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Pre-infection of pigs with *Mycoplasma hyopneumoniae* modifies outcomes of infection with European swine influenza virus of H1N1, but not H1N2, subtype

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ABSTRACT

Swine influenza virus (SIV) and *Mycoplasma hyopneumoniae* (Mhp) are widespread in farms and are major pathogens involved in the porcine respiratory disease complex (PRDC). The aim of this experiment was to compare the pathogenicity of European avian-like swine H1N1 and European human-like reassortant swine H1N2 viruses in naïve pigs and in pigs previously infected with Mhp. Six groups of SPF pigs were inoculated intra-tracheally with either Mhp, or H1N1, or H1N2 or Mhp + H1N1 or Mhp + H1N2, both pathogens being inoculated at 21 days intervals in these two last groups. A mock-infected group was included. Although both SIV strains induced clinical signs when singly inoculated, results indicated that the H1N2 SIV was more pathogenic than the H1N1 virus, with an earlier shedding and a greater spread in lungs. Initial infection with Mhp before SIV inoculation increased flu clinical signs and pathogenesis (hyperthermia, loss of appetite, pneumonia lesions) due to the H1N1 virus but did not modify significantly outcomes of H1N2 infection. Thus, Mhp and SIV H1N1 appeared to act synergistically, whereas Mhp and SIV H1N2 would compete, as H1N2 infection led to the elimination of Mhp in lung diaphragmatic lobes. In conclusion, SIV would be a risk factor for the severity of respiratory disorders when associated with Mhp, depending on the viral subtype involved. This experimental model of coinfection with Mhp and avian-like swine H1N1 is a relevant tool for studying the pathogenesis of SIV-associated PRDC and testing intervention strategies for the control of the disease.

1. Introduction

Swine influenza is an acute respiratory tract infection in pigs, characterized by high fever, depression, loss of appetite, tachypnoea, dyspnea and coughing. At the microscopic level, the infection induces interstitial pneumonia and bronchiolitis. Pathogens causing swine flu are type A influenza viruses. Three swine influenza virus (SIV) subtypes, i.e. H1N1, H1N2 and H3N2, are circulating among pigs worldwide, whereas lineages may vary within each subtype depending on the region, i.e. North America, Europe and Asia. The disease is highly contagious. The morbidity can be high (near 100%) but the mortality rate is usually low (less than 1%) (Olsen et al., 2006). A flu outbreak has an important economic impact particularly because of the medications costs and the growth rate

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decrease (Bardini et al., 2011; Brons et al., 2011). These financial losses vary depending on the severity of the disease, which is influenced by many factors: the virus strain, the age and immune status of the infected pig, the presence of concurrent infections, climatic conditions, housing and infection pressure.

SIVs have been also recognized to be viral pathogens involved in the porcine respiratory disease complex (PRDC). PRDC is characterized by decreased rate of growth, decreased feed efficiency, anorexia, fever, cough and dyspnea. It is a multifactorial disease of pigs caused by sequential or concurrent infections with several viral or bacterial respiratory pathogens. Together with SIVs, some pathogens involved in PRDC have been identified to be Porcine Reproductive and Respiratory Syndrome Virus (PRRSV), Porcine Circovirus type-2 (PCV-2), Porcine Respiratory Coronavirus (PRCV), *Mycoplasma hyopneumoniae* (Mhp), *Pasteurella multocida, Streptococcus suis, Haemophilus parasuis* and *Actinobacillus pleuropneumoniae* (Choi et al., 2003; Sorensen et al., 2006; Thacker, 2001). Several studies based on experimental dual infections of pigs have been conducted to understand interactions between SIVs and PRRSV, PCV-2, Mhp, PRCV or *Bordetella bronchiseptica*, pathogens that are frequently detected in herds (Lanza et al., 1992; Loving et al., 2010; Thacker et al., 2001; Van Reeth et al., 1996, 2001; Van Reeth and Pensaert, 1994; Wei et al., 2010; Yazawa et al., 2004). Among them, Mhp is one of the pathogens that is most commonly isolated from pigs with clinical signs of PRDC. Alone, Mhp induces chronic pneumonia with dry, non productive cough beginning 7–14 days after infection (Thacker, 2006). The hallmark lesions associated with Mhp are hyperplasia of the bronchus associated lymphoid tissue (BALT) and bronchointerstitial pneumonia. Experimental co-infections with Mhp and SIVs from the "classical swine H1N1" lineage mostly circulating in America suggested that the dual infection results in increased overall pathogenicity as compared to outcomes of individual infections (Thacker et al., 2001; Yazawa et al., 2004).

Despite the extensive use of *M. hyopneumoniae* vaccination in France, Mhp is widespread in French farms. An epidemiological survey in French farms showed that Mhp was detected in 1/3 of herds at 4 weeks of age (Fablet et al., 2012). Also, SIVs of the European "avian-like swine H1N1" and "human-like reassortant swine H1N2" lineages are currently circulating in nearly half of French herds (Kuntz-Simon and Madec, 2009; Kyriakis et al., 2011) and epidemiological investigations suggested that the presence of Mhp may influence the outcomes of subsequent infections with SIVs (Fablet et al., 2012). In order to go further in understanding interactions between these two pathogens, we intended to compare outcomes of infection with European H1N1 and H1N2 SIVs, in naïve pigs or in pigs previously infected with Mhp.

2. Materials and methods

2.1. Viruses and Mycoplasma strain

The SIV strains A/Sw/Cotes d'Armor/0231/06 (H1N1) and A/Sw/Cotes d'Armor/0113/06 (H1N2) were isolated from nasal swabs taken from pigs from outbreaks of acute respiratory disease in French herds. They were isolated onto Madin Darby Canine Kidney (MDCK) cells and further propagated in the allantoic cavity of 9-day-old embryonated chicken eggs at 36 °C for 3 days for inoculum production, following standard procedure (OIE, 2008). Allantoic fluids were tested for haemagglutinating activity with 0.5% chicken erythrocytes. Virus titer was determined by inoculating 5 embryonated chicken eggs with 150 μl of 10-fold serial dilutions of the virus stock. After 9 days of incubation at 36 °C, the embryonic lethal dose (ELD_{50}/ml) was calculated by the method of Reed and Muench.

M. hyopneumoniae (Mhp, strain 116) was isolated from an outbreak of enzootic pneumonia in France and cultivated in Friis broth medium (FBM) at 37 °C (Marois et al., 2007). Three 10-fold serial dilutions of the Mhp stock were prepared in FBM and incubated at 37 °C. After 5–10 days, color changes of medium were observed and the Mhp stock titer was calculated and expressed as color-changing units per milliliter (CCU/ml).

2.2. Animals and experimental design

Forty specific pathogen-free 6-week-old pigs were randomly allocated into study groups. The animals were obtained from the experimental pig herd of the French Agency for Food, Environmental and Occupational Health and Safety (Anses) at Ploufragan. These animals were known to be free from SIV and Mhp at the beginning of the study. Experiments were performed in accordance with the animal welfare experimentation recommendation granted by the Direction des Services Vétérinaires des Côtes d'Armor (Anses registration number B-22-745-1), under the responsibility of G. Simon (authorization number 22-26).

Twenty pigs were inoculated intra-tracheally with Mhp 116 (5×10^8 CCU in a total of 5 ml), while twenty others were inoculated intra-tracheally with 5 ml of FBM. These operations were repeated twice during a 24 h period. Three weeks later, i.e. at day 21, 5 Mhp-infected animals and 5 mock-infected animals were inoculated intra-tracheally with 5×10^5 ELD_{50} in a total of 5 ml of SIV H1N1 (MH1N1 and H1N1 groups, respectively). 5 other Mhp-infected animals and 5 other mock-infected animals were inoculated intra-tracheally with 5×10^5 ELD_{50} in a total of 5 ml of SIV H1N2 (MH1N2 and H1N2 groups, respectively). Simultaneously, the last 10 Mhp-infected and the last 10 mock-infected animals were inoculated intra-tracheally with 5 ml of allantoic fluid (M and C groups, respectively) (Table 1).

2.3. Clinical observation and sampling

Clinical signs including cough (number of coughs for 15 min), respiratory rate (number of breathing of 2 pigs per group for 1 min) and rectal temperature were evaluated daily, throughout the study. Pigs with rectal temperatures >40 °C were considered to be febrile. Pigs were weighted weekly for four weeks. Nasal swabs were taken at days 23, 25 and 28, i.e. 2, 4 and 7 days post-infection (DPI) with SIV. After collection, swabs were suspended in 2 mL of Eagle's