

## Porcine Respiratory Disease Complex and Biofilms

Abraham Loera-Muro<sup>1</sup>, Flor Y Ramírez-Castillo<sup>2</sup>, Francisco J Avelar-González<sup>2</sup> and Alma L Guerrero-Barrera<sup>3\*</sup><sup>1</sup>Centro de Investigaciones Biológicas del Noroeste (CIBNOR), La Paz, Baja California Sur, México<sup>2</sup>Centro de Ciencias Básicas, Universidad Autónoma de Aguascalientes, Aguascalientes, Ags., México<sup>3</sup>Laboratorio de Biología Celular y Tisular, Departamento de Morfología, Centro de Ciencias Básicas, Universidad Autónoma de Aguascalientes, Aguascalientes, Ags., México**\*Corresponding author:** Alma L Guerrero-Barrera, Laboratorio de Biología Celular y Tisular, Departamento de Morfología, Centro de Ciencias Básicas, Universidad Autónoma de Aguascalientes, Aguascalientes, Ags., México, C. P. 20131, Tel: 0201113532752; E-mail: [alguerre@correo.uaa.mx](mailto:alguerre@correo.uaa.mx)**Received date:** Sept 15, 2015; **Accepted date:** Oct 29, 2015; **Published date:** Nov 02, 2015**Copyright:** © 2015 Loera-Muro, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

### Abstract

The Porcine respiratory disease complex (PRDC) is a term used to describe polymicrobial respiratory infections in pigs. Respiratory diseases in pigs are common in modern pork production worldwide and are the responsible for major economic losses in the swine industry. Pathogens involved in respiratory disease in pigs vary significantly among farms, production sites, regions and countries, making generalizations about the PRDC treatment and difficult to control it. The interactions that occur on the cellular and molecular levels during concurrent infection of pigs with two or more respiratory pathogens are multi-faceted and convoluted. There are a variety of bacterial and viral pathogens commonly associated with the PRDC. The main associated bacteria include: *Actinobacillus pleuropneumoniae*, *Streptococcus suis*, *Pasteurella multocida*, *Bordetella bronchiseptica*, *Haemophilus parasuis*, and *Mycoplasma hyopneumoniae*. Currently, it is known among microbiologists that biofilm formation is an universal attribute of microorganisms and the main way of life in nature that are causing problems such as developing diseases in animals and humans. Here, is reviewed the current knowledge of the major bacteria involved in this disease, their ability to form biofilms, as well as their importance on the infection process.

**Keywords** Porcine respiratory disease complex (PRDC); Respiratory infections; Respiratory pathogens; Biofilmpathogens or co-factors, in order to induce substantial lesions in the respiratory system (*S. suis*, *P. multocida* and *H. parasuis*) [1-3].

### Introduction

Respiratory disease in pigs is common in modern pork production worldwide and is often referred as porcine respiratory disease complex (PRDC). PRDC is polymicrobial in nature, and results from infection with various combinations of primary and secondary respiratory pathogens. As a true multifactorial disease; environmental conditions, population size, management strategies and pig-specific factors such as age and genetics play critical roles in the outcome of PRDC. Pathogens involved in respiratory disease in pigs vary significantly among farms, production sites, regions and countries, difficulting the treatment and control of the PRDC. The interaction that occurs on the cellular and molecular levels during concurrent infection of pigs with two or more respiratory pathogens is multi-faceted and complex. Rates of morbidity related to PRDC range from 30% to 70%, and mortality rates vary between 4 and 6 percent, which could be even higher in affected farms [1-2].

There are a variety of viral and bacterial pathogens commonly associated with PRDC. The main bacteria associated to PRDC includes: *Actinobacillus pleuropneumoniae*, *Streptococcus suis*, *Pasteurella multocida*, *Bordetella bronchiseptica*, *Haemophilus parasuis* and *Mycoplasma hyopneumoniae* [2]. Respiratory pathogens can be further distributed into primary pathogens that are capable of inducing severe lesions in respiratory tissues as a result of their own virulence (*A. pleuropneumoniae*, *B. bronchiseptica* and *M. hyopneumoniae*), and secondary or opportunistic infectious pathogens, which typically need help from others co-infecting

### Biofilms in the Porcine Respiratory Disease Complex

Generally, bacteria operate in complex associations, communities or consortia called biofilms. These associations are responsible for the maintenance of the biosphere biogeochemical and in other cases could cause serious illness. Currently, the ability to form biofilms is considered a universal attribute of all microorganisms [4-5]. Biofilms are bacterial community attached on an inert surface or living tissue and are embedded in an exopolysaccharide matrix [6]. This matrix may be composed of polysaccharides, nucleic acids and proteins, in which can also coexist different bacterial species [4,7-11].

Biofilms, especially multi-specie biofilms, are the most common form to microbial growth in nature [5,9-10,12-13]. Biofilms formation is a complex event that could involve many bacterial species and numerous factors [14]. The life cycle is a dynamic process that involves several stages. The initial stage corresponds to the bacterial cell adhesion to a substrate live or dead. The appendices as flagella, fimbriae or pili (Gram negative), help to move and adhere to substrates [10,14]. In Gram positive, proteins associated with the cell wall, called adhesins, play an important role in bacterial adhesion and coaggregation [15]. Adhesion and biofilm formation are coordinated elements. For both, bacterial secreted small chemicals substances of low molecular weight and rapid diffusion that can modulate the activities of neighboring cells. These molecules are called "quorum sensing" or autoinducers [16]. Early in the process primary adhesion is giving by early settlers to the surface, forming multiply these microcolonies that eventually accordance with microenvironmental conditions, colonized completely and cover the surface, facilitating the

arrived of colonizers secondary or later inducing development multi-species consortia [17]. This step is marked by cell division and expansion of the daughter cells surrounding the site of primary adhesion. Is secreted the biofilm matrix: an exopolysaccharide, which can be composed of N-acetylglucosamine, alginate, cellulose, glucose, galactose, among others [18]. The composition of this matrix depends on the bacteria strains involved and the environmental conditions [4]. The last step is the detachment or separation of the biofilm bacterial cells. At this stage, some bacteria are released from the biofilm matrix, alone or in bacterial clusters, and begin to colonize new environments. This release can be for: the lack of the exopolysaccharide synthesis for some bacteria (*Staphylococcus aureus*), for the enzymes synthesis (alginate lyases, dispersin B), which degrades the exopolysaccharide matrix, allowing bacterial release (*Aggregatibacter actinomycetemcomitans* and *Pseudomonas aeruginosa*) [8]; or for bacteriophages activity within the biofilm. Other mechanisms involved in this phenomenon are: a) erosion or sliding: continuous removal of small parts of the biofilm; b) separation: rapid and massive remotion; and c) abrasion: liberation by collisions with particle in the fluid with the biofilm [19].

Approximately 80% of the world's microbial biomass resides as biofilm populations. The National Institutes of Health (NIH) estimates that up to 75% of human infections are caused by the formation and persistence of biofilms. Considering the extensive involvement of biofilms in infections diseases in humans, biofilms are likely responsible for a wide variety of infections in veterinary medicine [4,20]. However, only a few studies are related on the importance of biofilm formation in pathogens of veterinary importance. In the case of PRDC, it is known that several bacteria involved in it are able to form biofilms but the importance of biofilms on the development of these infections is unknown.

## Bacteria Involved in the PRDC and Biofilms Formation

### *Actinobacillus pleuropneumoniae*

*Actinobacillus pleuropneumoniae* is a Gram negative coccobacillus bacteria, pleomorphic, facultative anaerobic, non-spore-forming, encapsulated and belonging to the family Pasteurellaceae [21-23]. *A. pleuropneumoniae* is the etiologic agent of porcine contagious pleuropneumonia, an infectious respiratory disease of swine, which causes important worldwide economic losses in the swine industry [24-29]. This disease is highly contagious and could be even deadly, which are causing enormous economic losses. Injuries caused by these bacteria are characterized by hemorrhage and necrosis in lungs [28]. Two biotypes have been described based on dependence of nicotinamide adenine dinucleotide (NAD). Similarly, fifteen serotypes have been recognized by the capsular antigens compositions with distribution in different countries [30]. Serotypes 1 to 12 and 15 usually belong to biotype 1, which contain NAD-dependent strains and are commonly found in pneumonia diseases. Serotypes 13 and 14 are usually biotype 2 and are independent of NAD [26-27,31]. However, variants have been reported belonging to biotype 2 serotype 2, 4, 7, 9 and 11 [26-27,30]. All serotypes are obligate pathogens, but have differences in virulence and geographic distribution [28,30,32]. Serotypes 1, 5 and 7, are found predominantly in North America, the serotype 2 is the most commonly found in Europe and serotypes 1, 3, 4, 5 and 7 are typically isolated in China [28,32,33]. Moreover, atypical *A. pleuropneumoniae* serotype 13 has been described in North

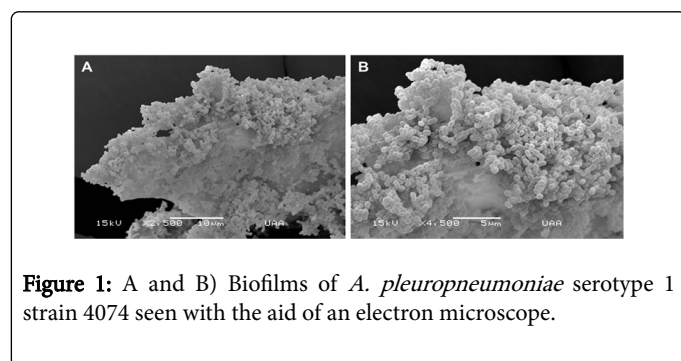
America, Canada and USA, these strains being dependent NAD (Biotype 1) [30].

Many virulence factors have been reported in *A. pleuropneumoniae* that contribute to the disease; these are including lipopolysaccharide (LPS), exotoxins (Apx), capsule polysaccharide, proteases [29,31], urease, iron acquisition systems and enzymes involved in anaerobic respiration [21]. Recently, some adhesion structures as type IV pilus [34], Flp pilus [25,34], autotransporters of adhesins [34] and biofilm formation [22,25,35] were also found to be associated with the infection processes. However, Apx toxins, are major virulence factors involved in pathogenesis *pleuropneumoniae* [21], which are also primarily responsible for the lesions observed in swine lungs [21,36], being ApxI and ApxII toxins the most damaging. Therefore, it is known serotypes 1, 5, 9 and 11 are among the most virulent serotypes since they exhibit both toxins [25]. The Apx toxin belonging to RTX toxin family ("repeat in toxin"). These toxins are widely distributed in Gram negative bacteria. These toxins are called Apx by "*A. pleuropneumoniae* RTX toxin"; existing four of them, the first, ApxI is strongly hemolytic and cytotoxic, the second ApxII is weakly hemolytic and moderately cytotoxic, the third ApxIII, is not hemolytic but is strongly cytotoxic, and the fourth, the ApxIV, is weakly hemolytic but cytotoxic. It has been shown that the latter toxin ApxIV, is only expressed in animal infections but not in vitro. Different serotypes secrete different set of toxins causing variations in hemolytic and cytotoxic activity. These toxins are encoded by apx operon, which consists of four genes usually arranged as continuous apxCABD [37-38].

Bacterial surface polysaccharides, lipopolysaccharides (LPS) and capsular polysaccharides (CPS), usually play an important role in virulence. Lipopolysaccharides are complex molecules composed of 3 well-defined regions: (i) lipid A; (ii) the core oligosaccharide, containing 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo); and (iii) the O-antigen, a polysaccharide consisting of repeating units. The LPS of *A. pleuropneumoniae* are involved in the adherence of the bacteria to porcine respiratory tract cells [32]. The structure of *A. pleuropneumoniae* serotype 1 O-antigen was previously described by Altman, Brisson, and Perry as branched tetrasaccharide repeating units composed of 2  $\alpha$ -L-rhamnopyranosyls, 1  $\alpha$ -D-glycopyranosyl and 1 2-acetamido-2-deoxy- $\beta$ -D-glucose residues [32].

The ability of *A. pleuropneumoniae* to form biofilms in vitro has been well established [22-23,35,39-40] (Figure 1). In addition, biofilm formation is a stress response in *A. pleuropneumoniae* [41] and it has been suggested to contribute to colonization and persistence of this pathogen in vivo [25,42]. Previous works from our group have been demonstrated that *A. pleuropneumoniae* biofilms are formed in vivo in drinking water of swine farms as a strategy for survival and/or transmission (unpublished data) and it is able to grow in unsuitable environments by forming multi-species biofilms with other respiratory pathogens of pigs that are also part of the PRDC (unpublished data). The major component of the extracellular matrix of the biofilm that forms *A. pleuropneumoniae* is poly-N-acetylglucosamine (PGA). However, recent studies have been shown the existence of protein and extracellular DNA (eDNA) in the matrix of this pathogen [33]. The PGA is dependent biosynthesis of proteins encoded within the pgaABCD operon [39]. This operon can be regulated by various proteins, for example, H-NS regulates synthesis operon by repression of pga [40]. Also, the sigma factor  $\sigma E$  positively regulates expression of this operon, which indicates that the formation of biofilms in *A. pleuropneumoniae* is part of an extracytoplasmic stress response [41].

Other genes associated with biofilm formation in *A. pleuropneumoniae* are: luxS [43], the two-component system ArcAB [28] and a serine protease autotransporter [40] (Table 1). It also has been established that *A. pleuropneumoniae* biofilm present a greater resistance to different antibiotics. Archambault et al. [44] reports that cells of this pathogen in biofilms form are 100 to 30,000 times more resistant to antimicrobial agents compared to their planktonic state. Hathroubi et al. [35] evaluated the effect of sub-minimum inhibitory concentration (MIC) of penicillin G on the biofilm formation of *A. pleuropneumoniae*. They found that sub-MICs of penicillin G significantly induce biofilm formation due to the result of a cell envelope stress sensed by the CpxRA system resulting in an increased production of PGA and other matrix components. However, reports mention that biofilm formation was inhibited by zinc [22,33]. Finally, Luna-Castro et al. [45] report that bovine iron-free Lactoferrin (BapoLs) decreased biofilms formation (60-70%) in the three strains of *A. pleuropneumoniae* used in the study (two serotype 1, strains S4074 and the isolate BC52, and a serotype 7 reference strain WF83).



**Figure 1:** A and B) Biofilms of *A. pleuropneumoniae* serotype 1 strain 4074 seen with the aid of an electron microscope.

### ***Streptococcus suis***

*Streptococcus suis* is a Gram positive bacteria, facultative anaerobic, endemic to most countries, and is recognized as one of the leading pig respiratory pathogens and emerging zoonotic agent [46-50]. This pathogen is also responsible for great economic losses in the swine industry. In addition, outbreaks in humans in different parts of the planet have been presented, by de transmission of this pathogen by contact with infected pigs or derived products [48,51]. Moreover, this pathogen can be isolated from other ruminant animals such as cats, dogs, goats and horses [46]. *S. suis* infections also are associated with a variety of clinical conditions such as encephalitis, meningitis, arthritis, sepsis, endocarditis and abortions in pigs, and in human, the infection could lead to septicemia and meningitis [49], and even death [52]. Thirty-five serotypes have been described (1-34 and 1/2), based on its capsular antigen [50]. Nevertheless, only a few serotypes are responsible for infections in pigs, which include serotypes 1-9 and 14, where the serotype 2 is considered the most pathogenic for pigs and humans [46-47,53]. In addition to serotype 2, *S. suis* serotype 14 has been described as being an important swine pathogen and an emerging zoonotic agent [54-55]. Serotypes 1, 2, 7, 9 and 14 are most commonly isolated from diseased pigs in Europe and serotypes 3 and 8 in North America [49]. In Asia, commonly found serotypes are 1/2, 2, 3, 4 and 7 [56].

Little is known about the virulence factors of this pathogen, these include: the capsular polysaccharide (CPS), the toxin suilysin (SLY), muramidase protein (MRP), extracellular proteins (EF), adhesins, fibronectin, fibronectin binding proteins (FBP), serum opacity factor and arginine deiminase system and biofilm [57]. Some factors that are

encoded by the mrp gene for MRP, epf encodes an EF and sly to cytotoxin (hemolysin) SLY [58]. The cytotoxin SLY (suilysin) seems to protect bacteria against complement-mediated uptake and killing by neutrophils, macrophages and dendritic cells [54]. The CPS, which defines the serotype, is considered as the major virulence factor [53,55]. The structures of types 2 and 14 *S. suis* CPSs comprise the monosaccharides glucose, galactose, N-acetylglucosamine, and rhamnose (for type 2 only) arranged into a unique repeating unit that also contains a side chain terminated by sialic acid [53]. A previous study analyzing 15 *S. suis* cps gene clusters indicated that all the cps gene clusters are located between the orfZ (conserved hypothetical protein gene) and aroA (3-phosphoshikimate 1-carboxyvinyltransferase gene) genes on the chromosome [59]. Moreover, sialic acid of bacterial polysaccharides has been suggested to be involved in immune evasion via several mechanisms [53]. Overexpression of the sialic acid rich capsule in the bloodstream is required to avoid phagocytosis and killing by innate immune cells, but the pathogen needs to reduce capsule expression to avoid hindering adhesins important for attachment to epithelial/endotelial cells and formation of biofilms [60]. Therefore, this pathogen has appeared in recent years as a major public health problem, especially in Asia and European countries [46-48,60,61].

Some bacterial factors have been extensively used to try to predict the virulence of *S. suis* strains. Two proteins known as MRP (muramidase-released protein) and EF (extracellular factor) protein have historically been used as virulence markers. MRP is a 136-kDa cell-wall anchored protein also released into the culture supernatant during bacterial growth, while EF is a 110-kDa secreted protein [60].

Several studies have been showed that the ability of *S. suis* to form biofilms is a property restricted to only a few strains. The matrix of the *S. suis* biofilm is composed of an exopolysaccharide highly hydrated called glycocalyx [56]. Studies have been demonstrated that the luxS gene is involved in biofilm formation in these species (Table 1). Wang et al. [56] reported that luxS mutant exhibited a significant reduction in their ability to form biofilms, cell adhesion, hemolytic activity and transcript levels of several virulence factors. However, overexpressed luxS is not able to increase the level of pfs expression and produce additional AI-2, leading to slower growth and slightly increases into biofilm formation [62]. Furthermore, AI-2 supplemented exogenously acted as a concentration-dependent signaling molecule to regulate *S. suis* biofilm formation, host-cell adherence, and transcription levels of many virulence genes [63]. Moreover, the capsular polysaccharide (CPS), besides to be an important virulence factor, is also a vital component of the extracellular matrix responsible for the formation of the biofilm [63].

### ***Pasteurella multocida***

*Pasteurella multocida* is a Gram negative *coccobacillus* belonging to the family Pasteurellaceae, facultative anaerobic and immobile, and can be alone, in pairs or as short chains [64-65]. There are three subspecies (ssp.): *P. multocida* ssp. *multocida*, *P. multocida* ssp. *gallicida* and *P. multocida* ssp. *septica* [64,66]. *P. multocida* is a pathogen associated with a variety of animal species. In pigs, the pathogen induces pneumonia and progressive atrophic rhinitis (PAR) [67], which are cause of symptoms such as shortening, twisting of the mouth, tears, and dark spots [68-69]. Atrophic rhinitis rarely causes death, but it has economic importance because significantly reduces the growth of infected animals [67,70]. However, the protection of

animals with an efficient vaccination has been considered the most important and attractive method to control this disease [69].

The strains of this microorganism could be classified into two ways, first into the groups: A, B, D, E and F (cap genes) based on the antigenicity of the capsules; and secondly into serotypes 1 to 16, based on their lipopolysaccharide antigens [64,70-72]. The composition and structure of capsular material found in subgroups A, D and F are similar to those found in mammalian glycosaminoglycans [73]. All the data of *P. multocida* knowledge that these strains produce LPS molecules without the O-antigen that is common to many enteric Gram-negative bacterial species. Instead, the outer core region of LPS is the most distal and highly variable component of the *P. multocida* LPS [74]. The structure of the L4 LPS outer core produced by the serovar 6 type strain consisted of  $\beta$ -Gal-(1-3)- $\beta$ -N-acetylgalactosamine (GalNAc)-(1-4)- $\beta$ -GalNAc3OAc-(1-4)- $\alpha$ -GalNAc3OAc-(1-3)- $\beta$ -Gal, whereas the serovar 7 type strain produced a highly truncated LPS outer core containing only a single  $\beta$ -Gal residue. The structure of the L8 LPS outer core produced by the serovar 16 type strain consisted of  $\beta$ -Gal-(1-3)- $\beta$ -GalNAc-(1-4)-( $\alpha$ -GalNAc-(1-3)- $\alpha$ -GalNAc [75]. The longest outer core oligosaccharide, produced by the serovar 12 type strain, contained a terminal region consisting of  $\beta$ -Gal-(1,4)- $\beta$ -GlcNAc-(1,3)- $\beta$ -Gal-(1,4)- $\beta$ -Glc that was identical in structure to the vertebrate glycosphingolipid, paragloboside. Mimicry of host glycosphingolipids has been observed previously in *P. multocida* strains belonging to L3 LPS genotype, which produce LPS similar in structure to the globo-series of glycosphingolipids [74].

Atrophic rhinitis in pigs is usually caused by *P. multocida* typically belonging to serogroup D and expressing a toxin (PMT). PMT toxin is a 146 kDa monomeric protein encoded by the gene *toxA* [67,69]. The PMT is an AB-type toxin, dermonecrotic, that causes pleiotropic effects in intoxicated host cells. PMT protein has four functionally defined regions: a N-terminal region that contains the receptor-binding/translocation domains (residues 1-568) and a C-terminal region that contains a C1 domain (residues 569-719) for membrane localization; a C2 domain (residues 720-1104) of unknown function; and a catalytic C3 domain (residues 1105-1284) with G $\alpha$ -protein-deamidase activity [76]. PMT has many properties that mark it out as a potential carcinogen. PMT is a highly potent mitogen and has been demonstrated to block apoptosis. PMT modifies and activates members of three of the four families of heterotrimeric G-proteins, all of which have potential roles in carcinogenesis. Many signalling components downstream of these G-proteins are known proto-oncogenes and have been shown to be activated by PMT. On the evidence so far, it would appear unlikely that PMT contributes significantly to human cancer. It is of interest that some human respiratory infections with *P. multocida* are suggested to be chronic, as this is a further characteristic that is shared by many carcinogenic agents [77].

*P. multocida* isolates are known to possess type IV fimbriae (pili) as one of the major virulence factors. Type IV fimbriae, encoded by the gene *ptfA* (~435 bp), is one of the major surface components of *P. multocida* known to frequently mediate colonization of host surfaces by adhesion [67].

There are not many studies on biofilm formation in *P. multocida* [78-80]. Olson et al. [78] reported that in order to reach the biofilm formation it is necessary to add fetal bovine serum to the medium. Additionally, Olson et al. [78] found that there is only a greater tolerance to the antimicrobial agent trimethoprim sulphadoxine as biofilm in comparison to their planktonic counterparts. The strain

diversity among *P. multocida* involved in serious pulmonary disease is completely consistent with an opportunist role for *P. multocida* and the establishment of a multicellular, multi-species bacterial biofilm [81]. Recently, our group found that *P. multocida* is able to form multi-species biofilm with *A. pleuropneumoniae* in vitro, allowing *A. pleuropneumoniae* to grow without the supply of NAD and changing the composition of the extracellular matrix (unpublished data). Likewise, during the formation of this multi-species biofilms it was observed that the eDNA became an important part of the structure, since treatment with DNase dispersed biofilm formed by *A. pleuropneumoniae* and *P. multocida* (unpublished data).

### ***Bordetella bronchiseptica***

*Bordetella bronchiseptica* is a Gram negative bacteria pathogen of the respiratory tract and can infect a wide range of mammals [82-83]. *B. bronchiseptica* is also being increasingly isolated from humans, mainly from immunocompromised patients [84]. It is considered one of the main causative agents of atrophic rhinitis in pigs [85] along with *P. multocida* [86]. Infections with this microorganism are transmitted by aerosol and bacteria can be localized invading the epithelial cells of the respiratory tract [83], where the damage to the respiratory tract is fundamentally caused by dermonecrotic toxins [86]. Several recent reports have demonstrated the isolation of *B. bronchiseptica* from cystic fibrosis (CF) patients [87].

A majority of its virulence determinants are controlled by a two-component signal transduction system, BvgAS (for *Bordetella* virulence gene) [82]. This locus comprises a sensor kinase protein, BvgS, and a DNA-binding response-regulator protein, BvgA. In response to environmental cues, BvgAS controls the expression of a spectrum of phenotypic phases transitioning between a virulent (Bvg+) and a non-virulent phase (Bvg-), a process referred to as phenotypic modulation [84]. In the virulent Bvg+ phase, the response regulator BvgA becomes highly phosphorylated, and the transcription of various Bvg-activated genes confers on the bacterium its virulent phenotype. Virulence factors expressed in the Bvg+ phase include adhesins such as filamentous hemagglutinin (FHA) and fimbriae as well as toxins/toxin delivery systems such as adenylate cyclase/hemolysin (CyaA) and type III secretion system [84].

The LPS structures are regulated by the BvgAS virulence control system. *B. bronchiseptica* expresses LPS molecules that are very similar antigenically and electrophoretically to *B. pertussis* bands A and B, as well as a form containing an O antigen-like homopolymer of 2,3-dideoxy-2,3-di-N-acetylgalactosaminuronic acid (2,3-di-NacGalA), primarily in the Bvg2 phase. The *wlb* gene cluster, composed of 12 genes, is required for biosynthesis and addition of the trisaccharide in band A and the O-antigen-like repeat in *B. bronchiseptica* [88]. In addition, the *wbm* and *pagP* genes, whose expression results in LPS modifications, are also regulated by BvgAS, suggesting they may be involved in the infectious process. The *pagP* gene encodes a palmitoyl transferase that is responsible for transferring a palmitate group to the lipid A region of LPS. The lipid A acylation state may influence outer membrane permeability and have been shown to affect complement activation and opsonization, as well as TLR-4 recognition in vitro. The LPS structure of the wild-type RB50 strain of *B. bronchiseptica* is primarily hexa-acylated in the Bvg-phase [89]. Finally, *B. bronchiseptica* demonstrated *pagP*-dependent resistance to immune serum complement killing at low serum concentrations. These data suggest that increased lipid A acylation in *B. bronchiseptica* confers

resistance to antibody-mediated complement lysis during infection [89].

Studies with *B. bronchiseptica* have been shown that these bacteria are capable of living as biofilms on a number of abiotic surfaces [90]. Filamentous Hemagglutinin (FHA) is required for *B. bronchiseptica* biofilm formation and proposed that the expression of CyaA suppresses biofilm via its association with FHA. The biofilm phenotype being predominantly expressed only when *B. bronchiseptica* is grown in the Bvgi phase but not in the Bvg+ or Bvg-phase [82]. Flagella enhance the initial cell-surface interactions, thereby providing mechanistic information on the initial stages of biofilm development for *B. bronchiseptica*. Biofilm formation by *B. bronchiseptica* involves the production of both Bvg-activated and Bvg-repressed factors followed by the repression of factors that inhibit formation of mature biofilms [84]. Sisti et al. [91] shows the presence of c-di-GMP regulatory signalling in *B. bronchiseptica*. The extracellular components of *B. bronchiseptica* biofilm matrix revealed that the major sugar component in the matrix was xylose, and linkage analysis indicated a majority of it to be in a 4-linked polymeric form. The production of xylose was independent of Bvg regulation but instead was dependent on bacterial growth phase. In addition, N-acetylglucosamine (or Bps polysaccharide) in the matrix was found to be important for the initial development of the biofilm. These results suggest that *B. bronchiseptica* biofilm formation is growth phase dependent in addition to being regulated by the Bvg virulence system [82]. Bordetella Bps polysaccharide encoded by the bpsABCD locus is critical for the stability and maintenance of three-dimensional structures of biofilms (Table 1). Bps is essential for the formation of efficient nasal biofilms and is required for the colonization of the nose. Likewise, the Bps polysaccharide has an essential role in the biofilm lifestyle for Bordetella in mammalian respiratory tracts and in the persistence of the nares [92-93]. BpsB contains a polysaccharide N-deacetylase domain and is homologous to IcaB and PgaB, which are involved in the deacetylation of PIA/PNAG and PGA, respectively. IcaB has crucial roles in biofilm formation, immune evasion, and virulence. BpsC is homologous to the glycosyltransferase 2 family of proteins and is predicted to encode a processive glycosyltransferase. IcaC of *S. epidermidis* and PgaC of *E. coli* are required for the complete synthesis of the longer oligomeric chains of PIA. Finally, IcaD and PgaD are required for optimal production of the PIA and PGA polysaccharides, respectively [93]. Like Bps polysaccharide, DNA is a significant component of *B. bronchiseptica* biofilm matrix. Addition of DNaseI at the initiation of biofilm growth inhibited biofilm formation. Treatment of pre-established mature biofilms formed under both static and flow conditions with DNaseI led to a disruption of the biofilm biomass. These results suggest that eDNA is a crucial structural matrix component of both in vitro and in vivo formed *B. bronchiseptica* biofilms [94].

### *Haemophilus parasuis*

*Haemophilus parasuis* is a commensal bacterium that inhabits the airways of pigs, also belongs to the family Pasteurellaceae and like another members of the same family, its growth requires V factor (nicotinamide adenine dinucleotide, NAD) but not X factor (hemin) [95-97]. *H. parasuis* is a Gram negative bacterium that causes Glässer's disease or polyserositis, can also cause septicemia, arthritis, meningitis and pneumonia [98], and under certain circumstances, could have the behavior of an opportunistic pathogen [96]. Fifteen serotypes have been described, but above 25% of isolates are usually not established [97]. Nevertheless, there is no clear correlation between virulence and

serotypes. Little is known of the specific virulence factors of *H. parasuis* and recent studies are just beginning to reveal components involved in the mechanisms that cause the disease. Several genes are involved in the virulence, including a variety of carriers, metabolic and biosynthetic enzymes, surface membrane proteins and some homologous genes apparently expressed by other members of the family Pasteurellaceae [97].

Bacteria	Genes	References
<i>A. pleuropneumoniae</i>	<i>hns, potD2, ptsI, tig, rpmF, pgaABCD, rseA, arcA, aasP, luxS, ftpA, clpP, dus, cpxA, cpxR, rseA, tolC, fdxG, fdnI, fdxH, dmsB, fruK, glpK, crp, wecABD, wzz.</i>	[10,22,23,34,35,40,41,42,120, 121,122,123]
<i>S. suis</i>	<i>luxS, pfs, gdh, cps2, sly, mrrp, ef, fbps, gapdh,</i>	[56,62,63]
<i>B. bronchiseptica</i>	<i>bpsABCD, bpsR, bvgAS, flaA, cheW, flIA, flgB, cyaA, fhaB.</i>	[82,84,87,90,92,93,124]
<i>P. multocida</i>	Unknown	
<i>H. parasuis</i>	<i>galE, galU, pilQ, fhaB, fhaC (tpsB), ompW, plp4.</i>	[97,102]
<i>M. hyopneumoniae</i>	Unknown	

**Table 1:** Reported genes involved in the biofilms formation in bacteria belonging to the Porcine respiratory disease complex.

*H. parasuis* contains a short LPS or lipooligosaccharide (LOS) reported to play a partial role in interaction with host cells. The presence of capsule has been phenotypically demonstrated in certain *H. parasuis* strains and its role in virulence has been suggested [99]. The structure of capsular polysaccharide (CPS) and LOS from virulent strains ER-6P and Nagasaki was studied by NMR spectroscopy, mass spectrometry and chemical methods. CPS from both strains had the same main chain with disaccharide repeating unit, substituted with  $\alpha$ -Neu5R-(2-3)- $\alpha$ -GalNAc-(1-P-(strain ER-6P) or  $\alpha$ -Neu5R-(2-3)- $\alpha$ -Gal-(1-P-strain Nagasaki) side chains, where R is the N-acetyl or N-glycolyl group. Glycolyl-neuraminic acid is widely found in animal glycoproteins, but it apparently has not been found in bacteria before, and might be important for the biology of this microorganism. Both strains produced the same LPS of a rough type with a single phosphorylated Kdo linking core and lipid A parts. LOS structure was similar to some strains of *H. influenzae* and contained a globotetraose terminal sequence [99-100].

*H. parasuis* can form biofilms, however, the relationship between biofilm formation and pathogenesis or persistent *H. parasuis* infection is unknown [101]. Jin et al. [101] tested the ability to form biofilms for a total of 80 field isolates and 15 reference strains of *H. parasuis*. A total 43% of field isolates, including strains representing 13 serotypes (except serotypes 3 and 8) and non-typable strains, exhibited the ability to form biofilms at different levels via polystyrene microtiter plate assays. Among the reference strains representing fifteen serotypes, only serotypes 2, 9, 12, 13 and 15 could not form biofilms on the polystyrene surface. Generally, non-virulent serotypes showed a higher degree of biofilm formation than virulent serotypes. Bello-Ortí et al. [102] found that non-virulent strains of *H. parasuis* have the ability to form robust biofilms in contrast to virulent, systemic strains. Therefore, biofilm formation might allow the non-virulent strains to

colonize and persist in the upper respiratory tract of pigs. Zou et al. [97] found that the lack of expression of GalU protein by the galU mutant increased its tendency to autoagglutinate, indicating that galU gene plays a significant role in autoagglutination and biofilm formation, while galE may affect the biofilm production indirectly (Table 1). Genes galU and galE are known as important virulence factors in a number of Gram-negative pathogens. GalU is a UDP-glucose pyrophosphorylase responsible for the synthesis of UDP-glucose from glucose 1-phosphate and UTP, whereas UDP-glucose 40-epimerase (GalE) converts UDP-glucose to UDP-galactose. As substrates, UDP-glucose and UDP-galactose could participate in the biosynthesis of capsular polysaccharide (CPS) which could contribute to complement resistance or extracellular polysaccharide (EPS) which was also proposed to be involved in the formation of biofilm [97]. Furthermore, Zhang et al. [103] mentions that biofilm positive strains had positive correlation with resistance to  $\beta$ -lactams antibiotics. Thus, biofilm formation may play important roles during *H. parasuis* infections.

### *Mycoplasma hyopneumoniae*

*Mycoplasma hyopneumoniae* is the pathogen with a major contribution to the PRDC [104-105]. The mycoplasmas are prokaryotic pathogens of humans and other animals, distinguished by the lack of a cell wall, diminutive size, and a limited genome. They are parasitic obligate scavengers of numerous host factors required for growth. Colonization occurs predominantly at the mucosal surfaces of the genital and respiratory tracts and is a prerequisite for infection [106]. Mycoplasmas are unable to synthesize peptidoglycans or their precursors, and therefore, non-cell wall is present in this microorganism. The mycoplasma cell membrane is formed from proteins, phospholipids and cholesterol; cholesterol is an essential nutrient to bacterial growth and is responsible for membrane rigidity and stability [107]. *M. hyopneumoniae* is the primary cause of enzootic pneumonia in pigs, a disease that causes significant worldwide losses in the swine industry due to reduced performance and increased the amount and the time of medication [108-110]. Like other members of the class *Mollicutes*, *M. hyopneumoniae* evolved by a process of reductive evolution from the low G + C Firmicutes. The genome of *M. hyopneumoniae* is small (893-920 kb) and lacks the genetic repertoire to construct a cell wall or perform oxidative phosphorylation via the TCA cycle, and is reliant on swine for the availability of macromolecular building blocks to assemble proteins, nucleic acids and lipid membranes for growth [111-112]. As such, *M. hyopneumoniae* is armed with enzymes that degrade nucleic acids and proteins and membrane associated transporters that facilitate uptake of the products of these degradative processes [111-114].

In pigs, *M. hyopneumoniae* is found to be attached to the cilia of the tracheal epithelial cells, causing a reduction in ciliary action, and predisposing the swine to infection by other pathogens, such as *Pasteurella multocida* and porcine reproductive and respiratory syndrome virus (PRRSV) [107,112]. *M. hyopneumoniae* adhesion to the swine tracheal epithelial cells is essential to disease establishment, and the characterization of adhesion-mediating molecules has been the focus of most studies on the bacterial mechanisms of virulence and pathogenesis. Bacterial adhesive capability is related to several proteins, such as the well-described P97 adhesin and adhesin-like proteins, such as P216, P159, P102, P146 and P116 [107].

In *M. hyopneumoniae*, biofilm formation has not been well studied. Some mycoplasmas have been shown to form biofilms on glass and

plastic surfaces [115-120], and it has been determined whether they form biofilms on the tracheal epithelium [115]. Biofilms formed in vitro protect mycoplasmas from the lytic effects of not only complement but also the small antimicrobial peptide gramicidin [115].

### Conclusion and Future Challenges

The Porcine respiratory disease complex (PRDC) is a serious problem that brings to the swine industry worldwide. It is now known that in order to development the disease, there must be several interactions between different pathogenic and commensal bacteria present in the respiratory tract of pigs. The majority of the pathogenic bacteria causing respiratory diseases possess the ability to form biofilms that exacerbates the problem and hinder their study. Moreover, given that the way in which bacteria exist in nature is through multi-species biofilm, the formation of these communities could explain how they are interacting and causing disease. However, more studies are needed to perceive the importance of biofilm formation, whether single or multi-species, by pathogenic bacteria that are part of the porcine respiratory disease complex during the development, persistence and/or transmission of the disease.

### Acknowledgment

The authors would like to thank to editors and the reviewers for their helpful comments. We are gratefully acknowledging the grant from CONACYT, Mexico (No. 258863).

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