Update on *Mycoplasma hyopneumoniae* infections in pigs: knowledge gaps for improved disease control

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Summary

Mycoplasma hyopneumoniae (*M. hyopneumoniae*) is the primary pathogen of enzootic pneumonia, a chronic respiratory disease in pigs. Infections occur worldwide and cause major economic losses to the pig industry. The present paper reviews the current knowledge on *M. hyopneumoniae* infections, with emphasis on identification and analysis of knowledge gaps for optimizing control of the disease. Close contact between infected and susceptible pigs is the main route of *M. hyopneumoniae* transmission. Management and housing conditions predisposing for infection or disease are known, but further research is needed to better understand *M. hyopneumoniae* transmission patterns in modern pig production systems, and to assess the importance of the breeding population for downstream disease control. The organism is primarily found on the mucosal surface of the trachea, bronchi, and bronchioles. Different adhesins and lipoproteins are involved in the adherence process. However, a clear picture of the virulence and pathogenicity of *M. hyopneumoniae* is still missing. The role of glycerol metabolism, myo-inositol metabolism and the *Mycoplasma* Ig binding protein (MIB) - *Mycoplasma* Ig protease (MIP) system should be further investigated for their contribution to virulence. The destruction of the mucociliary apparatus, together with modulating the immune response, enhances the susceptibility of infected pigs to secondary pathogens. Clinical signs and severity of lesions depend on different factors, such as management, environmental conditions and likely also *M. hyopneumoniae* strain. The potential impact of strain variability on disease severity is not well defined. Diagnostics could be improved by developing tests that may detect virulent strains, by improving sampling in live animals and by designing ELISA assays allowing discrimination between infected and vaccinated pigs. The currently available vaccines are often cost-efficient, but the ongoing research on developing new vaccines that confer protective immunity and reduce transmission should be continued, as well as optimization of protocols to eliminate *M. hyopneumoniae* from pig herds.

Keywords: pig – *Mycoplasma hyopneumoniae* - review

Introduction

Mycoplasma hyopneumoniae (*M. hyopneumoniae*) is the primary pathogen of enzootic pneumonia (EP), a chronic respiratory disease in pigs, and one of the primary agents involved in the porcine respiratory disease complex (PRDC; Thacker and Minion, 2012). Infections with *M. hyopneumoniae* are highly prevalent worldwide, and cause tremendous financial losses to the pig industry. Losses are mainly due to costs for treatment and vaccination, decreased performance and increased mortality derived from secondary infections (Holst et al., 2015).

Similar to other Mycoplasmas, *M. hyopneumoniae* has a small genome, lacks a cell wall and is pleomorphic. *M. hyopneumoniae* is very difficult to isolate because of its slow growth and potential overgrowth with other swine mycoplasmas. Bacterial culture is usually attempted when an isolate is deemed, but not for routine diagnostics. The organism is primarily identified on the mucosal surface of the trachea, bronchi, and bronchioles (Blanchard et al., 1992). It affects the mucosal clearance system by disrupting the cilia on the epithelial surface and, additionally, the organism modulates the immune system of the respiratory tract (Thacker and Minion, 2012). Therefore, *M. hyopneumoniae* predisposes animals to concurrent infections with other respiratory pathogens including bacteria, parasites and viruses. Infections also lead to increased use of antimicrobials. While *M. hyopneumoniae* can be virtually recovered from pigs of all sites in segregated production systems, the clinical presentation of EP is mainly observed in growing and finishing pigs. Vaccination against *M. hyopneumoniae* is widely practiced and is often cost-efficient in affected herds. Additional control measures include optimizing management and biosecurity, reducing other risk factors and disease elimination.

The present paper reviews the current knowledge on *M. hyopneumoniae* infections, with emphasis on identification and analysis of knowledge gaps for optimizing control measures.

Epidemiology

To date, domestic pigs and wild boar are the only hosts known to become infected with *M. hyopneumoniae*. In pigs, there is no clear indication of susceptibility based on age, although clinical presentation of the disease is usually evident in grow-finishing stages. Close contact between infected and susceptible pigs is the main route of *M. hyopneumoniae* transmission. Piglets are considered free from *M. hyopneumoniae* at birth, as *in utero* transmission has not been documented, and first exposure events occur during the lactation period, when piglets are in contact with dams shedding the microorganism (Calsamiglia and Pijoan, 2000; Nathues et al., 2013). In fact, the length of the lactation period has been suggested as one risk factor for piglet colonization with *M. hyopneumoniae* prior to weaning (Pieters et al., 2014).

Piglet colonization with *M. hyopneumoniae* at weaning age is of special importance in segregated production systems, where pigs are transferred to clean facilities for the growing and finishing phases. It has been proposed that the initial group colonization with *M. hyopneumoniae* determines downstream clinical presentation and disease severity (Fano et al., 2007; Sibila et al., 2007), although the effect of other factors needs to be considered for disease presentation as well. The influence of the lactation period duration in piglet colonization is evidenced by the successful application of segregated early weaning (SEW) to obtain *M. hyopneumoniae*-free pigs born to positive dams (Alexander et al., 1980). Research data has shown that transmission of *M. hyopneumoniae* among pen-mates is slow (Meyns et al., 2004; Villarreal et al., 2011b; Roos et al., 2016), fitting the picture that disease presentation can be the result of early group colonization and subsequent transmission events. However, a clear understanding of *M. hyopneumoniae* transmission in the field is still needed in order to improve infection models used in experimental research.

Dams and piglets in the breeding herds are considered the reservoir of *M. hyopneumoniae* infections for the entire production system. Circulation of *M. hyopneumoniae* is thought to

occur among existing sows and be transmitted to incoming gilts, which are capable of maintaining the pathogen within the farm and are responsible for the majority of bacterial shedding to newborn pigs (Calsamiglia and Pijoan, 2000; Fano et al., 2005). In this manner, the constant addition of gilts and birth of piglets provide critical susceptible populations needed to maintain pathogen transmission. On the other hand, infection with *M. hyopneumoniae* has a long duration, reaching up to 240 days (Pieters et al., 2009), complicating the already slow disease transmission scenario observed in sow herds. A critical aspect of the epidemiology of *M. hyopneumoniae* is based on the long pathogen persistence, which determining factors are poorly understood. Nevertheless, management practices at the sow farm such as minimizing cross-fostering and limiting the length of the lactation period may help mitigating the effect of *M. hyopneumoniae* transmission by decreasing risk factors for piglet colonization (Nathues et al., 2013a; Nathues et al., 2013b; Pieters et al., 2014; Vangroenweghe et al., 2015). Therefore, identification of risk factors for disease transmission at the sow farm is key for downstream disease control. In continuous flow production systems, *M. hyopneumoniae* colonization in piglets at weaning may not be as influential as in segregated systems. In continuous flow systems, there is a fairly constant contact between animals of different ages, facilitating transmission of *M. hyopneumoniae*. In this respect, the colonization of piglets at weaning is not the only determining factor for downstream infection.

An additional source of variability regarding epidemiology of *M. hyopneumoniae* lies on the demonstration of distinct strains circulating in the field. Partial sequencing of the P146 gene (Mayor et al., 2007), and Multiple-Locus Variable number tandem repeat Analysis (MLVA; Vranckx et al., 2011; Dos Santos et al., 2015) are commonly used to discriminate *M. hyopneumoniae*. The use of *M. hyopneumoniae* molecular characterization has aided outbreak and area spread investigations, by tracking specific strains. In addition, it has enabled research directed at understanding pathogen variability and its relationship with disease presentation

(Michiels et al., 2017). However, current tools for *M. hyopneumoniae* characterization are based on the sole evaluation of similarities in specific loci, and do not indicate strain virulence and/or antigenicity. Development of molecular characterization methods allowing strain virulence evaluation would be beneficial in order to tailor control strategies at the farm level, and could shed light on understanding potential cross-protection among strains.

Vectors do not seem to play an important role in the transmission of *M. hyopneumoniae* in pigs, however, little research has been performed on this topic. Data from Nathues et al. (2012) showed that personnel working with pigs for several hours could harbor the bacterium in their upper respiratory tract, as detected via PCR in nasal swabs; however, the potential for pathogen transmission was not evaluated. Airborne transmission of *M. hyopneumoniae* has been suspected for decades (Goodwin, 1985). Cardona et al. (2005) demonstrated that the microorganism could travel for at least 300 m, and Otake at al. (2010) showed that air samples recovered *M. hyopneumoniae* DNA from as far as 9.2 km from an infected farm. In the latter case, the air sample was shown to be infectious when inoculated to animals using a swine bioassay.

The geographical distribution of *M. hyopneumoniae* infections is assumed to be worldwide. The prevalence is low in some Scandinavian countries, and Switzerland is free after the application of a national eradication program (Stark et al., 2007). Specific data on *M. hyopneumoniae* prevalence by country is not available in the literature, as the disease is not considered of obligatory report and does not limit commercial trade.

Wild-boar has been shown to harbor *M. hyopneumoniae* and may carry identical genotypes found in EP outbreaks (Sibila et al., 2010; Kuhnert et al 2014). However, this might be rather the result of spillover from domestic pig outbreaks than representing wild boars as a reservoir.

Incubation period and kinetic pattern

Under experimental conditions, where usually seronegative animals are challenged with a high infectious dose of *M. hyopneumoniae*, lung lesions and onset of coughing can appear as early as 7-14 days post-infection (dpi; Blanchard et al., 1992; Lorenzo et al, 2006), with maximum severity and extension of lung lesions occurring around 28 dpi (Villarreal et al., 2011a). The minimal infectious dose to induce lung lesions was established at 10^5 color-changing units per ml (CCU/ml) per pig (corresponding to 10^8 mycoplasmas; Marois et al., 2010). Since differences in virulence among strains do exist, this minimal infectious dose is probably strain dependent.

Under natural conditions, the incubation period is difficult to predict as it depends on production system, presence of secondary infections, immune status of animals, virulence of the *M. hyopneumoniae* strain and infectious pressure (Sibila et al., 2009). Indeed, it was postulated that a critical mass of pigs should be infected to elicit the appearance of clinical signs (Calsamiglia et al., 1999). Nevertheless, in clinically affected farms, seroconversion as well as coughing would appear after, approximately, 1 to 6 weeks post infection (Leon et al., 2001).

M. hyopneumoniae is excreted from the respiratory tract of infected individuals through exhalation of microscopic droplets during coughing episodes and /or by nose-to-nose direct contact (Hermann et al., 2008). *M. hyopneumoniae* DNA has also been detected in different samples from the oral cavity such as oro-parhyngeal swabs (Hermann et al., 2008; Fablet et al., 2010), oral fluids (Roos et al., 2016) and tonsilar samples (Sibila et al., 2007, Fablet et al., 2010).

It is known that sows of different parity number may shed the organism, the younger ones being the main shedders (Sibila et al., 2007; Boonsoongnern et al., 2012). Practices directed at segregation of parity, giving special care to parity-one dams and piglets and separating them from the rest of the herd, have been reported to provide significant control of *M. hyopneumoniae*

infections in young parity dams (Moore, 2003). Nonetheless, it remains unclear whether this excretion is uniform and continuous or, on the contrary, is of variable intensity and intermittent. An experimental longitudinal study showed the onset of excretion at 7-14 dpi, followed by an irregular and inconsistent shedding (detection of *M. hyopneumoniae* DNA at nasal cavities) up to 91 dpi (Fano et al., 2005). Indeed, longer excretion was indirectly demonstrated by the transmissibility of *M. hyopneumoniae* to naïve contact pigs at 214 dpi (Pieters et al., 2009). Under natural conditions, shedding duration and kinetics are more difficult to be ascertained, since a low bacterial load is usually present at upper respiratory tract sites (Hermann et al.,2008; Sibila et al., 2009). In a study where *M. hyopneumoniae* detection was assessed in nasal swabs at 9 sampling points through the productive life of pigs, two out of 33 (6%) non-vaccinated animals were continuously nested PCR positive in the last five sampling points (from 12 to 25 weeks of age; Sibila et al., 2007). On the contrary, in a longitudinal study in sows, only 2.4% of them had at least one of the three assessed respiratory sites (nasal, tonsillar and oro-pharyngeal swabs) positive by PCR. For all these positive sows, *M. hyopneumoniae* was detected at one out of eight sampling occasions (Fablet et al., 2011). Takeuti et al. (2017) followed 44 gilts longitudinally from their weaning until after the weaning of their first offspring, collecting laryngeal swabs every 30 days in an endemically infected farm, and identified approximately 20% of gilts negative throughout the study, while other gilts were positive for *M. hyopneumoniae* by real-time mostly once, with a proportion of gilts being positive at multiple samplings. Therefore, considering that the sow population is the origin of *M. hyopneumoniae* circulation, shedding pattern in such population deserves further investigations.

Pathogenicity

Adhesins and lipoproteins

Adhesion along the entire length of cilia of ciliated epithelium of the respiratory tract (trachea, bronchi and bronchioles) of pigs is the first step of infection with *M. hyopneumoniae* followed by induction of ciliostasis and loss of cilia (Debey and Ross, 1994). The primary adhesin of *M. hyopneumoniae* is the P97 and its paralogues (Hsu et al. 1997; Hsu et al. 1998). The other family of adhesins, related with P97, is formed by P102 and its paralogues (Adams et al., 2005). Finally, P159 is an adhesin unrelated to the other two (Burnett et al., 2006). Adhesin receptors on the eukaryotic cell are mainly glycosaminoglycanes and fibronectin. Most of the proteins from the P97/P102 paralog families and P159 are post-translationally processed and cleaved, a system observed with many other surface-associated proteins (Seymour et al., 2010). Like that, Tacchi et al. (2016) identified 35 proteins that are endoproteolytically cleaved in *M hyopneumoniae*. These include not only adhesins but also lipoproteins and even multifunctional cytosolic proteins "moonlighting" at the cell surface. This massive processing and cleavage leads to a very dynamic surface topography of *M. hyopneumoniae* that could well be involved in host evasion and modulation of the immune response. The cleaved fragments of the P97/P102 paralog families and P159 remain on the cell surface and function as receptors of heparin, plasminogen and fibronectin, thereby influencing interaction of *M. hyopneumoniae* with its host (Bogema et al., 2012, Simionatto et al., 2013).

P146 (LppS) is an adhesion lipoprotein containing a serine rich region, the genetic basis of it is also used for genotyping (Mayor et al., 2007). The homolog in *Mycoplasma conjunctivae* was hypothesized to be involved in pathogenesis and in *M. hyopneumoniae* it is also proteolytically processed (Belloy et al., 2003, Bogema et al., 2012). This together with the fact that *M. hyopneumoniae* can alter the number of consecutive serine repeats in P146 (so far observed

from 9 up to 48) could indicate that it is also involved in antigen variation and immune evasion (Dos Santos et al. 2015).

Cell-surface lipoproteins, alternatively called lipid associated membrane proteins (LAMP), have also been found to be implicated in apoptosis. Whole membrane lipoprotein fractions were shown to induce apoptosis via caspases 3 and 8 activation *in vitro* in various cell types, including porcine peripheral blood mononuclear cells (PBMC; Bai et al., 2015; Ni et al., 2015). Furthermore, LAMPs activate production of nitric oxide (NO) and reactive oxygen species (ROS) in the host cell.

H_2O_2 production

Mycoplasmas in general lack classical virulence factors like toxins. It has remained obscure for a long time how these smallest self-replicating organisms could elicit toxic effects. Recently the production of toxic metabolites like H_2O_2 was found as a virulence mechanism of certain mycoplasma species. The bovine pathogen *Mycoplasma mycoides* subsp. *mycoides* SC is able to take up glycerol efficiently, which is then metabolized by glycerolphosphate-oxidase (GlpO) leading to the production of hydrogen peroxide. Similar metabolic pathways account for virulence in the human pathogen *Mycoplasma pneumoniae* (Hames et al., 2009). For *M. hyopneumoniae,* Ferrarini et al. (2016) reconstructed a metabolic model based on its genome. They postulated an ability of *M. hyopneumoniae* to use glycerol as a carbon source, thereby enabling the production of hydrogen peroxide. In fact, the homologous gene *glpD* is present in *M. hyopneumoniae*. However, functional assays for testing production of hydrogen peroxide were so far all negative for various strains of *M. hyopneumoniae* (Kuhnert, personal communication). Thus, whether production of toxic metabolic compounds from glycerol is a possible virulence mechanism in *M. hyopneumoniae* remains to be investigated.

Comparative studies with other Mycoplasmas

Differences in strain virulence have been observed for *M. hyopneumoniae* (Vicca et al., 2003; Villarreal et al., 2009; Woolley et al., 2012,). Moreover, the most closely related *Mycoplasma flocculare* is a commensal of the pig not causing any disease (Siqueira et al., 2013). This allows for large scale comparisons on the genome, transcriptome, proteome, metabolome and secretome level in order to find differences related to virulence and pathogenesis (Pinto et al., 2009; Liu et al., 2013; Siqueira et al., 2013; Siqueira et al., 2014; Ferrarini et al., 2016; Paes et al., 2017). In a study comparing the pathogenic strain 168 with its attenuated derivative, Liu et al. (2013) found that besides the known virulence associated proteins (mainly adhesins), also mutations in genes involved in metabolism and growth contribute to virulence. In a genome-reduced organism like *M. hyopneumoniae* that lost most of its biosynthetic capacity, it is not surprising that further loss of gene functions has a dramatic influence on survival and growth of the microorganism. This also holds true for *e.g.* lipoproteins involved in nutrient acquisition (Browning et al., 2011). Nevertheless, such attenuated strains could be a basis for live vaccines. A genome comparison of *M. hyopneumoniae* and *M. flocculare* revealed differences in genome structure and organization (Siqueira et al., 2013). Some genes of the P97 adhesin family were absent in *M. flocculare*, showed sequence differences or were missing domains involved in adhesion to host cells. However, there were no specific factors identified that could explain the pathogenic nature of *M. hyopneumoniae* compared to the commensal *M. flocculare*.

An integrative conjugal element (ICE) has been identified in the genomes of pathogenic strains 7448 and 232, while it is absent in the non-pathogenic strain J (Pinto et al., 2007). The ICE from *M. hyopneumoniae*, called ICEH, was also found in the pathogenic strain 168 but likewise in its attenuated variant (Liu et al., 2013). While ICE, acting as self-replicating mobile genetic elements, are generally recognized to be involved in virulence, their role in the pathogenicity of *M. hyopneumoniae* remains unclear. The fact that there are notable differences of ICEH

267 between the strains indicate that they are at least involved in horizontal gene transfer, thereby
268 maintaining genome plasticity and variability of *M. hyopneumoniae*.
269 Ferrarini et al. (2016) used a genome-scale metabolic modeling approach to get insight into the
270 virulence of *M. hyopneumoniae*. Besides a glycerol conversion pathway potentially leading to
271 peroxide production in *M. hyopneumoniae*, another significant metabolic pathway was
272 identified to be absent in *M. flocculare* which is the myo-inositol uptake and catabolism.
273 Functional and *in vivo* assays have to be conducted to proof whether these *in silico* findings
274 play a role in virulence and pathogenicity.
275 Differences in virulence could also be due to variation in expression levels of virulence
276 associated genes like adhesins. This was observed in a proteome analysis of *in vitro* cultures
277 comparing the non-pathogenic strain J to virulent strains 7448 and 7422 (Pinto et al., 2009).
278 They identified 64 proteins being overexpressed in the pathogenic strains compared to the non-
279 pathogenic strain, the most prominent being P97.
280 Finally, the secretomes of *M. hyopneumoniae* and *M. flocculare* grown in a serum reduced
281 medium were compared recently, looking more precisely at factors directly involved in
282 mycoplasma-host interaction (Paes et al., 2017). A higher number of secreted proteins was
283 found with *M. hyopneumoniae* compared to *M. flocculare* (62 vs 26). While *M. hyopneumoniae*
284 secreted adhesins, methylases, nucleases and lipoproteins, *M. flocculare* secreted only two
285 adhesins that were also found in the *M. hyopneumoniae* secretome. There was a difference
286 between the transcriptome and the secretome using the same Mycoplasma strains (Siqueira et
287 al., 2014).
288 These comparative “omics” studies confirmed that adhesins play a major role in virulence and
289 pathogenesis. However, there are no clear-cut factors that differ between pathogenic and non-
290 pathogenic strains or differ between the pathogen *M. hyopneumoniae* and the commensal *M.*
291 *flocculare*.

Mycoplasma-host interaction and immune modulation

Infection with *M. hyopneumoniae* is often chronic what indicates a certain degree of immune evasion of the microbe. In a study looking at introgression of domestic swine genes into the wild boar population, an increased *M. hyopneumoniae* disease susceptibility in hybrid animals was observed (Goedbloed et al., 2015). The authors hypothesized that the large-scale use of antibiotics in the swine breeding sector may have led to selecting deleterious properties of domestic swine immune genes. These in turn lead to the increased disease susceptibility if introgression to the wild boar occurs. These observations confirm a certain host-predisposition for *M. hyopneumoniae* which in the opposite direction can also be achieved by selecting for more resistant breeds (Borjigin et al., 2016). Furthermore, it indicates certain "immune degeneration" of domestic pigs, allowing *M. hyopneumoniae* to cause disease in a well-protected animal. The destruction of the mucociliary apparatus together with down-modulating the immune response at later stages enhances the susceptibility of *M. hyopneumoniae* infected pigs to secondary pathogens (Shen et al., 2017).

The host immune response is considered to be the main driver of pulmonary lesions. At the same time, *M. hyopneumoniae* modulates the immune response in order to persist in the host. Pro-inflammatory cytokines like IL-1, TNF-α and IL-6 are induced during infection and plasmin is recognized as central to the regulation of inflammatory responses (Woolley et al., 2013). A number of the P97/P102 adhesin family members interact with plasminogen of the porcine host and enhance its activation to plasmin, a serine protease which in turn stimulates macrophage signaling resulting in production of ROS and cytokine release, thereby contributing to inflammation (Syrovets et al., 2012). A leucine aminopeptidase has recently been shown to "moonlight" as a multifunctional adhesin, including binding and cleaving of plasminogen, on the *M. hyopneumoniae* cell surface (Jarocki et al., 2015). Plasminogen is readily available in the ciliated airways affected by *M. hyopneumoniae* (Seymour et al., 2012).

Interaction with surface accessible actin on the epithelial cells and causing cytoskeletal rearrangements allows the organism being phagocytosed. It is hypothesized that *M. hyopneumoniae* can survive within the phagolysosome, escape it and reside within the cytoplasm (Tacchi et al. 2016). Thereby it can not only evade the immune system but could disseminate to internal organs and persist within its host without causing disease. Isolation of *M. hyopneumoniae* from other tissue than lung has in fact been reported (Le Carrou et al., 2006; Marois et al., 2007; Marchioro et al., 2013).

Immune evasion by specifically cleaving immunoglobulins has been described for *M. mycoides* subsp. *capri* (Arfi et al., 2016). This two-protein system consists of a *Mycoplasma* Ig binding protein (MIB) and a *Mycoplasma* Ig protease (MIP), where the MIB is necessary for the proteolytic activity of MIP. The two proteins are encoded by two genes organized in tandem and often found in multiple copies in various mycoplasmas including *M. hyopneumoniae* (Arfi et al., 2016*)*. How far this MIB-MIP system plays a role in virulence and immune evasion of *M. hyopneumoniae*, deserves further studies.

A clear picture of virulence and pathogenicity of *M. hyopneumoniae* is still not available The role of glycerol metabolism, myo-inositol metabolism and the MIB-MIP system should be further investigated for their contribution to virulence.

Interaction of *M. hyopneumoniae* with other pathogens

Different interactions have been described between *M. hyopneumoniae* and other pathogens. *M. hyopneumoniae* predisposes pigs to infections with secondary bacteria. Combined experimental infections with *M. hyopneumoniae* and either *Pasteurella multocida* (*P. multocida*) (Amass et al., 1994) or *Actinobacillus pleuropneumoniae* (*A. pleuropneumoniae*) (Marois et al., 2009) result in more severe lesions compared to the single infections. Co- or subsequent infections with *P. multocida* and *A. pleuropneumoniae*, and with other bacteria such

as *Bordetella bronchiseptica, Haemophilus parasuis*, *Trueperella pyogenes*, streptococci or staphylococci are commonly found in field outbreaks of EP.

Initial studies focussing on the interaction between *M. hyopneumoniae* and porcine reproductive and respiratory syndrome virus (PRRSV) could not demonstrate a potentiating effect of both pathogens (Van Alstine et al., 1996). Subsequently, it was shown that *M. hyopneumoniae* significantly prolonged and increased the severity of PRRSV-induced pneumonia under experimental conditions (Thacker et al., 1999). Dual infection studies with *M. hyopneumoniae* and swine influenza virus (SIV) could not show the potentiating effects of both pathogens as observed with PRRSV. The effect was less pronounced and only transitory (Thacker et al. 2001; Yazawa et al. 2004). Deblanc et al. (2012) showed that *M. hyopneumoniae* infection increased the severity of H1N1 SIV but not that of H1N2 SIV. Opriessnig et al. (2004) indicated using an experimental study that *M. hyopneumoniae* infection potentiates the severity of porcine circovirus type 2 (PCV2)-associated lung and lymphoid lesions, increases the amount of PCV2-antigen and prolongs its presence, and increases the incidence of post-weaning multisystemic wasting syndrome in pigs. Sibila et al. (2012) however, could not demonstrate an interaction between *M. hyopneumoniae* and PCV2 infection.

Flesja and Ulvesaeter (1980) reported that the extent of pneumonia was associated with the presence of liver lesions due to migrating *Ascaris suum* larvae.

Finally, Pósa et al. (2013) showed that pigs receiving feed contaminated with Fumonisin B elicited more severe lung lesions upon *M. hyopneumoniae* challenge infection compared to pigs fed with non-contaminated feed. Pigs that received feed contaminated with the mycotoxin deoxynivalenol (DON) did not develop more severe disease and lesions upon experimental *M. hyopneumoniae* infection than pigs fed with non-contaminated feed (Michiels et al., 2016). A full review of all interactions between different respiratory pathogens has been published by Opriessnig et al. (2011).

Clinical signs and lesions

Infections with *M. hyopneumoniae* are clinically characterized by an intermittent, variable in intensity, dry non-productive cough (Sibila et al., 2009), which may last from weeks to months. When this type of coughing affects simultaneously many animals of different ages, also other pathogens such as SIV or PRRSV may be involved in the problem (Nathues et al., 2012). Under an endemic scenario, disease course implies high morbidity but low mortality. When *M. hyopneumoniae* enters into a naïve population, the disease may be more severe, affecting animals of all ages and increasing the morbidity up to 100% (Thacker and Minion, 2012). If other bacterial and/or viral agents are involved, or in case of poor air quality due to particulate matter and ammonia (Michiels et al., 2015), clinical signs may be aggravated including labored breathing, pyrexia, anorexia, lethargy and even death (Maes et al., 1996). In uncomplicated cases, a variable proportion of animals might remain subclinically infected for several weeks (Fano et al., 2005), with no evidence of coughing or pulmonary lesions at slaughter.

Coughing is the direct consequence of the lung lesions observed in affected animals, which consist of purple to gray consolidated areas affecting the apical and middle lobes and, eventually, cranial part of diaphragmatic lobes (García-Morante et al., 2016). In case of secondary bacterial infections, pigs show heavier and firmer lungs with higher proportion of tissue affected, and gray-to-white mucopurulent exudate in the airways. In recovered lesions, whitish firm and thickened interlobular connective tissue formation (scars) can be observed (Thacker and Minion, 2012).

Both clinical signs and macroscopic lesions are suggestive, but not exclusive, of *M. hyopneumoniae* infection. Other pathogens such as SIV or *P. multocida* should be considered within the most probably differential diagnoses (Sibila et al., 2009). *Pasteurella multocida* is considered a secondary pathogen, following infection with *M. hyopneumoniae* or another pathogen. Infections with *P. multocida* may cause catarrhal-purulent pneumonia, which is

392 grossly seen as cranioventral pulmonary consolidation (Register et al., 2012). At microscopic
393 level, *M. hyopneumoniae* produces a well differentiated broncho-interstitial pneumonia. At
394 early stages of infection, perivascular and peribronchiolar lymphoplasmacytic hyperplasia,
395 pneumocyte type II hyperplasia and edema fluid in the alveolar spaces with presence of
396 neutrophils, macrophages and plasma cells is observed (Blanchard et al., 1992). As disease
397 progresses, these lesions are aggravated with an evident peribronchial and perivascular
398 lymphoid follicles (Sibila et al., 2007), with and increased number of goblet cells and
399 hyperplasia of submucosal glands (Thacker and Minion, 2012).
400 Intensity of clinical signs and severity of lesions may depend on different factors, such as
401 management, environmental conditions and *M. hyopneumoniae* strain. Information on the
402 impact of strain variability may exert on lung lesions severity and clinical signs onset and
403 duration is still not well defined. Whereas it has been reported that co-infection with more than
404 one strain resulted in more severe lesions (Villarreal et al., 2009; Michiels et al., 2017), such
405 potentiation was not observed in others (Charlebois et al., 2014). To date, the lack of a known
406 virulence marker together with the coexistence of different strains at individual and farm levels
407 (Nathues et al., 2011; Vranckx et al., 2011, 2012a; Pantoja et al., 2016), prevent knowing the
408 impact of a particular strain on the severity of clinical signs and lung lesions.
409

410 ## Socio-economic impact

411 *M. hyopneumoniae* is a species-specific pathogen of suids. Therefore, it is not of zoonotic
412 concern and no impact on public health is considered. However, infections with *M.*
413 *hyopneumoniae* cause major economic losses to the pig industry, mainly because of reduced
414 performance, uneven growth, increased number of days to reach slaughter weight, treatment
415 and control costs, and increased mortality in case of complicated infections (Holst et al., 2015).
416 Unfortunately, few updated information on the economic impact of EP is available, and existing

417 data have been mostly generated by assessing the relationship between evaluation of lung
418 lesions at slaughterhouse with average daily weight gain (ADWG). Whereas a reduction of 6-
419 16% in the growth rate in finishing pigs was reported by some authors (Pointon et al., 1985,
420 Rautiainen et al., 2000), no impact of lung lesions on ADWG at experimental (Escobar et al.,
421 2002; García-Morante et al., 2016) or natural (Scheidt et al., 1990; Straw et al., 1990) conditions
422 was detected by other authors. The economic impact of *M. hyopneumoniae* subclinical infection
423 has been inferred only once from the difference in ADWG (38 g/d) between seropositive and
424 seronegative pigs from 18 different cohorts (Regula et al., 2000). The importance of subclinical
425 infections should be further studied, especially in eradication programs.
426

427 ## Diagnostics

428 Clinical signs and lungs lesions can lead to a tentative diagnosis, but laboratory testing is
429 necessary for a conclusive diagnosis. The use of remote systems for cough recording at the barn
430 level and analysis appears to be a potential tool for early EP detection, although such systems
431 have not been validated for *M. hyopneumoniae* infections in the field.
432 Bacterial isolation remains a confirmatory method for pathogen detection; however the
433 requirement to use specialized media, the high cost associated with the technique, the common
434 overgrowth of other bacteria in the sample, and the low sensitivity of the method make
435 significant detractors for this approach. Nevertheless, recent work from Cook et al. (2016)
436 promises to aid controlling bacterial overgrowth with *M. hyorhinis,* and data from Ferrarini et
437 al. (2016) may help with development of the highly needed media specifically suited for *M.*
438 *hyopneumoniae* growth. Improving *M. hyopneumoniae* culture and isolation methods will help
439 increasing strain collections, which can be used for research, diagnostics and vaccine
440 development, and evaluation of antimicrobial sensitivity testing.

441 Tissue detection of *M. hyopneumoniae* can be accomplished with several techniques, including
442 immunohistochemistry (IHC; Oppriesnig et al., 2004), *in situ* hybridization (ISH; Boye et al.,
443 2001) and PCR (Dubosson et al., 2005; Strait et al, 2008). *In situ* hybridization and ISH allow
444 for detection of *M. hyopneumoniae* in the target tissue, however; this feature can be considered
445 a limiting factor, as only small tissue sections can be evaluated. The application of PCR has
446 allowed for a significant increase in detection of *M. hyopneumoniae* in multiple sample types
447 and, nowadays, real-time PCR constitutes one of the most common methods for *M.*
448 *hyopneumoniae* detection (Dubosson et al., 2005; Strait et al., 2008). Real-time PCR is
449 characterized for its high specificity and sensitivity. Samples collected from the *M.*
450 *hyopneumoniae* target tissue, such as bronchi and bronchioles in the lower respiratory tract,
451 exhibit a higher sensitivity compared to samples obtained from the upper respiratory tract. *In*
452 *vivo* sampling by means of tracheo-bronchial (Fablet et al., 2010) or laryngeal (Pieters et al.,
453 2017) swabs tested by real-time PCR appears to offer a high sensitivity, at least during the early
454 stages of EP. Oral fluid samples tested by PCR have shown low sensitivity when compared to
455 other sample types (Pieters et al., 2017), especially before clinical signs are evident. Further,
456 Hernandez-Garcia et al. (2017) showed the lack of consistency of *M. hyopneumoniae* detection
457 with the use of oral fluids, even after long periods of time post-infection. Ideally, an *in vivo*
458 sample obtained from the bronchioles, and of easy collection, is envisioned by swine
459 practitioners.
460 Circulating antibodies (IgG) can be detected in pigs exposed to *M. hyopneumoniae*. Antibodies
461 are developed several weeks after initial infection (Thacker and Minion, 2012), can be the result
462 of maternal antibody absorption in piglets (Bandrick et al., 2008), or can be generated after
463 vaccination (Maes et al., 2008). Antibodies against *M. hyopneumoniae* can be detected with
464 ELISA tests regardless of origin, which complicates interpretation. Also, in the chronic phase
465 of infection, antibodies against *M. hyopneumoniae* wane and are no longer detected.

Commercial ELISA kits are available for *M. hyopneumoniae* antibody detection, and although based on different antigens and ELISA platforms, they exhibit similar accuracy (Erlandson et al., 2005; Pieters et al., 2017) and are equally unable to differentiate pigs that have been vaccinated from those that have suffered from the disease. The use of several serological parameters to forecast lifetime pneumonia was assessed under natural and experimental conditions. The IgG2 OD-values at systemic levels showed the best correlation with *M. hyopneumoniae* associated lung lesions severity (García-Morante et al. 2017). Further research is warranted to improve ELISA assays, including discrimination between infected and vaccinated pigs, and providing higher specificity.

Prevention and Control

Management and biosecurity

Avoiding the introduction of *M. hyopneumoniae* into negative farms is crucial in order to remain free from the infection. While *M. hyopneumoniae*-specific biosecurity measures have not been developed, the use of general strategies is recommended to maintain a *M. hyopneumoniae*-free status or to elude the introduction of new bacterial strains into herds. For example, the use of farm air filtration systems, usually in place to avoid infections with PRRSV, is becoming popular in sow farms in North America. However, the specific effect of air filtration systems on reduction of *M. hyopneumoniae* infections or the value in area control programs has not been evaluated.

Introduction of *M. hyopneumoniae* via fomites does not appear to be a high risk for farms, and regular disinfection and decontamination systems are thought to be effective against this bacterium. Of special importance is the fact that *M. hyopneumoniae* survival outside of the host is limited, based on the pathogen dependence on the host environment. Nevertheless, data from Browne et al. (2017) suggests that *M. hyopneumoniae* can survive outside of host on certain

plastic surfaces and in dust for up to 8 days when temperatures are low (4°C). This information highlights the need to keep decontamination systems in place in order to avoid pathogen introduction.

The highest risk for pathogen introduction to swine farms resides in incoming pigs and this is especially important for *M. hyopneumoniae* infections. The epidemiological features of this bacterium make it difficult for early detection and clinical presentation, although testing protocols, generally based on serological testing, are in place and are widely used to screen unvaccinated incoming pigs for *M. hyopneumoniae* infections. However, clinical outbreaks have been reported months after the suspected pathogen introduction, when control is far from being effective. Therefore, the development of diagnostic protocols aimed at early detection and surveillance, are of paramount importance.

Therapeutics

Antimicrobials active against *M. hyopneumoniae* include tetracyclines, 15- and 16-membered ring macrolides, lincosamides, pleuromutilins, fluoroquinolones, florfenicol, aminoglycosides and aminocyclitols (Hannan et al., 1997). *M. hyopneumoniae* is intrinsically resistant to antibiotics which interfere with the polymerization of cell wall precursors, such as beta-lactam antibiotics, and to polymyxins, 14-membered ring macrolides (such as oleandomycin and erythromycin), rifampin, trimethoprim and sulfonamides. Acquired resistance has been documented for tetracyclines, 16-membered ring macrolides (tylosin, tilmicosin), lincosamides (lincomycin) and fluoroquinolones (Stakenborg et al., 2005; Le Carrou et al., 2006; Vicca et al., 2007; Thongkamkoon et al., 2013; Tavío et al., 2014). The susceptibility to valnemulin and tiamulin may have decreased in the period 1997 (Hannan et al., 1997) to 2013 (Thongkamkoon et al., 2013; Tavío et al., 2014). However, taken together, acquired antimicrobial resistance does currently not seem to constitute a major problem for treatment of *M. hyopneumoniae* infections

(Klein et al., 2017), although the situation may be different for other bacteria complicating these infections. Finally, within a farm, strains with differences in antibiotic susceptibility may co-exist (Thongkamkoon et al. 2013).

Many different studies have shown the efficacy of various antimicrobials for the treatment and control of *M. hyopneumoniae* infections (reviewed by del Pozo Sacristán, 2014). For most antimicrobials tested, performance improved, and lung lesions as well as clinical signs decreased. Results of treating field cases of EP may be disappointing because the disease signs and the shedding of micro-organisms tend to reappear after cessation of the therapy.

Vaccines

Vaccination is widely applied worldwide to control *M. hyopneumoniae* infections. Commercial vaccines mostly consist of inactivated, adjuvanted whole-cell preparations that are administered intramuscularly (Maes et al. 2008). Table 1 gives an overview of different commercially available *M. hyopneumoniae* bacterin vaccines. Recently, an inactivated vaccine based on soluble antigens of *M. hyopneumoniae* is also commercially available (USA: Fostera PCV MH, Zoetis; in Europe: Suvaxyn Circo + MH RTU, Zoetis). It is a one-shot vaccine combined with PCV2 that can be administered to piglets from three weeks of age onwards. In addition, attenuated vaccines against *M. hyopneumoniae* have been licensed in China (Feng et al. 2013) and Mexico. The vaccine in Mexico is a thermosensitive mutant of *M. hyopneumoniae* (VaxSafe MHYO, Avimex).

Vaccination reduces clinical signs and lung lesions and improves performance. Also, commercial vaccines reduce the number of organisms in the respiratory tract (Meyns et al., 2006; Vranckx et al., 2012b) and decrease the infection level in a herd (Sibila et al., 2007). However, studies under experimental (Meyns et al., 2006) and field conditions (Pieters et al.,

2010; Villarreal et al. 2011b) showed that vaccination conferred only a limited reduction of the transmission ratio of *M. hyopneumoniae*.

The exact mechanisms of protection are not yet fully understood. Studies suggest that systemic cell-mediated immune responses are important for protection (Marchioro et al., 2013). Vranckx et al. (2012b) reported a lower infiltration of macrophages in the lung tissue in vaccinated animals upon infection with *M. hyopneumoniae*, indicating that vaccination modulates the immune response following infection. The importance of local mucosal antibodies remains unclear. The serum *M. hyopneumoniae* specific antibodies raised after vaccination are not suited to evaluate protective immunity (Djordjevic et al., 1997).

Different vaccination strategies have been adopted, depending on the type of herd, production system and management practices, infection pattern and preferences of the pig producer. Vaccination of piglets, applied once or twice, is most commonly used. Single vaccination at either 7 or 21 days of age was efficacious in a pig herd with clinical respiratory disease during the second half of the fattening period (Del Pozo Sacristan et al., 2014). Recent experimental (Arsenakis et al. 2016) and field studies (Arsenakis et al. 2017a) showed that vaccinating piglets three days prior to weaning conferred slightly better results than vaccination at weaning, possibly because of less interference of weaning stress.

Vaccination confers beneficial effects in most infected herds, but variable effects are obtained. This may be due to different factors such as improper vaccine storage conditions and administration, vaccination compliance, infection level, diversity of *M. hyopneumoniae* strains and co-infections. The influence of maternally derived antibodies on vaccine responses in piglets is not fully elucidated. Martelli et al. (2006) showed that passively acquired antibodies have little or no effect on either a vaccine induced priming or subsequent anamnestic response. Sibila et al. (2008) and Arsenakis et al. (2017b) showed that vaccination of sows at the end of gestation, resulted in a lower number of *M. hyopneumoniae* colonized piglets at weaning.

Breeding gilt vaccination is recommended in endemically infected herds to avoid destabilization of the breeding stock immunity (Bargen 2004).

Constant effort is being directed towards the investigation of new vaccines that may offer a better protection against *M. hyopneumoniae* infections. In one study (Villarreal et al., 2009), infection with a low virulent *M. hyopneumoniae* isolate did not protect piglets against infection with a highly virulent *M. hyopneumoniae* isolate one month apart, suggesting that low virulent strains might not be suitable as such to be used as vaccines. Further research however is needed. Several studies have evaluated recombinant proteins of *M. hyopneumoniae* in various forms of administration and formulations. Table 2 summarizes the antigens, adjuvants, vectors and routes of immunization used in the studies on experimental *M. hyopneumoniae* vaccines. Most of the recombinant proteins were evaluated only in mice, and only a few of them were tested in challenge experiments in pigs. Some of the vaccines conferred protection, and may represent a promising tool for controlling *M. hyopneumoniae* infections in pigs. However, validation in pigs under experimental and practical circumstances is needed.

Elimination

Successful elimination of *M. hyopneumoniae* from swine herds has been reported over decades (reviewed by Holst et al., 2015) and several protocols have been developed. Besides depopulation and repopulation, initial efforts for disease elimination were based on the Swiss method (Zimmerman et al., 1989). This method includes partial depopulation (i.e. culling of all animals younger than 10 months of age) and whole herd medication, along with a two-week farrowing pause towards the end of the protocol. While this method has proven highly successful, its application in large herds is difficult. Thus, other protocols such as herd closure and medication, and whole herd medication, keeping young animals at the farms and piglet

590 production undisturbed, have been developed (Holst et al., 2015). The herd closure and
591 medication strategy allows for the introduction of replacement gilts at the beginning of the
592 process, relies on early exposure of the incoming females, and calls for no further introduction
593 of susceptible animals for a period of at least 240 days, or when pathogen persistence has no
594 longer been observed after initial infection (Pieters et al., 2009). In addition, quarterly mass
595 vaccination is included as part of herd closure for disease elimination. On the other hand, the
596 whole herd medication protocol is usually applied after clinical outbreaks of the disease, and
597 does not require the culling of animals or pause in farrowing. However, the reported success of
598 disease elimination is lower for this protocol compared to herd closure (Holst et al., 2015).
599 Although elimination of *M. hyopneumoniae* is becoming increasingly common in the USA,
600 several aspects of the protocols and the potential success could be questioned. For example,
601 concerns exist on the likelihood to eliminate *M. hyopneumoniae* in high pig dense areas due to
602 potential lateral infection of the herd, although this does not seem to occur frequently. Scientific
603 information on lateral transmission of *M. hyopneumoniae* infections is largely missing, and
604 previous evidence of pathogen airborne transmission can make a difficult case otherwise
605 (Goodwin, 1985; Otake et al., 2010). Also, refinement of the elimination protocols is necessary
606 for practicality and application in combination with other disease elimination programs.
607 Nevertheless, elimination of *M. hyopneumoniae* from commercial herds, whether alone or in
608 combination with the elimination of other pathogens, appears to be justified from the ethical
609 and economical points of view (Yeske, 2016).
610

Conclusion

M. hyopneumoniae is a very important pathogen that causes major losses to the pig industry. Research has focused on the epidemiology and transmission in different production systems, partly elucidated the very complex interaction of the pathogen with the respiratory tract, and has developed and tested many commercial and experimental vaccines. However, important knowledge gaps remain and, therefore, further research is needed to achieve a better control and possible elimination of the pathogen. The focus should be on better understanding of the virulence mechanisms and the interaction of the pathogen with the host and with other pathogens, and the development of vaccines that confer better protection and can be administered easily. Finally, research on how to successfully eliminate the pathogen from pig herds should also be stimulated.

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Table 1: Most commonly used commercially available *M. hyopneumoniae* bacterin vaccines (2017) – Bacterin vaccines available in only one or a few countries are not included the table.

Vaccine	Antigen / Strain	Adjuvant	Route of administration	Age of administration (days)	Boosts needed after …weeks
Hyogen (Ceva)	Ceva strain BA 2940-99	Imuvant (W/O J5 LPS)	IM	≥21	-
HYORESP (Merial)	NI [a]	Aluminium hydroxide	IM	≥5	3-4
INGELVAC MYCOFLEX (Boehringer Ingelheim)	J strain isolate B-3745	Impran (water-in-oil adjuvant emulsion)	IM	≥21	-
M+Pac (Intervet Int.) [b]	NI [a]	Mineral oil and Aluminium hydroxide	IM	≥7	3-4
MYPRAVAC SUIS (Hipra Lab)	J strain	Levamisole and carbomer	IM	≥7-10	3
PORCILIS M. HYO (Intervet)	Strain 11	dl-α-tocopherol acetate	IM	≥7	3
Porcilis PCV M. HYO (MSD-Intervet Int) [c]	J Strain	Mineral oil and Aluminium hydroxide	IM	≥21	-
Porcilis MHYO ID Once (MSD-Intervet Int.)	Strain 11	Paraffin oil and dl-α-tocoferylacetaat	ID	≥14	-
STELLAMUNE MYCOPLASMA (Eli Lilly)	NL 1042	Mineral oil and lecithin	IM	≥3	2-4
STELLAMUNE ONE (Eli Lilly)	NL 1042	Amphigen Base, and Drakeol 5 (mineral oil)	IM	≥3	-
SUVAXYN M.HYO [d] (Zoetis)	P-5722-3	Carbopol	IM	≥7	2
SUVAXYN MH-ONE [e] (Zoetis)	P-5722-3	Carbopol and squalane	IM	≥7	-
SUVAXYN M.HYO – PARASUIS [f] (Zoetis)	P-5722-3	Carbopol and squalane	IM	≥7	2

a No information available
b Vaccination scheme when one ml is used for each administration. No boost vaccination needed if a 2 ml dose is used the first time.
c Combination vaccine with Porcine Circovirus type 2
d Named Suvaxyn RespiFend MH in USA
e Same name is used in the USA, but Amphigen is used as adjuvant in the USA, and vaccine can be administered from day one of age onwards
f Combination vaccine with *Haemophilus parasuis* - Named Suvaxyn RespiFend MH HPS in USA

Table 2: Overview of experimental vaccines against *M. hyopneumoniae*

Antigen	Vacinne type	Vector/Adjuvant	Species	Route	Challenge infection	Reference
P97	Recombinant Subunit	Complete Freund's adjuvant	pig	IM**	yes	King et al. 1997
NrdF (R2)	Recombinant Vector	*Salmonella* Typhimurium aroA SL3261	mice	Oral	no	Fagan et al. 1997
P97 (R1)	Recombinant Vector	Pseudomonas aeroginosa exotoxin A	mice and pig	SC*** and IM	no	Chen et al. 2001
NrdF (R2)	Recombinant Vector	*Salmonella* Typhimurium aroA SL3261	pig	Oral	yes	Fagan et al. 2001
Strain PRIT-5	Inactivated whole cell	Spray dried microspheres	pig	Oral	yes	Lin et al. 2003
P42	DNA	pcDNA3	mice	IM	no	Chen et al. 2003
P97 (R1R2)	Recombinant Vector	*Erysipelothrix rhusiopathiae* YS-1	mice and pig	SC and IN****	no	Shimoji et al. 2003
P97 (R1)	Recombinant Vector	*Salmonella* Typhimurium aroA CS332	mice	Oral	no	Chen et al. 2006a
NrdF (R2)	Recombinant Vector	*Salmonella* Typhimurium aroA CS332	mice	Oral	no	Chen et al. 2006b
P97 (R1)	Recombinant Subunit	LTB	mice	IM and IN	no	Conceição et al. 2006
P97 (R1)	Recombinant Vector	Adenovirus	mice	IM and IN	no	Okamba et al. 2007
P97 (R1R2)	Recombinant Vector	*Erysipelothrix rhusiopathiae* Koganei	pig	Oral	yes	Ogawa et al. 2009
P97 (R1)	Recombinant Vector	Adenovirus	pig	IN	yes	Okamba et al. 2010
P36	Recombinant Vector	*Actinobacillus pleuropneumoniae* SLW36	mice	IM	no	Zou et al. 2011
34*	Recombinant Subunit	Aluminun	mice	IM	no	Simionatto et al. 2012

P37, P42, P46, P95	Recombinant Subunit and DNA	Aluminium and pcDNA3	mice	IM	no	Galli et al. 2012
P97 (R1,R2)	Recombinant chimeric Subunit	Heat-labile enterotoxin *E. coli* (LTB) and Montanide IMS	mice	IM	no	Barate et al. 2014
P46, HSP70,MnuA	Recombinant Subunit and DNA	Complete Freund's adjuvant	mice	Intra-peritoneal	no	Virginio et al. 2014
P97, P42, NrdF	Recombinant chimeric Subunit	Heat-labile enterotoxin *E. coli* (LTB)	pig	IM and IN	no	Marchioro et al. 2014
HSP70	Recombinant Subunit	Mesoporous silica nanoparticles SBa-15 and SBa-16, Aluminium	mice	Intra-peritoneal	no	Virginio et al. 2017

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