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REVIEW ARTICLE

The challenges with Glässer's disease in technified pig production

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ABSTRACT. The swine upper respiratory tract is early colonised by *Haemophilus parasuis*, a bacteria which causes Glässer´s disease under favorable conditions. Glässer's disease is a septicemic infectious disease characterised by causing polyserositis. The prevention of Glässer disease still represents a big challenge for the production chain, since the mechanism of systemic infection in pigs and virulence factors that prevent phagocytosis are not yet well understood. Even in swine herds with high sanitary standard, it is the main cause of mortality that has led to productive and economic losses in the pig industry worldwide. Although the *H. parasuis* genome sequence has been completed already, diagnosis is still difficult due to the existence of non-virulent strains and the early colonisation of the upper respiratory tract of healthy swines. This review aims to provide up-to-date information about the etiology, epidemiology, pathogenesis, clinical signs, gross and microscopic lesions, diagnosis, treatment and control of Glässer's disease.

Key words: epidemiology, swine, virulence factors, vaccines.

RESUMEN. El tracto respiratorio superior del cerdo es colonizado inicialmente por *Haemophilus parasuis*, bacteria que en condiciones favorables causa la enfermedad de Glässer. La enfermedad de Glässer es una afección infecciosa que se caracteriza por el desarrollo de poliserositis septicémica. La prevención de esta enfermedad sigue siendo un problema en la producción porcina tecnificada, ya que los mecanismos de virulencia de este microorganismo y los factores sistémicos que impiden la fagocitosis no son bien conocidos. Inclusive en rebaños con un alto estándar de salud, *H. parasuis* es la principal causa de mortalidad, generando así pérdidas productivas y económicas en la industria porcina a nivel mundial. A pesar de que la secuencia del genoma de *H. parasuis* se ha completado recientemente, el diagnóstico aún se complica por la existencia de cepas no virulentas y la temprana colonización del tracto respiratorio superior de cerdos sanos. El objetivo de esta revisión es entregar información actualizada respecto de la etiología, epidemiología, patogénesis, signos clínicos, lesiones macroscópicas y microscópicas, diagnóstico, tratamiento y control de la enfermedad de Glässer.

Palabras clave: cerdos, epidemiología, vacunas, virulencia.

INTRODUCTION

Glässer's disease is considered a major bacterial infection with worldwide distribution that has caused considerable economic losses even in high health status farms worldwide (Oliveira and Pijoan 2004). *Haemophilus parasuis (H. parasuis)* is the etiological agent of Glässer's disease in swine. This bacterium colonises healthy pigs and, under certain circumstances, some strains are able to invade the host and cause severe lesions. The initial acquisitions of *H. parasuis* occur right after piglet birth during direct contact of the nut with the piglet (Aragon *et al* 2012). Systemic invasion is characterised by fibrinous polyserositis inflammation, polyarthritis and fibrinous meningitis (Oliveira and Pijoan 2004, Moleres *et al* 2015), and cause significant losses to producer due to reduction in weight gain, increases in the use of drugs, dead animals, and carcass depreciation (Castilla 2012).

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Nowadays, the increase in the occurrence of Glässer's disease is being more associated to the current practices in animal production and with the emergence of immunosuppressive viruses (Aragon *et al* 2012). Usually, the diagnosis of *H. parasuis*-associated disease is done according to clinical signs, pathological findings and bacterial isolation. However, since it is a commensal in the respiratory tract of pigs and there are non-virulent strains, an inconclusive diagnosis is common which difficult its control and improvements in vaccination programs. Based on that, it is evident the importance of developing a standardised diagnostic technic to improve disease control (Castilla 2012).

More information is still needed to better understand the defense mechanisms of *H. parasuis* because involves the activation of several elements of the innate and acquired porcine immune system. The factors responsible for colonization and systemic infection are not enlightened, while prevention and control of Glasser's disease continues to be challenging. Therefore the objective of this review was to update the main characteristics about the etiology, epidemiology, pathogenesis, clinical signs, macroscopic and microscopic lesions, diagnosis, treatment and control of Glässer's disease.

ETIOLOGY

The *H. parasuis* is the causative agent of Glässer's disease decribed by Glässer in the exudate of pig with fibrinous polyserositis around 1910 (Aragon *et al* 2012,

Castilla 2012). However, its isolation was performed for the first time by Schermer and Ehrlich in 1922 (Little 1970). This is a gram-negative bacterium, small, rod-shaped, pleomorphic, nonhemolytic, non-motile and microaerophile of *Haemophilus* genus of *Pasteurellaceae* family (Castilla 2012).

There are seven members in the of family *Pasteurellaceae* dependent on the V factor (NAD - nicotinamide adenine dinucleotide) found in the upper respiratory tract of pigs: *Actinobacillus pleuropneumoniae* (App) - agent of porcine pleuropneumonia (PP); *Haemophilus parasuis* – agent of Glässer's disease; and *Haemophilus* taxon *minor* group, and taxons C, D, E and F (Dousse *et al* 2008).

The first causal agent identified was the *H. suis*, and then, the *Haemophilus influenzae*. Initially, the biochemical characterisation concluded that they were very similar due to the requirement of both growth factor X (iron porphyrin) and V (NAD; Lewis and Shope 1931). Thus, different classification of *H. parasuis* species was classified based on the nomenclature accepted to *Haemophilus* genus (Oliveira and Pijoan 2004).

Due to nutricius demands of *H. parasuis* its cultivation has been hampered, several media enriched with V-growth factor are used for the cultures making necessary the use of media supplemented with NAD (chocolate agar, Levinthal agar, PPLO agar supplemented with NAD). Another possibility is the use of agar of sheep blood with a groove of *Staphylococcus aureus* to obtain the factor V. In this case, the *H. parasuis* grows around *Staphylococcus aureus* in the phenomenon called sathelitism (Castilla 2012). The identification of species is based on morphological characteristics and biochemical tests such as urease production, indole, oxidase, capacity of reduce nitrate to nitrite, catalase, glucose fermenter, sucrose, fructose, galactose and mannose (Kielstein *et al* 2001, Oliveira 2007).

According to the currently worldwide accepted classification, only 15 serotypes of *H. parasuis* have been identified. However, due to the large amount of non-typable samples, there is a high probability of existence of serological varieties that are different from those already described (Howell *et al* 2013). The development of genotyping techniques such as MLST (Olvera *et al* 2006, Mullins *et al* 2013), 16S rRNA gene sequencing (Christensen *et al* 2004) and analysis of selected outer-membrane proteins (Mullins *et al* 2009) helps to better characterise the strains from *H. parasuis*.

PRINCIPAL EPIDEMIOLOGICAL CHARACTERISTICS

The identification of serovars has practical applications in local and global epidemiology such as the quantification of how many serovars are causing a single outbreak and discovering a link between particular strains with those found in others geographical points. There is not direct association between genotype and serovar. Isolates from the same serovar may include different strains, whereas strains with identical genotypes may differ regarding their serovars (Turni *et al* 2010). The severity of Glässer's disease is associated to the immune status of the herd. There is no certainty which factors are responsible for different degrees of virulence in this bacterium (Kielstein and Rapp-Gabrielson 1992).

Several studies to identify the prevalence profiles have been performed worldwide. The serovar 4 was identified as the dominant while serovar 5 was usually isolated to *H. parasuis* in most countries such as China (Li *et al* 2009), USA (Rapp-Gabrielson and Gabrielson 1992), Canada (Tadjine *et al* 2004), Brazil (Macêdo *et al* 2009), Spain (Rubies *et al* 1999), and Japan (Morikoshi *et al* 1990). The results differ to isolations in Australia (Blackall *et al* 1997, Rafiee and Blackall 2000) and Denmark (Angen *et al* 2004) with the prevalence of serovars 5 and 13.

Virulence factors of *Pasteurellaceae* strains that colonise the upper respiratory tract are the capsule, protein profiles of membrane protein, fimbriae and lipopolysaccharides (Ruiz *et al* 2001). Oliveira and Pijoan (2004) demonstrated the presence of a group of proteins with molecular weight between 36 and 38 kDa in a study of 98 samples of *H. parasuis*. The authors also observed the presence of these proteins in 90.7% samples isolated from systemic sites while they were absent in 83.4% samples isolated from the upper respiratory tract of healthy animals.

The prevalence of *H. parasuis* has increased for worldwide. In Brazil, its prevalence has increased nearly 2% per year. However, since small farms do not have the financial resources to maintain efficient sanitary control, this prevalence might be higher than suggested (Teixeira *et al* 2011). To prevent the herd from dissemination and infection, even in small farms, some practices such as early weaning and segregated production are recommended. According to Bello-orti (2014) found some virulent strains in lung and a biofilm-like growth in nasal turbinates and trachea. Indeed, some virulent strains were detected in association with macrophages, neutrophils and inside pneumocyte-like cells, while non-virulent strains were not detected in lung.

Most of the epidemiological studies on *H. parasuis* were done using serotyping (Zhang *et al* 2012). The relationship between serotype and virulence is unclear, and the cross-protection between different serotypes and the same serotype is variable (Hill *et al* 2003). Serotyping does not provide a correct discrimination of isolates to epidemiological studies, mainly because 15-41% of the isolates are not typeable (Oliveira *et al* 2003). However, the development of new molecular methods have boosted epidemiological studies on *H. parasuis* (Oliveira and Pijoan 2004), due to the possibility of characterising the isolates, compare them to others genotypes, and carry out association of some DNA profiles to virulence profile (Oliveira *et al* 2003).

UPDATE ABOUT THE PATHOGENESIS

Bacterial pathogenesis is multifactorial, requiring multiple mechanisms to cause infection and produce the

clinical signals. The pathogenesis factors include to invade the host, bacterial multiplication and damage to host tissue (Bouchet *et al* 2009). Comparisons in functional assays between virulent and non-virulent strains of *H. parasuis* allow the identification of several virulence mechanisms that help the bacterium colonise and initiate the infection by adhesion and invasion of epithelial cells (Frandoloso *et al* 2012). As other members of Pasteurellaceae family, *H. parasuis* can avoid phagocytosis, but the bacterial factors involved in the virulence are still unknown (Costa-hurtado *et al* 2013).

The infection of *H. parasuis* occurs by aerosols and suspended particles in the air (Barcellos *et al* 2008). Firstly, the agent colonises the mucosa of nasal cavity, and membranes such as synovial, meningeal and pulmonary parenchyma, which cause severe inflammatory injuries induced by the agent infection (Smart *et al* 1993). The most virulent strains of *H. parasuis* invade endothelial cells more efficiently than nasal strains and support the role of invasion in the virulence of this bacterium, however, invasion of endothelial cells is not completely required (Aragon *et al* 2010).

According to Bouchet *et al* (2009), the bacterial adhesion to epithelial cells induces apoptosis, and the release of cytokines that may be important events for colonization. Antigenic properties of *H. parasuis* have been studied by evaluating the immune response against phenotypic markers, the capsule, fimbriae and outer membrane proteins (OMP), lipopolysaccharides (LPS) and capsular polysaccharides which have been associated with the colonization of the respiratory tract (Biberstein 1990, Oliveira and Pijoan 2004). Interference of phagocytosis by *H. parasuis* virulent strains is likely associated with presence of capsule (Olvera *et al* 2009).

Another pathogenesis-related aspect are the oligopolissacarideos that follow similar patterns in virulent and non-virulent strains (Zucker *et al* 1996). The adherence on the surface of epithelial cells of host through fimbriae or adhesins factors is an important point for colonization and pathogenicity of several bacteria. When present in the blood flow, the lipopolysaccharide is considered a virulence factor, because it disseminates intravascular coagulation and thrombosis (Macinnes and Desrosiers 1999). The mechanism of growth and persistence in the host cells is also benefited by neuramidases that are associated to the production of sialic acids, neuraminidase activity could be detected, quantified and correlated with the nanH gene sequence in the set of *H. parasuis* strains under analysis, although this activity did not display an apparent correlation with virulence (Martínez-Moliner *et al* 2012).

According to Olvera *et al* (2009), different susceptibilities to phagocytosis were observed in strains of different clinical origins. Strains isolated from the nose of healthy pigs were easily phagocytosed by alveolar macrophages (AMP), while those isolated from systemic lesions were resistant to this interaction. A possible explanation to this was the presence of a separate capsule identified after interaction with AMP, which can represent a resistance factor.

According to Oliveira and Pijoan (2004), acute septicemia and disseminated intravascular coagulation are often observed in cases of *H. parasuis* infection. The *Cytolethal distending toxin* can be an important virulence factor of *H. parasuis* due to its characteristic regarding adhesion and invasion in host cells (Zhang *et al* 2012).

METHODS FOR DETECTING THE SICK ANIMAL

Usually, diagnosis of Glässer's disease at farm is done according to clinical signs, presence of lesions at necropsy, and bacteriologic culture (Vahle *et al* 1995). Clinical signs showed by infected pigs are highly variable and depend on immune status of herd, strain virulence and stage of infection (Santos *et al* 2012). *Haemophilus parasuis* causes Glässer's disease (fibrinous polyserositis), pneumonia and sudden death in pigs, but the bacterium can also be found in the upper respiratory tract of healthy piglets (Rapp-Gabrielson *et al* 2006).

At the beginning, pigs may show increased body temperature, apathy and inappetence, progressing to coughing, dyspnoea, body weight loss, lameness, incoordination, cyanosis, decubitus and death in some cases. In the acute form, sick pigs show abruptly anorexia, fever (temperature above 40 °C), inappetence, cyanosis, and lethargy. Due to the tropism for membrane serosase depending on the site of occurrence of the injury, it can cause coughing, dyspnea, nasal discharge, abdominal breathing and claudication (Nedbalcova *et al* 2006). On the other hand, when the disease progress to chronic form, clinical signs such as chronic arthritis, adherence of serous, increased scrap animals with respiratory signs, hair creepy and opaque, or death may be observed (Santos *et al* 2012). Neurological clinical signs such as tremors, incoordination, paddling, convulsion and lateral position can be found in both forms (Macinnes and Desrosiers 1999, Nedbalcova 2006). Although it is not common, some animals may present swollen and cyanotic head as a result of acute myositis of the masseter muscle, where the subcutaneous fascia and fat become dilated with fibrin-purulent content (Hoefling 1991).

MACROSCOPIC AND MICROSCOPIC LESIONS

During *post-mortem* examination at farm characteristics as serofibrinous or fibrino-purulent exudate on mucosal surface and characteristics of pleuritis, pericarditis, peritonitis, meningitis, and polyarthritis are observed (Menin *et al* 2005). *H. parasuis* can cause three clinical forms of Glässer's diseases. The sporadic one occurs in young pigs due to stress factors and is known as the classic form of Glässer's disease. The lesions observed in the first form are fibrinous and purulent exudate on serosal surface (polyserositis) of synovium, pericardium, peritoneum, pleura and meninges (Menin *et al* 2005, Santos *et al* 2012). In

the second one, certain characteristics such as septicemia without polyserositis, sub-capsular kidney bleeding and sudden death are found. In the third form, *H. parasuis* can cause pneumonia and be isolated as primary or secondary agent in infections of Circovirosis (PCV2) and virus from swine Reproductive and Respiratory Syndrome (PRRS) (Santos *et al* 2012).

The progress of *H. parasuis* infection is gradual. In pigs challenged by the respiratory route, signals as lethargic and high rectal temperature were found after 16 h post-inoculation. The first macroscopic lesions present in necropsy, were described by moderate amount of turbid liquid into the pleural, pericardial and peritoneal cavities. Pigs inspected after 36 hours post-infection showed fibrin clots in the pericardium, pleura, peritoneal fluid and joint and fibrin-purulent exudate into pericardial, pleural and peritoneal cavity found between 96 and 108 hours (Vahle *et al* 1995, Oliveira and Pijoan 2004).

According to Nedbalcova *et al* (2011) and Santos *et al* (2012), the necropsy of dead pigs infected by *H. parasuis* showed serofibrinous or fibrin purulent exudate on the surfaces of the peritoneum, pericardium, pleura and joints. Hyperemic liver, splenomegaly, catarrhal bronchopneumonia, acute and purulent hepatitis and encephalitis are commonly finding in infected pigs.

Microscopic lesions include inflammation of serous membrane with the presence of neutrophils and macrophages infiltrated (Menin *et al* 2005). Sometimes, *H. parasuis* infection may result in acute septicemia, cyanosis, subcutaneous and pulmonary edema, and death can occur without the typical serosal inflammation (Desrosiers *et al* 1986). Fasciitis and myositis (Hoefling 1991) and purulent rhinitis (Vahle *et al* 1995) have also been described.

DIAGNOSIS

Usually, diagnosis of Glässer's disease is based on clinical signs, presence of lesions at necropsy, and bacteriologic culture. As previously commented, isolation of *H. parasuis* depends on special needs, which can difficult the confirmation of this agent in the laboratory. Routinely, the identification is done using culture of clinical samples onto blood agar, NAD-supplemented media and chocolate agar. Some alternatives for diagnosis are the use of technics such as immunohistochemistry (Amano *et al* 1994, Segales *et al* 1997), oligonucleotide specific capture plate hybridization (Calsamiglia *et al* 1999), serological diagnosis (Oliveira and Pijoan 2004) and PCR (Oliveira *et al* 2001).

Serological diagnosis of *H. parasuis* is inconsistent and inaccurate. Nowadays, the development of molecular biology has been improving diagnosis techniques and providing new alternatives. The PCR has been far more sensitive and specific in *H. parasuis* detection than the other techniques (Oliveira *et al* 2001, Oliveira and Pijoan 2004) especially the sensitivity of the real-time PCR combined with high specificity makes it a very valuable tool for the diagnosis of Glässer's disease (Turni *et al* 2010).

The culture from clinical samples can be done from fibrin-purulent contents of the pericardium, pleura, peritoneum, joints and cerebrospinal fluid samples (Menin *et al* 2005). However, due to particular characteristics of *H. parasuis*, the bacterium isolation from clinical samples is difficult. A possible solution is the use of supplement media with antibiotics (bacitracin, lincomycin or crystal violet) to improve growth, isolation and recovery from contaminated samples. The growth is noted within 24-48 hours of incubation at 37 °C as a small, translucent and non-hemolytic colony (Nedbalcova *et al* 2006). Since samples may contain the agent in low quantity, pigs may have been medicated, or mistakes during collecting, handling and transporting of the sample may occur, negative results in bacterial culture do not discard the herd from *H. parasuis* (Oliveira and Pijoan 2004, Menin *et al* 2005).

The presence of *H. parasuis* as commensal of upper respiratory tract makes necessary not only isolation but also the identification of strains to determine its prevalence in herd (Menin *et al* 2005). Molecular methods give a better characterisation of isolates for analysis of profiles associated to virulence, and greater accuracy to monitor the distribution, prevalence and the emergence of new highly virulent isolates in swine herds (Macedo *et al* 2009). The use of molecular techniques as Enterobacterial Repetitive Intergenic Consensus (ERIC-PCR) allows the definition of prevalent strains that affects the herd (Oliveira and Pijoan 2004). The ERIC-PCR technic uses primers helps to detect different genotypes in isolates from the same serotype, which is important to understand better the epidemiology of the disease (Macedo *et al* 2009).

Systemic samples are indicated for bacterium isolation, while nasal and lung samples are not recommended. Genomic studies are elucidating problem of cross-immunity between strains, identifying virulent clones, and developing alternative methods of control. The differential diagnosis should be done for *Streptococcus sp*, *Erysipelothrix rhusiopathia*, *Mycoplasma hyorhinis*, *Actinobacillus suis*, *Salmonella cholerasuis, Escherichia coli* to conclude diagnosis of pigs with polyserositis in young pigs (Castilla 2012).

HOW PREVENT PROBLEMS WITH *H. parasuis* IN PIG PRODUCTION

Protection against *H. parasuis* depends on several factors of innate and acquired immune system, and serovar virulence. An effective prevention and control of *H. parasuis* have to be done according to an epidemiological study in each herd. The serotyping is an important tool to characterise strains and select the specific immunization to protect pigs. Vaccination of sows reduced not only the colonization of upper respiratory tract of piglets, but also the variability of strains of *H. parasuis* colonizing piglets (Cerdà-Cuéllar *et al* 2010). Sow-reared pigs are commonly

protected against *H. parasuis* infection, whereas colostrum deprived piglets are susceptible to systemic infection (Oliveira and Pijoan 2004).

Usually, commercial (Riising 1981, Solano-Aguilar *et al* 1999, Baumann and Bilkei 2002, Bak and Riising 2002) or autogenous (Smart *et al* 1993, Mocellin *et al* 2010) vaccines are available to control and prevent *H. parasuis* infections. If commercial vaccines are not effective, the use of autogenous vaccines produced from clinical cases isolated from the own herd is recommended as an efficient alternative (Oliveira and Pijoan 2004), which has been mostly effective (Smart *et al* 1993). However, several studies have demonstrated that heterologous protection between different serotypes is restricted to specific serotypes, and the diversity in serovar existent has difficult the development of effective-cross protective immunity (Oliveira and Pijoan 2004).

The protocol of vaccine should receive special attention to achieve success in the immunization of herd. Because of the interference in the development of active immunity by vaccine, the period of application is an important issue. Reverse vaccinology and immunoproteomic analysis identified several putative virulence-associated genes and immunogenic proteins in different *H. parasuis* strains (Hong *et al* 2011). In order to avoid the development of clinical signs or lesions characteristic of systemic infection after be challenged, it is recommended to vaccinated at least sows. Some studies (Solano-Aguilar *et al* 1999; Baumann and Bilkei 2002) recommend the vaccination of gilts and piglets to prevent the development of clinical disease. Vaccinating gilts and piglets showed no or fewer macroscopic lesions than vaccinated piglets born from non-vaccinated gilts that had neurological and lameness signs (Solano-Aguilar *et al* 1999; Baumann and Bilkei 2002). Maternal immunity not only protects piglets against *H. parasuis* infection, but could also interfere with the response after vaccination. Maternally derived antibodies against Glässer's disease were above the positive level until thereabout three weeks of life in pigs, but an examination of the serological profile of the herd is powerfully recommended before immunization (Pomorska-mól *et al* 2011).

RECOMMENDATIONS FOR TREATMENT

Antimicrobials are normally used in the swine industry to treat and control Glasser's disease, but some antimicrobials have been shown to reduce colonization by *H. parasuis,* development of effective immune responses and immunomodulation (Macedo *et al* 2015). Because of the inefficacy of vaccination protocols, use of antimicrobials during livestock is necessary to treat *H. parasuis* infections. Usually, pigs treated early during infection are able to recover from the systemic damage. Therefore, treatment should start as soon as the first clinical signs appear.

Treatment using cephalosporins has been preconized instead of penicillin due concerns of resistance to drugs. In laboratory, *H. parasuis* showed an increase of 40% in resistance to the use of tetracyclines (De la Fuente *et al* 2007). The protocols and dose recommendations vary from case to case (Nedbalcova *et al* 2006). Olvera *et al* (2007) monitored a swine herd during one year evaluating the susceptibility to antimicrobial and observed that all isolates were resistant to amoxicillin (30 mg), sensitive to enrofloxacin (10 mg), doxycycline (80 mg), sulfa-trimethoprim (5.2 + 240 mg), and tylosin (150 mg). Although antibiotic treatment can be very effective at controlling *H. parasuis* infections, it may also interpose with the development of protective immune responses against *H. parasuis* (Macedo *et al* 2015). Even though many serovars are considered sensitive to most antibiotic cited, monitoring susceptibility patterns of the agent and the judicious use of antimicrobials to treat Glasser's disease are still important criteria to be considered before administration of therapy.

Antibiotic treatments and vaccination may be used to control infection caused by *H. parasuis,* but the permanent use of antibiotics may result in an increased cost of production and may create resistance to these antibiotics (Oliveira and Pijoan 2004).

FINAL CONSIDERATIONS

Glässer's disease has been a challenge for the swine production. Although *H. parasuis* usually emerged as a major cause of nursery mortality, the several factors involved in prevalence and control of infections remain unknown. Some stressful practices such as weaning, transportation, and numerous sites of production may have affect the epidemiology of *H. parasuis* within herds. A possibility to prevent and control *H. parasuis* is the use of uniform age to weaning, prevent segregation in different ages and decrease stressor agents that cause immunosuppression. The development of molecular techniques has improved identification of virulence factors, differentiated and genotyped strains, defined the true prevalence of systemic infection, and helped to better understand infection an diseases mechanisms. However, further studies are needed to better understand this agent and have an active control over the pig herd.

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