The Journal of Veterinary Medical Science

FULL PAPER

Wildlife Science

Characterization of Myxovirus resistance protein in birds showing different susceptibilities to highly pathogenic influenza virus

Katsuro HAGIWARA[1)]*, Takaaki NAKAYA[2)] and Manabu ONUMA[1,3)]*

[1)]School of Veterinary Medicine, Rakuno Gakuen University, 582 Bunkyodai, Ebetsu, Hokkaido 069-8501, Japan
[2)]Department of Infectious Diseases, Kyoto Prefectural University of Medicine, Kajii-cho, Kawaramachi-Hirokoji, Kamigyo-ku, Kyoto 602-8566, Japan
[3)]Ecological Risk Assessment and Control Section, Center for Environmental Biology and Ecosystem Studies, National Institute for Environmental Studies, 16-2, Onogawa, Tsukuba, Ibaraki 305-8506, Japan

ABSTRACT. We compared the *Mx* expression and anti-viral function and the 3D structure of Mx protein in four species: chicken (*Gallus gallus*), whooper swan (*Cygnus cygnus*), jungle crow (*Corvus macrorhynchos*), and rock dove (*Columba livia*). We observed different mortalities associated with highly pathogenic avian influenza virus (HPAIV) infection to understand the relationship between *Mx* function as an immune response factor and HPAIV proliferation in bird cells. Different levels of *Mx* were observed among the different bird species after virus infection. Strong *Mx* expression was confirmed in the rock dove and whooper swan 6 hr after viral infection. The lowest virus copy numbers were observed in rock dove. The virus infectivity was significantly reduced in the BALB/3T3 cells expressing rock dove and jungle crow *Mx*. These results suggested that high *Mx* expression and significant *Mx*-induced anti-viral effects might result in the rock dove primary cells having the lowest virus copy number. Comparison of the expected 3D structure of Mx protein in all four bird species demonstrated that the structure of loop L4 varied among the investigated species. It was reported that differences in amino acid sequence in loop L4 affect antiviral activity in human and mouse cells, and a significant anti-viral effect was observed in the rock dove *Mx*. Thus, the amino acid sequence of loop L4 in rock dove might represent relatively high anti-viral activity.

KEY WORDS: highly pathogenic avian influenza virus, *Mx* gene, Mx protein, loop L4

J. Vet. Med. Sci.
82(5): 619–625, 2020
doi: 10.1292/jvms.19-0408

Received: 22 July 2019
Accepted: 5 March 2020
Advanced Epub:
16 March 2020

Interferon-stimulated genes (ISGs) are upregulated by type I interferons (IFNs) as an innate immune system response. Several hundred species have been confirmed to express ISGs, and they have been the subject of antiviral activity studies [6]. ISGs include genes coding for proteins, such as myxovirus resistance protein (Mx protein), protein kinase R, 2'-5'-oligoadenylate synthetase, and IFN-inducible transmembrane protein [17]. Mx protein has been identified in many animals from fish to primates. Mx protein is a crucial module in type I IFN-induced antiviral effect in many species, and it is mainly known for its antiviral activity against RNA viruses like influenza virus [17, 18]. Mx protein is classified into five subgroups, MxA-like, MxB-like, rodent Mx, avian Mx, and Fish Mx. It is thought that the interaction of the C-terminal effector domain with the N-terminal GTPase domain is necessary for its antiviral action [9]. Mx gene (*Mx*) is single gene in birds, and the gene has been confirmed in several species such as chickens, ducks, and geese [1, 4, 24]. Although an *Mx* has been reported in poultry, there is little information about this gene in wild birds [3, 7] especially its anti-viral function against highly pathogenic avian influenza virus (HPAIV) infections in wild birds.

HPAIV has been previously identified in wild birds, poultry, and their infections worldwide. HPAIV infection prevention is important for the poultry industry, but various migratory birds and wild birds are also potential virus carriers. Therefore, it is important to understand HPAIV infection sensitivity in wild birds as previously reported [14, 16]. Several findings in recent HPAIV experimental infection reports suggest differences in the susceptibility of wild birds. For example, HPAIV was shown to be highly pathogenic in whooper swans (*Cygnus cygnus*) [5]. Two whooper swans were inoculated intranasally with 10^6 EID_{50} of A/whooper swan/Mongolia/244/2005 (H5N1) (clade 2.2). The results showed that all birds died within four days of infection. Furthermore, two birds housed with inoculated birds also died within four days after being housed together. According to the results of the experimental infection in the jungle crow (*Corvus macrorhynchos*) [10], the mortality rate varies depending on

*Correspondence to: Hagiwara, K.: k-hagi@rakuno.ac.jp, Onuma, M.: monuma@nies.go.jp
(Supplementary material: refer to PMC https://www.ncbi.nlm.nih.gov/pmc/journals/2350/)

the virus lineage. Four jungle crows were inoculated intranasally with 10^6 EID_{50} of A/peregrine falcon/Hong Kong/810/2009 (H5N1) (clade 2.3.4). The results showed that all four birds died within two weeks of infection. Four jungle crows were inoculated intranasally with 10^6 EID_{50} of A/muscovy duck/Vietnam/OIE-559/2011 (H5N1) (clade 1.1). The results showed that the two birds died within two weeks of infection. However, no mortality was reported after intranasal inoculation with 10^6 EID_{50} of A/whooper swan/Hokkaido/4/2011 (H5N1) (clade 2.3.2.1). Furthermore, 12 pigeons (*Columba livia domesticus*) were inoculated intranasally with 10^3–10^6 EID_{50} of A/chicken/Miyazaki/K11/2007 (H5N1) (clade 2.2) or A/whooper swan/Akita/1/2008 (H5N1) (clade 2.3.2). The results showed that there were no mortalities after two weeks of observation [23]. As mentioned above, the HPAIV in wild birds showed variable pathogenicity from species to species. The differences might be due to the differences in immune responses to virus proliferation in each species.

Thus, we compared *Mx* expression, anti-viral function, and Mx protein 3D structure as immune response factors in four species with different mortalities in response to HPAIV infection to understand the relationship between *Mx* function and HPAIV proliferation in the bird cells.

MATERIALS AND METHODS

Bird cells

Bird cells (primary cells mainly consisting of fibroblasts) from chicken (*Gallus gallus*), jungle crow, rock dove (*Columba livia*), and whooper swan were derived from skin tissue (dead body of jungle crow), muscle tissues (dead body of rock dove and whooper swan), or embryo (chicken).

The primary explant method was used to prepare bird cells. The tissues were cut into 1–2-mm pieces using scissors. The culture medium KAv-1 [12] was added to the tissues and mixed well in a culture dish. The mixtures were transferred to a cell culture flask. The samples were incubated at 38°C with air gas phase. The medium was replaced every three days. Primary cell subculturing was conducted when the cells reached 80–90% confluence. After subculturing three to four times, the bird cells were cryopreserved in a liquid nitrogen tank with 10% dimethyl sulfoxide (DMSO) in FBS until virus infection.

Virus infection

Bird cells (4×10^5 cells/well) were cultured in medium with 10% FBS in 24-well plates. After washing with FBS-free medium, the cells were infected with A/crow/Kyoto/53/2004 (H5N1) using a multiplicity of infection (MOI) of 0.1 for 1 hr at 37°C in a 5% CO_2 incubator. The cells were cultured in medium with 10% FBS at 37°C with 5% CO_2 for 24 hr.

Evaluation of viral copy number and Mx expression

Viral RNA was extracted from the culture supernatant of the virus-infected cells using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany). Viral multiplication was measured using the Rotor-Gene SYBR Green RT-PCR Kit with Rotor-Gene cyclers (Qiagen) using the M region primer of the influenza A virus according to the WHO protocol [22] (Table 1). Mx expression was measured by lysing the cells with RLT buffer at 0- and 6-hr post-infection (hpi) and extracting RNA from the cells using the RNeasy Mini Kit (Qiagen). RNA was measured using the Rotor-Gene SYBR Green RT-PCR Kit. The expression values were normalized to that of *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) to evaluate *Mx* expression. The primers used for quantitative *Mx* expression are shown in Table 1.

Table 1. The primers set for amplification of *Mx* gene and influenza a gene

Primer	Forward	Reverse
Avian Mx1	5′- GTGATTGGAGACCGGAACTCTGG-3′ a)	5′-GCTTTTCCCAGAGTTCCGGTCTCCAATCAC-3′
Avian Mx2	5′- GACCTGACACTAATTGATCTTCCTGGA-3′ b)	5′-TCCAGGAAGATCAATTAGTGTCAGGTC-3′
Avian Mx3	5′- CCATGTAATGTGGATATTGCA-3′	5′-TGCAATATCCACATTACATGG-3′ c)
Avian Mx4	5′- GAGGACATTATAAAAGAGCA-3′	5′-TTGCTCTTTTATAATGTCCTC-3′ d)
Avian Mx5	5′- AATCAGATTCCTCTGATCATCC-3′	5′-GGATGATCAGAGGAATCTGATT-3′ e)
Influenza A M region	5′-ATGAGYCTTYTAACCGAGGTCGAAACG-3′	5′-TGGACAAANCGTCTACGCTGCAG-3′
Mx qRT-PCR*		
Chick	5′-GACCTGAACCTAATTGATCTCC-3′	5′-TTCAGCGCTTCTGTTGTTGC-3′
Jungle Crow	5′-GACCTGAACCTAATTGATCTCC-3′	5′-TTCAATGCTTCTGTTGTTGC-3′
Rock Dove	5′-GATCTGACATTAATTGATCTTC-3′	5′-TTCAGTGCTTCTGTTGTTGC-3′
Whooper Swan	5′-GACCTGAACCTAATTGATCTCC-3′	5′-TTCAGTGCTTCTGTTGTTGC-3′
GAPDH	5′- CCTCTCCTGGCAAAGTCCAAG-3′	5′-CATCTGCCCATTTGATGTTG-3′

a) This primer was used for 3′-RACE as outer gene specific primer. b) This primer was used for 3′-RACE as inner gene specific primer. c) This primer was used for 5′-RACE as inner gene specific primer in rock dove and whooper swan. d) This primer was used for 5′-RACE as outer gene specific primer in rock dove and used as innner gene spcific prime in chicken and jungle crow. e) This primer was used for 5′-RACE as outer gene specific primer in chicken, jungle crow and whooper swan . *The primer pairs used for Mx gene expression in the bird cells. The amplification size of Mx gene is 173 bp, and GAPDH is 200 bp. *GAPDH*: *glyceraldehyde-3-phosphate dehydrogenase*.

Mx cloning

Cells from chicken, jungle crow, rock dove, and whooper swan were treated with polyinosinic-polycytidylic acid (Poly (I:C), 50 *µ*g/m*l* in culture medium) (Sigma-Aldrich, St. Louis, MO, USA) overnight. Total RNA was extracted from treated cells using the EZ1 RNA Universal Tissue Kit (Qiagen, Hilden, Germany). 5′ RACE and 3′ RACE were conducted using the SMARTer^{TM}RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA) according to the manufacturer's instructions. Inner and outer gene specific primers for 5′ RACE and 3′ RACE are shown in Table 1. The primers were designed based on the reported *Mx* sequences in birds (accession No.: EF575641, EF575606, EF575605, EF575604, Z21550, Z21549, Z23168, and EF575607). The forward primer for each full length *Mx* sequence was designed based on 5′ RACE results (Supplementary Table 1). The reverse primer for each full length *Mx* sequence was designed based on 3′ RACE results (Supplementary Table 1). cDNAs to amplify the full length *Mx* from each bird were synthesized from the total RNA extracted from Poly (I:C) treated bird cells with PrimeScript RT Master Mix (Perfect Real Time) (Takara Bio Inc., Kusatsu, Japan). The PCR reaction mixture to amplify *Mx* was prepared using AccuPrime^{TM} Taq DNA Polymerase High Fidelity (Invitrogen, Waltham, MA, USA). PCR amplification of *Mx* was conducted using the following conditions with the Gene Amp PCR System 9700 (Applied Biosystems, Waltham, MA, USA): 94°C for 1 min, 40 cycles of 94°C for 30 sec, 52°C for 30 sec, and 68°C for 2 min, and hold at 4°C. The PCR products were cloned into pGEM-T Easy vector (Promega, Madison, WI). Cloned PCR product sequencing was performed using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) and the 3130 Genetic Analyzer (Applied Biosystems) with the primers Avian Mx1 (forward), Avian Mx2 (forward), Avian Mx3 (forward), Avian Mx4 (forward-2), Avian Mx5 (forward), Avian Mx1 (reverse), Avian Mx2 (reverse), Avian Mx3 (reverse), Avian Mx4 (reverse), Avian Mx5 (reverse), SP6, and T7 (Table 1). The full length *Mx* from each bird was deposited in Genbank with the accession numbers LC413895 (chicken), LC413896 (jungle crow), LC413897 (rock dove), and LC413898 (whooper swan).

Constitutive Mx expression in 3T3 cells

The expression vectors of the bird-derived *Mx* were constructed using pTARGET^{TM} Vector Mammalian Expression Vector System (Promega, Madison, WI, USA) with the *Mx* of chicken (Accession No. LC413895), jungle crow (Accession No. LC413896, rock dove (Accession No. LC413897), and whooper swan (Accession No. LC413898). The vectors were transfected into mouse fibroblast BALB/3T3 cells using Lipofectamine 2000 Transfection Reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The transfected cells were selected with the antibiotic G-418 (Promega). The BALB/3T3 cells lack functional Mx [11, 19], enabling evaluation of the synthesized endogenous Mx proteins. The BALB/3T3 cells were cultured in Dulbecco's Modified Eagle's Medium containing 10% FBS and G-418 for stable *Mx* expression. These studies were approved and implemented by the Genetic Recombination Committee of the Institution (NIES: accession no. H29-08, RGU accession no. 141).

The following experiment was performed to confirm *Mx* expression in BALB/3T3 cells. Total RNA was extracted from each transfected cell using EZ 1 advance (Qiagen) with DNase treatment. The extracted total RNA (200 *n*g) was used to synthesize cDNA using the PrimeScript RT Master Mix (Perfect Real Time) in a total volume of 20 *µl* according to the manufacturer's instructions (Takara Bio). One *µl* of each cDNA, which was equivalent of 10 *n*g of total RNA, was used for PCR amplification to confirm *Mx* expression in each of the transfected cells. The primer sets for the full length *Mx* (approximately 2 kb) of each bird were used for PCR (Supplementary Table 1). The PCR reaction mixture contained 5 *µl* of 10x Buffer for KOD-Plus-Ver.2, 5 *µl* of 2 mM dNTPs, 3 *µl* of 25 mM $MgSO_4$, 1.5 *µl* of forward specific primer (10 pmol 1.5 *µl* of reverse specific primer (10 pmol), 1 *µl* of KOD-Plus- (1 U/m*l*), 32 *µl* of autoclaved distilled water, and 1 *µl* of the cDNA templates. PCR amplification was performed using the Biometra T3000 Thermocycler (Biometra GmbH, Gottingen, Germany) with the following conditions: 94°C for 2 min, 35 cycles of 98°C for 10 sec, 55°C (60°C for chicken) for 30 sec, and 68°C for 2 min 30 sec, and a hold step at 4°C. The PCR product was electrophoresed on a 2% gel to confirm product size and amplification. The partial sequence of the mouse *GAPDH* (366 bp) was used as the internal control.

Virus infectivity

Virus infectivity was determined based on the focus-forming units (FFU)/m*l* in each of the Mx-3T3 cells using the micro-focus method according to previous report [13] with some modifications. The FFU assay is a useful method to compare the anti-viral effect at early infection stages. The virus-infected Mx-3T3 cells at 24 hpi were fixed with ice-cold methanol/acetone, and permeability treatment was performed using 0.1% Triton x-100. The virus antigen on the cells was detected using the C43 monoclonal antibody to influenza A virus nucleoprotein (ab128193; Abcam, Tokyo, Japan), followed by FITC-conjugated anti-mouse IgG to detect the antigen. The infectivity was confirmed by observing the samples under a fluorescence microscope (Zeiss, Oberkochen, Germany).

3D structure analysis of Mx protein

The amino acid sequences of chicken, jungle crow, rock dove, and whooper swan Mx protein were aligned using the CLUSTAL W program [20]. Sequence identity was analyzed using DNASTAR Lasergene version 15.2 (DNASTAR, Madison, WI, USA).

The functional domain of Mx protein from each bird was identified based on the previously reported functional domains, GTPase domain (G-domain), bundle signaling elements (BSE), middle stalk domain, GTPase effector domain, and the loop regions in chicken [8]. The aligned sequences were used for protein structure prediction. Protein structure prediction was performed using the Raptor X protein structure predication server (http://raptorx.uchicago.edu/StructurePrediction/). The predicted 3D structures

were visualized using DNASTAR Lasergene version 15.2 (DNASTAR). The RMSD values of the four structures were calculated by DNASTAR Lasergene version 15.2 (DNASTAR).

Statistical analysis

Statistically significant differences were assessed using Kruskal-Wallis, Tukey-Kramer, and Student's *t*-tests compared to the control using R software (R-3.2.1).

RESULTS

Virus proliferation and Mx expression in bird primary cells

The number of virus copies in the culture supernatant at 24 hpi was assessed to compare the proliferation of viruses in each bird's primary cell cultures. As a result, the viral copy number showed a difference depending on the bird species with an average log 6.1 to 7.1/m*l* values. The birds with the highest number of virus copies were chicken and jungle crow with log 7/m*l*, followed by rock dove with log 6.5/m*l* and whooper swan with log 6.2/m*l*. The copy number was significantly lower in the rock dove and whooper swan compared to the jungle crow (Fig. 1). *Mx* expression in the infected cells was compared at 6 hpi. *Mx* expression was highly expressed in rock dove and whooper swan at 0 hpi. *Mx* expression was significantly different in bird types. (Kruskal-Wallis P=0.0055 (0 hpi), P=0.0079 (6 hpi)). The *Mx* expression increased significantly in the cells from the rock dove after infection (P=0.02, Fig. 2).

Infectivity evaluation in cells expressing bird Mx

The amino acid sequences of the Mx protein from the four birds were compared. A sequence identity of 68.4% was found between the rock dove and jungle crow. On the other hand, the identity between the Mx protein of chicken and jungle crow was 60.4%, and it was 57.7% between the chicken and rock dove (Table 2, Supplementary Fig. 2). Constitutive expression of bird *Mx* was confirmed in 3T3 cells using PCR. The results showed a clear positive *Mx* signal at the expected size, and the expression of the *Mx* in each bird was confirmed in the BALB/3T3 cells (Supplementary Fig. 1). Next, we evaluated HPAIV infectivity in these cells. Virus infectivity was evaluated as the focus foaming unit (FFU)/m*l* at 24 hpi. The viral infectivity varied depending on the *Mx* type in the birds. *Mx*-expressing cells derived from chicken, jungle crow, and rock dove significantly decreased virus infectivity (Fig. 3). The cells with the lowest infectivity showed a decrease of about 60% at 150 FFU/m*l* or less in the pigeon (P=0.0002). Both the chicken and jungle crow showed an infectivity below 200 FFU/m*l* (P=0.006), and the whooper swan showed an infectivity of 200 FFU/m*l* with no significant differences compared to the vector control (Fig. 3).

Mx protein 3D structure analysis

The amino acid sequence alignments from the four bird species were analyzed for sequence identity (Table 2). The rock dove sequence showed the highest similarity to the jungle crow sequence (68.4%), and the chicken sequence showed the lowest similarity (57.7%). The 3D structure models of the Mx protein from each species are shown in Fig. 4. Diversity in the Mx 3D structure was confirmed in the birds (Fig. 5). According to amino acid sequence alignment between the four species, the functional domains start at the 90th amino acid. Thus, 616 residues in chicken, 615 residues in whooper swan, 614 residues in jungle crow,

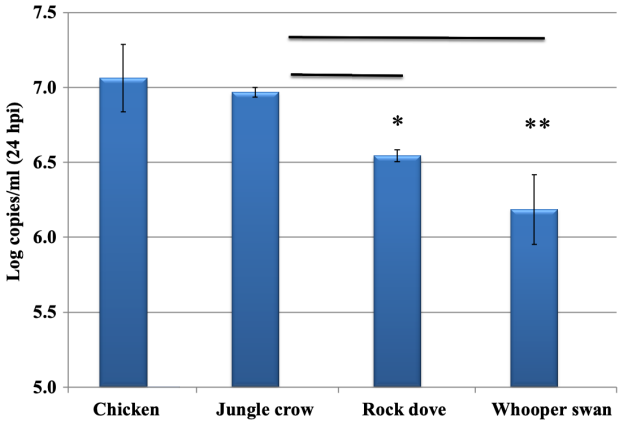

Fig. 1. Virus copy numbers 24 hr after infection. The primary cells from each bird were infected with H5N1 virus at multiplicity of infection (MOI) of 0.1, and the virus copy number was determined using qRT-PCR in the culture supernatant. The result is the mean and standard deviation of the two experimental results. Pigeons and swans showed significantly reduced viral copy numbers compared to crows (**P<0.01, *P<0.05).

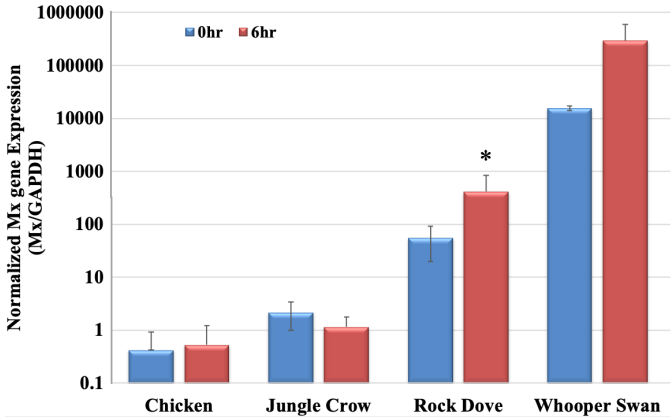

Fig. 2. *Mx* expression 0 and 6 hr after HPAIV infection. The data show the *Mx* expression normalized to *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) in the infected primary cells. *P<0.05.

Table 2. Bird Mx amino acid sequence identity (%) and 3D structure comparison using Root mean square deviation (Å, lower column)

	Chicken	Whooper swan	Jungle crow	Rock dove
Chicken		65.1%	60.4%	57.7%
Whooper swan	2.743 Å		66.3%	68.6%
Jungle crow	2.634 Å	1.676 Å		68.4%
Rock dove	2.327 Å	1.864 Å	1.686 Å	

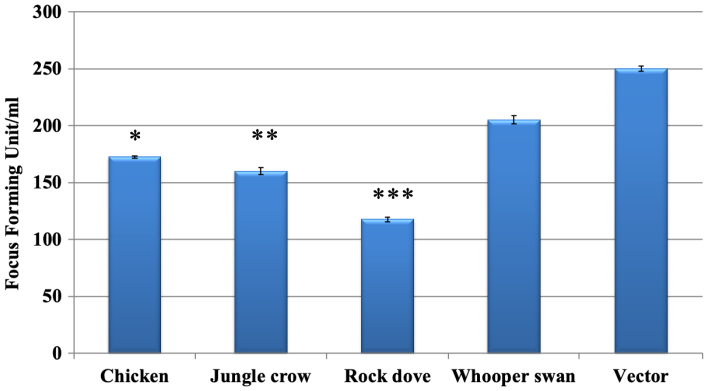

Fig. 3. Infectivity of the virus in Mx-expressing 3T3 cells. FFU was evaluated in Mx-expressing cells derived from each bird at 24 hpi. The infection rate was significantly different compared to the vector control (*P<0.05, **P<0.01 or ***P<0.001).

and 616 residues in rock dove were used for 3D structure predictions. The results of the 3D structure prediction identified the crystal structure of human myxovirus resistance protein 2 (Mx B) (PDB ID: 4WHJA) as the best template (P-value: 9.32e−11 in chicken, 6.41e−11 in whooper swan, 1.01e−10 in jungle crow, and 1.39e−10 in rock dove).

DISCUSSION

This study showed that the proliferation of the H5N1 virus varied in different bird-derived cells after infection. The viral copy number was significantly lower in rock dove and whooper swan than jungle crow cells (Fig. 1). *Mx* expression in bird cells showed different levels during early infection depending on the species compared to chicken and jungle crow, and the highest levels were in rock dove and whooper swan. These results suggested that virus multiplication in primary bird cells seemed to be regulated by *Mx* expression at early infection. However, it is necessary to confirm whether each bird-derived Mx protein has an antiviral effect.

Therefore, we evaluated infectivity against HPAIV in BALB/ 3T3 cells expressing four avian *Mx* genes. Chicken Mx protein was previously shown to localize to the cytoplasm [2], and it has been reported that the antiviral activity of chicken and duck Mx proteins is weak [1, 4]. Our results showed that the antiviral activity of chicken *Mx* was weak compared to rock dove and jungle crow (Fig. 3). Thus, our findings indicate that the low *Mx* expression in response to HPAIV infection and low antiviral activity of chicken *Mx* might cause high H5N1 virus proliferation in primary chicken cells.

The antiviral activity of whooper swan *Mx* was weak compared to the other three species (Fig. 3). However, the virus copy numbers in the primary cells of the whooper swan were relatively lower 24 hr after infection than those in other species (Fig. 1). The robust expression of *Mx* (Fig. 2) at the early infection (0−6 hpi) could supplement the low antiviral activity of *Mx* in the primary cells of whooper swan.

Although the antiviral effect of the jungle crow-derived *Mx* was relatively high (Fig. 3), the virus copy number was the same level as the chicken primary cells (Fig. 1). The phenomena might be due to insufficient *Mx* expression at the early infection stages in the jungle crow cells (Fig. 2).

The *Mx* of the rock dove had a significant anti-viral effect in response to HPAIV (Fig. 3). The high gene expression in the rock dove primary cells (Fig. 2) and significant anti-viral effect of *Mx* might contribute to the lowest virus copy number at 24 hr after infection in the rock dove primary cells. The species showed limited susceptibility to HPAIV infection [23]. Thus, the high level of *Mx* expression and the significant anti-viral effect of *Mx* might result in relatively low virus replication in the primary cells and could be the molecular basis of HPAIV infection resistance in the species.

Our results showed that the antiviral activity of *Mx* differs by species based on focus-forming units in the *Mx*-3T3 cells (Fig. 3). The *Mx* of the rock dove had a significant anti-viral effect among the four species. On the other hand, the whooper swan *Mx* showed the lowest antiviral activity among the species. According to previous reports, the C-terminus of loop L4 plays an important role in antiviral activity. In human MxA protein, mutations at positions 561, 562, and 577 affect MxA activity against influenza A virus strain A/Thailand/1/04 (H5N1) [15]. In addition, loop L4 of the mouse Mx1 protein is a key region for antiviral activity [21]. For example, V516A substitution was found to decrease the antiviral activity of *Mus spretus* Mx1. On the other hand, the E540G mutation increased the antiviral activity against influenza A virus. The results of the 3D structure comparison demonstrated that the structure of loop L4 varied among the four bird species (Fig. 5). The variation in the structure of loop L4 could result from amino acid sequence variation because the sequence directly affects the protein structure. The association between influenza A virus infectivity and the amino acid sequence of loop L4 in wild birds remains unclear. Further research is essential to elucidate the mechanisms of underlying the antiviral activity of loop L4 in wild birds. However, based on the relation between loop L4 amino acid sequence and anti-viral effect observed in human MxA protein and mouse Mx1 protein and a

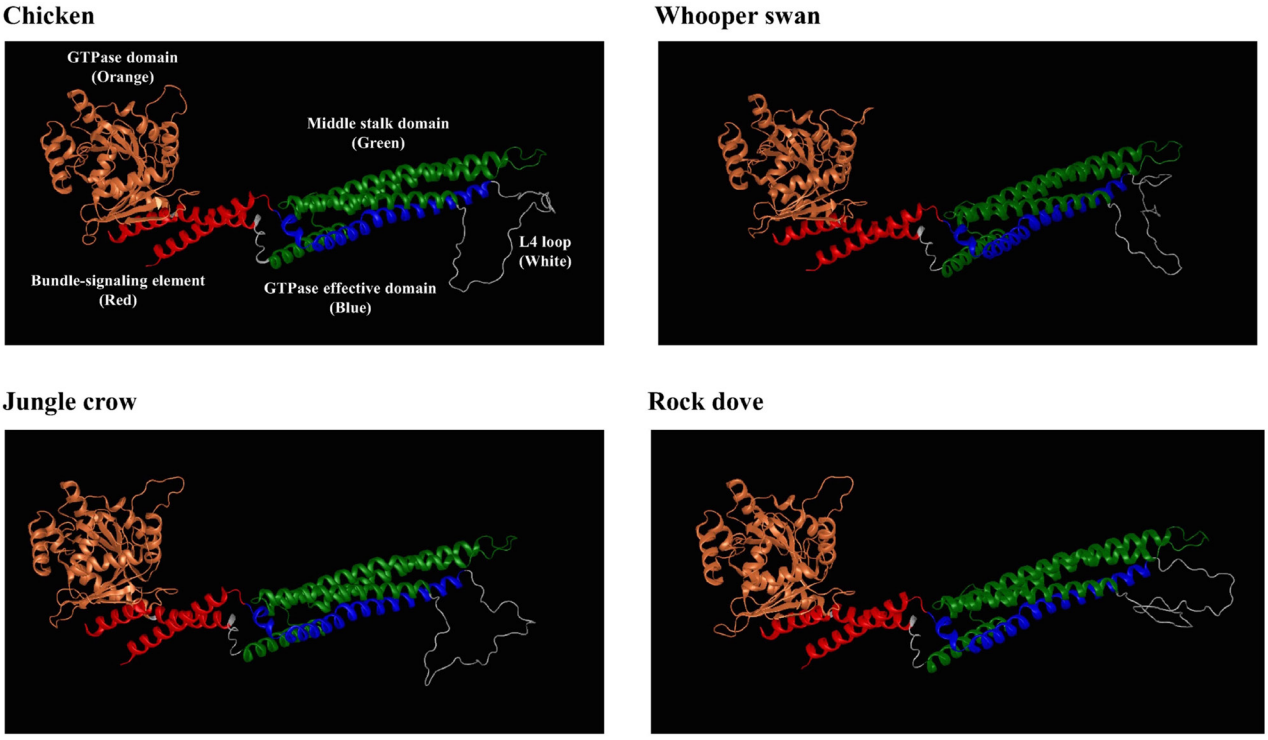

Fig. 4. Mx protein 3D structure comparison between the four bird species. The structure of the L4 loop clearly differed between the four species.

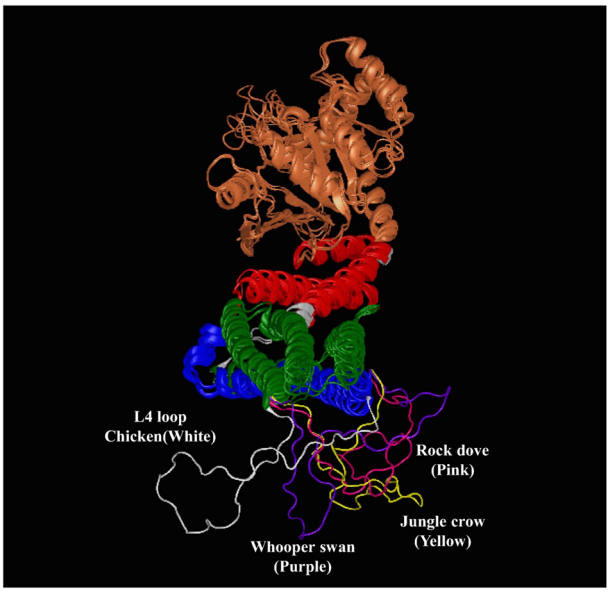

Fig. 5. Comparisons of the structure of L4 loop among the four bird species. Four different 3D structures of Mx are merged in the Figure.

significant anti-viral effect observed in rock dove, the amino acid sequence of loop L4 in rock dove might represent relatively high anti-viral activity.

The study revealed that Mx expression patterns in birds cells showed difference, and the antiviral activity of Mx varied depending on bird species. There could be complex factors relating the resistance against HPAIV infection in birds. However, the bird species showing resistance against HPAIV infection might have robust Mx expression in the early stages of the virus infection, and have an anti-viral effect of Mx protein reflecting characteristic amino acid sequences in the loop L4 region.

ACKNOWLEDGMENTS. We would like to thank Dr. Yuko Kato-Mori at RGU for her expert technical assistance and Dr Keisuke Saito and Dr. Yukiko Watanabe at Institute for Raptor Biomedicine Japan for providing the tissue samples of whooper swan. This work was partly supported by the JSPS Core-to-Core Program, B. Asia-Africa Science Platforms (2016–2018) and by the Environment Research and Technology Development Fund (SII-1) of the Environmental Restoration and Conservation Agency of Japan.

REFERENCES

1. Bazzigher, L., Schwarz, A. and Staeheli, P. 1993. No enhanced influenza virus resistance of murine and avian cells expressing cloned duck Mx protein. *Virology* **195**: 100–112. [Medline] [CrossRef]
2. Benfield, C. T., Lyall, J. W. and Tiley, L. S. 2010. The cytoplasmic location of chicken mx is not the determining factor for its lack of antiviral activity. *PLoS One* **5**: e12151. [Medline] [CrossRef]
3. Berlin, S., Qu, L., Li, X., Yang, N. and Ellegren, H. 2008. Positive diversifying selection in avian Mx genes. *Immunogenetics* **60**: 689–697. [Medline] [CrossRef]
4. Bernasconi, D., Schultz, U. and Staeheli, P. 1995. The interferon-induced Mx protein of chickens lacks antiviral activity. *J. Interferon Cytokine Res.* **15**: 47–53. [Medline] [CrossRef]
5. Brown, J. D., Stallknecht, D. E. and Swayne, D. E. 2008. Experimental infection of swans and geese with highly pathogenic avian influenza virus (H5N1) of Asian lineage. *Emerg. Infect. Dis.* **14**: 136–142. [Medline] [CrossRef]
6. de Veer, M. J., Holko, M., Frevel, M., Walker, E., Der, S., Paranjape, J. M., Silverman, R. H. and Williams, B. R. 2001. Functional classification of interferon-stimulated genes identified using microarrays. *J. Leukoc. Biol.* **69**: 912–920. [Medline]
7. Dillon, D. and Runstadler, J. 2010. Mx gene diversity and influenza association among five wild dabbling duck species (Anas spp.) in Alaska. *Infect. Genet. Evol.* **10**: 1085–1093. [Medline] [CrossRef]
8. Fulton, J. E., Arango, J., Ali, R. A., Bohorquez, E. B., Lund, A. R., Ashwell, C. M., Settar, P., O'Sullivan, N. P. and Koci, M. D. 2014. Genetic variation within the Mx gene of commercially selected chicken lines reveals multiple haplotypes, recombination and a protein under selection pressure. *PLoS One* **9**: e108054. [Medline] [CrossRef]
9. Haller, O., Stertz, S. and Kochs, G. 2007. The Mx GTPase family of interferon-induced antiviral proteins. *Microbes Infect.* **9**: 1636–1643. [Medline] [CrossRef]
10. Hiono, T., Okamatsu, M., Yamamoto, N., Ogasawara, K., Endo, M., Kuribayashi, S., Shichinohe, S., Motohashi, Y., Chu, D. H., Suzuki, M., Ichikawa, T., Nishi, T., Abe, Y., Matsuno, K., Tanaka, K., Tanigawa, T., Kida, H. and Sakoda, Y. 2016. Experimental infection of highly and low pathogenic avian influenza viruses to chickens, ducks, tree sparrows, jungle crows, and black rats for the evaluation of their roles in virus transmission. *Vet. Microbiol.* **182**: 108–115. [Medline] [CrossRef]
11. Horisberger, M. A., Staeheli, P. and Haller, O. 1983. Interferon induces a unique protein in mouse cells bearing a gene for resistance to influenza virus. *Proc. Natl. Acad. Sci. USA* **80**: 1910–1914. [Medline] [CrossRef]
12. Kuwana, T., Hashimoto, K., Nakanishi, A., Yasuda, Y., Tajima, A. and Naito, M. 1996. Long-term culture of avian embryonic cells in vitro. *Int. J. Dev. Biol.* **40**: 1061–1064. [Medline]
13. Okuno, Y., Tanaka, K., Baba, K., Maeda, A., Kunita, N. and Ueda, S. 1990. Rapid focus reduction neutralization test of influenza A and B viruses in microtiter system. *J. Clin. Microbiol.* **28**: 1308–1313. [Medline] [CrossRef]
14. Pandit, P. S., Bunn, D. A., Pande, S. A. and Aly, S. S. 2013. Modeling highly pathogenic avian influenza transmission in wild birds and poultry in West Bengal, India. *Sci. Rep.* **3**: 2175. [Medline] [CrossRef]
15. Patzina, C., Haller, O. and Kochs, G. 2014. Structural requirements for the antiviral activity of the human MxA protein against Thogoto and influenza A virus. *J. Biol. Chem.* **289**: 6020–6027. [Medline] [CrossRef]
16. Reed, C., Bruden, D., Byrd, K. K., Veguilla, V., Bruce, M., Hurlburt, D., Wang, D., Holiday, C., Hancock, K., Ortiz, J. R., Klejka, J., Katz, J. M. and Uyeki, T. M. 2014. Characterizing wild bird contact and seropositivity to highly pathogenic avian influenza A (H5N1) virus in Alaskan residents. *Influenza Other Respi. Viruses* **8**: 516–523. [Medline] [CrossRef]
17. Schneider, W. M., Chevillotte, M. D. and Rice, C. M. 2014. Interferon-stimulated genes: a complex web of host defenses. *Annu. Rev. Immunol.* **32**: 513–545. [Medline] [CrossRef]
18. Staeheli, P., Haller, O., Boll, W., Lindenmann, J. and Weissmann, C. 1986. Mx protein: constitutive expression in 3T3 cells transformed with cloned Mx cDNA confers selective resistance to influenza virus. *Cell* **44**: 147–158. [Medline] [CrossRef]
19. Staeheli, P., Grob, R., Meier, E., Sutcliffe, J. G. and Haller, O. 1988. Influenza virus-susceptible mice carry Mx genes with a large deletion or a nonsense mutation. *Mol. Cell. Biol.* **8**: 4518–4523. [Medline] [CrossRef]
20. Thompson, J. D., Higgins, D. G. and Gibson, T. J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**: 4673–4680. [Medline] [CrossRef]
21. Verhelst, J., Spitaels, J., Nürnberger, C., De Vlieger, D., Ysenbaert, T., Staeheli, P., Fiers, W. and Saelens, X. 2015. Functional Comparison of Mx1 from two different mouse species reveals the involvement of loop L4 in the antiviral activity against influenza a viruses. *J. Virol.* **89**: 10879–10890. [Medline] [CrossRef]
22. World Health Organization (WHO). WHO information for molecular diagnosis of influenza virus-update (http://www.who.int/influenza/gisrs_laboratory/molecular_ diagnosis_influenza_virus_humans_update_201403rev2015 05.pdf) [accessed on March 30, 2017].
23. Yamamoto, Y., Nakamura, K., Yamada, M. and Mase, M. 2012. Limited susceptibility of pigeons experimentally inoculated with H5N1 highly pathogenic avian influenza viruses. *J. Vet. Med. Sci.* **74**: 205–208. [Medline] [CrossRef]
24. Zeng, M., Chen, S., Wang, M., Jia, R., Zhu, D., Liu, M., Sun, K., Yang, Q., Wu, Y., Chen, X. and Cheng, A. 2016. Molecular identification and comparative transcriptional analysis of myxovirus resistance GTPase (Mx) gene in goose (*Anser cygnoide*) after H9N2 AIV infection. *Comp. Immunol. Microbiol. Infect. Dis.* **47**: 32–40. [Medline] [CrossRef]