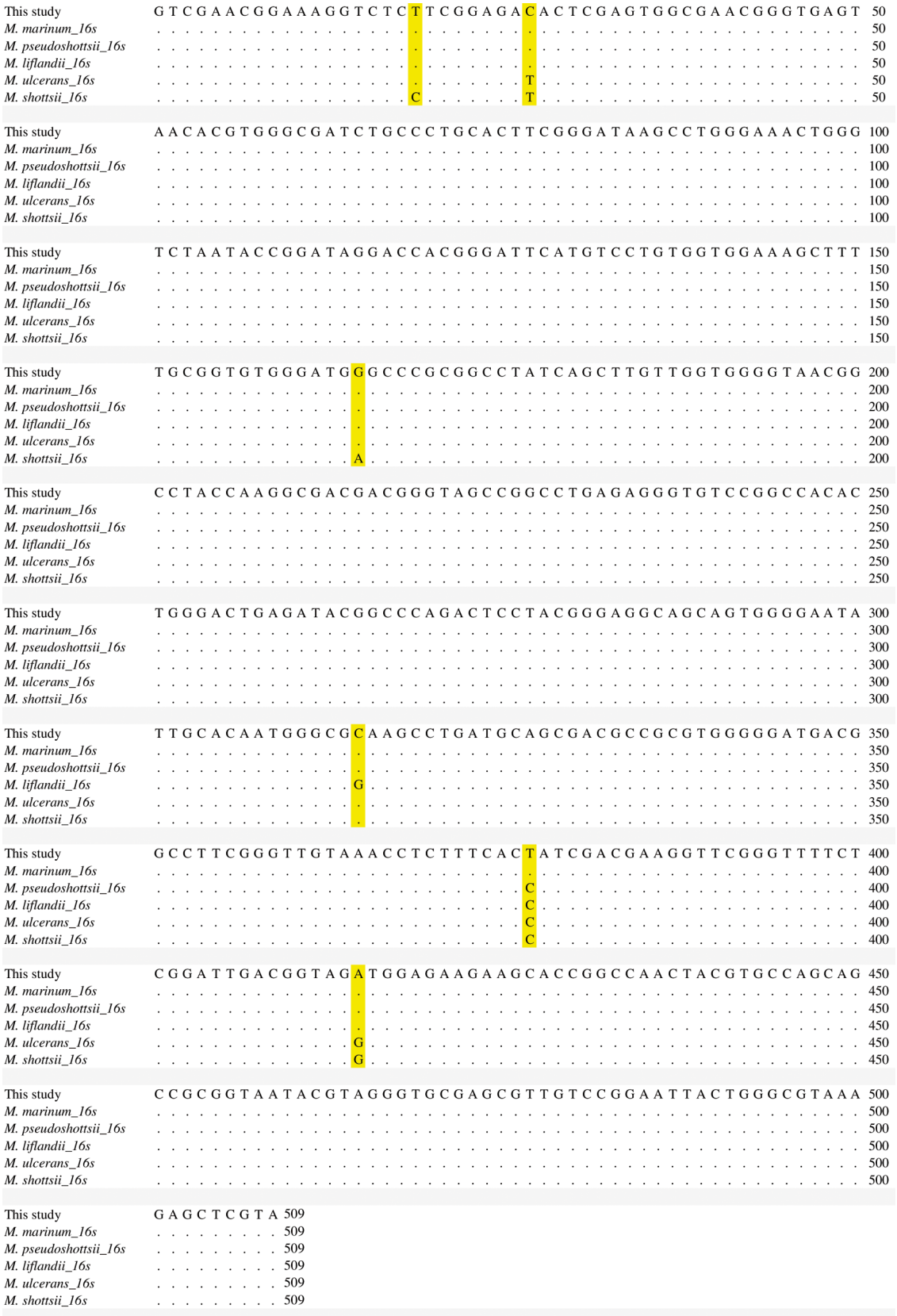

Fig. 6 (continued)

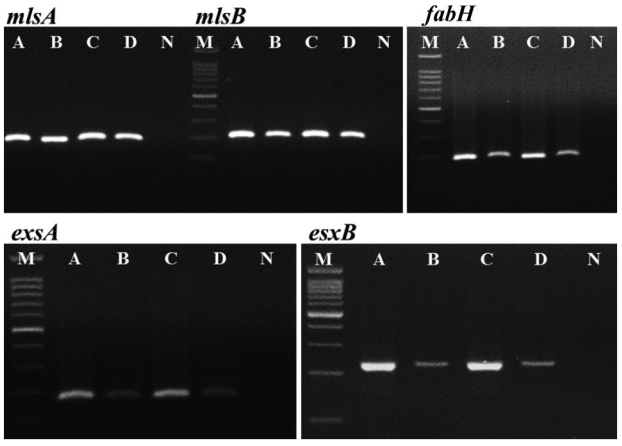

Fig. 7. Agarose gel electrophoresis (1.5%) of PCR for detecting the *mlsA*, *mlsB*, *fabH*, *esxA*, and *esxB* genes. Lanes: M, 100 bp ladder marker (Star-100bp DNA Ladder, OneStar); A, DNA template from newt 10; B, DNA template from newt 11; C, DNA template from newt 12; D, DNA template from newt 14; N, negative control.

also detected, suggesting that the current *M. marinum* is able to produce highly antigenic proteins. Therefore, lack of granulomatous inflammation in our case appears not to be completely dependent on the influence of mycolactone and *esxA* and *esxB* genes.

Table 2. Results for 14 Hong Kong warty newts including the histopathological findings, acid-fast staining, and PCR for mycobacteria and ranavirus. A positive result for acid-fast staining indicates that acid-fast positive bacilli were noted with necrotic lesions. na: not applicable

No.	Hepatitis	Splenitis	Nephritis	Water mold disease	Acid-fast staining	PCR for mycobacteria	PCR for ranavirus
1	+	+		na[b]	Positive	na	na
2	+		+	+	Positive	na	na
3	+			+	Positive	na	na
4	+	+	+	+	Positive	na	na
5	+	+		+	Positive	na	na
6	+	+		na[b]	Positive	na	na
7	+	+		na[b]	Positive	na	na
8	+			+	Positive	na	na
9	+			+	Positive	na	na
10[a]	+	+	+	+	Positive	*M. marinum*[c]	Negative
11[a]	+			+	Positive	*M. marinum*[c]	Negative
12[a]	+	+	+	+	Positive	*M. marinum*[c]	Negative
13	+	+		+	Positive	na	na
14[a]	+	+	+	+	Positive	*M. marinum*[c]	Negative

[a]Fresh samples were collected from these 4 individuals
[b]Skin tissues were not received
[c]The PCR results were 100% identical to those of *Mycobacterium marinum* by *hsp65/16s rRNA*, and 99% identical to that of *M. marinum* by *ITS*. The *M. marinum* was positive for *mlsA*, *mlsB*, *fabH*, *esxA*, and *esxB* genes

To our knowledge, mycobacteriosis has not been reported in salamanders, but has been reported in other amphibians, including *M. chelonae* in South African clawed frogs *Xenopus laevis* (Green et al. 2000); *M. gordonae*, *M. liflandii*, and *M. szulgai* in African clawed frogs *Xenopus tropicalis* (Chai et al. 2006, Sánchez-Morgado et al. 2009, Fremont-Rahl et al. 2011); and *M. marinum* in bullfrogs *Rana catesbeiana*, Japanese forest green tree frogs *Rhacophorus arboreus*, and leopard frogs *Rana pipiens* (Ramakrishnan et al. 1997, Ferreira et al. 2006, Haridy et al. 2014). Granulomatous inflammation with the presences of epithelioid macrophages, multinucleated giant cells, and intralesional acid-fast positive bacilli is a consistent finding in these studies (Ramakrishnan et al. 1997, Green et al. 2000, Trott et al. 2004, Chai et al. 2006, Ferreira et al. 2006, Sánchez-Morgado et al. 2009, Fremont-Rahl et al. 2011, Haridy et al. 2014). Previous *in vitro* studies have suggested that salamanders may have a weak adaptive immune response compared to *Xenopus* spp. (e.g. Godwin & Rosenthal 2014), but several case reports of salamanders have also demonstrated normal inflammatory response in association with bacterial, fungal, and parasitic infections (Migaki & Frye 1975, Ware et al. 2008, Martel et al. 2012). A case of novel Chlamydiaceae disease in salamanders revealed severe hepatitis characterized by abundant melanomacrophages and marked infiltration of granulocytes (Martel et al. 2012). In a case of tiger salamanders *Ambystoma tigrinum* affected by an unidentified brown-pigmented fungus, granulomatous myositis was observed (Migaki & Frye 1975). Furthermore, a case involving spotted salamanders *A. maculatum* also demonstrated granulomatous inflammation elicited by an Ichthyophonus-like organism (Ware et al. 2008). These studies demonstrate that various inflammatory responses including granulomatous inflammation occur in salamanders. Therefore, the non-granulomatous necrotic lesions in the present study cannot be fully explained by putative weak immune responses of salamanders.

M. marinum has been reported to cause multiple granulomatous lesions in different species of frogs (Ramakrishnan et al. 1997, Ferreira et al. 2006, Haridy et al. 2014), but it also induces a non-granulomatous lesion in experimentally immunosuppressed leopard frogs (Ramakrishnan

et al. 1997). Such non-granulomatous lesions in amphibians have not been reported in a field study, possibly indicating that a host with immunosuppression might impede the development of granulomatous inflammation. Furthermore, one study also suggested that the density of intralesional acid-fast positive bacilli markedly increased in hydrocortisone-treated frogs (Ramakrishnan et al. 1997). As above, the density of intralesional acid-fast positive bacilli and the integrality of granuloma can be indicators for host immune status. Therefore, the abundant intralesional acid-fast positive bacilli and the absence of granulomatous inflammation are morphological evidence of impaired host immune function in our case.

The water mold disease caused by *Saprolegnia* spp. has been considered an opportunistic infection that usually affects stressed, injured, or immunosuppressed individuals (Ruthig 2009, van den Berg et al. 2013). Hence, the presence of water mold in the present animals is also considered an indicator of impaired host immune function. If water mold is established on the skin, it may cause extensive skin ulceration and necrosis of underlying soft tissues without a marked inflammatory response (Green 2001). Antemortem fungal hyphae infiltration usually causes a tissue response such as degeneration and necrosis, which can be differentiated from the postmortem growth of the fungal hyphae (Green 2001). As mentioned above, the water mold disease, with the deep invasiveness of its fungal hyphae, may also be evidence of impaired host immune function.

Several factors have been considered to cause endogenous production of corticosteroid hormones in amphibians, which may be a benefit to short-term survival but cause immunosuppression under long-term exposure (Gray et al. 2009). Generally, these factors, called stressors, can be categorized as natural (decreased temperature and poor water quality/hygiene) and anthropogenic (inappropriate manipulation) (Gray et al. 2009). The sudden environmental changes due to the malfunction of the thermostat and water circulation systems was likely a significant stressor for these newts and may have been a trigger for establishing the infection of mycobacteriosis and water mold disease. A previous study also suggested that pathogens such as ranaviruses and *Batrachochytrium* spp. may cause physiological stress and thereby impair host immune function, which further exacerbates the disease status (Rollins-Smith 2017).

All MPM have highly similar genetic features, and can be re-assigned as strains of *M. ulcerans* (Pidot et al. 2010). Therefore, a previous study also suggested *M. ulcerans* and the other MPM should be considered as the same mycobacterial species (Pidot et al. 2010). Because other MPM have been reported to cause diseases in humans, the zoonotic potential of MPM should be of concern (Yip et al. 2007, Pidot et al. 2010). Mycolactone-producing *M. marinum* has been found only in the regions around western Asia (Israel, the Mediterranean Sea, and the Red Sea), and has never been reported in other regions. To our knowledge, this is the first study to report the mycolactone-producing *M. marinum* infection in East Asia, and this information indicates that MPM may be undergoing trans-boundary transmission by an undetermined pathway. Therefore, further monitoring and investigation of MPM in aquatic and/or wildlife animals in different regions is recommended.

In the present study, the necrotic lesions in amphibians could be misdiagnosed as ranavirus infection because of the absent granulomatous inflammation; hence, the possibility of zoonotic potential in such cases may be overlooked (Densmore & Green 2007, Geng et al. 2011). *M. marinum* infection commonly causes ulcerative and nodular skin lesions, but can also cause more aggressive diseases, such as septic arthritis and osteomyelitis, especially in cases of delayed treatment or an immunocompromised condition (Nguyen et al. 2015, Riera et al. 2016). Therefore, amphibian mycobacteriosis should always be assessed with differential diagnosis when non-granulomatous necrotic lesions in multiple organs are observed.

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