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Mycoplasma gallisepticum modifies the pathogenesis of influenza A virus in the avian tracheal epithelium

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ABSTRACT

Multiple respiratory infections have a significant impact on health and economy. Pathogenesis of co-infecting viruses and bacteria and their interaction with mucosal surfaces are poorly characterized. In this study we established a co-infection model based on pre-incubation of tracheal organ cultures (TOC) with Mycoplasma (M.) gallisepticum and a subsequent infection with avian influenza virus (AIV). *Mycoplasma gallisepticum* modified the pathogenesis of AIV as demonstrated in TOC of two different avian species (chickens and turkeys). Co-infection promoted bacterial growth in tracheal epithelium. Depending on the interaction time of *M. gallisepticum* with the host cells, AIV replication was either promoted or suppressed. *M. gallisepticum* inhibited the antiviral gene expression and affected AIV attachment to the host cell by desialylation of α-2,3 linked sialic acids. Ultrastructural analysis of co-infected TOC suggests that both pathogens may attach to and possibly infect the same epithelial cell. The obtained results contribute to better understanding of the interaction dynamics between *M. gallisepticum* and AIV. They highlight the importance of the time interval between infections as well as the biological properties of the involved pathogens as influencing factors in the outcome of respiratory infections.

1. Introduction

The upper respiratory tract is the target tissue for a variety of different viruses, bacteria and fungi. Mycoplasma (M.) gallisepticum and avian influenza virus (AIV) are two primary pathogens that may co-infect the avian respiratory tract leading to severe respiratory disease (Roussan et al., 2015; Sid et al., 2015; Stipkovits et al., 2012) and significant economic losses in chicken and turkey production (Stipkovits and Kempf, 1996; Alexander, 2000). Little is known about host-pathogen interaction during co-infection with these pathogens at the epithelial surfaces. In vivo studies in chickens showed that *Mycoplasma gallisepticum* predisposes birds for subsequent secondary invading pathogens such as H3N8, which may result in more severe clinical signs compared to mono-infected birds with either H3N8 or *M. gallisepticum* (Stipkovits et al., 2012). Pre-infection with *M. gallisepticum* followed by subsequent H3N8 infection modified the acquired immunity and led to a decrease in anti-*M. gallisepticum* antibody levels in co-infected groups (Stipkovits et al., 2011). Differences in the susceptibility to *M. gallisepticum* or AIV mono-infections between chickens and turkeys were reported. AIV-in vitro infection of tracheal organ cultures (TOC) of turkeys (TOC-tu) resulted in faster ciliostasis and higher virus replication compared to chicken TOC (TOC-ch) (Petersen et al., 2012). In addition, turkeys are known to be more susceptible to *M. gallisepticum* than chickens (Ley, 2008). Differences between chickens and turkeys following *M. gallisepticum* and AIV co-infection were not investigated. Knowledge about possible mechanisms leading to these differences in susceptibility during mono- and co-infections may help to develop more species-adapted prophylactic strategies.

M. gallisepticum adheres and colonizes the surfaces of the tracheal epithelium causing a complete destruction of cilia as well as hyperplasia of epithelial cells (Dykstra et al., 1985; Ley, 2008). Experimental infection of chickens with *M. gallisepticum* downregulated the mRNA expression of CCL20, IL-1β, IL-8 and IL-12p40 (Mohammed et al., 2007). On the other hand, in vitro studies in chicken tracheal epithelial cells demonstrated that lipid associated membrane proteins of *M. gallisepticum* induced significant upregulation of the previously mentioned inflammatory cytokines and chemokines (Majumder et al., 2014). Furthermore, *M. gallisepticum* has the ability to biologically synthesize active neuraminidase which is suggested to be responsible for desialylation of tracheal epithelial cells (Berčič et al., 2008).

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The initial replication of AIV also takes place in the respiratory epithelium (Halvorson et al., 1998). Lesion development after AIV infection may vary depending on host species, pathogenicity of the infecting virus, and presence of secondary pathogens (Perkins and Swayne, 2003). AIV infection is initiated by binding of the virus to specific viral receptors of the host cell. Major determinants for AIV infection are the terminal α-2,3 and α-2,6 sialic acid linked residues (Suzuki et al., 2000). The antiviral immune response mediated by type I interferon (IFN) including IFNα and IFNβ was shown to play a protective role during AIV infection in chickens (Meng et al., 2011). Likewise, IFNλ (IFN type III) was demonstrated to prevent AIV infection by limiting viral infection at mucosal surfaces in chickens (Reuter et al., 2014). The role of IFNλ in AIV-infected turkeys, which show different AIV susceptibility compared to chickens, has not been described yet.

In vivo models have not provided sufficient information about possible mechanisms responsible for the exacerbation of pathogenesis during co-infection with *M. gallisepticum* and AIV. In addition, it may be more difficult to investigate the differences in pathogenesis between chickens and turkeys after exposure to *M. gallisepticum* and AIV under in vivo conditions. It is unclear how *M. gallisepticum* would affect the innate immune response during co-infection with AIV.

Suitability of TOC as a model for the investigation of mono-infection with H9N2 or *M. gallisepticum* was previously demonstrated (Abdul-Wahab et al., 1996; Petersen et al., 2012). This organ culture model provides the epithelial cells, in their natural context and their maintained polarity, as the main target cells of both pathogens. The trachea is lined by a pseudostratified columnar epithelium that consists of ciliated epithelial cells as well as non-ciliated goblet cells (Randall and Reece, 1996). In addition, subepithelial tissue (lamina propria and perichondrium) is also present in this organ culture, but these cell types are not directly affected by *M. gallisepticum* and H9N2 (Tajima et al., 1979; Kolesnikova et al., 2013). Different parameters evaluated in the context of this investigation will focus mainly on the epithelial layer. Therefore we used the TOC model to investigate the effect of pre-infection with *M. gallisepticum* S6 strain on the outcome of subsequent H9N2 AIV inoculation. We compared ciliary activity, pathogen replication, interferon type I and III gene expression and apoptosis rates after mono- and co-infection of chicken and turkey TOC. Furthermore, we examined the effect of *M. gallisepticum* on the sialic acid expression, which may subsequently affect AIV infection.

Our results demonstrated that TOC is a valid model for the investigation of co-infection with viral and bacterial pathogens. Interaction of *M. gallisepticum* with the respiratory epithelium led to histo-pathological changes and affected the innate immune response, which may have subsequently affected the outcome of AIV infection. Depending on the length of the time interval between *M. gallisepticum* and H9N2 infection, viral replication was either promoted or suppressed. These in vitro results provide new information on pathogen-host interaction at the epithelial surface in the face of co-infecting pathogens, which may explain the disturbances in the pathogenesis of AIV frequently observed in the face of bacterial pathogens.

2. Material and methods

2.1. Preparation of TOC

TOC were prepared as previously described (Petersen et al., 2012). Briefly, tracheae were isolated from humanely sacrificed 25-days-old turkey embryos and 20-days-old chicken embryos and cut into 0.8 mm rings. TOC-ch and TOC-tu were transferred into 5 ml tubes with 800 μl of 199 medium with Hanks' salts supplemented with 1% L-glutamine (200 mM, Biochrom, Berlin, Germany) and 1% of Penicillin 10.000 U/ml/Streptomycin 10.000 μg/ml (Biochrom) and incubated at 37 °C. Five days after preparation, TOC were assessed for ciliary activity using an inverted microscope (Zeiss, Germany). TOC with 100% ciliary activity were assigned to different groups (Petersen et al., 2012). The diameter of turkey and chicken rings did not differ significantly ($p > 0.7$, data not shown), therefore, we consider a comparable epithelial surface being available for infection studies for TOC of both species.

2.2. Virus and bacterium

Viral and bacterial stocks were stored in aliquots at −70 °C. A/chicken/Saudi Arabia/CP7/1998 (H9N2) AIV, a field isolate from a meat-type chicken flock, was kindly provided by Hans-Christian Philipp from Lohmann Tierzucht (Cuxhaven, Germany) and had been propagated in embryonated chicken eggs as previously described (Woolcock, 2008). This strain was selected since it was shown to induce a different pathogenesis in TOC-ch compared to TOC-tu (Petersen et al., 2012). *M. gallisepticum* S6 laboratory strain, which was stored in Frey's broth without thallium acetate, was thawed and serially diluted in TOC medium (Abdul-Wahab et al., 1996; Frey et al., 1968). Based on preliminary experiments, 10^2 focus forming units (FFU) of H9N2 were used to infect the TOC. 10^3 CFU (colony forming units) of *M. gallisepticum* were used to infect the TOC unless otherwise stated.

2.3. Experimental design

The experimental design is presented in Table 1. TOC were assigned to different groups of 5 TOC. They were infected with *M. gallisepticum* and subsequently 24 or 72 h later with H9N2 (Experiment 1 and Experiment 2, respectively). Mono-infected TOC received only one pathogen. Negative control TOC received pathogen-free medium at same time points. After 30 min incubation time with the primary pathogen, inocula were aspirated and replaced by 1 ml medium supplemented with 1% L-glutamine (Biochrom, Berlin, Germany), Penicillin (1650U/mg) (Sigma Aldrich, Steinheim, Germany) and 0.2% bovine serum albumin (BSA) (Carl Roth®, Karlsruhe, Germany). Ciliary activity was assessed at 12, 20, 24, 28, 32, 36 and 48 h post AIV infection (hpAIVi). For *M. gallisepticum* mono-infected TOC, ciliary activity was controlled at indicated hours post *M. gallisepticum* infection (hpMGi). At 24 hpAIVi (equivalent to 48 or 96 hpMGi), TOC were embedded in paraffin for histological investigation and snap-frozen in liquid nitrogen for the detection of antigens by immunofluorescence and apoptotic cells by TUNEL assay. The selection of this time point was based on preliminary results which indicated that 8 and 12 h after AIV mono-infection (Petersen et al., 2012) or 12 hpMGi did not lead to a significant increase in the apoptosis rate of epithelial cells (data not shown). At 4, 12 and 24 hpAIVi (equivalent to 28, 36, 48, 76, 84, 96 hpMGi, depending on the experiment), TOC were collected for RNA isolation and supernatants were collected for pathogen quantification.

In the 3rd experiment, the ability of *M. gallisepticum* to desialylate sialic acid moieties (α-2,3 linked sialic acids) in the tracheal epithelium of chickens and turkeys was investigated. TOC were infected with *M. gallisepticum* as described in the 1st experiment and collected at 24 and 72 hpi. They were snap-frozen in liquid nitrogen. Cryosections were prepared and co-stained for *M. gallisepticum* and α-2,3 linked sialic acids.

In the 4th experiment, ultrastructure of co-infected TOC-ch and TOC-tu was investigated by electron microscope. TOC-ch and TOC-tu were pre-infected with *M. gallisepticum* at 10^6 CFU/TOC and subsequently infected 24 h later with H9N2 at 10^2 FFU/TOC.