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Effect of combined infection with *Salmonella* and influenza virus on their respective proliferation in chicken embryonated eggs

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Abstract

Background: *Salmonella* is a major food-borne bacterial pathogen that causes food poisoning related to the consumption of eggs, milk, and meat. Food safety in relation to *Salmonella* is particularly important for eggs because their shells as well as their contents can be a source of contamination. Chicken can also be infected with influenza virus, but it remains unclear how co-infection of *Salmonella* and influenza virus affect each other.

Aim: The potential influence of co-infection of *Salmonella* and influenza virus was examined.

Methods: *Salmonella* Abony and influenza virus were injected into chicken embryonated eggs. After incubation, proliferation of *Salmonella* and influenza virus was measured using a direct culture assay for bacteria and an enzyme-linked immunosorbent assay for influenza virus, respectively.

Results: Our findings indicate that the number of colony-forming units (CFUs) of *Salmonella* did not vary between chicken embryonated eggs co-infected with influenza A virus and *Salmonella*-only infected eggs. Furthermore, we found the proliferation of influenza A or B virus was not significantly influenced by co-infection of the eggs with *Salmonella*.

Conclusion: These results suggest that combined infection of *Salmonella* with influenza virus does not affect each other, at least in terms of their proliferation.

Keywords: Co-infection, Combined infection, Influenza virus, *Salmonella*, Secondary infection.

Introduction

The annual consumption of poultry eggs continues to increase worldwide (Mottet and Tempio, 2017), especially in Asia (International Egg Commission, 2023). Indeed, the consumption of poultry eggs in Asia is the highest in the world (International Egg Commission, 2023). In recent years, the use of eggs has been further accelerated by the diversification of processed foods. Eggs are also increasingly used in the medical field, e.g., where embryonated eggs are needed for vaccine production (Sakudo *et al.*, 2010). Unfortunately, eggs are susceptible to microbial contamination (Agunos *et al.*, 2016). Microorganisms isolated from chickens that potentially cause food poisoning include *Salmonella*, *Escherichia coli*, *Staphylococcus*, *Campylobacter*, *Yerseina*, *Bacillus*, *Streptococcus*, and *Corynebacterium* (Switaj *et al.*, 2015). The most common microorganism isolated from eggs that is associated with food poisoning is *Salmonella* (Switaj *et al.*, 2015; Antunes *et al.*, 2016).

Salmonella spp. can cause severe diarrheal illness with annual estimated infections of 1.35 million, resulting in an average of 26,500 hospitalizations, and 420 deaths per year in the United States (Centers for Disease Control and Prevention, 2023). Approximately 30% of all food poisoning cases in the United States are caused by *Salmonella* spp. (Ekperigin and Nagaraja, 1998; Crump *et al*., 2004; Crump and Mintz, 2010). The recent emergence of various serotypes with antimicrobial resistance has exacerbated the problem of *Salmonella* infections (Nair *et al*., 2018).

The pathogenesis of microorganisms frequently involves multiple bacterial species and viruses that interact in a complex manner (Bettelheim *et al*., 1999). Both *Salmonella* and influenza viruses are commonly found in poultry farms (Arafat *et al.*, 2020). Moreover, chicken eggs can be infected not only with *Salmonella* but also with influenza viruses. However, it is not known how co-infection of influenza virus and bacteria such as *Salmonella* affects the growth of each pathogen in eggs.

There are numerous reports of co-infection of bacteria and viruses. For example, periodontal pathogens are known to increase the risk of influenza virus infection (Tada and Senpuku, 2021). Periodontopathic bacteria have been implicated as a risk factor for severe coronavirus disease 2019 (COVID-19) caused by the severe acute respiratory syndrome

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coronavirus 2 (SARS-CoV-2) (Patel and Sampson, 2020). *Staphylococcus aureus* is reported to enhance influenza virus replication (Goncheva *et al*., 2020) and accelerate influenza virus-associated pneumonia in humans (Shen *et al*., 2022). Moreover, modulation of the immune system following virus infection often influences bacterial infection and proliferation. Indeed, opportunistic infections frequently occur after infection with human immunodeficiency virus (Ong, 2008).

These observations are consistent with experiments in which bacterial (*Streptococcus oralis* and *mitis*) culture supernatant was found to enhance the proliferation of influenza virus *in vitro* (Nishioka *et al*., 2021). Nonetheless, other groups have reported that infection of *Streptococcus pneumoniae* inhibits influenza virus replication (Brown *et al*., 2022), while influenza virus infections promote bacterial growth and transmission (Mina *et al.*, 2015). A study of co-infection of *Salmonella* and influenza virus in chicken suggested that *Salmonella* infection increased avian influenza virus H9N2 in adult chicken birds (Arafat *et al.*, 2010). Taken together, these studies demonstrate that the growth of some bacteria and influenza viruses can affect each other (Morris *et al*., 2017) and this may be related to the morbidity and mortality of viral infections (Gupta *et al.*, 2008), at least in adult humans and animals.

However, it has yet to be clarified how influenza virus and *Salmonella* affect the growth of each pathogen when they coexist in eggs, which is a major source of infection. In this study, we investigated the effect of co-infection of influenza virus and *Salmonella* by comparing the amounts of virus particles and bacteria in chicken embryonated eggs to examine the effect of the combined infection with *Salmonella* and influenza virus on their respective proliferation in the eggs.

Materials and Methods

Bacteria and virus strains

Salmonella enterica subsp. *enterica* serovar Abony ATCC NCTC 6017, *Stenotrophomonas maltophilia* ATCC 13637, influenza A virus (A/PR/8/34, H1N1), and influenza B virus (B/Shangdonng/7/97, Yamagata-like) were used in this study.

Preparation of bacterial and virus inoculates

Bacterial suspensions were prepared from EZ-CFU™ One Step *S. enterica* subsp. *enterica* serovar Abony ATCC NCTC 6017 (Microbiologics Inc., St Cloud, MN) and *S. maltophilia* ATCC 13637 (Microbiologics Inc.) according to the instructions supplied by the manufacturer. Briefly, dried cultures of *Salmonella* and *Stenotrophomonas* were revived in a hydration buffer and then grown in buffered peptone water (BPW) (Merck KGaA, Darmstadt, Germany) at 35°C with shaking for 24 hours. Virus suspensions were prepared by infection of influenza virus into 11-day-old chicken embryonated eggs and collected as allantoic fluid after 48 hours incubation. The suspensions of bacteria (~2×10^6 colony f orming units (CFU) per 100 µl) and virus (hemagglutination (HA) titer units = ~2^{11} per 25 µl) were diluted 10^6-fold with phosphate-buffered saline and subsequently used to inoculate eggs.

Bacteria and virus proliferation in embryonated eggs

A 100-µl inoculum of bacteria (~2 CFU per 100 µl) and/or influenza virus (~0.01 HA titer units per 100 µl) was injected into 11-day-old or 14-day-old chicken embryonated eggs by injection of the inoculum into the allantoic cavity using a needle. The number of embryonated eggs was kept to a minimum from a bioethical perspective. Allantoic fluid was collected from the eggs 12 or 24 hours post-inoculation. The eggs were then chilled at 4°C for at least 4 hours before removing the shell over the air sac and opening the chorioallantoic membrane. Allantoic fluid was collected from the space under the chorioallantoic membrane as previously described (Brauer and Chen, 2015).

Calculation of viable cell number of bacteria using a direct culture assay

After injection of the embryonated eggs with bacterial suspension, the eggs were incubated. The bacterial viable cell number was determined using a direct culture assay. Specifically, samples of *Salmonella* were spread on BPW agar medium and incubated at 35 °C for 24 hours. Similarly, samples of *S. maltophilia* were spread on a BPW agar medium and incubated at 37°C for 24 hours. The number of CFU per ml was measured by counting the colonies of bacteria. The experiments were performed in triplicate.

Enzyme-linked immunosorbent assay (ELISA)

The number of influenza virus particles in the allantoic fluid of the eggs was measured by performing an ELISA. Specifically, an Influenza A virus Nucleoprotein (NP) Antigen Capture ELISA kit (Cat IAV-142-2; Virusys Corporation, Taneytown, MD) was used in accordance with the manufacturer's instructions. NP concentration was compared with concentrations of density gradient purified influenza A virus antigen (HyTest Ltd., Turku, Finland) and estimated using a standard curve of the purified influenza virus antigen by measuring the absorbance at 450 nm.

Statistical analysis

All experiments were performed at least in triplicate. Differences between the two groups were assessed using the Mann–Whitney U-test, while those among more than three groups were assessed by the Kruskal–Wallis test. The p value less than 0.05 was considered to be statistically significant. Statistical analyses were carried out with GraphPad Prism 7 software (GraphPad Prism Software Inc., La Jolla, CA).

Ethical approval

Not needed for this study.

Results

Effect of combined infection with Salmonella and influenza A virus in chicken embryonated eggs

First, the number of viable cells of *Salmonella* after injection of *Salmonella* Abony and/or influenza A virus into 14-day-old embryonated eggs and egg incubation for 24 or 12 hours was investigated using the direct culture assay (Fig. 1). In 24 hours egg incubation, the results showed that the viable cell number of *Salmonella* was 8.25 $\log_{10}$ CFU/100 µl (SEM (standard error of the mean) = 0.07 $\log_{10}$ CFU/100 µl) in single *Salmonella*-injected eggs and 8.08 $\log_{10}$ CFU/100 µl (SEM= 0.03 $\log_{10}$ CFU/100 µl) in *Salmonella* and influenza A virus-co-injected eggs (Fig. 1a). No significant difference in viable cell number of *Salmonella* was observed between the two groups, i.e., *Salmonella* and *Salmonella* + influenza A virus. Moreover, a similar result was obtained when the eggs were incubated for 12 hours instead of 24 hours (Fig. 1b).

Next, we examined the proliferation of influenza virus in 11-day-old embryonated eggs after incubation of the eggs for 24 hours. To quantify virus proliferation, quantitative analysis was performed using ELISA (Fig. 2). Allantoic fluid from embryonated eggs after injection of influenza A virus with or without *Salmonella* and incubation were subjected to ELISA for influenza A virus NP. The average amount of influenza virus NP in the single influenza A virus-injected embryonated eggs was 1181.1 ng/ml (SEM = 240.3 ng/ml), while that of influenza A virus+*Salmonella*-injected embryonated eggs was 1511.2 ng/ml (SEM = 290.6 ng/ml). Thus, these indicate no significant difference between the two groups (Fig. 2).

Effect of combined infection with Salmonella and influenza A/B virus in chicken embryonated eggs

Next, the number of viable cells of *Salmonella* after injection of *Salmonella* with or without influenza A/B virus into 11-day-old embryonated eggs followed by 24 h incubation was determined using a direct culture assay (Fig. 3). The results showed that the viable cell number of *Salmonella* was 8.25 $\log_{10}$ CFU/100 µl (SEM = 0.08 $\log_{10}$ CFU/100 µl) in single *Salmonella* injected eggs, 8.32 $\log_{10}$ CFU/100 µl (SEM = 0.07 $\log_{10}$ CFU/100 µl) *Salmonella* and influenza A virus-co-injected eggs, and 7.86 $\log_{10}$ CFU/100 µl (SEM = 0.24 $\log_{10}$ CFU/100 µl) *Salmonella* and influenza B virus-co-injected eggs. Thus, no significant difference in viable cell number was found between the *Salmonella*, *Salmonella* + influenza A/B virus, and *Salmonella* + influenza B virus groups (Fig. 3).

Effect of combined infection with Stenotrophomonas and influenza virus in chicken embryonated eggs

Finally, the potential effect of different bacteria was investigated. Here, we chose to use *S. maltophilia* as the test organism because it has been reported to exacerbate influenza virus infection of human, pig, and horse host cells (Mancini et al., 2005). The number of viable cells of *S. maltophilia* after injection of *S. maltophilia* and/

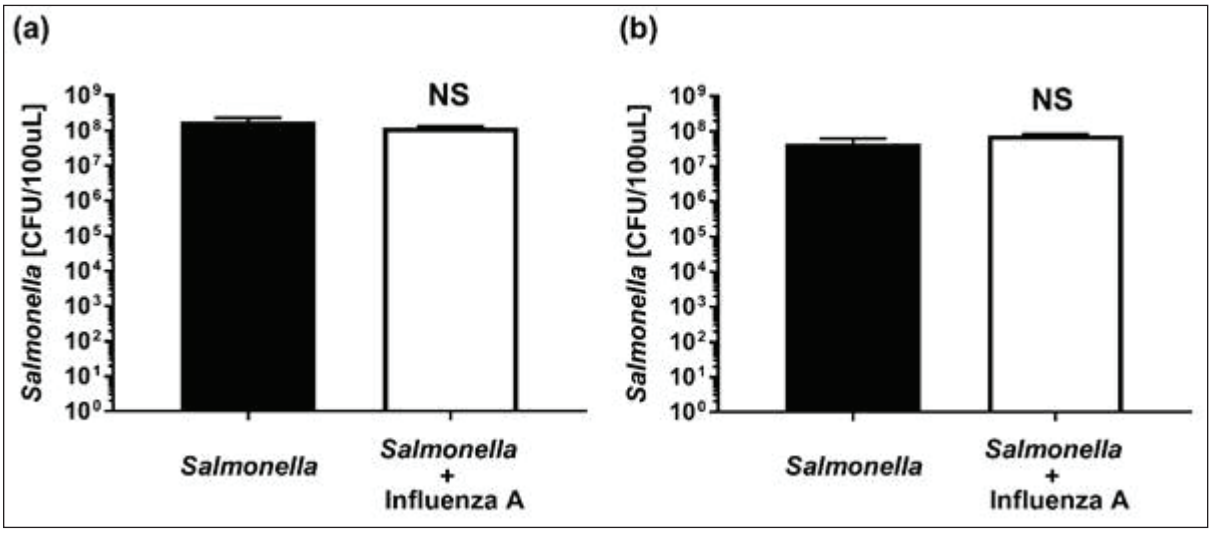

Fig. 1. Viable cell number of *Salmonella* in *Salmonella*-injected group (Black bar) and *Salmonella*+influenza A virus-injected group (White bar) after co-infection of *Salmonella* and influenza A virus into 14-day-old chicken embryonated eggs after incubation for (a) 24 hours and (b) 12 hours. Chicken embryonated eggs (14 days old) were injected with a suspension of *S. enterica* subsp. *enterica* serovar Abony with or without allantoic fluid containing influenza virus (A/PR/8/34). The eggs were incubated for (a) 24 hours or (b) 12 hours. After incubation, samples were spread on BPW agar medium and incubated at 35°C for 24 hours. The number of viable bacteria was then determined by counting the colony-forming units (CFUs)/100 µl. Data show the mean ± SEM (standard error of the mean) of results obtained from experiments. No significant differences were observed between the two groups by the Mann–Whitney U test. NS: no significant difference compared to the *Salmonella* group.

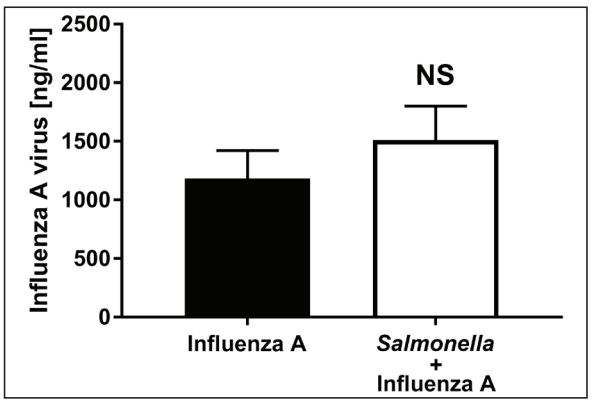

Fig. 2. The concentration of influenza A virus in the influenza A virus-injected group (Black bar) and *Salmonella*+influenza A virus-injected group (White bar) of chicken embryonated eggs after 24 hours incubation. Chicken embryonated eggs (11 days old) were injected with allantoic fluid infected with influenza virus (A/PR/8/34) plus or minus a suspension of *S. enterica* subsp. *enterica* serovar Abony. The eggs were incubated for 24 hours. The quantity of influenza A NP was then measured by ELISA (Influenza A virus NP Antigen Capture ELISA kit). The average quantity of viral protein ± SEM in each group is shown. There were no significant differences between the two groups using the Mann–Whitney U test. NS: no significant difference compared to influenza A group.

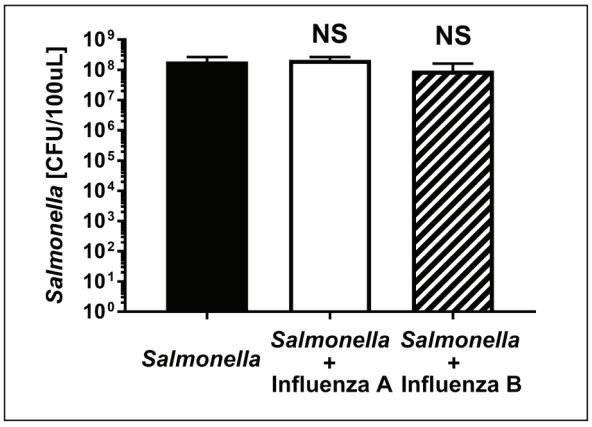

Fig. 3. Viable cell number of *Salmonella* in *Salmonella*-injected group (Black bar), *Salmonella*+influenza A virus-injected group (White bar), and *Salmonella*+influenza B virus-injected group (Hatched bar) after injection into chicken embryonated eggs following 24 hours incubation. Chicken embryonated eggs (11 days old) were injected with a suspension of *S. enterica* subsp. *enterica* serovar Abony alone or mixed with allantoic fluid infected with either influenza A virus (A/PR/8/34) or influenza B virus (B/Shangdonng/7/97, Yamagata-like). The eggs were incubated for 24 hours. Samples were spread on BPW agar medium and incubated at 35°C for 24 hours. The bacterial cell number was determined as CFU/100 µl. Data are shown as the mean ± SEM of experiments. There are no significant differences among the three groups by the Kruskal-Wallis test. NS: no significant difference compared to *Salmonella* group.

or influenza A virus into 14-day-old embryonated eggs and incubation for 24 hours was investigated (Fig. 4). The results showed that the viable cell number of *Stenotrophomonas* was 8.15 $\log_{10}$ CFU/100 *µl* (SEM = 0.20 $\log_{10}$ CFU/100 *µl*) in single *Stenotrophomonas*-injected eggs and 8.08 $\log_{10}$ CFU/100 *µl* (SEM = 0.08 $\log_{10}$ CFU/100 *µl*) in *Stenotrophomonas* and influenza A virus-co-injected eggs. Thus, there was no significant difference in viable cell number between the *Stenotrophomonas* and *Stenotrophomonas* + influenza A virus group (Fig. 4).

Discussion

Salmonella and influenza virus are common infectious foodborne agents that negatively impact human health worldwide. In particular, the poultry industry is often the source of these types of infections. Although, as far as the author is aware, there are no confirmed reports of co-infection with *Salmonella* and influenza virus in embryonated eggs in the industry, the high risks of *Salmonella* contamination in eggs suggest the potential for co-infection of both infectious agents. Furthermore, there is evidence that some antibacterial proteins present in egg whites are progressively impaired during the incubation of both embryonated and unfertilized eggs, possibly due to their degradation (Guyot *et al*., 2016). Several antibacterial proteins such as ovalbumin-related protein X (Réhault-Godbert *et al*., 2013) and avian β-defensin 11 (AvBD11) (Hervé-Grépinet *et al*., 2010) have been isolated from egg white. However, any

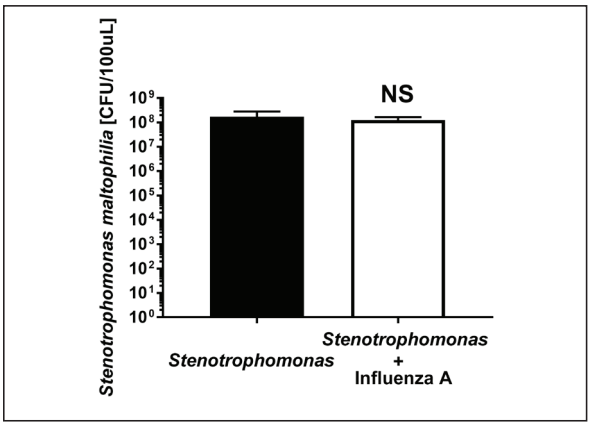

Fig. 4. Viable cell number of *S. maltophilia* in *S. maltophilia*-injected group and *S. maltophilia* +influenza A virus-injected group after injection into chicken embryonated eggs following 24 hours incubation. Embryonated eggs (14 days old) were injected with a suspension of *S. maltophilia* alone or mixed with allantoic fluid infected with the influenza virus (A/PR/8/34). The eggs were incubated for 24 hours. Samples were spread on a BPW agar medium and incubated at 37°C for 24 hours. The bacterial cell number was determined as CFU/100 µl. Data show the mean ± SEM of experiments. No significant differences were found between the two groups by the Mann–Whitney U test. NS: no significant difference compared to *Stenotrophomonas* group.

interactions between these two pathogenic infectious agents in chicken eggs remain unclear. In this study, we examined the potential interplay of *Salmonella* and influenza A/B virus co-infection in chicken embryonated eggs.

Our findings suggest that *Salmonella* and influenza virus infections in chicken embryonated eggs are unaffected by co-infection, at least in terms of proliferation. Similar results were also obtained for the co-infection of *S. maltophilia* and influenza A virus. However, there are a number of limitations in this study. First, a single strain of *Salmonella* and *S. maltophilia* was used in the present study, but the effect of bacterial co-infection on the influenza virus might differ among different strains and subspecies. Previous reports have demonstrated that the proliferation of bacteria such as *Streptococcus* species, *Haemophilus influenzae*, and *Staphylococcus aureus* can also be influenced by influenza virus infection both *in vitro* and *in vivo* (Nishioka *et al*., 2021; Brown *et al*., 2022). In addition, infection of *Salmonella* was reported to enhance the proliferation of avian influenza virus H9N2 in oropharyngeal and cloacal swabs of adult chicken birds (Arafat *et al*., 2020). Specifically, influenza virus infection was found to prompt the growth of *Salmonella* Typhimurium. However, in the present study, only one strain of influenza A or B virus as well as one strain of *Salmonella* was used. Thus, it remains unclear whether the results are applicable to all strains/subtypes of *Salmonella* and influenza virus. This is also the case for the relationship between *S. maltophilia* and influenza virus. Therefore, further studies on the co-infection of bacteria and influenza viruses using various strains are required. Immunoregulation induced by influenza virus infection enhances *Salmonella* colonization in adult chicken birds (Arafat *et al*., 2017) and *Salmonella*-induced immune response in mice (Deriu *et al.*, 2016). In addition, influenza virus infection is aggravated by the protease (elastase) secreted by *S. maltophilia* (Mancini *et al*., 2005). Nonetheless, it remains unclear whether such immunoregulation and protease secretion occur in chicken embryonated eggs. Moreover, when harvested allantoic fluid contains some bacterial endotoxin derived from bacterial lipopolysaccharide in influenza virus vaccine, immunoregulation by endotoxin tolerance is conceivable (Kox *et al*., 2011; Koch *et al*., 2017). Further analysis of the interaction between bacteria and viruses as well as immune response at the molecular level, such as gene expression profiling, will help reveal the underlying mechanism of interaction and immunoregulation.

Robust microbial control measures in the poultry industry are essential to ensure food safety. A better understanding of bacterial-virus interactions will assist in the development of improved control measures. Furthermore, these studies will be directly relevant to the medical field where eggs are used for vaccine production. Consumer awareness of food safety has increased in recent years following several widely reported incidents involving foodborne pathogens. Moreover, the emergence of zoonotic viruses, such as avian influenza viruses as well as coronaviruses, has highlighted the potential dangers of such events and increased demands for vaccine safety. Thus, further research leading to the development of improved microbial control measures is urgently needed.

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Conflict of interest

The Author declares that there is no conflict of interest.

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Data availability

All data are provided in the manuscript.

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