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1 ***Actinobacillus pleuropneumoniae* biofilms: Role in pathogenicity and potential impact**
2 **for vaccination development.**

3

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20

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22

23 **Running title:** *A. pleuropneumoniae* biofilm, reviewing a decade current research

24

25 **ABSTRACT.**

26

27 *Actinobacillus pleuropneumoniae* is a Gram-negative bacterium that belongs to the family
28 *Pasteurellaceae*. It is the causative agent of porcine pleuropneumonia, a highly contagious
29 respiratory disease that is responsible for major economic losses in the global pork
30 industry. The disease may present itself as a chronic or an acute infection characterized by
31 severe pathology including hemorrhage, fibrinous and necrotic lung lesions, and, in the
32 worst cases, rapid death. *A. pleuropneumoniae* is transmitted via aerosol route, direct
33 contact with infected pigs, and by the farm environment. Many virulence factors associated
34 with this bacterium are well characterized. However, much less is known about the role of
35 biofilm, a sessile mode of growth, that may have a critical impact on *A. pleuropneumoniae*
36 pathogenicity. Here we review the current knowledge on *A. pleuropneumoniae* biofilm,
37 factors associated with biofilm formation and dispersion, and the impact of biofilm on the
38 pathogenesis *A. pleuropneumoniae*. We also provide an overview of current vaccination
39 strategies against *A. pleuropneumoniae* and consider the possible role of biofilms vaccines
40 for controlling the disease.

41

42 **Keywords:** *Actinobacillus pleuropneumoniae*, pleuropneumonia, biofilm, antimicrobial
43 therapy and vaccine.

44

45 **INTRODUCTION.**

46 Respiratory diseases in pigs are common global problems for modern pork producers and
47 are frequently associated with the porcine respiratory disease complex (PRDC) (Opriessnig
48 *et al.*, 2011). PRDC is a multifactorial syndrome caused by the interaction of bacteria,
49 viruses and stresses associated with management practices, environmental conditions and
50 genetic predispositions (Opriessnig *et al.*, 2011; Schmidt *et al.*, 2016). Within PRDC,
51 *Actinobacillus pleuropneumoniae* is one of the most commonly identified bacterial
52 pathogen that causes respiratory infections in pigs (Opriessnig *et al.*, 2011; Dayao *et al.*,
53 2016). *A. pleuropneumoniae* is a Gram-negative rod-shaped bacterium belonging to the
54 *Pasteurellaceae* family (Chiers *et al.*, 2010; Gómez-Laguna *et al.*, 2014) and is the
55 etiologic agent of porcine pleuropneumonia (Frey, 1995; Buettner *et al.*, 2011). This
56 respiratory infection is the major cause of morbidity and mortality, and is responsible for
57 substantial economic losses worldwide (Chiers *et al.*, 2010; Bossé *et al.*, 2014). The disease
58 is characterized by an exudative, fibrinous, hemorrhagic, and necrotizing pneumonia and
59 associated pleuritis (Chen *et al.*, 2011). Porcine pleuropneumonia is transmitted via
60 aerosols or direct contact with infected animals including asymptomatic carriers (i.e.
61 animals with a sub-clinical infection). Clinical infections may result into a chronic and
62 persistent form, an acute form with the pathology described above or a peracute form
63 associated with severe pathology and rapid death (Gottschalk, 2015).

64

65 In 1964, Shope was the first to described a disease affecting pigs in Argentina as porcine
66 contagious pleuropneumonia (PCP) and he named the causative agent *Haemophilus*
67 *pleuropneumoniae* (Shope, 1964; Shope *et al.*, 1964). In 1983, Pohl and coworkers
68 transferred the causative agents of PCP or similar infections to the genus *Actinobacillus*

69 based on the higher DNA-sequence homology to the genus *Actinobacillus* (*Actinobacillus*
70 *lignieresii*, 72-75%) (Pohl *et al.*, 1983; Nicolet, 1988). In 1986, O'Reilly and Niven
71 identified the pyridine nucleotides, the precursors that were needed to satisfied the V-factor
72 requirement, and the nicotinamide adenine dinucleotide (NAD) was identified as a
73 supplement that supported *in vitro* growth (O'Reilly and Niven, 1986). *A.*
74 *pleuropneumoniae* is now divided into two biovars based of their NAD requirement for
75 growth: biotype 1 is NAD-dependent, and biotype 2 is NAD-independent (Turni *et al.*,
76 2014; Gottschalk, 2015; Ito, 2015).

77

78 *A. pleuropneumoniae* is further divided into 16 serotypes (or serovars) based on the
79 antigenic properties of the capsular polysaccharides and the O-chain of the
80 lipopolysaccharides (LPS) (Sárközi *et al.*, 2015; Kim *et al.*, 2016; Bossé *et al.*, 2017).
81 Serotypes 1 to 12 and 15 typically belong to biotype 1 whereas serotypes 13 and 14 are
82 typically biotype 2 (Serrano *et al.*, 2008; Gottschalk, 2015). The serotype 16 is not yet
83 officially grouped in any biotype. However, this is not an absolute rule since variants of
84 serotype 2, 4, 7, 9 and 11 have been identified as NAD-independent (biotype 2) (Perry *et*
85 *al.*, 2012). Furthermore, there has been an increase in the prevalence of isolates that are
86 untypable (UT) (Morioka *et al.*, 2016). Despite the global distribution of *A.*
87 *pleuropneumoniae*, the prevalence of different serotypes varies between countries (Morioka
88 *et al.*, 2015). Specifically, serotypes 1, 5 and 7 are predominantly found in North America,
89 serotype 2 is the most common type in Europe and serotypes 1, 3, 4, 5 and 7 are typically
90 isolated in China (Buettner *et al.*, 2011; Gottschalk and Lacouture; 2015; Morioka *et al.*,
91 2016). For South America, serotypes 4, 6 and 7 are reported as the dominant serotypes in
92 the region (Gómez-Laguna *et al.*, 2014).

93

94 Infection and persistence of *A. pleuropneumoniae* is mediated by multiple virulence factors.

95 Well characterized virulence factors of *A. pleuropneumoniae* include: the Apx toxins

96 (ApxI, ApxII, ApxIII and ApxIV), lipopolysaccharide (LPS), capsule polysaccharide

97 (CPS), proteases (e.g. LonA), urease, iron acquisition systems (e.g. transferrin-binding

98 protein [Tbp], haemoglobin-binding protein [HbpA]), enzymes involved in anaerobic

99 respiration (e.g. two-component signal transduction system [TCSTS] *arcB* and *arcA*), type

100 IV pilus, Flp pilus, autotransporters (e.g. Trimeric Autotransporter Adhesin [TAA]), and

101 more recently biofilms (Chiers *et al.*, 2010; Tremblay *et al.*, 2017). The role of biofilm in

102 persistence, survival and pathogenesis of *A. pleuropneumoniae* is relatively new and the

103 importance of biofilm is not fully understood. It has now been demonstrated that biofilms

104 can develop during an infection and a recent report describes the growth of *A.*

105 *pleuropneumoniae* as aggregates in lungs obtained from natural pig infections (Tremblay *et*

106 *al.*, 2017). In this review, our aim is to highlight and summarize the current knowledge on

107 *A. pleuropneumoniae* biofilm formation and suggest its possible role in pathogenesis.

108 Furthermore, we will also talk about vaccination and new strategies based on recent biofilm

109 findings.

110

111 **BIOFILMS AND ANIMAL HEALTH**

112 It is well accepted by the scientific community that most bacteria can produce biofilms in

113 their natural ecosystem as well as in artificial *in vitro* ecosystems (Briandet *et al.* 2012).

114 Biofilms are defined as structured communities enclosed in a self-produced matrix that is

115 attached to a surface (biotic or abiotic); however, recent evidences have demonstrated that

116 *in vivo* biofilms and bacterial aggregates are not necessarily attached to the surface and are

117 often embedded in host material (Bjarnsholt *et al.*, 2013; Kragh *et al.*, 2016). Our group has
118 extensively reviewed biofilm formation by animal and zoonotic pathogens, and we will not
119 cover general information about biofilm in this review (see Jacques *et al.*, 2010). Several
120 members of the *Pasteurellaceae* family, which include many important animal pathogens,
121 are able to form biofilms and several studies in the past decade have demonstrated the
122 ability of its members such as *Haemophilus influenzae*, *Pasteurella multocida*,
123 *Aggregatibacter actinomycetemcomitans*, *Mannheimia haemolytica* *Histophilus somni*, and
124 *Haemophilus parasuis* to produce a biofilm (Olson *et al.*, 2002; Kaplan and
125 Velliyagounder, 2004; Jin *et al.*, 2006; Sandal *et al.*, 2007; Wu *et al.*, 2013; Bello-Ortí *et al.*
126 2014; Boukahil and Czuprynski, 2015). For several members of the *Pasteurellaceae*
127 family, it has been suggested that biofilm formation is crucial for the persistence of these
128 obligate inhabitants (Jin *et al.*, 2006; Sandal *et al.*, 2007; Bello-Ortí *et al.* 2014; Boukahil
129 and Czuprynski, 2015). For example, non-virulent isolates of *H. parasuis* formed stronger
130 and more robust biofilms than virulent isolates, suggesting that the biofilm phase favors
131 colonization and the planktonic phase allows for the dissemination within the host (Jin *et*
132 *al.*, 2006; Bello-Ortí *et al.* 2014).

133

134 ***ACTINOBACILLUS PLEUROPNEUMONIAE* BIOFILMS.**

135 The ability of *A. pleuropneumoniae* to form biofilms *in vitro* was first studied using a 96-
136 well microtiter plate model (Coffey and Anderson, 2014) (Fig. 1). Kaplan *et al.* (2004)
137 were the first to report that serotype 5b and 11 are producers of biofilms *in vitro* (Kaplan *et*
138 *al.*, 2004). *A. pleuropneumoniae* biofilms have also been assessed in glass tubes and under
139 agitation. Biofilms form a ring at the air-liquid interface in this closed system model that
140 incorporates shear force (Kaplan and Mulks, 2005). The ability to form biofilms appears to

141 be common among *A. pleuropneumoniae* isolates since studies demonstrate that isolates
142 from every serotype are able to produce biofilms in microtiter plates and/or glass tubes
143 (Kaplan and Mulks, 2005; Labrie *et al.*, 2010). In the case of the newly reported serotype
144 16, the ability to form biofilms has yet to be studied.

145

146 **Biofilm formation in microtiter plates**

147 In general, the production of biofilm by *A. pleuropneumoniae* in microtiter plates is
148 described as a rapid process with the detection of biomass as early as 3 hours for serotype 1
149 type strain S4074 and 6 hours for serotype 5b type strain L20 and clinical isolates (Labrie *et*
150 *al.*, 2010; Tremblay *et al.*, 2013a). Interestingly, the biofilm cycle of serotype 1 type strain
151 S4074 is completed within 8 hours. Specifically, biomass becomes detectable after 3 hours
152 and reaches its peak at 5 hours, which correspond to the mature form of the biofilm
153 (Tremblay *et al.*, 2013a). Dispersion of the biofilm begins between 5-6 hours and the
154 biomass is no longer detectable after 8 hours (Tremblay *et al.*, 2013) (Fig. 2). The biofilm
155 persistence can be extended if the spent medium is removed and fresh culture medium is
156 added to a 4-hour old biofilm (i.e., a maturing biofilm). The change of growth medium can
157 cause an increase in biomass and delay biofilm dispersion by 1 hour. This suggests that
158 depletion of the culture medium or the accumulation of one or several signals molecules
159 can activate biofilm dispersal (Tremblay *et al.*, 2013a). These observations provide a good
160 example for the limitations of closed biofilm systems.

161

162 **Biofilm formation in models with biologically relevant parameters**

163 To overcome the limitations of the microtiter plates, dynamic models are often used and
164 these systems are thought to be more representative of the conditions encountered by

165 bacteria in their natural environment (Coenye and Nelis, 2010). For example, the “drip
166 flow” reactor is a continuous flow system that continuously irrigates biofilms with fresh
167 medium and allows biofilms to form on a coupon of choice (e.g., glass, stainless steel,
168 PVC) that is deposited inside a sealed chamber (Goeres *et al.*, 2009). In this model,
169 biofilms are formed at the air-liquid interface in the presence of low shear forces that mimic
170 the environment found in the lung and oral cavities (Goeres *et al.*, 2009; Schwartz *et al.*,
171 2010). Unlike the results obtained with the microtiter plates, *A. pleuropneumoniae* S4074 is
172 able to establish and maintain a biofilm for up to 48 hours (Tremblay *et al.*, 2013a). To
173 grow biofilms under these conditions, the growth medium (Brain Heart Infusion [BHI] with
174 NAD) is diluted to 50% and the flow can be set from 50 mL to 200 mL per hour per
175 chamber (Tremblay *et al.*, 2013a; Hathroubi *et al.*, 2016a). After 24 hours, *A.*
176 *pleuropneumoniae* forms an important biomass on a glass slide that is visible with the
177 naked eye (Fig. 2). This biofilm contains 10^9 - 10^{10} colonies forming units (CFU) per
178 chamber with an average dry weight of 10 mg (Tremblay *et al.*, 2013a; Hathroubi *et al.*,
179 2016a). Although the “drip-flow” reactor provides a dynamic environment that resembles
180 the lung cavity, the surface used was a microscopic slide, a substrate that *A.*
181 *pleuropneumoniae* would never encounter *in vivo*.

182

183 In order to see if a biotic surface could be used by *A. pleuropneumoniae*, Tremblay and
184 colleagues (2013b) investigated biofilm formation on a SJPL cell line by a non-hemolytic,
185 non-cytotoxic mutant of strain S4074, called MBHPP147. This mutant has deletions in both
186 the *apxIC* and *apxIIC* genes which prevents the acylation (and hence activation) of the
187 protoxins ApxIA and ApxIIA. As observed with strain S4074, MBHPP147 is able to form a
188 biofilm on polystyrene in microtiter plates. Furthermore, a robust biofilm is observed after

189 24 and 48 hours of contact with the SJPL cells (Tremblay *et al.*, 2013b). These studies are
190 consistent with the notion that *A. pleuropneumoniae* can form biofilms on biotic surfaces
191 during host colonization.

192

193 Recently, *A. pleuropneumoniae* biofilm formation was studied using an embedded model
194 created with 0.5% agarose. This porous substrate is thought to simulate the conditions
195 found in the lungs during a natural infection (Tremblay *et al.*, 2017). Biofilm formation in
196 this model was tested with two clinical isolates of *A. pleuropneumoniae* (one serotype 5,
197 and one serotype 7) that were previously shown to form biofilms in a 96-wells plates and
198 aggregates in the lungs of naturally infected pigs. In the embedded models, both isolates
199 developed aggregates ranging from 20-30 microns within the porous matrix formed by the
200 agarose. The size of the aggregates (30-45 microns) and their structure were similar to
201 those observed in the lungs of pigs naturally infected by either isolates (Tremblay *et al.*,
202 2017). The use of this new model that mimic the pulmonary alveolus environment during
203 an infection has a promising future and could provide a new platform to test the sensitivity
204 of *A. pleuropneumoniae* biofilm to several antibiotics.

205

206 **Factors involved in the formation and dispersion of *A. pleuropneumoniae* biofilms.**

207 Several strategies have been used to identify genetic factors associated with biofilm
208 formation. For example, a library of mini-*Tn10* transposon mutants in *A. pleuropneumoniae*
209 S4074 was screened in a 96-wells microplate assay and 16 genes affecting biofilm
210 formation were identified (Grasteau *et al.*, 2011). Otherwise, microarrays have also been
211 used to gain insight into the transcriptome of maturing or dispersing biofilms formed under
212 static or dynamic conditions (Tremblay *et al.*, 2013a). These approaches provide different

213 insight into the biofilm formation process. The results are summarized in the sections
214 below.

215

216 *1) Composition of the biofilm matrix*

217 Poly-N-acetyl-glucosamine (PGA) is the major component and an essential element of the
218 *A. pleuropneumoniae* biofilm matrix regardless of the growth conditions and surfaces used
219 (Fig. 1) (Izano *et al.*, 2007; Bossé *et al.*, 2010; Labrie *et al.*, 2010; Tremblay *et al.*, 2013a;
220 Tremblay *et al.*, 2013b; Hathroubi *et al.*, 2015; Hathroubi *et al.*, 2016a). The proteins
221 responsible for PGA synthesis are encoded by the *pgaABCD* operon (Kaplan *et al.*, 2004;
222 Izano *et al.*, 2007). This operon is highly prevalent among *A. pleuropneumoniae* serotypes
223 and appears to have been preserved in every studied serotype (Izano *et al.*, 2007). In studies
224 by Izano *et al.* (2007), PCR analysis of the gene coding for the biosynthesis of PGA, *pgaC*,
225 demonstrated that it was present in every reference strains investigated (serotypes 1 to 12)
226 and in 76 of the 77 field isolates tested. The synthesis of PGA is essential for the biofilm
227 formation process and deleting one gene in the operon, *pgaC*, completely abolishes the
228 production of PGA and, thus, prevents biofilm formation (Izano *et al.*, 2007; Bossé *et al.*,
229 2010; Hathroubi *et al.*, 2016a).

230

231 *A. pleuropneumoniae* can also control the degradation of the self-produced PGA polymers
232 using a glycoside hydrolase, dispersin B (Izano *et al.*, 2007). This enzyme can detach
233 biofilms formed on difference surfaces, under different conditions and in different model
234 systems (Izano *et al.*, 2007; Labrie *et al.*, 2010; Tremblay *et al.*, 2013a; Tremblay *et al.*,
235 2013b; Hathroubi *et al.*, 2015; Hathroubi *et al.*, 2016a).

236

237 Other components, such as extracellular DNA (eDNA) and proteins, may also provide
238 building blocks for the matrix. Proteins and eDNA have been stained and observed by
239 confocal microscopy in the biofilm formed by *A. pleuropneumoniae* (Wu *et al.*, 2013;
240 Hathroubi *et al.*, 2016a). Under most conditions tested, these components do not appear to
241 be required for the integrity of the biofilm matrix since proteinase K or DNase does not
242 disperse pre-established biofilms (Grasteau *et al.*, 2011; Hathroubi *et al.*, 2016a). However,
243 eDNA might contribute to the integrity of the biofilm under certain conditions such as in
244 the presence of sub-minimal inhibitory concentration of penicillin B or in multi-species
245 biofilms (Hathroubi *et al.*, 2016b; Loera-Muro *et al.*, 2016).

246

247 2) *Growth medium and other conditions inducing biofilm formation*

248 The composition of the culture medium affects *A. pleuropneumoniae* biofilm formation.
249 For example, Li and collaborators in 2008 demonstrated that the reference strain S4074
250 only produced a biofilm in TSB (Tryptic Soy Broth) medium in the absence of serum
251 although the mechanism of this inhibition remains to be determined (Li *et al.*, 2008). Later,
252 Labrie *et al.* (2010) demonstrated that BHI medium favored biofilm formation of *A.*
253 *pleuropneumoniae* S4074 when compared to TSB. Further, 54% serotypes 1, 5, 7 and 15
254 strains produced biofilms in BHI reinforcing the idea that BHI would be better for the study of
255 biofilms *in vitro*. However, the source of the BHI medium also has an impact on biofilm
256 formation. For example, BHI from Oxoid enhanced the production of a robust biofilms
257 whereas BHI from Difco does not promote biofilm formation (Labrie *et al.*, 2010).

258

259 When the composition of both media was analyzed the concentration of zinc was identified
260 as a key difference with higher levels in BHI-Difco than BHI-Oxoid (Labrie *et al.*, 2010).

261 In support of these observations, researchers have shown that the addition of zinc to BHI-
262 Oxoid inhibits biofilm formation in a dose-dependent manner without affecting bacterial
263 growth (Labrie *et al.*, 2010; Wu *et al.*, 2013). Thus, zinc appears to specifically inhibit the
264 production of biofilm by *A. pleuropneumoniae*. A similar inhibitory effect has also been
265 observed for other porcine pathogens such as *Escherichia coli*, *Salmonella* Typhimurium,
266 *Staphylococcus aureus* and *Streptococcus suis* (Wu *et al.*, 2013). In *A. pleuropneumoniae*,
267 the presence of zinc might interfere with the expression or biosynthesis of the major
268 polymer found in the biofilm matrix, PGA, since the expression of the *pgaABCD* operon is
269 up-regulated in BHI-Oxoid (Labrie *et al.*, 2010) and zinc inhibits the activity of PgaB in *E.*
270 *coli* (Little *et al.*, 2012).

271

272 In addition to growth medium, anaerobic conditions also appear to induce biofilm
273 formation (Li *et al.*, 2014). Indeed, exposure to anaerobic conditions result in an increase in
274 biofilm formation that is associated with the upregulation of the fine tangled pili major
275 subunit gene (*ftpA*) and *pgaA* (Li *et al.*, 2014).

276

277 Other growth conditions appear to induce the expression of biofilm-associated genes. For
278 example, direct contact of *A. pleuropneumoniae* with epithelial cells results in an increased
279 expression of the *pgaABCD* operons (Auger *et al.*, 2009). Further, epinephrine and
280 norepinephrine affect expression of *pgaB* and Apa1, an auto-transporter adhesin (Li *et al.*,
281 2012). However, only norepinephrine induces enhance attachment to SJPL cells and neither
282 catecholamine has an impact on biofilm formation (Li *et al.*, 2012). It is conceivable that
283 different factors play a role during the attachment of *A. pleuropneumoniae* to a biotic
284 surface (e.g. SJPL cells) and an abiotic surface (e.g. polystyrene or glass). In support of this

285 statement, *A. pleuropneumoniae* does not form a biofilm on polystyrene when grown in a
286 cell culture medium (Dulbecco's modified Eagle's medium [DMEM]) and was only able to
287 form biofilm in the presence of SJPL in DMEM (Tremblay *et al.*, 2013b).

288

289 3) *The biofilm transcriptome*

290 The transcriptomes of maturing (static 4h), mature (drip-flow) and dispersing (static 6h)
291 biofilms have been analysed and compared to each other and to their planktonic
292 counterparts. In a study by Tremblay *et al.* (2013a), only 47 and 117 genes were
293 differentially up or down-regulated in static biofilms when compared to planktonic cells.
294 For example, biofilm bacteria down-regulated the expression of their energy metabolism
295 gene when compared to planktonic bacteria (Tremblay *et al.*, 2013a). Indeed, the majority
296 of energy metabolism genes such as the genes encoding the key enzymes of the anaerobic
297 metabolism appeared to be repressed in the biofilm (Tremblay *et al.*, 2013a).

298

299 Major differences have also been observed when the maturing biofilm is compared to a
300 dispersing biofilm. Specifically, 456 genes were differently regulated when a maturing
301 biofilm and a dispersing biofilm were compared (Tremblay *et al.*, 2013a). Furthermore, the
302 maturing biofilm appears to be under an iron-rich condition because several major genes in
303 iron acquisition, including *tbpB*, are repressed in the maturing biofilm (Tremblay *et al.*,
304 2013a).

305

306 Interestingly, a comparative analysis reveals that the transcriptome of drip-flow biofilms
307 share few differentially expressed genes with static biofilms. On the other hand, the drip-

308 flow transcriptome has several genes that has also been identified in natural or experimental
309 infections of pigs (Tremblay *et al.*, 2013a). Transcriptome and cross-referencing analyses
310 indicate that biofilms formed in a drip-flow models require a different sub-set of genes than
311 biofilms grown in microtiter plates (Tremblay *et al.*, 2013a). Based on these results, it has
312 been suggested that the drip-flow apparatus might provide a more relevant model to study
313 biofilm formation by *A. pleuropneumoniae* (Tremblay *et al.*, 2013a).

314

315 4) Regulators of biofilm formation

316 While environmental conditions and growth medium composition that are optimal for
317 biofilm formation and induce production of PGA have been identified, other studies have
318 identified potential regulators and molecular mechanism associated with biofilm formation.
319 In addition to growth conditions, the expression of the *pgaABCD* genes and, consequently,
320 PGA production are regulated by the histone type H-NS (histone-like protein), which acts
321 as a repressor of expression and hence a suppressor of biofilm production (Dalai *et al.*,
322 2009; Bossé *et al.*, 2010; Grasteau *et al.*, 2011). *Tn* insertions in the *hns* gene of *A.*
323 *pleuropneumoniae* serotype 1 results in a sharp increase in biofilm formation and a loss of
324 virulence (Dalai *et al.*, 2009). Indeed, H-NS specifically represses the expression of the
325 operon by binding sequences upstream the *pgaA* gene (Bossé *et al.*, 2010). The importance
326 of *hns* in repressing biofilm formation has also been independently confirmed in a screen
327 that identified three *Tn*-mutants with an increase biofilm production (Grasteau *et al.*, 2011).
328 Unlike H-NS, the alternative sigma factor RpoE (or σ^E) is a transcriptional activator of the
329 *pgaABCD* operon (Bossé *et al.*, 2010).

330

331 Deletion of the gene encoding the negative regulator of the σ^E factor, RseA (regulator of

332 sigma-E), results in increased expression of the *pgaABDC* operon and higher biofilm
333 production (Bossé, *et al.*, 2010). Additionally, expression of the *pgaABCD* operon is also
334 under the control of the RNA chaperone Hfq (Subashchandrabosea *et al.*, 2013). Disruption
335 of *hfq* decreases PGA production, biofilm formation, virulence and fitness
336 (Subashchandrabosea *et al.*, 2013).

337

338 Deletion of the quorum-sensing (QS) gene also results in an increase in *pgaABC*
339 expression, a strong increase in biofilm production and a decrease in virulence (Li *et al.*,
340 2008; Li *et al.*, 2011). S-ribosylhomocysteine lyase (LuxS), is a protein involved in the
341 production of the auto-inducer type 2 (AI-2) and in the QS mechanism. QS is involved in
342 the biofilm formation in many bacteria (Prouty *et al.*, 2002; Merritt *et al.*, 2003; Ethapa *et*
343 *al.*, 2013). The increase biofilm production in *A. pleuropneumoniae* appears, however, to be
344 independent of the production of AI-2 since the addition of AI-2 to the culture medium
345 results in an increase biofilm production in the absence of LuxS (Li *et al.*, 2011). Enhanced
346 biofilm formation has also been observed in a mutant lacking the *relA*, a gene encoding the
347 stringent response regulatory protein responsible for synthesis of (p)ppGpp (Li *et al.*,
348 2015). This deletion results in the up-regulation of a fimbrial biogenesis protein and tight
349 adherence protein, proteins thought be important for adhesion to surfaces (Li *et al.*, 2015).

350

351 In addition to quorum sensing and the stringent response, two-component regulatory
352 system also controls biofilm formation in *A. pleuropneumoniae*. For example, deletion of
353 the ArcA, which belongs to the ArcAB two-component system, causes a defect in
354 autoaggregation and biofilm formation (Buettner *et al.*, 2008). Furthermore, the expression
355 of the *cpxA*, a gene encoding the histidine kinase of the CpxRA stress response system, is

356 induced in bacteria grown in biofilm when compared to their planktonic counterparts
357 (Tremblay, *et al.*, 2013a). In *E. coli*, this system is induced during the biofilm maturation
358 phase (Otto and Silhavy, 2002) and the CpxRA system can be activated by mechanical
359 pressure (Vogt and Raivio, 2012). It has been suggested that such pressure could be
360 encountered by bacteria during the initial attachment and biofilm formation and could
361 activate the CpxRA stress response. Interestingly, an O-antigen mutant, which lost its
362 ability to produce a biofilm, exhibits reduced expression of *cpxRA* (Hathroubi *et al.*,
363 2016a). Furthermore, enhanced biofilm production induced by sub-MIC of penicillin G is
364 associated with increased *cpxRA* expression (Hathroubi *et al.*, 2015). In both cases
365 described above, the expression of *pgaA* is also affected in the same direction suggesting a
366 link between the CpxRA response and *pgaABCD* expression. Overall, activation of the *A.*
367 *pleuropneumoniae* CpxRA system appears to occur during biofilm formation; however, the
368 link between the CpxRA system, *pgaABCD* expression and biofilm formation requires
369 further investigation before this could be said definitively.

370

371 5) Surface-associated proteins and polysaccharides

372 Proteins and polysaccharides located at the bacterium/surface interface are crucial for
373 facilitating attachment, microcolonies formation, and/or subsequent maturation of the
374 biofilm. Several proteins and polysaccharides have been identified and characterized as
375 important for biofilm formation. In addition to the biofilm matrix polysaccharides, other
376 surface polysaccharides have an impact on biofilm formation. For example, inactivation of
377 *galU* results in an increase biofilm production (Grasteau *et al.*, 2011). The *galU* gene
378 encodes an UTP- α -D-glucose-1-phosphate uridylyltransferase, an enzyme involved in the
379 biosynthesis of the lipopolysaccharide core oligosaccharide in *A. pleuropneumoniae*

380 (Ramjeet *et al.*, 2008). Further, the *wecABD* operon and the genes encoding proteins
381 involved in the biosynthesis of lipopolysaccharide O antigen are induced in a mature
382 biofilm (Tremblay *et al.*, 2013a).

383

384 Recently, it was demonstrated that the absence of the O antigen markedly reduces the
385 ability of *A. pleuropneumoniae* to form a mature biofilm. This decrease is associated with a
386 reduction in *pgaA* expression and, consequently, PGA production (Hathroubi *et al.*, 2016a).

387 Interestingly, LPS and O-antigen truncated LPS specifically bind PGA suggesting that
388 interactions between LPS and PGA may help bacterial cells attached to the biofilm matrix.

389 Taken together, these observations reinforce the idea that LPS may play a role in biofilm
390 formation of *A. pleuropneumoniae*. Several studies have shown the importance of O chains
391 in biofilm formation by other Gram negative such as *Stenotrophomonas maltophilia*
392 (Huang *et al.*, 2006), *Xanthomonas citri* ssp. *citri* (Li and Wang, 2011), *Xanthomonas*
393 *oryzae* pv. *oryzicola* (Wang *et al.*, 2013), and *Xylella fastidiosa* (Clifford *et al.*, 2013).

394 Although LPS may have a key role in biofilm formation, the capsule polysaccharides do
395 not appear to affect biofilm formation despite an increase in adherence to epithelial cells
396 and polystyrene by a capsule mutant (Rioux *et al.*, 2000; Hathroubi *et al.*, 2016a). The

397 capsule may mask critical adhesion factors such as adhesins. Several surface proteins have
398 been associated with biofilm formation in *A. pleuropneumoniae*. For example, deletion of

399 the autotransporter serine protease, AasP, results in increased adherence and biofilm
400 formation (Tegetmeyer *et al.*, 2009). The outer membrane protein VacJ is also involved in

401 biofilm formation and outer membrane integrity (Xie *et al.*, 2016a); deletion of this gene
402 reduces the ability of *A. pleuropneumoniae* to form biofilms. Interestingly outer membrane

403 efflux proteins, such as TolC or a TolC-like homologue, have also been associated with
404 biofilm formation. Moreover, it has been observed that the deletion of *tolC1* causes a
405 reduction in surface adherence, auto-aggregation and biofilm production but the second
406 *tolC* homologue, *tolC2*, does not have any effect on biofilm formation (Li *et al.*, 2016a; Li
407 *et al.*, 2016b). The cell hydrophobicity is also changed in the *tolC1* deletion mutant and
408 *pgaA* and *cpxR* expression is down-regulated in the mutant (Li *et al.*, 2016a). As a side
409 note, the *tolC2* gene is up-regulated in dispersing biofilms and it has been suggested that
410 this protein with MacAB-like proteins could mediate secretion of a dispersal signal
411 (Tremblay *et al.*, 2013a). Interestingly, the efflux pump inhibitor, phenylalanine-arginine
412 beta-naphthylamide (PA β N), is able to repress biofilm formation of *A. pleuropneumoniae*
413 and enhance the inhibitory effect of several antibiotics on pre-established biofilms (Li *et*
414 *al.*, 2016b).

415

416 Two trimeric autotransporter adhesins, Apa1 and Apa2, are also involved in
417 autoaggregation and biofilm formation of *A. pleuropneumoniae* (Xiao *et al.*, 2012; Wang *et*
418 *al.*, 2016). In the case of Apa1, the adhesion functional domain located at the head of the
419 protein is required for autoaggregation, biofilm formation and adherence to SJPL (Wang *et*
420 *al.*, 2015). Apa1 is a Hsf-like trimeric autotransporter adhesin that has been identified to be
421 differentially regulated under several conditions. For example, Apa1, also identified as
422 APL_0443, is up-regulated when *A. pleuropneumoniae* is cultured in a growth medium
423 favoring biofilm formation (Labrie *et al.*, 2010), in the presence of norepinephrine (Li *et*
424 *al.*, 2012) and in the presence of porcine bronchoalveolar lavage fluid (Lone *et al.*, 2009)
425 while it is down-regulated in *A. pleuropneumoniae* attached to SJPL cells (Auger *et al.*,

426 2009), in a maturing biofilm (Tremblay *et al.*, 2013a) and in the presence of epinephrine (Li
427 *et al.*, 2012). Based on these observations, it was suggested that APL_0443 is involved in
428 the early reversible attachment step during biofilm formation of *A. pleuropneumoniae*
429 (Tremblay *et al.*, 2013a).

430

431 6) Other factors identified

432 Factors involved in biofilm formation are not limited to regulators and structures at the
433 bacteria-surface interface; the periplasm and cytoplasm have also been identified as the
434 location of key processes for biofilm formation. For example, ClpP, a protease of the CLP
435 (caseinolytic protease) family, plays an important role in biofilm formation of *A.*
436 *pleuropneumoniae*. Indeed, a *clpP* deletion mutant has been shown to have a defect in
437 biofilm production (Xie *et al.*, 2013). Other proteases also influence biofilm formation by
438 *A. pleuropneumoniae*. Specifically, two homologues of the Lon proteases, LonA and LonC,
439 have been identified but only the deletion of LonA results in decrease biofilm production
440 (Xie *et al.*, 2016b). The Lon proteases belong to a family of ATP-dependent proteases
441 involved in the degradation of abnormal proteins created when bacteria are exposed to
442 environmental stresses.

443

444 Furthermore, mutations in genes such *potD2*, a dihydrouridine tRNA that binds
445 polyamine/spermidine, and *rpmF*, a ribosomal L32 protein, caused a decrease in the
446 production of *A. pleuropneumoniae* biofilm (Grasteau *et al.*, 2011). Homologues of these
447 genes have been associated with *Pseudomonas aeruginosa* biofilm and their mutations
448 decrease biofilm production (Musken *et al.*, 2010). Other genes such *pyrF* (decarboxylase
449 orotidine-5-phosphate), *ptsI* (phosphotransferase) and *ribA* (synthesis of riboflavin), are

450 also associated with a decrease in biofilm formation in *A. pleuropneumoniae* (Grasteau *et*
451 *al.*, 2011). Also, riboflavin synthesis appears to be an important element in biofilm
452 formation since the expression of certain genes in this pathway are modulated during
453 biofilm formation (Tremblay *et al.*, 2013a).

454

455 **BIOFILMS: ADVANTAGES AND BENEFITS FOR *A. PLEUROPNEUMONIAE***

456 It is recognized that biofilms provide various advantages to bacteria including survival in
457 harsh environments and resistance to stresses such as the presence of antibiotics or
458 disinfectants (Jefferson, 2004; Nadell *et al.*, 2015; Olsen, 2015; Hathroubi *et al.*, 2017). For
459 example, *A. pleuropneumoniae* grown as a biofilm is less sensitive to antibiotics and
460 concentrations 100 to 30 000 times higher than the minimal inhibitory concentrations
461 (MIC) required to kill planktonic cells (Archambault *et al.*, 2012) are needed to kill biofilm
462 cells. This decrease in sensitivity has been observed with antibiotics frequently used on pig
463 farms including ampicillin, florfenicol, tiamulin and tilmicosin (Archambault *et al.*, 2012).
464 It has been suggested that a decrease in sensitivity to antibiotics is due to the sequestration
465 of antibiotics by extracellular matrix components such as PGA which is found in the
466 biofilm matrix of *A. pleuropneumoniae* (Nadell *et al.*, 2015; Olsen, 2015; Hathroubi *et al.*,
467 2017). Indeed, pretreatment of biofilms with dispersin B increases the sensitivity of *A.*
468 *pleuropneumoniae* cells to ampicillin suggesting that PGA can limit the diffusion of this
469 antibiotic (Izano *et al.*, 2007). In addition to decreasing antibiotic sensitivity, biofilms can
470 also protect against the immune response or decrease the inflammatory response. With *A.*
471 *pleuropneumoniae*, pro-inflammatory genes are down-regulated in porcine pulmonary
472 alveolar macrophages exposed to biofilm cells when compared to planktonic cells
473 (Hathroubi *et al.*, 2016b). Furthermore, biofilm bacteria reduce the proliferation of porcine

474 peripheral blood mononuclear cells. Interestingly, biofilm cells modify their lipid A
475 structures, and these modifications are absent in planktonic cells. Overall, the immune
476 response towards cells isolated from *A. pleuropneumoniae* biofilms is weaker and this
477 change could be partially driven by lipid A modification (Hathroubi *et al.*, 2016b).

478

479 The advantages conferred by biofilm formation might not be limited to stress resistance.
480 During an infection or colonization, biofilms are generally formed as mixed population of
481 several microorganisms resulting in competitive and/or mutualistic relationships (Peters, *et*
482 *al.*, 2012; Willems *et al.*, 2016). In some cases, polymicrobial interactions in mixed
483 biofilms can provide fertile ground for the exchange of resistance genes and/or increase
484 survival and persistence (Harriott and Noverr, 2009; De Brucker *et al.*, 2015; Hathroubi *et*
485 *al.*, 2017). Recently, it was demonstrated that *A. pleuropneumoniae* is able to form mixed
486 biofilms with other swine pathogens such as *S. suis*, *Bordetella bronchiseptica* and *P.*
487 *multocida* (Loera-Muro *et al.*, 2016). In this situation, *A. pleuropneumoniae* does not
488 require the addition of the essential co-factor NAD to the medium for growth and biofilm
489 formation. Furthermore, *S. suis*, *Bordetella bronchiseptica* and *P. multocida* form a weak
490 biofilm that is at near the detection limit of the assay in BHI and in the absence of *A.*
491 *pleuropneumoniae*. The association of *A. pleuropneumoniae* with other swine pathogens
492 appears to benefit both partners. The swine pathogens provide an essential co-factor to *A.*
493 *pleuropneumoniae* and, in exchange, *A. pleuropneumoniae* could provide components for
494 the biofilm structure (e.g., PGA, eDNA, proteins, lipids) (Loera-Muro *et al.*, 2016).

495

496 The benefits of biofilm formation may not be limited to the host environment. Indeed, as an
497 obligate parasite of the porcine respiratory tract, *A. pleuropneumoniae* can only survive for

498 a very short period of time outside its host and is unable to survive in the farm
499 environment. However, a recent study detected *A. pleuropneumoniae* in biofilms from the
500 drinking water found on swine farms in Mexico (Loera-Muro *et al.*, 2013).

501 *A. pleuropneumoniae* biofilms may also be advantageous for other microorganisms such as
502 important viral pathogens of pigs. Recently, it was demonstrated that the porcine
503 reproductive and respiratory syndrome virus and type 2 porcine circovirus can persist inside
504 an *A. pleuropneumoniae* biofilm for several days (Jacques *et al.*, 2015).

505

506 On a final thought, biofilm may be a contributing factor, to some extent, to the high
507 prevalence of *A. pleuropneumoniae* in both Canadian domestic pigs (70%) (MacInnes *et*
508 *al.*, 2008) and feral pigs in the United States (69.7%) by favoring persistent infections
509 (Baroch *et al.*, 2015).

510

511 **MANAGEMENT OF *A. PLEUROPNEUMONIAE* OUTBREAKS**

512 A wide variety of antimicrobial agents are used to treat *A. pleuropneumoniae*: β -lactams
513 (amoxicillin, penicillin, ampicillin, and ceftiofur), tetracyclines (tetracycline and
514 doxycycline), florfenicol, trimethoprim/sulfamethoxazole, tiamulin,
515 lincomycin/spectinomycin, fluoroquinolones (danofloxacin and enrofloxacin) and
516 gentamicin (Dayao *et al.*, 2014; Dayao *et al.*, 2016). In recent years, isolates with different
517 levels of antibiotics resistance have started to arise worldwide (Archambault *et al.*, 2011;
518 Bossé *et al.*, 2015; Dayao *et al.*, 2014).

519

520 The direct link between biofilm formation and levels of antibiotic resistance in *A.*
521 *pleuropneumoniae* is still unclear. However, it is worth mentioning that sub-MIC of

522 penicillin G may enhance biofilm production via the induction of PGA expression
523 (Hathroubi *et al.*, 2015). Since antibiotics are often used in North America at sub-
524 therapeutic doses for growth promotion and prevention, and *A. pleuropneumoniae* biofilms
525 are more tolerant to antibiotics (Archambault *et al.*, 2012), the judicious use of antibiotic in
526 pig production is highly advised.

527

528 Currently, antibiotics represent the most effective measure for controlling *A.*
529 *pleuropneumoniae* outbreaks (Gottschalk, 2015). *A. pleuropneumoniae* biofilm should be
530 taken into consideration for the development of new effective treatment strategies. These
531 strategies should combine antimicrobials with anti-biofilm molecules such as zinc (Wu *et*
532 *al.*, 2013) or PAβN (Li *et al.*, 2016b) to overcome persistent infections and reduce the cost
533 of treatment.

534

535 **PREVENTION AND VACCINE STRATEGIES AGAINST A.** 536 ***PLEUROPNEUMONIAE***

537 In the last decade, several vaccines have been developed to protect against *A.*
538 *pleuropneumoniae* infections. Most of vaccines are based on recombinant Apx toxins and
539 membrane proteins (such as OMP and type 4 fimbrial proteins) and provide protection
540 against some but not all serotypes (Shao *et al.* 2010; Lu *et al.*, 2011; Shin *et al.* 2011;
541 Sadilkova *et al.* 2012; Li *et al.*, 2013; Hur and Lee, 2014; Yang *et al.*, 2014; Hur *et al.*,
542 2016; Kim *et al.*, 2016; Li *et al.*, 2016c; To *et al.*, 2016). Inactivated/whole *A.*
543 *pleuropneumoniae* cell based vaccines are also used in many countries to prevent porcine
544 pleuropneumonia (Shao *et al.*, 2010; Lu *et al.*, 2011; Lee *et al.*, 2014; Lopez-Bermudez *et*

545 *al.*, 2014). These vaccines are widely distributed. However, these vaccines do not provide
546 complete protection against all serotypes of *A. pleuropneumoniae*.

547

548 Bacterins are typically prepared from bacteria grown as planktonic cells. Since biofilm cells
549 are known to exhibit phenotypes that are different than their planktonic counterparts
550 (Stewart and Franklin, 2008; O'May *et al.*, 2009) and *A. pleuropneumoniae* form biofilm
551 aggregates during an infection (Tremblay *et al.*, 2017), the vaccines described above may
552 not provide a full protection against *A. pleuropneumoniae* infections. Bacterins may help
553 the vaccinated pig developed a significant memory response against the planktonic form of
554 *A. pleuropneumoniae* but the antigenic nature of some targets are modified during growth
555 as biofilms. For example, the *A. pleuropneumoniae* lipid A molecular structure is modified
556 according to the mode of growth (Hathroubi *et al.*, 2016b). Indeed, cells grown as a biofilm
557 have unique lipid A structures that are absent in planktonic cells including an increase in
558 higher molecular weight lipid A entities (Hathroubi *et al.*, 2016b). Accordingly, it would
559 likely be best to create bacterins using both planktonic and biofilm cultures to provide a
560 better protection against *A. pleuropneumoniae* infections by presenting a larger set of
561 antigens that could be biologically relevant.

562

563 As with bacterins, commercially available recombinant vaccines based on Apx toxins
564 and/or other proteins have failed to provide a complete protection against every *A.*
565 *pleuropneumoniae* isolates (Del Pozo-Sacristán *et al.* 2014; Sjölund *et al.* 2010). The
566 development of new vaccines based on antigens specifically associated with *A.*
567 *pleuropneumoniae* biofilms in combination with the Apx toxins and other antigens could

568 help improve the protection but further investigations are required to identify relevant
569 molecules expressed in biofilms and during infection.

570

571 Such strategies have been successful for the development of new vaccines against other
572 pathogens. For example, a proteomic analysis of *Bordetella pertussis* biofilm and
573 planktonic cells identified a biofilm-derived membrane protein called BipA as a potential
574 vaccine antigen (de Gouw *et al.*, 2014). Vaccination of mice with this antigen showed
575 promising results that included induction of a specific antibodies response and a significant
576 reduction in the colonization of lungs by *B. pertusis* (de Gouw *et al.*, 2014). Moreover,
577 anti-BipA antibodies have been detected in the serum of convalescent whooping cough
578 patients (de Gouw *et al.*, 2014). In another example, Gil *et al.* (2014) performed an
579 intradermal administration of an exoproteome extract derived from an exopolysaccharide-
580 dependent biofilm to develop an efficient antibiofilm vaccine against *Staphylococcus*
581 *aureus*. The biofilm exoproteome induced a humoral immune response and elicited the
582 production of interleukin (IL) 10 and IL-17 in mice. Furthermore, vaccination with the
583 exoproteome extract significantly reduced the number of bacteria within biofilms and
584 surrounding tissue in *in vivo* mesh-associated biofilm infection model (Gil *et al.*, 2014).

585

586 The strategy of using biofilm-specific antigen is not limited to *B. pertussis* and *S. aureus*;
587 others have begun to use similar strategies against bacterial pathogens of importance in
588 veterinary and human health. These pathogens include: *S. aureus* (Speziale *et al.*, 2014;
589 Gogoi-Tiwari *et al.*, 2015), *Campylobacter jejuni* (Theoret *et al.*, 2012), *Mycobacterium*
590 *tuberculosis*-complex (Flores-Valdez, 2016), *Streptococcus mutans* (Huang *et al.*, 2013),
591 *Staphylococcus epidermidis* (Shahrooei *et al.*, 2012; Speziale *et al.*, 2014), *Bacillus subtilis*

592 (Vogt *et al.*, 2016), *Acinetobacter baumannii* (Fattahian *et al.*, 2011), and *Streptococcus*
593 *equi ssp. zooepidemicus* (Yi *et al.*, 2016) (Table 1).

594

595 In the context of biofilm infections, two different types of antigens exist: bacterial cells
596 within the biofilm and the biofilm matrix. The biofilm matrix may be composed of
597 polysaccharides, proteins, and extracellular DNA and the composition of the matrix is
598 dependent on the bacterial genera, species, and strains (Harro *et al.*, 2010). Different studies
599 have focused on identifying antigens from the bacteria, the matrix or both as the best
600 strategy for the development of effective vaccines (Table 1).

601

602 Another factor that must be considered is that biofilm consortia typically exist as
603 communities of bacteria, viruses, protozoans, and fungi and the overall biofilm architecture
604 is affected by specific intermicrobial and host interactions (Harro *et al.*, 2010). These
605 consortia can allow colonization and subsequent infection by opportunistic pathogens that
606 exploit unique niches found in these polymicrobial environments resulting in the
607 development of polymicrobial infections.

608

609 Finally, vaccine research and design should take advantage of new techniques such as RNA
610 sequencing, bioinformatics, proteomics, and lipidomics to identify molecules specifically
611 expressed and/or secreted during biofilm formation. In our opinion, this should greatly
612 improve the efficacy of future vaccines and ensure better protection of pigs against *A.*
613 *pleuropneumoniae*.

614

615 **CONCLUSION AND FUTURE CHALLENGES**

616 Despite different strategies and years of prevention and control, *A. pleuropneumoniae*
617 remains one of the main respiratory pathogens of pigs and is responsible for great economic
618 losses to the worldwide pork industry. Although some countries such as the USA and
619 Canada can manage *A. pleuropneumoniae*, this pathogen remains present in farms and,
620 thus, resurgence in new outbreaks are always possible. These new outbreaks could emerge
621 from isolates with increase resistance to antibiotics. Great efforts have been made to
622 prevent infections with this pathogen through optimal farm management and through major
623 investments in research and development of new and better vaccines. However, neither
624 management nor vaccines have been 100% effective at controlling *A. pleuropneumoniae*
625 infections. Fortunately, new research is shedding light on the pathogenesis of *A.*
626 *pleuropneumoniae* and it is improving our understanding of this old acquaintance.
627 Importantly, recent studies have revealed that *A. pleuropneumoniae* forms biofilm
628 aggregates in the lung (Tremblay *et al.*, 2017) and can form multi-species biofilms with
629 other respiratory pathogens (Loera-Muro *et al.*, 2016). Using these new findings, it will be
630 possible to identify novel vaccine candidates to improve the next generation of vaccines
631 and to develop better strategies to control *A. pleuropneumoniae*. These new developments
632 could hopefully help prevent the persistent problems cause by this pathogen in the
633 worldwide production of pigs for the last 50 years.

634

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639

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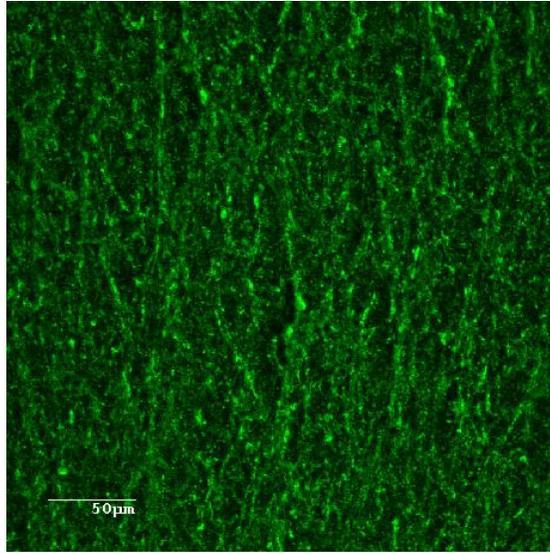
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1087 **Figure 1** Confocal laser scanning microscopy image of *A. pleuropneumoniae* 4074 biofilm
1088 stained with WGA-Oregon Green 488.
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1096 **Figure 2** Coupon with *A. pleuropneumoniae* 4074 biofilm from Drip flow system.

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Table 1. Examples of vaccines based on biofilm-specific antigens produced by pathogenic bacteria of importance in veterinary and human health.

Bacterial species	Disease	Antigens	Reference
<i>Acinetobacter baumannii</i>	Nosocomial pathogen and causes severe infections such as bacteremia, pneumonia, meningitis, urinary tract and wound infections.	Biofilm associated protein (Bap), a 371 amino acid subunit.	Fattahian <i>et al.</i> , 2011.
<i>Acinetobacter baumannii</i>	Nosocomial pathogen and causes severe infections such as bacteremia, pneumonia, meningitis, urinary tract and wound infections.	Bap with Outer Membrane Vesicles (without lipid A or Outer Membrane Protein A).	Badmasti <i>et al.</i> , 2015.
<i>Bordetella pertussis</i>	Whooping cough or pertussis.	Bordetella intermediate protein A (BipA).	de Gouw <i>et al.</i> , 2014.
<i>Burkholderia pseudomallei</i>	The causative agent of melioidosis (category B select agent).	mAbs namely BURK24 and BURK37.*	Pedayelachagiri <i>et al.</i> , 2014.
<i>Campylobacter jejuni</i>	Food-borne bacterial gastroenteritis.	Oral vaccination with a recombinant attenuated <i>Salmonella enterica</i> strain synthesizing the <i>C. jejuni</i> Dps protein.	Theoret <i>et al.</i> , 2012.
<i>Enterococcus faecalis</i>	Cause catheter-associated urinary tract infections.	Heteropolymeric surface long hair-like fiber known as the endocarditis-and biofilm-	Flores-Mireles <i>et al.</i> , 2014.

			associated pilus (Ebp).	
<i>Staphylococcus aureus</i>	Associated with biofilm-mediated and infections (endocarditis, osteomyelitis, medical devices, etc.).	Phosphonate ABC transporter substrate binding protein (PhnD).		Lam <i>et al.</i> , 2014
<i>Staphylococcus epidermidis</i>				
<i>Staphylococcus aureus</i>	Associated with biofilm-mediated and infections (endocarditis, osteomyelitis, medical devices, etc.).	The Major amidase (Atl-AM, functional non-covalently associated protein involved in biofilm formation).		Nair <i>et al.</i> , 2015.
<i>Staphylococcus epidermidis</i>				
<i>Staphylococcus aureus</i>	Associated with biofilm-mediated infections (endocarditis, osteomyelitis, medical devices, etc.).	Exoproteome extract of an exopolysaccharide-dependent biofilm.		Gil <i>et al.</i> , 2014.
<i>Staphylococcus aureus</i>	Persistent and chronic forms of mastitis in cows.	Formalin killed whole cell vaccine of <i>S. aureus</i> in a biofilm state.		Gogoi-Tiwari <i>et al.</i> , 2015.
<i>Staphylococcus aureus</i>	Persistent and chronic forms of mastitis in cows.	Protein A (in biofilm formation contributing to the severity of <i>S. aureus</i> associated infections).		Gogoi-Tiwari <i>et al.</i> , 2016.
<i>Staphylococcus epidermidis</i>	Medical implants associated infections.	Accumulation associated protein (Aap) C-terminal single B-repeat construct followed		Hu <i>et al.</i> , 2011.

				by the 79-aa half repeat (AapBrpt1.5).	
<i>Staphylococcus epidermidis</i>	Medical implants associated infections.			Vaccination with a recombinant truncated SesC (hypothetical LPXTG motif-containing proteins).	Shahrooei <i>et al.</i> , 2012.
<i>Staphylococcus epidermidis</i>	Medical implants associated infections.			Accumulation associated protein (Aap).	Yan <i>et al.</i> , 2014
<i>Streptococcus mutans</i>	Predominant microorganism in the etiology and pathogenesis of dental caries.			DNA vaccine-induced salivary secretory immunoglobulin A (S-IgA) antibodies (DNA vaccine pGJA-P/VAX).	Huang <i>et al.</i> , 2013.
<i>Streptococcus equi ssp. zooepidemicus</i>	Opportunistic pathogen infecting a wide variety of animals and human.			Recombinant chaperonin GroEL protein.	Yi <i>et al.</i> , 2016.

* Murine Monoclonal Antibodies (mAbs) against *Burkholderia pseudomallei* biofilms.