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Artículo de revisión

Biofilm in *Actinobacillus pleuropneumoniae*, gene regulation and virulence role

Biofilm en *Actinobacillus pleuropneumoniae*, regulación génica y su papel en la virulencia

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RESUMEN

Esta revisión presenta los principales hallazgos sobre la formación de biopelículas de *Actinobacillus pleuropneumoniae*, algunos de los genes más conocidos involucrados, así como el papel del biofilm en la virulencia de este patógeno. El manuscrito pretende ofrecer al lector una panorámica de los trabajos realizados en este campo.
Palabras clave: APP, biofilm cerdos, neumonía.

ABSTRACT

This review presents the main findings about Actinobacillus pleuropneumoniae biofilm formation, some of the better known genes involved as well as the role of biofilm in the virulence of this pathogen. The manuscript pretends to give the reader a panoramic of the work done in this field.
Keywords: APP, biofilm, pigs, pneumonia.

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Introducción

Pleuropneumonia in pigs, also known as porcine contagious pleuropneumonia, is a disease caused by *Actinobacillus pleuropneumoniae*, a Gram-negative rod (Gottschalk and Taylor, 2006; Chiers *et al.*, 2010), encapsulated and with coccobacillary morphology (formerly known as *Haemophilus pleuropneumoniae* or also *H. parahaemolyticus*) (Gottschalk and Taylor, 2006).

The organism has been classified into two different biotypes (biotype I or biotype II) which differentiate easily by the growth in laboratory: biotype I only grows in blood agar around colonies of *Staphylococcus aureus* or when it is supplemented with NAD while biotype II grow easily on blood agar plates without the presence of that. *A. pleuropneumoniae* has 15 serotypes, of which 13 belong to biotype I and 2 to biotype II (Gottschalk and Taylor, 2006). There are differences in the virulence capacity between the different serotypes (Rosendal *et al.*, 1985; Jolie *et al.*, 1994). *A. pleuropneumoniae* is isolated with relative frequency from the upper respiratory tract of healthy pigs (Sidibé *et al.*, 1993), and constitutes a relevant problem for swine industries in most countries of the world that keep swine (Rosendal *et al.*, 1985). The disease is relevant economically because of the cost that it generates in acute outbreaks (Gottschalk and Taylor, 2006) when the disease is established. That is a really undesirable factor in a herd (Sidibé *et al.*, 1993).

The way of entrance of the pathogen is by aerosol. After inhalation *A. pleuropneumoniae* colonizes tissue, were the lower respiratory tract is more damaged, leading to fibrinohemorrhagic necrotizing bronchopneumonia and fibrinous pleuritis due to some of the virulence factors *A. pleuropneumoniae* produces, and which often lead to a fatal outcome (Chiers *et al.*, 2010).

A. pleuropneumoniae is able to produce a biofilm phenotype (Kaplan and Mulks, 2005; Labrie *et al.*, 2010). Biofilm can be defined as bacterial cells communities, surrounded by a matrix that gives adherent properties to different surfaces or tissues (Costerton *et al.*, 1999; O´Toole *et al.*, 2000).

The biofilm formation provides the microorganism a set of tools to endure the different environmental conditions experienced in order to establish and proliferate (Parsek and Singh, 2003; Hall-Stoodley *et al.*, 2004). Biofilms could be related with body dissemination of disease (Costerton *et al.*, 2003) and may have a role in bacterial colonization and/or persistence (Auger *et al.*, 2009). Even more, the ability to form biofilm is related to chronic infections (Costerton *et al.*, 2003). The objective of this review is to present the main findings

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reached by numerous researchers, giving the reader a panoramic of the work done in this field, based on the works related to biofilm production, quorum sensing, and virulence gene expression in *A. pleuropneumoniae* (Izano *et al.*, 2007; Li *et al.*, 2008; Buettner *et al.*, 2008; Dalai *et al.*, 2009; Tegetmeyer *et al.*, 2009; Bosse *et al.*, 2010; Grasteau *et al.*, 2011; Li *et al.*, 2011; Xie *et al.*, 2013).

What is a biofilm, quorum sensing and the relevance in disease?

The definition of bacterial biofilms can be stated as bacterial cells living in arranged communities (single or multiple species), surrounded by a matrix produced by themselves, that gives adherent properties to different surfaces or tissues (Costerton *et al.*, 1999; O´Toole *et al.*, 2000).

The development of biofilm is a process that involves multiple steps evolving from adherence between bacterial cells up to generating multiple layers that could have accumulation of bacteria and host components (Hall-Stoodley *et al.*, 2004), and seems to be triggered by diverse environmental conditions (x. e. nutrient supply) which drives changes in bacterial surfaces in response to the presented challenges (O´Toole *et al.*, 2000).

An example that clearly demonstrate this fact is seen in the work done by Kaplan and Mulks (2005) which uses trypticase soy agar or mueller hinton broth and show that only reference strains of *A. pleuroneumoniae* 5B and 11 are capable of biofilm formation. They also showed that after broth passage of bacteria, the biofilm phenotype is lost and suggest that this process was irreversible. However, their findings are in contrast with Labrie *et al.* (2010), who found that growth conditions (growth cultures) drive the biofilm formation. This work found that other serotypes also are able to produce biofilm. Additionally, the microorganism´s transition of biofilm positive to negative is reversible.

Bacteria with biofilm capacity must have different abilities; among those is the capacity of sensing cell densities (O´Toole *et al.*, 2000). The ability of bacteria to sense their population in order to act jointly, is known as ¨quorum sensing (QS). QS is a term that explains a mechanism or mechanisms used by bacteria to establish communication between them. Bacteria are able to secrete molecules with the role of signaling the others about the population density. When signals reach certain levels, they activate expression of genes that will modify the general behavior of all the population (Boyen *et al.*, 2009).

The signaling molecules are known as (autoinducers [AIs]) (Xavier and Bassler, 2003). An AI-2 autoinducer, initially detected in *V. harvey* was also reported in multiple bacteria and related with the production of a protein known as LuxS (Miller and Bassler, 2001).

At the same time, the biofilm formation provides the microorganism with a set of tools to endure different environmental conditions experienced in order to establish and proliferate. Biofilm was mention that could enable the bacteria with characteristics such as resistance to antimicrobials, potential for genetic exchange, ability to develop adaptative mutations, persistence and tolerance to host defenses and stress situations (mediating transition from acute to chronic infection) (Parsek and Singh, 2003; Hall-Stoodley *et al.*, 2004).

Archambault *et al.* (2012) clearly showed in a study the advantage biofilms confer to *A. pleuroneumoniae* in respect to antimicrobial resistance, when showed that cells in biofilm were up to 30,000 times more resistant than cells that were not in the biofilm. This correlation was also done by Izano *et al.* (2007) although the levels of resistance found in that study were just 10 fold higher than the one exhibited by planktonic cells.

Related to the biofilm field, it is suggested that chronic infections are commonly associated with biofilm formation (Costerton *et al.*, 2003). However, *A. pleuropneumoniae* is able to form biofilm as early as 6 hours after incubation, suggesting a possible role for it, in acute infections also (Labrie *et al.*, 2010).

Parsek and Singh (2003) established some criteria in order to delimit biofilm-caused infections: 1. Adherence of the pathogenic bacteria to a location (surface or substratum), 2. Presence of bacterial clusters or microcolonies in host tissue, surrounded by extracellular matrix, 3. Confinement of the infection to a singular place, and 4. Difficulty or impossibility to eliminate infection by antimicrobial treatment.

Analysis of the aforementioned criteria, added to other properties of biofilm such as role of biofilms in systemic dissemination of disease (Costerton *et al.*, 2003) and possible role in vivo, in the colonization and/or persistence of the bacteria (Auger *et al.*, 2009), will allow the researchers to understand expectations being studying in this relatively new field in the bacterial world. The understanding of why and how biofilm is produced and their properties, will open a new world to develop possible new tools to manage and control the diseases produced by biofilm positive organisms, such as *A. pleuropneumoniae*.

Biofilm formation, virulence and genes associated.

Virulence factors are substances that play a relevant role in the pathogenesis of *A. pleuropneumoniae*, for example: capsular polysaccharide, lipopolysaccharide, outer membrane proteins, transferrin binding proteins, RTX-toxins (ApxI, ApxII, and ApxIII), proteases and adhesins (Haesebrouck *et al.*, 1997; Bossé *et al.*, 2002). However, Parsek and Singh (2003) have also suggested that biofilm has a relevant role in the pathogenesis of infections caused by numerous bacterial pathogens, and Labrie *et al.* (2010) mentioned that biofilm formation for bacteria, including *A. pleuropneumoniae* is relevant for virulence.

The first biofilm formation report of *A. pleuropneumoniae* was conducted by Kaplan *et al.* (2004). They showed that cells of this bacterium produce a intercellular adhesin base on polysaccharide containing hexosamine (PGA), and PGA is an important matrix adhesin component of biofilm that seems to be basic for its formation, and appears to increase the antibiotic

resistance of this bacteria when is compared to planktonic cells (Izano *et al.*, 2007). The capacity of resistance to antimicrobial agents by biofilm forming bacteria, is suggested to be related with the failure of the substance to penetrate the biofilm completely (Costerton *et al.*, 1999).

Biofilm may have relevance in some activities of *A. pleuropneumoniae* such as: colonization, pathogenesis, and transmission (Kaplan *et al.*, 2004). The pathogenesis of *A. pleuropneumoniae* in porcine contagious pleuropneumonia is a complex that involves many virulence factors of the bacterium, and it has been proposed that the capacity of biofilm formation mediates the *in vivo* colonization (Chiers *et al.*, 2010).

Kaplan and Mulks (2005), showed that field isolates of *A. pleuropneumoniae* display a biofilm phenotype, and proposed that it could have a role in the natural infection at early stages in the upper respiratory tract.

There are many research works done that correlate *A. pleuropneumoniae* with virulence factors, gene expression and/or pathogenesis. Work by Baltes and Gerlach (2004) tried to correlate certain findings in the course of disease with transcription of genes, which identify genes expressed by the bacteria in necrotic tissue. Since the first report of biofilm formation by *A. pleuropneumoniae*, numerous works have studied the role of diverse genes that could be related to biofilm production, quorum sensing regulation and expression of virulence factors by this pathogen (Izano *et al.*, 2007; Buettner *et al.*, 2008; Li *et al.*, 2008; Dalai *et al.*, 2009; Tegetmeyer *et al.*, 2009; Bosse *et al.*, 2010; Grasteau *et al.*, 2011; Li *et al.*, 2011; Xie *et al.*, 2013).

One of the first works in *A. pleuropneumoniae* that correlates biofilm with virulence, was done by Izano *et al.* (2007). Kaplan *et al.* (2004) demonstrated that genes *pgaCD* are used for the bacterial production of a polysaccharide adhesin implied in biofilm. Based on Kaplan's study, Izano *et al.* (2007) demonstrated that a *pgaC* mutant is deficient in biofilm production and proved that the *pgaC* expression is relevant for biofilm formation. The authors suggested a major biofilm adhesin role for the pgaC related product (PGA), with possible relevance in colonization and pathogenesis of disease caused by this bacteria.

Another important gene implied in biofilm formation and virulence is the *hns* gene. Dalai *et al.* (2009) found that this gene, which codes for an H-NS protein with histone-like characteristics, when inactivated by the effect of mutated strains, increased the ability to produce biofilm and weakened the virulence related with up-regulation or repression of the apx genes. They also showed that strains with down-regulation of *hns*, were not able to form biofilm and the virulence was also attenuated, when tested in mice models. Using transposon insertion mutagenesis, Bossé *et al.* (2010) studied the role of *hns* and another gene *rseA* (regulator of sigma factor "anti σ^E", a stress protein analoge to *E. coli rseA* (Alba and Gross, 2004) in the regulation of biofilm expression and virulence. It was shown that rseA and hns role in biofilm formation is related to inactivation or repression of pga operon transcription. However, their findings contrast with the study of Dalai *et al.* (2009) in the role of *hns* in virulence, because contrary to that study, Bossé *et al.* (2010) found that none of *hns* mutants were avirulent, when tested in a pig model, and demonstrated full virulence.

luxS is a comprehensively studied gene for its role in biofilm formation. luxS encodes a protein related to the production of AI-2 autoinducers, implied in QS activities (Miller and Bassler, 2001). Studies developed initially in the related bacteria *Actinobacillus actinomycetemcomitans*, showed that this bacteria has a *luxS* gene and is able to produce a signal molecule similar to AI-2. It was shown that this bacterium secretes a leukotoxin, whose levels increase in association with LuxS presence, suggesting that it could have a virulence role in vivo (Fong *et al.*, 2001). Novak *et al.* (2010), showed that part of the virulence activity of *A. actinomycetemcomitans* in vivo and biofilm formation properties linked to AI-2 were related with the regulation of a *qseBC* operon.

A. pleuropneumomiae's luxS role also was investigated by Li *et al.* (2008). They found that AI-2 production is mediated by *luxS*. Mutants deleted in *luxS* were able to increase biofilm production although, it was reported that the virulence decreases, associated with downregulation of *apxIIA* gen. Li *et al.* (2011) studied the genes regulated by *luxS* mutant along different bacterial growth status, and they were able to find that mutation in this gene was related with differential expression of multiple genes. Some of the genes related with iron acquisition, biofilm and adherence.

Another gene implied in studies of biofilm formation and virulence in *A. pleuropneumoniae* is the regulatory gene *arcA*. *arcA* plays an important role in the control of aerobic respiration of *E. coli* (Iuchi and Lin, 1988). Studies of this regulatory system in *V. cholera* showed that mutants were attenuated in virulence in comparison to the wild type strain (Sengupta *et al.*, 2003).). By means of a mutant, Buettner *et al.* (2008) studied the role of the gene *arcA* in aggregation, biofilm formation and virulence of *A. pleuropneumoniae*. They found that the mutant carrying a deletion in this gene diminished its capacity for aggregate and form biofilm. They observed that animals affected by the mutants, also showed lower clinical findings represented as clinical score, when were compared with the wild strain. The authors suggested *arcA* plays a role in the persistence of this pathogen in respiratory tract.

The most recent study related with biofilm formation and virulence in *A. pleuropneumoniae* was performed by Xie *et al.* (2013), who studied the role of the stress protein ClpP protease, encoded by the gene *clpP*. The authors found that *clpP* mutation decreases the biofilm formation. They suggested that ClpP also may have a relevant role in regulation of virulence because *clpP* mutants improve their iron use, two iron-acquisition proteins expression were affected by this mutant.

Genes with a minor role in virulence and/or adherence, also have been identified. Tegetmeyer *et al.* (2009) studied the role of AasP in biofilm formation and virulence in *A. pleuropneumomiae*. AasP is an autotransporter serine protease

encoded by *aasP* gene (Baltes *et al.*, 2007). In the research developed by Tegetmeyer *et al.* (2009), a role for Aasp in adhesion to abiotic surface was discovered. Mutants in this protein presented an altered biofilm formation, however, they reported that this protein does not have a role in pathogenicity. Finally, one of the most recent studies done by Grasteau *et al.* (2011) using a transposon insertion study, was able to identify 24 mutants with defects in biofilm formation, implying a minimum of 16 genes from which at least the half has been previously identified associated with biofilm formation in *A. pleuropneumomiae* and/or other bacteria. The authors suggest the need to study the role of those genes in biofilm formation and the need to generate a more extensive study in order to identify more genetic determinants related to biofilm formation in that bacteria.

Conclusion

It can be seen that there are multiples studies studying relating diverse genes and biofilm production and virulence, although some have shown contradictory results. It is relevant to mention that in spite of the multiple knowledge acquired recently in this field, as Grasteau *et al.* (2011) have shown, it is needed to continue studying the role of these genes in biofilm formation and/or virulence, in order to get a better understanding of pathogenicity of this disease in our livestock systems.

After researching and analyzing the information available about this important pathogen for the swine industry, I am in agreement with the conclusion of the work done by Haesebrouck *et al.* (1997). They stated that "the pathogenesis of porcine pleuropneumonia is very complex", and despite the multiple technologies displayed in order to understand all the aspects related with the pathogenesis of this microorganism, the complexity of the variables implied in this process, has hampered our full comprehension of the same.

The multiple works presented in this review, should encourage researchers interested in this topic, toward developing new integrative tools to fully comprehend how this bacteria, the biofilm and virulence factors function in a host in order to become pathogenic, develop disease and their consequences.

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