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Estimation of the sensitivity of four sampling methods for *Mycoplasma hyopneumoniae* detection in live pigs using a Bayesian approach

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ABSTRACT

Four sampling techniques for *Mycoplasma hyopneumoniae* detection, namely nasal swabbing, oral-pharyngeal brushing, tracheo-bronchial swabbing and tracheo-bronchial washing, were compared in naturally infected live pigs. In addition, a quantitative real-time PCR assay for *M. hyopneumoniae* quantification was validated with the same samples. 60 finishing pigs were randomly selected from a batch of contemporary pigs on a farm chronically affected by respiratory disorders. Each pig was submitted to nasal swabbing, oral-pharyngeal brushing, tracheo-bronchial swabbing and tracheo-bronchial washing. Nested-PCR and real-time PCR assays were performed on all samples. A Bayesian approach was used to analyze the nested-PCR results of the four sampling methods (*i.e.* positive or negative) to estimate the sensitivity and specificity of each method. *M. hyopneumoniae* was detected by nested-PCR in at least one sample from 70% of the pigs. The most sensitive sampling methods for detecting *M. hyopneumoniae* in live naturally infected pigs were tracheo-bronchial swabbing and tracheo-bronchial washing, as compared to oral-pharyngeal brushing and nasal swabbing. Swabbing the nasal cavities appeared to be the least sensitive method. Significantly higher amounts of *M. hyopneumoniae* DNA were found at the sites of tracheo-bronchial sampling than in the nasal cavities or at the oral-pharyngeal site ($p < 0.001$). There was no difference between the tracheo-bronchial washing and the tracheo-bronchial swabbing results ($p > 0.05$). Our study indicated that tracheo-bronchial swabbing associated with real-time PCR could be an accurate diagnostic tool for assessing infection dynamics in pig herds.

1. Introduction

Mycoplasma hyopneumoniae is the primary aetiological agent of enzootic pneumonia in pigs, a chronic respiratory disease of worldwide distribution (Thacker, 2006). *M. hyopneumoniae*, in association with bacteria and viruses of the respiratory tract, is also involved in the pathogenesis of Porcine Respiratory Disease Complex (PRDC) (Sibila et al., 2009). Monitoring *M. hyopneumoniae* contamination in live pigs provides useful information on the dynamics of infection within a herd together with insight into the factors influencing the infection pattern and the design of suitably timed preventive and/or control strategies. These investigations rely on the availability of accurate and reliable sampling sites and laboratory analyses. Although the detection of *M. hyopneumoniae* by bacteriological culture is considered as the "gold standard", difficulties in culturing this micro-organism have led to the development of other assays, especially PCR based technology (Thacker, 2006; Sibila et al., 2009). The PCRs currently performed on samples from live pigs have some limitations since they only provide qualitative results. Little is known about the bacterial load carried by the animals and whether this differs in different parts of the respiratory tract. This information is important when assessing (i) the potential of different sampling techniques to detect

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contaminated animals and (ii) the ability of these animals to shed bacteria as high levels are more likely to result in more rapid spreading. Recently, a quantitative real-time PCR assay was developed and validated on samples taken from experimentally infected pigs (Marois et al., in press).

Different sampling sites and types were used to detect *M. hyopneumoniae* infection by PCR techniques on live pigs subjected to experimental and natural challenges *i.e.* nasal, tonsillar, tracheal swabs or brushes and tracheo-bronchial and broncho-alveolar washings (Baumeister et al., 1998; Calsamiglia et al., 1999a, 1999b; Verdin et al., 2000b; Kurth et al., 2002; Otagiri et al., 2005; Moorkamp et al., 2008, 2009). For practical reasons, the nasal cavities are the most frequently chosen sampling sites to assess *M. hyopneumoniae* contamination of live pigs, under field conditions. However, in experimental trials, tracheal and tracheo-bronchial washings are the most efficient samples for detecting *M. hyopneumoniae* (Kurth et al., 2002; Marois et al., 2007).

The aims of this study were therefore (1) to compare 4 sampling techniques: nasal swabbing, oral-pharyngeal brushing, tracheo-bronchial swabbing and tracheo-bronchial washing for the detection of *M. hyopneumoniae* in naturally infected live pigs and (2) to validate the use of a quantitative real-time PCR to assess the amount of *M. hyopneumoniae* in samples taken at different levels of the airways.

2. Material and methods

2.1. Animals and study design

2.1.1. Herd selection

The study was carried out on a two-sites farrow-to-finish herd located in Brittany (France), managed all-in all-out by room with a 3-week batch interval. According to the farm's veterinarian, the herd was chronically affected by respiratory disorders. Coughing was typically expressed during the finishing phase and respiratory disorders were the main reason for medication. Pigs were vaccinated against *M. hyopneumoniae* at 4 and 7 weeks of age. Pneumonia was regularly observed at the slaughterhouse. The farm was visited before beginning the experiment, to confirm the clinical signs and *M. hyopneumoniae* infection of a batch of 180 days old finishing pigs. Tracheo-bronchial swabs from a sample of 10 randomly selected pigs gave 6 positive results in a nested-PCR detection of *M. hyopneumoniae* DNA (as detailed below). The study was carried out on a subsequent batch of 87 finishing pigs, 3 weeks after the first check visit.

2.1.2. Animals and sampling scheme

The pigs were housed in a mechanically ventilated finishing room, containing eight pens. No treatment was administered during the 3 weeks before the study. Sample size calculations were based on expected prevalence and sensitivity parameters by applying the method for diagnostic accuracy of two paired tests described by Zhou et al. (2002). Data from an experimental study were used to produce hypotheses on nasal swabbing, tracheo-bronchial swabbing and tracheo-bronchial washing sensitivities and correlations (Marois et al., 2007). The expected prevalence, based on the first visit, was 55%. Sample size calculations were based on a significance level of 5% and a power of 75%. A minimum sample size of 61 pigs was required to estimate a difference in sensitivity of 0.4 between nasal swabbing and tracheo-bronchial swabbing and between nasal swabbing and oral-pharyngeal brushing with a correlation coefficient of 0.3. A minimum sample size of 55 pigs was needed to detect a suspected difference in sensitivity of 0.35 between tracheo-bronchial swabbing and tracheo-bronchial washing with a correlation coefficient of 0.8. A minimal sample size of 54 pigs was required to detect a difference in sensitivity of 0.35 between oral-pharyngeal brushing and tracheo-bronchial swabbing and between oral-pharyngeal brushing and tracheo-bronchial washing, with a correlation coefficient of 0.7. A sample was constituted of 60 pigs randomly selected from the 8 pens.

2.1.3. Sampling techniques in live pigs

2.1.3.1. Preliminary assessment under experimental conditions. Before their use in live pigs under field conditions, the feasibility and the characteristics of the four sampling techniques were tested in preliminary experimental studies, in both specific-pathogen-free (SPF) and *M. hyopneumoniae* experimentally infected SPF pigs, which served as negative and positive control groups respectively (Marois et al., 2007, in press). For the positive control group, data on day 21 post-inoculation of pigs intratracheally infected with 10^9 UCC of *M. hyopneumoniae* strain were considered. Results obtained from isolation of *M. hyopneumoniae* by bacteriological culture in the sampling specimens were used in the positive control group to test the ability of the four sampling techniques to detect viable *M. hyopneumoniae* in each sampling site. For the negative control group, results from both culturing and PCR technique were considered to assess the probability of false positive results from each sampling technique in negative SPF pigs.

2.1.3.2. Sample collection under field conditions. The animals were restrained with a conventional cable snare over the maxilla. Each pig was subjected to 4 samplings, performed by previously trained technicians, in the following order: oral-pharyngeal brushing, tracheo-bronchial swabbing, tracheo-bronchial washing and nasal swabbing. The pig's mouth was held open with a gag to obtain the oral-pharyngeal and tracheo-bronchial samples. Oral-pharyngeal samples were obtained by swabbing the surface of the oral-pharyngeal cavity thoroughly but gently with a brush protected by a catheter (Ori Endometrial BrushTM, Orifice Medical AB, Ystad, Sweden). Tracheo-bronchial swabs were collected with a sterile catheter used for tracheal intubations (Euromedis, Neuilly-sous-Clermont, France). The catheter was deeply inserted into the trachea as the pig inspired, then rotated and moved up-and-down. Tracheo-bronchial washing samples were collected by transtracheal aspiration: 10 ml of 0.1 M PBS pH 7.4 containing 0.15 M NaCl were introduced into the trachea as deeply as possible with a sterile catheter and immediately aspirated. For nasal sampling, both nasal