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# A newly developed temperature-sensitive *Mycoplasma synoviae* live attenuated strain prevents pathological lesions of the respiratory and reproductive tracts in chickens caused by a wild-type *M. synoviae* strain

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**ABSTRACT.** A field isolate of *Mycoplasma synoviae*, designated D14-383, caused pathogenic lesions in the air sac, trachea, lung, and ovary and induced a decline or stop in egg production and eggshell apex abnormalities in chickens after intratracheal inoculation. A live *M. synoviae* vaccine candidate was developed after *in vitro* passaging at 32°C from a wild-type *M. synoviae* strain originally isolated from the trachea of a 5-month-old asymptomatic layer chicken. In this study, the vaccine efficacy of the attenuated vaccine candidate strain against *M. synoviae* infection caused by the D14-383 strain was investigated. Eye-drop vaccination of 4-week-old chickens with $10^{5.9}$ colony-forming units (CFU)/dose of the attenuated strain induced high levels of anti-*M. synoviae* antibodies, which were tested by serum plate agglutination, and a 95% seroconversion rate was maintained in the vaccinated birds for 108 weeks after vaccination. In the vaccinated birds, the air sac lesion score was 0.93 versus 4.0 in the unvaccinated group, as revealed by postmortem examination 7 days after challenge infection with the D14-383 strain. Moreover, the percentage of ovarian lesions was 15.9% (7/44 birds) and 73.3% (11/15 birds) in the vaccinated and unvaccinated groups, respectively. During the observation period after vaccination, egg production stopped in the unvaccinated birds but not in the vaccinated birds. Thus, the attenuated strain was proven to induce 2 years of protective immunity against *M. synoviae* infection in chickens.

**KEYWORDS:** live vaccine, *Mycoplasma synoviae*

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## INTRODUCTION

*Mycoplasma synoviae* is an avian pathogen associated with infectious synovitis and the development of lesions in the respiratory tract [8]. In chickens, *M. synoviae* can cause severe clinical symptoms in combination with infection by Newcastle disease virus, infectious bronchitis virus, or both [7, 12, 14]. *M. synoviae* infection in chickens also results in a reduction in body weight gain, poor feed conversion, and decreased egg production in infected flocks, causing severe economic losses to the chicken industry [8]. Since the 2000s, eggshell apex abnormality (EAA) syndrome has been observed in commercial layer farms in association with *M. synoviae* infection in the Netherlands [6], Italy [1], France [4], and Korea [9].

*M. synoviae* strains vary in their pathogenicity and show different tissue tropisms, i.e., respiratory and reproductive tracts, viscera, joints, tendons, and footpads [2, 8, 11, 15]. Experimental infection of chickens with *M. synoviae* can reproduce airsacculitis and induce a noticeable decrease in egg production after inoculation [2, 6, 7, 12, 13]; however, other pathogens, including *Escherichia coli,* can act as complicating factors in the development of the reproductive diseases observed in *M. synoviae* infection [19].

Effective strategies to control *M. synoviae* infection include good practices such as biosecurity regulation, eliminating the infected flock by culling, and introducing clean chicks or eggs from *M. synoviae*-free breeder farms [10]. For vaccination, inactivated, oil emulsion bacterin and live attenuated vaccines are commercially available. Currently, only two live vaccines are available for *M. synoviae* infection: MS-H, a temperature-sensitive mutant strain developed by chemical mutagenesis from an Australian isolate [17],

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and Nobilis® MS live (Intervet International B.V., Boxmeer, The Netherlands), a spontaneously attenuated NAD-independent mutant. These vaccines are widely used in most major poultry-producing countries to control *M. synoviae* infection.

In this study, two trials were conducted: the first trial established an experimental *M. synoviae* infection model in chickens via a wild-type strain. In the second trial, we developed a low-temperature-adapted *M. synoviae* strain and examined its vaccine efficacy against challenge infection with the wild-type *M. synoviae* strain. To determine whether the vaccine candidate strain can induce lifelong immunity in commercial layer chickens, 4-week-old chickens were vaccinated via eye-drop administration and challenged with the wild-type strain at 108 weeks after vaccination.

## MATERIALS AND METHODS

### *M. synoviae strains*

The *M. synoviae* strain D14-383, which was isolated from an 80-day-old laying hen at a commercial layer farm in Yamaguchi Prefecture, Japan, was used as the challenge strain. The *M. synoviae* attenuated strain was established at Vaxxinova Japan K.K. (Nikko, Japan) from a wild-type strain, an isolate from the trachea of a 5-month-old asymptomatic layer chicken in Gunma Prefecture, Japan, by 3 passages at 32°C in medium containing beef heart extract (4.62 g), meat peptone (7.7 g), sodium chloride (3.85 g), horse serum (100 mL), inactivated porcine serum (50 mL), 25 w/v% homemade yeast extract solution (50 mL), β-NAD (0.1 g), L-cysteine hydrochloride monohydrate (0.1 g), 1 w/v% phenol red (2 mL), glucose (1 g), L-arginine hydrochloride (1 g), sodium hydrogen L-glutamate monohydrate (0.1 g), benzylpenicillin potassium (500 units/mL), and thallium acetate (0.02 w/v%) in 1,000 mL of distilled water, and further 7 passages at 32°C in medium containing vegetable peptone (20.0 g), sodium chloride (3.7 g), inactivated porcine serum (120 mL), 10 w/v% homemade yeast extract solution (100 mL), β-NAD (1.0 g), L-cysteine hydrochloride monohydrate (1.0 g), 1 w/v% phenol red (2 mL), glucose (3.3 g), sodium hydrogen phosphate (1.4 g), and benzylpenicillin potassium (1,000 units/mL) in 1,000 mL of distilled water. The attenuated strain samples for vaccination were prepared by freeze-drying and stored at 4°C until use.

### *M. synoviae isolation and identification by PCR*

Tracheal and oviduct swab samples were inoculated in Frey mycoplasma broth base (Frey's broth) and cultivated at 37°C for 2 weeks. The samples showing color changes were subjected to a TaKaRa PCR Mycoplasma detection set (TaKaRa Bio Inc., Kusatsu, Japan) and the *M. synoviae* vaccine candidate-specific PCR for differentiating from *M. synoviae* and other Mycoplasma strains. The vaccine candidate-specific PCR was carried out with the primer sets MSC11-5b (5′-TGCTTGCTATTGCAGTAACT-3′) and MSC11-31b (5′-TCTTCCAACAGTTGGTACTT-3′). The primer sequences were designed on the basis of strain-specific sequences in the *vlhA* gene, which encodes variable lipoprotein hemagglutinin, to specifically amplify a 356-base pair fragment. Briefly, the partial sequence of the *vlhA* gene of the attenuated strain was compared with the sequences of three *vlhA* genes deposited in the NCBI database. The specificity of the primer set was experimentally verified using genomic DNA of other *M. synoviae* strains, WVU1853, MS-H, D14-383, a wild-type field strain, and two passaged strains of the vaccine candidate. The PCR reagent was prepared by mixing with TaKaRa Taq DNA polymerase (TaKaRa Bio, Inc.) containing 5 µL of 10 × PCR buffer ($Mg^{2+}$ plus), 4 µL of dNTP mixture (2.5 mM each), 1 µL of each primer mixture (MSC11-5b, MSC11-31b) (2 µL in total), 0.25 µL of TaKaRa Taq, and 2 µL of template (sample), with the total volume adjusted to 50 µL with distilled water. The prepared PCR mixture was subjected to PCR under the following protocol: 94°C for 3 min for predenaturing the bacterial template, followed by 30 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec. The partial nucleotide sequence of the *vlhA* gene of the vaccine candidate strain has been deposited in the DDBJ/EMBL/GenBank databases under accession number PV425004.

### *Serological response monitoring*

The antibody response against *M. synoviae* was monitored via the serum plate agglutination (SPA) test. The SPA test was conducted using the commercial *M. synoviae* diagnostic SPA test antigen (Nisseiken Co., Ltd., Tokyo, Japan). The serological response was determined with scores from 0 to 3 on the basis of the manufacturer's specification, and a score of ≥1 was considered positive.

### *Gross examination*

Vaccine efficacy was evaluated by scoring gross lesions of the air sac, trachea, lung, and ovary caused by *M. synoviae* infection. All tested chickens were euthanized 7, 14, or 22 days postinoculation (dpi) with the wild-type strain D14-383 and necropsied to observe lesions in the target organs. According to a previously described method [12], scoring was performed using different scales to rate lesion progression depending on the organ, as shown in Table 1.

### *Histopathological examination*

The nasal cavity, trachea, air sac, lung, heart, liver, kidney, spleen, and ovary were collected for microscopic examination. Tissues were fixed with 10% neutral buffered formalin, embedded in paraffin, cut into 2–4 µm sections, and the sections were stained with hematoxylin and eosin (H&E).

### *Experimental infection study*

Animal experiments performed in this study were approved by the Animal Ethics Committee of Vaxxinova Japan K.K. (permit numbers were AC015-C-EX-332 and AC015-C-EX-206 for Trial 1 and Trial 2, respectively).

The chickens used in this study were hatched from specific pathogen-free (SPF) Leghorn eggs obtained from a commercial SPF

**Table 1.** Criteria for scoring gross lesions

| Organ | Score | Description |
|---|---|---|
| Trachea | 0 | No abnormal lesions observed |
| | 1 | Trachea thickened, cloudiness, yellowish exudate, or hemorrhage |
| Air sac | 0 | No abnormal lesions observed |
| | 1 | Membranes slightly cloudy with few yellowish foci |
| | 2 | Slight thickening with small accumulation of caseous exudate |
| | 3 | Air sac obviously thickened, usually with accumulation of clotted exudates confined to a single air sac |
| | 4 | As for score 3 but found in two or more air sacs |
| Lung | 0 | No abnormal lesions observed |
| | 1 | Partial necrosis observed on one side of lobe |
| | 2 | Predominant necrosis on one side or partial necrosis on both sides of lobe |
| | 3 | Predominant necrosis on both sides of lobe |
| Ovary | 0 | No abnormal lesions observed |
| | 1 | Ovarian regression or cyst, multiple follicle hierarchy or other abnormalities |
| | 2 | Egg yolk peritonitis observed |

egg company (VALO Biomedia, Osterholz-Scharmbeck, Germany) and housed in positive-pressure isolator units until use. Tracheal and cloacal swabs were collected from every bird and confirmed to be negative by pathogen-specific PCR for Newcastle disease virus [18], infectious bronchitis virus [16], infectious laryngotracheitis virus [22], *Avibacterium paragallinarum* [3], *Mycoplasma gallisepticum* (TaKaRa Bio Inc. Product No. 6601), and *M. synoviae* [20]. Chickens were also confirmed to be free of *M. synoviae* infection by the SPA test before vaccination and/or inoculation.

*Trial 1 (Pathogenetic study of M. synoviae D14-383)*: Thirty 52-week-old female chickens were randomly assigned to three groups. Ten chickens in the high-dose group were intratracheally administered $10^{7.3}$ colony-forming units (CFU) of *M. synoviae* D14-383 in a 1 mL suspension. Moreover, 10 chickens in the low-dose group were intratracheally administered $10^{5.3}$ CFU of the strain. Ten uninoculated chickens served as negative controls and were kept in a positive-pressure room until necropsy. Clinical signs and egg production were recorded daily for 3 weeks. Blood samples for the SPA test and tracheal and cloacal swab samples for molecular biological examination and mycoplasma isolation were collected. For macroscopic and histopathological examination, three chickens from each group were euthanized at 7 and 14 dpi, four chickens from each group were euthanized at 22 dpi, and air sac, trachea, lung, and ovary samples were collected. The severity of the macroscopic tissue lesions was assessed via a scoring system based on the criteria listed in Table 1.

*Trial 2 (Efficacy of the M. synoviae attenuated strain)*: One hundred female chickens were randomly assigned to two groups. At 4 weeks of age, fifty chickens in the vaccinated group were inoculated with 30 µL of the *M. synoviae* attenuated strain ($10^{5.9}$ CFU/dose) by eye-drop administration; the inoculation dose was determined based on the data obtained from graded-doses of inoculation of the strain. The other 50 chickens in the unvaccinated control group were left untreated. All chickens were observed daily for clinical signs, and blood samples were collected monthly for 24 months. Chickens exhibiting depression or severe health problems were euthanized in consideration of the animals' welfare; gross lesions were observed, and molecular biological tests were performed to determine the cause of death. All blood samples were subjected to the SPA test to monitor the antibody response. Tracheal swabs were collected from the vaccinated group at 24 months (103 weeks of age) and cultured in Frey's broth at 37°C for *M. synoviae* isolation and PCR identification. At 108 weeks of age, all birds in the vaccinated group and 15 randomly selected birds from the unvaccinated control group were challenged with 1 mL of D14-383 culture ($10^{7.0}$ CFU/mL) by intratracheal inoculation and observed for clinical signs. At 7 dpi, all chickens were euthanized for gross examination of the air sac, trachea, lungs, and ovaries.

### *Statistical analysis*

The gross lesion scores of the air sac, trachea, lung, and ovary were analyzed via the Mann–Whitney *U* test. The daily egg production rate was analyzed via the Kruskal–Wallis test. $P \leq 0.05$ was considered statistically significant.

## RESULTS

### *Trial 1: Pathogenetic study of M. synoviae D14-383*

*Clinical signs*: Depression was observed beginning at 5 dpi in all chickens in the high-dose group and in 5 out of 10 chickens in the low-dose group. Respiratory symptoms such as open mouth breathing, rales, and nasal discharge were also observed after 7 dpi in 7 and 3 chickens in the high-dose and low-dose groups, respectively. Egg production decreased gradually from 7 dpi to 22 dpi in the low-dose group, while in the high-dose group it decreased sharply at 6 dpi. The chickens in the high-dose group stopped laying eggs from 13 dpi to 20 dpi; egg production recovered at 21 dpi, but did not fully recover during the observation period (Fig. 1). The daily egg production rate of the high-dose, low-dose, and control groups was 0.29 ± 0.3 (mean ± standard deviation), 0.5 ± 0.3, and 0.92 ± 0.2, respectively, indicating a significant difference between any of the inoculation groups and the control group ($P \leq 0.01$). Abnormal eggs were obtained from the D14-383-inoculated chickens; two EAA eggs, 2 shell-less eggs, and 2 crushed eggs were produced by

the high-dose inoculated chickens, whereas 1 EAA egg and 2 shell-less eggs were produced by the low-dose inoculated chickens. No abnormal eggs were obtained from the control group during the 22-day observation period. However, clinical signs of *M. synoviae* infection, such as synovitis, arthritis, hock joints, or swollen footpads, were not observed in this trial.

*Gross lesions and M. synoviae isolation from organs*: Ovarian lesions were observed in the high-dose and low-dose groups at 7, 14 and 22 dpi. At 14 dpi, severe tracheal lesions were observed in both groups, with slight hemorrhage and yellow exudate. At 22 dpi, all the *M. synoviae*-inoculated chickens in both groups presented the highest score of air sac lesions with airsacculitis. The chickens in the low-dose group clinically recovered earlier than those in the high-dose group did, and the gross lesions resolved earlier. In chickens in the control group, no lesions were observed in any of the organs examined within the 22-day observation period. The gross lesion score data are presented in Table 2.

*M. synoviae* D14-383 was isolated from all tracheal swabs from the high-dose and low-dose groups but not from those from the uninoculated group (Table 2). On the other hand, D14-383 was isolated from the oviduct of only one bird in the low-dose inoculation group at 7 dpi. All culture samples showing color changes were confirmed to be positive by *M. synoviae*-specific PCR (data not shown).

*Histopathological findings*: Inflammation and pathogenic lesions induced by *M. synoviae* infection were observed in chickens in both the high-dose and low-dose groups. In both groups, mild mononuclear cell infiltration widely occurred in the nasal cavity and air sacs during the experimental period. However, in the tertiary bronchi, granulomas were formed with severe infiltration of mononuclear cells and heterophil in all chickens. The tracheal lesions became more severe from 14 dpi to the end of the trial (22 dpi). In the heart, mononuclear cell infiltrates were observed in the epicardium and myocardium. In the liver, there were widespread diffuse infiltrates of heterophils and fibrinous exudate; meanwhile, hemorrhagic foci were observed at 7 dpi. In addition, fibrinous exudate was observed in the high-dose group at 14 dpi. In the spleen, lymphocyte depletion and fibrin exudation were observed only at 7 dpi in both groups. In the ovaries, mononuclear cell infiltrates, fibrinous exudates, and hemosiderin deposits were found in the

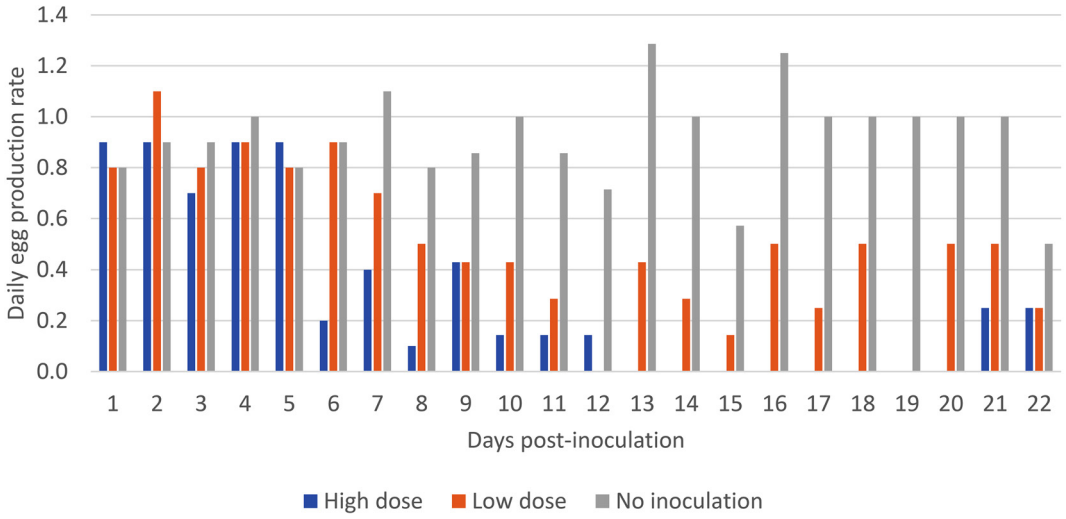


**Fig. 1.** The daily egg production rate in each group in trial 1. The blue bar represents the high-dose group, the orange bar represents the low-dose group, and the gray bar represents the uninoculated group. The daily egg production rate was calculated by dividing the total number of eggs laid per day by the total number of birds. Data are shown as means.

**Table 2.** Gross lesions and isolation of *Mycoplasma synoviae* D14-383 in trial 1

| Group | Dpi[a] | Results of macroscopic observation of | | | | Isolation of D14-383[b] | |
|---|---|---|---|---|---|---|---|
| | | Air sac | Trachea | Lung | Ovary | Trachea | Oviduct |
| High dose | 7 | 3/3[c] (4)[d] | 2/3 (0.7) | 1/3 (0.3) | 1/3 (0.7) | 10/10 | 0/10 |
| | 14 | 3/3 (4) | 3/3 (1) | 3/3 (1.3) | 3/3 (1) | | |
| | 22 | 4/4 (4) | 4/4 (1) | 0/4 (0) | 1/4 (0.3) | | |
| Low dose | 7 | 3/3 (4) | 1/3 (0.3) | 2/3 (0.7) | 2/3 (1) | 10/10 | 1/10 |
| | 14 | 3/3 (3.7) | 3/3 (1) | 0/3 (0) | 2/3 (0.6) | | |
| | 22 | 4/4 (2.5) | 2/4 (0.5) | 0/4 (0) | 1/4 (0.3) | | |
| No inoculation | 7 | 0/3 (0) | 0/3 (0) | 0/3 (0) | 0/3 (0) | 0/10 | 0/10 |
| | 14 | 0/3 (0) | 0/3 (0) | 0/3 (0) | 0/3 (0) | | |
| | 22 | 0/4 (0) | 0/4 (0) | 0/4 (0) | 0/4 (0) | | |

[a] Dpi: days postinoculation. [b] *M. synoviae* was isolated from tracheal swabs and oviduct, and then identified by species-specific PCR as positive. Results are shown as the no. of PCR-positive samples/no. of samples tested. [c] No. of lesion-positive samples/no. of samples tested. [d] Mean lesion score of target organs. Criteria are described in Table 1.

connective tissue in both groups, and the most severe lesions were observed at 22 dpi in the high-dose group. Intriguingly, localized inflammatory lesions were observed in the lungs, heart, and ovaries, which were located near the inflamed air sacs. The number of chickens affected by the challenge infection for each organ is shown in Table 3.

### *Trial 2: Efficacy of the M. synoviae attenuated strain*

*Clinical and macroscopic observations*: No deaths or adverse reactions following vaccination were observed. However, before challenge infection at 24 months postvaccination (mpv), 6 chickens in the vaccinated group died or were euthanized due to egg peritonitis at 3 mpv, cannibalism at 6 and 9 mpv, lameness at 16 mpv, depression and diarrhea at 17 mpv, and peritonitis caused by fecal contamination in the peritoneum at 21 mpv. In the unvaccinated group, 5 chickens died or were euthanized due to cannibalism at 5 and 6 mpv and accidental injury and depression at 7 mpv. Overall, 44 vaccinated chickens and 15 randomly selected unvaccinated chickens were challenged with *M. synoviae* D14-383 at 24 mpv to evaluate vaccine efficacy. Two unvaccinated chickens served as unchallenged controls.

At 7 days postchallenge (dpc), respiratory symptoms and depression were observed in all unvaccinated chickens. In the vaccinated group, thirteen out of 44 chickens presented with respiratory symptoms, 2 of which presented with depression. At necropsy (at 109 weeks of age), severe airsacculitis in which thickened air sacs and a large accumulation of cheese-like exudate in two or more air sacs were observed in all birds in the unvaccinated group. In the vaccinated group, air sac lesions were found in 18 out of 44 chickens (41%); the highest air sac lesion score of 4 occurred in 3 out of 44 chickens, while 4 chickens had a score of 3, 6 chickens had a score of 2, and 5 chickens had a score of 1. The air sac lesion score of the vaccinated group differed significantly from that of the unvaccinated group ($P \leq 0.01$). Lung necrosis was detected in 3 unvaccinated chickens in one lobe, whereas yellow exudate was detected in the trachea of 1 vaccinated chicken. The lesion scoring results are summarized in Table 4. Importantly, ovarian regression associated with peritonitis or hemorrhagic lesions was observed in 6 birds in the vaccinated group (13.6%) and 9 birds in the unvaccinated group (60.0%). Egg peritonitis was observed in 1 bird in the vaccinated group (2.3%) and 2 birds in the unvaccinated group (13.3%). *M. synoviae* was isolated from the tracheal swabs of 10 chickens in the vaccinated group, and all of the isolates were identified as *M. synoviae* attenuated strains via strain-specific PCR. Thus, the rate of positivity for the attenuated strain in the vaccinated group was 22.7% (10/44 birds) at 24 mpv (103 weeks of age) (Table 4).

In this trial, egg production was stopped at 5 dpi in all 15 birds in the unvaccinated group. At post-mortem examination, majority of the chickens in the unvaccinated group presented abnormal ovarian follicles (Fig. 2A–C). The chickens in the vaccinated group laid eggs without a significant decrease in egg number. These chickens presented apparently normal ovarian follicles (Fig. 2D); however, 3 abnormal eggs were produced in the vaccinated group at 6 dpc: 2 eggs had rough shells, and one had EAA (Fig. 2E and 2F), thus showing the importance of clarification of the mechanism for the occurrence of abnormal eggs in the vaccinated chickens. Two of the unvaccinated and unchallenged chickens presented no clinical signs.

*Serological monitoring*: Seroconversion of the vaccinated chickens, as determined by the SPA test, was detected at 2 weeks postvaccination (wpv) and reached the highest titer at 3 wpv (50 out of 50 chickens had a score of 3). The serum positive rate from

**Table 3.** Results of microscopic examination

| Group | Dpi | Number of chickens affected by the challenge infection[a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Nasal cavity | Trachea | Air sac | Lung | Heart | Liver | Spleen | Ovary |
| High dose | 7 | 1/3[b] | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 2/3 | 2/3 |
| | 14 | 2/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 0/3 | 0/3 |
| | 22 | 4/4 | 4/4 | 4/4 | 4/4 | 3/4 | 2/4 | 0/4 | 3/4 |
| Low dose | 7 | 2/3 | 3/3 | 3/3 | 3/3 | 3/3 | 2/3 | 2/3 | 2/3 |
| | 14 | 1/3 | 3/3 | 3/3 | 3/3 | 3/3 | 0/3 | 0/3 | 1/3 |
| | 22 | 2/4 | 4/4 | 4/4 | 2/4 | 1/4 | 3/4 | 0/4 | 2/4 |
| No inoculation | 7 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 |
| | 14 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 |
| | 22 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 |

[a] In all groups, three chickens were euthanized at 7 dpi and 14 dpi, or four chickens at 22 dpi. [b] No. of chickens affected/no. of chickens examined.

**Table 4.** Gross lesions and isolation of *Mycoplasma synoviae* vaccine candidate in trial 2

| Group | Gross lesion ratio (mean score) | | | | Isolation of vaccine candidate strain[a] |
|---|---|---|---|---|---|
| | Air sac | Trachea | Lung | Ovary | |
| Vaccination | 18/44[b] (0.93)[c,d] | 1/44 (0.02) | 0/44 (0) | 7/44 (0.18) | 10/44 |
| No vaccination | 15/15 (4) | 0/15 (0) | 3/15 (0.2) | 11/15 (0.87) | - |

[a] Tracheal swabs were collected before challenge infection. [b] No. of lesion-positive samples/No. of samples tested. [c] Mean gross lesion score. [d] Significant difference compared with no vaccination group ($P \leq 0.01$).

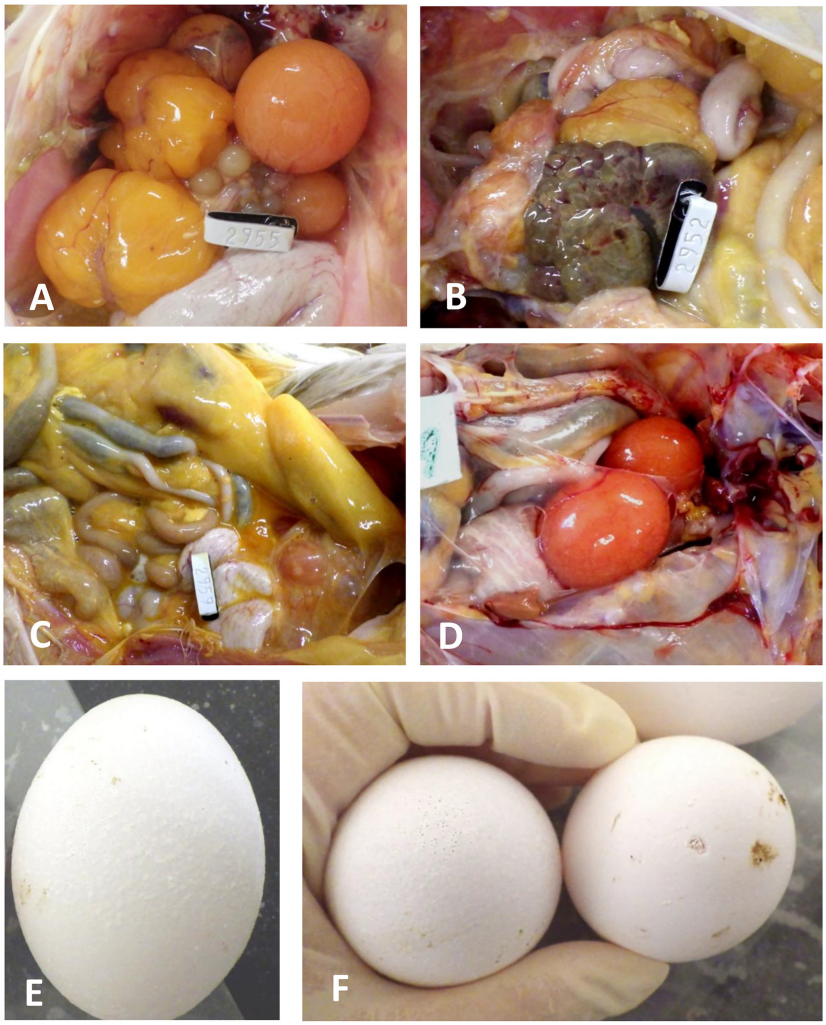


**Fig. 2.** Postmortem findings at 7 dpi (A–D) and abnormal eggs from chickens in the vaccinated group (E and F) in Trial 2. (A) Follicle hemorrhage and airsacculitis observed in the unvaccinated group, (B) ovarian regression and cystic right oviduct from the unvaccinated group, (C) airsacculitis and yolk peritonitis from the unvaccinated group, (D) normal ovary from the vaccinated group, (E) rough texture of eggshell, and (F) abnormal eggs.

1 to 9 mpv was 100% in the vaccinated group, and 45 of 47 birds at 10 mpv, 43 of 45 at 24 mpv, and 42 of 44 at 108 mpv were positive (Table 5). Moreover, all tested chickens in the unvaccinated group were negative during the 24-month observation period.

## DISCUSSION

Previous studies showed that intratracheal inoculation with *M. synoviae* does not necessarily cause reproductive diseases [6, 7]. In our challenge study with *M. synoviae* D14-383, which is an isolate from a joint of a layer chicken with lameness, intratracheal challenge infection of chickens with the D14-383 strain resulted in not only respiratory disease, such as airsacculitis, but also reproductive disease. In trial 1, the chickens showed clinical signs, and gross lesions were observed in the air sac, trachea, lung, ovary, and peritoneum in both the high-dose and low-dose group. Severe airsacculitis and caseous

**Table 5.** Serological test results of trial 2

| Age (weeks) | Group | Challenge | SPA[a] | Positive rate (%) |
|---|---|---|---|---|
| 4 | Vaccination | - | 0/50[b] (0.0)[c] | 0 |
| | No vaccination | - | 0/3 (0.0) | 0 |
| 8 | Vaccination | - | 50/50 (3.0) | 100 |
| | No vaccination | - | 0/3 (0.0) | 0 |
| 48 | Vaccination | - | 45/47[d] (2.9) | 96 |
| | No vaccination | - | 0/3 (0.0) | 0 |
| 108[e] | Vaccination | - | 42/44[f] (2.9) | 95 |
| | No vaccination | - | 0/3 (0.0) | 0 |
| 109[g] | Vaccination | + | 44/44 (3.0) | 100 |
| | No vaccination | + | 3/3 (3.0) | 100 |

[a] Serum plate agglutination (SPA). [b] No. of serum-positive samples/No. of samples tested. [c] Mean agglutination grade (from 0 to 3). [d] Including three dead chickens. [e] Blood collected before challenge. [f] Including three dead chickens. [g] One week post-challenge.

exudate in the respiratory tract and inflammation in other organs were also confirmed via microscopic examination. Furthermore, ovarian regression, ovarian cysts, yolk peritonitis, and decreased egg production were observed in these chickens. Thus, intratracheal infection of layer chickens with the D14-383 strain is a good model for studying not only respiratory diseases but also reproductive diseases caused by *M. synoviae* infection.

Using the D14-383 strain as a challenge strain, we examined vaccine efficacy of the low-temperature passaged *M. synoviae* live attenuated strain. We confirmed eye-drop vaccination of chickens with the strain induced high levels of anti-*M. synoviae* antibodies and significantly reduced respiratory and reproductive lesions, providing protection against the decrease in egg production observed in unvaccinated chickens challenged with the wild-type D14-383 strain. Importantly, the duration of immunity of the *M. synoviae* attenuated strain was experimentally confirmed to be at least 104 weeks; this duration may be sufficient to protect the flock for the whole production period in Japan, where the production periods of breeders and commercial layers are 1 and 2 years, respectively. The duration of immunity provided by live MS-H vaccine, which is only available *M. synoviae* vaccine in Japan, has been reported to be at least 55 weeks after vaccination [21]. Moreover, it appears that the duration of immunity of Nobilis® MS live, which is also a widely used *M. synoviae* vaccine, is 44 weeks after vaccination [5]. Thus, our vaccine candidate was confirmed to provide longer protection, showing that the strain could be useful in control of *M. synoviae* infection in Japan, with the ease of vaccine application and long-lasting immunity.

CONFLICT OF INTEREST. The authors declare that they have no conflict of interest.

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