



# HOKKAIDO UNIVERSITY

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The titles of theses and other information are as follows:

## **A study on mitochondrial genome and the glycolysis pathway of diminazene aceturate-resistant *Babesia gibsoni* isolate *in vitro***

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*Babesia gibsoni* is a blood protozoan of dogs and a causative pathogen of canine babesiosis. In the treatment of canine babesiosis, possible relapses and the development of drug-resistant isolates are matters of concern. Thus, the goal of this study was to understand the mechanism of DA resistance in the DA-resistant *B. gibsoni* isolate. In the present study, the changes in the mitochondrial genome and the glycolysis pathway were considered as possible candidates for the development of DA resistance in *B. gibsoni*. Therefore, the mitochondrial genome and the glycolysis pathway in the DA-resistant *B. gibsoni* isolate were analyzed and compared with that of the wild-type.

In chapter I, the mitochondrial genome in the DA-resistant *B. gibsoni* isolate was investigated. First, to observe the stability of the capacity of DA resistance, the DA-resistant isolate was cultured without DA for 4 weeks and then cultured with 200 ng/ml DA for 2 weeks. As a result, this isolate proliferated both with and without DA, indicating that the characteristic of DA resistance was stable in the DA-resistant isolate. From this result, it was speculated that structure of some genes in the DA-resistant isolate would be different from those in the wild-type; therefore, mitochondrial genes, such as

*cytochrome c oxidase subunit I (COXI)*, *cytochrome c oxidase subunit III (COXIII)*, and *cytochrome b (CYTb)*, were analyzed in the present study. According to the results, nucleotide sequences, deduced amino acid sequences, and transcription levels of the *COXI*, *COXIII*, and *CYTb* genes of the DA-resistant isolate were not altered compared to those of the wild-type. These results indicated that DA should not affect mitochondrial genes directly and mitochondrial genes should not play a central role in the development of DA resistance in *B. gibsoni*.

In chapter II, the energy generation and glycolysis pathway of the DA-resistant *B. gibsoni* isolate were investigated. The level of parasitemia in the DA-resistant isolate was comparatively lower than that in the wild-type, suggesting that the proliferation potential of the DA-resistant isolate would be lower than that of the wild-type. Thus, the potential for alterations in the energy metabolism of the DA-resistant isolate was expected. Interestingly, intracellular ATP and glucose concentrations in the DA-resistant *B. gibsoni* isolate were significantly higher than those in the wild-type, indicating that the DA-resistant *B. gibsoni* isolate would require more ATP than the wild-type to maintain its DA resistance. Meanwhile, the activity of lactate

dehydrogenase and the concentration of lactate in the DA-resistant isolate did not differ from those in the wild-type, suggesting that the activity of the glycolysis pathway in the DA-resistant isolate would not be altered. In addition, the activity of pyruvate kinase and the concentration of pyruvate in the DA-resistant isolate were also not different from those in the wild-type, indicating that the activity of metabolic pathways in which pyruvate is metabolized would not be changed in the DA-resistant isolate. From these results, it was considered that the glycolysis pathway would not be upregulated in the DA-resistant isolate, although the production

of ATP would be enhanced.

In conclusion, it is shown that mitochondrial genes are not directly involved in the development of DA resistance, and the glycolysis pathway is not activated in the DA-resistant isolate; however, in the present study, the activities of mitochondrial enzymes could not be determined. Therefore, in a further study, the metabolism of the mitochondria of the DA-resistant *B. gibsoni* isolate should be investigated to clarify why this isolate has high ATP concentration. These studies could lead to elucidation of the mechanism of DA resistance of *B. gibsoni*.

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The original papers of this thesis appeared in *Jpn. J. Vet. Res.*, **60**: 51-61, (2012) and *J. Vet. Med. Sci.*, in press.

## Toxic metal contamination in cattle of Zambia

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In the past decade, there has been a steady accumulation of toxic metals including Pb and Cd in the African environment. Studies of environmental pollution in Africa indicate that toxic metal pollution has reached unprecedented levels over the past decade. Therefore, human exposure to toxic metals has become a major health risk on the continent and is the subject of increasing attention from national and international environmentalists. In chapter 1, I reviewed the current status of metal pollution in Africa and found that pollution levels in many countries were at critical points, as the levels of toxic metals in water, fish, soils, edible vegetables and food animals exceeded acceptable limits for human consumption.

In chapter 2, I examined metal contamination in cattle from Kabwe, Zambia, and found that

cattle reared near the Pb-Zn mine in Kabwe, where Cd was produced as a by-product, accumulated higher concentrations of Pb and Cd than cattle from other towns in Zambia. The Pb-Zn mine was the likely source of metal pollution and had a negative environmental impact on livestock production in Kabwe and the surrounding regions like Chibombo town. No indications of metal toxicity on the health of cattle were observed but contribution of cattle offal to toxic metal exposure in humans could be significant. Continuous monitoring of Pb and Cd contaminations in offal of cattle in the vicinity of Pb-Zn mines was recommended in the interest of human consumers.

In chapter 3, I found that backyard local breed chickens reared in townships around the Pb-Zn mine in Kabwe accumulate extremely high

levels of Pb and Cd compared to commercial broiler chickens. These findings were important as even muscle tissue accumulated concentrations of Pb above the acceptable limits for human consumption. The toxic metal concentrations in chickens were higher than metal concentrations in offal of cattle from Kabwe. These results indicate that offal from backyard chickens in Kabwe presents a much higher health risk to human consumers than cattle offal since the chickens were reared in townships close to the mine. Therefore, there is a clear need to avoid consumption of contaminated offal of free-range chickens in Kabwe as well as restrict backyard chickens from roaming and scavenging for food near the mine.

In chapter 4, I examined metal accumulation

in offal of cattle from an agricultural area in Zambia. I observed lower concentrations of toxic metals including Pb and Cd than concentrations in cattle from Kabwe. However, the study revealed potential health risks for humans as accumulations of Ni and Cr were elevated and comparable in concentration to the cattle from Kabwe and higher than reports in other countries. Concentrations of Ni and Cr should be assessed regularly for the health of consumers as these metals are toxic and carcinogenic. Although metal concentration in soils was not determined, the prolonged application of fertilizers and pesticides on commercial farms in the study site could have contributed significantly to metal accumulations in cattle offal.

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The original papers of this thesis appeared in *Environ. Toxicol. Chem.*, **30**: 1892–1897 (2011), *J. Vet. Med. Sci.*, **74**: 1345–1347 (2012) and *J. Vet. Med. Sci.*, **72**: 1257–1263 (2010).

## **Studies on pathological changes of lung tissues in influenza A virus-infected mice and inhibitory effects of anti-M2 monoclonal antibody on different strains of influenza virus**

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Every year, influenza epidemics seriously affect all age group of people, but the highest risk of complications occurs among elderly individuals over the age of 65 years. The major subtypes of influenza A that are currently circulating among people worldwide include H1N1 and H3N2 viruses, resulting in a global burden of ~500,000 deaths every year. Following epidemics of influenza A virus and a recent 2009 H1N1 pandemic, counter-measures have been developed to minimize the damages caused by influenza virus infection, for example, the

addition of neutralizing antibodies to provide an immediate treatment option for influenza pandemic emergency, while more time-consuming developments of vaccine and drugs are ongoing. A number of anti-M2 ectodomain (M2e) antibodies that have been generated in recent years, showed promising anti-influenza A activities both in prophylactic and therapeutic settings. In the present thesis, I described the histopathological and immunohistochemical examination of the lungs of young and aged mice intranasally inoculated with influenza virus. In our results

young mice showed bodyweight loss after 4 days post-infection (dpi), meanwhile in aged mice decrease started from 9 dpi. This difference seems to be related to low levels of infiltration of inflammatory cells and mild pathological changes occurring at the early phase of infection in aged mice. This condition is associated with the delayed viral clearance and longer lasting period of infection. Secondly, I described about the antiviral activities of anti-M2e specific monoclonal antibody, rM2ss23, which binds to the M2 proteins of the influenza A viruses. The rM2ss23 bound to both A/Aichi/2/68 (H3N2) and A/PR/8/34 (H1N1) M2 proteins expressed on the cell surface, inhibiting plaque formation by the Aichi strain in a dose-dependent manner when infected cells were cultured in the presence of the

antibody. A reverse genetics approach revealed that the inhibitory effect of rM2ss23 on the Aichi virus was abolished by replacing the genes encoding the HA and/or M proteins with those of the PR8 strain. Therefore, through the present thesis, I provided timely and detailed insights into the age-specific course at the early phase of infection in aged mice by low levels of infiltration of inflammatory cells and reduced lung pathology associated with severe influenza virus infection. In addition, I demonstrated that rM2ss23 prevents virus release from infected cells and further suggested that the mechanisms underlying the budding of viruses mediated by HA and M2 proteins might differ between the Aichi and PR8 strains.