FULL PAPER *Avian Pathology*

Molecular Detection of Avian Pathogens in Poultry Red Mite (*Dermanyssus gallinae)* Collected in Chicken Farms

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ABSTRACT. Poultry red mite (PRM, *Dermanyssus gallinae)* is a blood-sucking ectoparasite as well as a possible vector of several avian pathogens. In this study, to define the role of PRM in the prevalence of avian infectious agents, we used polymerase chain reaction (PCR) to check for the presence of seven pathogens: Avipox virus (APV), Fowl Adenovirus (FAdV), Marek's disease virus (MDV), *Erysipelothrix rhusiopathiae* (ER), *Salmonella enterica* (SE), *Mycoplasma synoviae* (MS) and *Mycoplasma gallisepticum* (MG). A total of 159 PRM samples collected between 2004 and 2012 from 142 chicken farms in 38 prefectures in Japan were examined. APV DNA was detected in 22 samples (13.8%), 19 of which were wild-type APV. 16S ribosomal RNA (16S rRNA) of MS was detected in 15 samples (9.4%), and the *mgc2* gene of MG was detected in 2 samples (1.3%). Eight of 15 MS 16S rRNA sequences differed from the vaccine sequence, indicating they were wild-type strains, while both of the MG *mgc2* gene sequences detected were identical to the vaccine sequences. Of these avian pathogen-positive mite samples, three were positive for both wild-types of APV and MS. On the other hand, the DNAs of ER, SE, FAdV and MDV were not detected in any samples. These findings indicated that PRM can harbor the wild-type pathogens and might play a role as a vector in spreading these diseases in farms.

KEY WORDS: avian pathogens, *Dermanyssus gallinae,* DNA detection, poultry red mite

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Dermanyssus gallinae (Acari, Mesostigmata, Dermanyssoidea, Dermanyssidae), poultry red mite (PRM), is an obligatory blood-sucking parasite of both domestic and wild birds. The mite has been reported to parasitize at least 28 avian species [15] and is considered one of the major problems in poultry farms in the United States [7], Europe [2] and Japan [14]. The infestation of PRM in poultry results in stress, decreasing egg production, anemia and even mortality due to exsanguination.

PRMs carry and are potential vectors of several pathogens including *Salmonella enterica* (SE) [9, 25], *Erysipelothrix rhusiopathiae* (ER) [4] and Avipox virus (APV) [3, 18]. The mites can also be sites of *Salmonella* Enteritidis multiplication [24]. However, the prevalence of avian microbial pathogens in PRM over a wide region has not been studied.

In the present study, to define the role of PRM in transmitting and maintaining avian pathogens in poultry farms, PRMs were collected from chicken farms throughout Japan and examined for DNAs of APV, ER and SE. We also examined PRM for the presence of four other pathogens that are frequently detected in chicken farms, but that so far have not been detected in PRM: *Mycoplasma synoviae* (MS), *Mycoplasma gallisepticum* (MG), Fowl Adenovirus (FAdV) and Marek's disease virus (MDV).

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MATERIALS AND METHODS

Dermanyssus gallinae: A total of 159 samples (at least 50 for each sample) were obtained from 142 chicken farms in 38 prefectures throughout Japan from 2004 to 2012. In all of the farms, the chickens appeared healthy. Each sample was stored in 99.5% ethanol at room temperature until use.

DNA preparation: Mite DNA samples used in this study were obtained from 10 pooled adult mites. The mites were homogenized with zirconia beads using TissueLyser II (Qiagen Inc., Chatsworth, CA, U.S.A.) in 20 μl of buffer 1 provided by a Ten Minute DNA Release Kit −1 (Jacksun Easy Biotech Inc., New York, NY, U.S.A.), and the DNA samples were prepared according to the manufacturer's instructions. When a sample was found to be positive for one of the seven pathogens, another DNA sample was prepared as described below to estimate whether the pathogens exist on surface or internal side of the PRM. Ten mites prepared from the sample that was positive for any pathogens were washed out before the DNA preparation to remove the microorganisms on the surface of the mites as previously described [8]. Briefly, ten mites taken from the 99.5% ethanol were rinsed three times in 500 μl of sterile ultrapure water with vigorous shaking. Then, the washed mites were used for DNA preparation. If the PCRs using the DNA samples prepared from the washed mites were positive, it was speculated that

the pathogens existed internal side of PRM. DNA samples of commercial vaccines were also prepared with the same DNA extraction kit as a control. The DNA samples prepared were stored at −30°C until use.

PCR for detection of avian pathogens: The DNA samples were screened for seven avian pathogens including ER, MS, MG, SE, APV, FAdV and MDV by PCR as described below. The target genes and their PCR primers are shown in Table 1.

For detection of APV DNA, primers P1x and P2 were used to amplify part of the 4b core protein gene [10]. Primer P1x was designed based on the sequence of primer P1 [10] by adding one more T nucleotide to the 5′ end to improve the sensitivity (our unpublished data). If the gene coding for the 4b core protein was detected, the viral DNA was further examined to determine whether it was derived from the wild type APV or commercial vaccine strain. Because the wild type APV genome includes an intact reticuloendotheliosis (RE) provirus and the genome of the vaccine virus includes only the truncated LTR sequence of RE provirus [6], to detect the insertion of intact RE provirus sequence in APV genome, PCR using heterologous primer set that anneals to *env* gene of RE provirus (REVenv7F primer) [6] and FPV ORF 203 (FPV203 3R primer) [6] was conducted as semi-nested first PCR. When this PCR was negative, semi-nested second PCR using primers REVenv7F and TR2 [19] that anneals to the internal region flanking to FPV203 3R primer annealing site was conducted. When the first or second PCRs were positive, we concluded that intact RE provirus insertion, that was specifically found in wild type APV, was positive. Each primer sequence, target genes and expected length of the PCR products are shown in Table 1. DNAs prepared from the commercial vaccines used in Japan were used as negative controls for the presence of RE provirus.

Primers MSL1 and MSL2 [12] (Table 1) were used to detect the MS 16S rRNA sequence. Subsequently, the nucleotide sequences of PCR products were compared with the sequence derived from the MS-H vaccine strain used in Japan to differentiate between the MS wild type and the vaccine strain [1].

Primers Mgc2 2F and Mgc2 2R were used to detect the MG *mgc2* gene [5]. The obtained nucleotide sequences were used to differentiate MG from 5 commercial live vaccine strains used in Japan (ts-11, 6/85, MGS, G210 and K5831-B19) following a previous report [11].

PCRs were performed in a total volume of 25 μl containing 2 μl of DNA template (or 0.5 μl of the first PCR product in semi-nested second PCR) prepared as described above, 1 μl of forward and reverse primer mixture (final concentration was 0.5 μM each) and 12.5 μl of GoTaq Green Master Mix, 2X (Promega, Madison, WI, U.S.A.). The PCR conditions for each pair of primers were employed according to the previous reports listed in Table 1. Amplification products were confirmed by electrophoresis using 1.2% (w/v) agarose gels, stained with 0.1% Gel Red (Biotium, Inc., Hayward, CA, U.S.A.) and visualized with a UV transilluminator. To confirm the specificity of the PCR, nucleotide sequence of the PCR products was determined by Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems, Carlsbad, CA, U.S.A.) with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

RESULTS

Twenty-five (15.7%) of the 159 DNA samples prepared from the mites without washing prior to the DNA preparation were positive for a single pathogen, either APV or MS or MG, while the DNAs of ER, SE, FAdV and MDV were not detected (Table 2). Fifteen of these 25 samples were positive only for APV, and 9 samples and 1 sample were positive only for MS and MG, respectively. In addition, seven samples (4.4%) without washing were multiple pathogens positive. Of the seven DNA samples, six were positive for both APV and MS, and another was positive for both APV and MG. In total, 32 (20.1%) of the 159 DNA samples, 25 for single pathogen and 7 for multiple pathogens, were positive in PCRs conducted in this study. APV, MS and MG positive samples were detected from 12, 12 and 2 prefectures, respectively. Apparent geographical bias was not found in distribution of the positive samples.

The APV gene was totally detected in 22 of the 159 PRM DNA samples (13.8%) prepared from the mites without washing. Furthermore, 4 of the 22 DNA samples prepared from the mites after washing were also positive for APV. Nineteen samples positive for APV were also positive in PCR for REV *env* to FPV ORF 203 region.

In total, fifteen of the 159 DNA samples (9.4%) prepared from the mite without washing were positive for the MS 16S rRNA. In addition, two of the 15 DNA samples prepared from the mite after washing were positive for the rRNA. Of the fifteen positive samples, seven of these sequences were identical to the sequence of the vaccine MS-H used in Japan. The eight other sequences differed by at least 1 nucleotide from the sequence of MS-H vaccine. Two of these 8 sequences were identical to each other and differed by 2 nucleotides from the MS-H sequence. These two sequences were detected from different chicken houses in the same farm.

Two of the 159 DNA samples (1.3%) prepared from the unwashed mites were positive for the MG *mgc2* gene (Table 2). However, when the two DNA samples were prepared from the washed mites, no DNA samples were positive for the *mgc2* gene. Of the two positive DNA samples, one sequence was identical to that of vaccine strain G210, and the other was identical to that of vaccine strain K5831-B19.

Of the six samples positive for both APV and MS, 5 samples were positive in PCR for REV *env* to FPV ORF 203 region. In addition, MS 16S rRNA sequences detected from 3 of these 5 samples were different from the sequence of MS-H vaccine. In the case of another multiple pathogens positive sample, which was positive for both APV and MG, PCR for REV *env* to FPV ORF 203 region was positive, and the sequence of MG *mgc2* gene detected was identical to that of vaccine strain K5831-B19.

Table 1. List of primers used for detection of each pathogen

Pathogens	Primers[a)]	Primer sequences	Target sequences	Length of PCR products	References
Erysipelothrix rhusiopathiae	ER1F ER1R	5′-gttcatctctctaatgcactac-3′ 5′-tgttggactactaatcgtttcg-3′	23S rRNA	399 bp	[23]
Mycoplasma gallisepticum	Mgc2 2F Mgc2 2R	5′-cgcaatttggtcctaatccccaac-3′ 5′-taaacccacctccagctttatttc-3′	*mgc2*	236–302 bp	[5]
Mycoplasma synoviae	MSL1 MSL2	5′-gagaagcaaaatagtgatatca-3′ 5′-cagtcgtctccgaagttaacaa-3′	16S rRNA	211 bp	[12]
Salmonella enterica	Stn − 101 Stn − 111	5′-ctttggtcgtaaaataaggcg-3′ 5′-tgcccaaagcagagagattc-3′	*enterotoxin*	260 bp	[26]
	16S F 16S R	5′-tgttgtggttaataaccgca-3′ 5′-cacaaatccatctctgga- 3′	16S rRNA	574 bp	[26]
Avipox virus	P1x P2	5′-tcagcaggtgctaaacaaca-3′ 5′-cggtagcttaacgccgaata-3′	4b core protein	579 bp	In this study [10]
	REVenv7F FPV203 3R	5′-cctgactcgcattatccatgacaa-3′ 5′-ttcaaccaccaggctacataaagg-3′	REV *env* to FPV ORF203 for the semi-nested first PCR	740 bp	[6]
	REVenv7F TR2	5′-cctgactcgcattatccatgacaa-3′ 5′-cacacgaatataccaataagg-3′	REV *env* to FPV ORF203 for the semi-nested second PCR	579 bp	[6] [19]
Fowl Adenovirus	Hex L1-s Hex L1-as	5′-atgggagcSacctaYttcgacat-3′ 5′- aaattgtcccKRaaNccgatgta-3′	*hexon*	590 bp	[21]
Marek's disease virus	MEQ FP MEQ RP	5′-ggatcgcccaccacgattactacc-3′ 5′-actgcctcacacaacctcatctcc-3′	*meq*	400 bp	[16]

a) Forward and reverse primers.

DISCUSSION

In the present study, APV, MS and MG DNAs were detected in 22, 15 and 2 DNA samples prepared from the unwashed mites, respectively. Of the total, 25 samples were single pathogen positive, and 7 samples were multiple pathogens positive. These results indicate the possibility that the poultry red mite can transfer APV, MS and MG. In addition, 4 and 2 of 32 DNA samples prepared from the mites after washing were positive for APV and MS, respectively. Although the positive number decreased after washing, APV or MS was detected even after 3 times washing in the mites. Because the washing step is expected to remove microorganisms on the surface of the mites, this result implied that poultry red mite can harbor APV and MS both externally and internally.

Of the 22 DNA samples positive for APV, 19 were also positive for REV *env* to FPV ORF 203 region. This finding shows that the 19 samples have intact RE provirus reported as a virulence marker for wild-type APV [20]. In Japan, almost all chickens are vaccinated for APV infection, and only a few outbreaks of avian pox are reported each year. However, this finding indicates that wild-type APV is latently distributed even in apparently healthy farms and that PRM may play a role in transmission of the virus in chicken farms. In fact, outbreaks of avian pox were reported even in vaccinated chicken flocks in farms where the mites were present [3]. Because PRM is known as a mechanical vector of APV [18], in addition to vaccination against APV infection, eradiation of PRM is needed for effective prevention of the disease.

Of the 15 MS 16S rRNA sequences obtained in this study, eight were different from the MS vaccine sequence, indicating that the MS detected was the wild type (Table 2). This shows that PRM can harbor wild-type MS, which is a possible chicken pathogen. Although this finding raises the possibility that PRM can transmit MS among chickens, further studies, such as experimental infection of MS to chicken through a PRM, may be needed to confirm it. Moreover, the finding of two identical wild-type MS sequences in different chicken houses in the same farm implied that PRM can spread MS among chicken houses in a farm. To our knowledge, this is the first report to show the presence of a mycoplasma in PRM.

Of the six mite samples positive for both APV and MS, three samples were positive in PCR for REV *env* to FPV ORF 203 region, and the MS 16S rRNA sequences detected were different from the sequence of vaccine strain. These results indicate that both of the APV and MS detected from the 3 samples were wild-type (Table 2). These findings suggest that the mite can harbor and transmit more than one avian pathogen in poultry farms. This is the first report to show the presence of multiple pathogens in PRMs.

In European countries, ER was found in PRM collected from infected farms [4], and SE was found in mites collected from apparently healthy farms [9]. The infections of SE and ER among some chickens have been reported in Japan [17, 22]. However, these bacteria were not detected in any of the present mite samples. Furthermore, although FAdV and MDV are ubiquitous pathogens in chicken farms [13,

Table 2. Sample numbers and the distribution of the mite samples which showed positive in PCR for detection of avian pathogen DNAs from the mite samples without washing

Prefectures	Sample number	Single pathogen positive							Multiple pathogens positive					All negative (%)
		APV[a)]		MS[b)]		MG[c)]		Subtotal (%)	APV Wild & MS Vac (%)	APV Wild & MS Wild (%)	APV Vac & MS Vac (%)	APV Wild & MG Vac (%)	Subtotal (%)	
		Wild (%)	Vac[d)] (%)	Wild (%)	Vac (%)	Wild (%)	Vac (%)							
Akita	1	1 (100)	0	0	0	0	0	1 (100)	0	0	0	0	0	0
Aomori	2	0	0	1 (50.0)	0	0	0	1 (50.0)	0	0	0	0	0	1 (50.0)
Chiba	44	5 (11.4)	0	1 (2.3)	0	0	1 (2.3)	7 (15.9)	1 (2.3)	0	0	0	1 (2.3)	36 (81.8)
Fukui	2	0	0	0	0	0	0	0	0	1 (50.0)	0	0	1 (50.0)	1 (50.0)
Gunma	6	1 (16.7)	0	0	0	0	0	1 (16.7)	0	0	0	0	0	5 (83.3)
Hokkaido	1	0	0	0	0	0	0	0	0	1 (100)	0	0	1 (100)	0
Hyogo	11	2 (18.2)	0	1 (9.1)	0	0	0	3 (27.3)	0	1 (9.1)	0	0	1 (9.1)	7 (63.6)
Ibaraki	6	1 (16.7)	0	0	0	0	0	1 (16.7)	1 (16.7)	0	0	0	1 (16.7)	4 (66.7)
Iwate	7	0	0	1 (14.3)	0	0	0	1 (14.3)	0	0	0	0	0	6 (85.7)
Kanagawa	3	1 (33.3)	0	1 (33.3)	1 (33.3)	0	0	3 (100)	0	0	0	0	0	0
Mie	12	1 (8.3)	0	0	0	0	0	1 (8.3)	0	0	1 (8.3)	1 (8.3)	2 (16.7)	9 (75.0)
Nagasaki	6	0	1 (16.7)	0	1 (16.7)	0	0	2 (33.3)	0	0	0	0	0	4 (66.7)
Okayama	8	0	1 (12.5)	0	1 (12.5)	0	0	2 (25.0)	0	0	0	0	0	6 (75.0)
Shizuoka	3	1 (33.3)	0	0	0	0	0	1 (33.3)	0	0	0	0	0	2 (66.7)
Tochigi	3	0	0	0	1 (33.3)	0	0	1 (33.3)	0	0	0	0	0	2 (66.7)
Other 23 prefectures[e)]	44	0	0	0	0	0	0	0	0	0	0	0	0	44 (100)
Total	159	13 (8.2)	2 (1.3)	5 (3.1)	4 (2.5)	0	1 (0.6)	25 (15.7)	2 (1.3)	3 (1.9)	1 (0.6)	1 (0.6)	7 (4.4)	127 (79.9)

E*rysipelothrix rhusiopathiae*, *Salmonella enterica*, Fowl Adenovirus and Marek's disease virus were not detected. a) If the intact RE provirus integration was detected, such samples were considered as wild type. If the intact RE provirus integration was not detected, such samples were considered as vaccine. b) If 16S rRNA detected was different from the sequence of vaccine used in Japan, such samples were considered as wild type. If 16S rRNA detected was identical to the sequence of vaccine used in Japan, such samples were considered as vaccine. c) If *mgc2* sequence detected was different from the sequence of vaccine used in Japan, such samples were considered as wild type. If *mgc2* sequence detected was identical to the sequence of vaccine used in Japan, such samples were considered as vaccine. d) Vac: vaccine. e) Other 23 prefectures: Aichi, Ehime, Fukushima, Gifu, Hiroshima, Ishikawa, Kagoshima, Kumamoto, Kyoto, Miyagi, Miyazaki, Nara, Niigata, Oita, Saga, Saitama, Tokushima, Tottori, Toyama, Wakayama, Yamagata, Yamaguchi and Yamanashi.

16], they were not detected in any samples in this study. Although the reason why these pathogens were not detected in this study was not clear, because the PRM samples used in this study were stored in ethanol until use, some organisms could be lysed by ethanol and it may affect the results. Alternatively, PRMs may be rarely associated with the spread of these pathogens in Japan. To define the actual distribution of these organisms in PRM, further studies using PRM without fixation may be needed.

This study demonstrated that APV, MS and MG including wild type strains are prevalent in PRMs. Although further studies about the actual transmission activity of the mites for these pathogens are needed, these findings suggest that PRM may transfer these pathogens among chickens. The present results suggest that eradication of PRM will not only reduce the negative effects of blood-sucking but also decrease the transmission of these pathogens in poultry farms.

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