GIARLÃ CUNHA DA SILVA

# EFEITO DA PROTEÍNA Hfq EM VESÍCULAS DE MEMBRANA EXTERNA PRODUZIDAS POR *Actinobacillus pleuropneumoniae* SOROTIPO 8

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, para obtenção do título de *Magister Scientiae*.

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APROVADA: 9 de fevereiro de 2018.

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# SUMÁRIO

## RESUMO

DA SILVA, Giarlã Cunha, M.Sc., Universidade Federal de Viçosa, fevereiro de 2018. **Efeito da proteína Hfq em vesículas de membrana externa produzidas por *Actinobacillus pleuropneumoniae* sorotipo 8.** Orientadora: Denise Mara Soares Bazzolli. Coorientadores: Mateus Ferreira Santana, Marisa Viera de Queiroz e Janine Therèse Bossé.

A suinocultura é uma atividade de grande importância econômica no Brasil e no mundo. O Estado de Minas Gerais é um dos principais produtores de carne suína, apresentando constante crescimento. Na suinocultura, podem-se relatar diversas doenças respiratórias que acometem suínos, sendo um dos mais importantes a bactéria *Actinobacillus pleuropneumoniae*. Em *A. pleuropneumoniae* vários fatores de virulência como LPS, cápsula, toxinas RTX e proteínas de captação de ferro, contribuem de diferentes maneiras e de forma complexa para o sucesso desta bactéria durante a infecção. Um fator de virulência muito importante em diversas bactérias, porém pouco relatado em *A. pleuropneumoniae* são as vesículas de membrana externa (OMVs). As OMVs são vesículas produzidas geralmente por bactérias Gram-negativas, constituídas de proteínas, lipídeos e lipopolissacarídeos. Elas podem transportar diferentes moléculas como proteínas, íons, DNA e RNA, participando na virulência de diversas espécies. Além dos fatores mencionados, um fator muito importante para *A. pleuropneumoniae* é a proteína Hfq, uma chaperona de RNAs que possui papel central no crescimento e virulência desta bactéria. Desta forma, o objetivo deste trabalho foi caracterizar OMVs produzidas por *A. pleuropneumoniae* sorotipo 8 e sua respectiva linhagem isogênica Δ*hfq.* O perfil de proteínas foi diferencial entre as células e as OMVs, no entanto não houve diferença entre o perfil proteico encontrado nas OMVs produzidas pelas linhagens WT e Δ*hfq.* Não foi possível identificar a proteína Hfq em OMVs de *A. pleuropneumoniae*. As análises microscópicas realizadas evidenciaram que as vesículas possuem um tamanho predominante entre 20-30 nm podendo chegar até 100 nm. O ensaio de virulência não apresentou diferença em relação à sobrevivência para as larvas de *Galleria mellonella* infectadas com OMVs de WT em relação às infectadas com OMVs de Δ*hfq.* Porém, larvas infectadas com OMVs apresentaram melanização mais rápida e intensa em relação às larvas infectadas com as células. No interior das OMVs foi possível identificar RNAs, incluindo RNAs pequenos regulatórios, já previamente estudados por nosso grupo, porém não foi

observado diferença na composição destes encontrados em OMVs produzidos por WT e Δ*hfq*. A partir destes resultados pode-se concluir que a proteína Hfq não interferiu na virulência, morfologia e composição das OMVs produzidas por *A. pleuoropneumoniae*. Este estudo foi o primeiro a relatar a presença de RNAs regulatórios no interior de OMVs produzidas por uma bactéria patogênica de suíno da família Pasteurellaceae.

## ABSTRACT

DA SILVA, Giarlã Cunha, M.Sc., Universidade Federal de Viçosa, February, 2018. **Effect of Hfq protein in outer membrane vesicles produced by *Actinobacillus pleuropneumoniae* serotype 8.** Adviser: Denise Mara Soares Bazzolli. Co-advisers: Mateus Ferreira Santana, Marisa Viera de Queiroz and Janine Therèse Bossé.

Pig farming is an activity of great economic importance in Brazil and worldwide. Minas Gerais is one of the main producers of pork, showing constant growth. In pig industry, several respiratory diseases affecting pigs can be reported, one of the most important being the bacterium *Actinobacillus pleuropneumoniae*. In *A. pleuropneumoniae* several virulence factors such as LPS, capsule, RTX toxins and iron uptake proteins, contribute in different and complex ways to the success of this bacterium during infection. A very important virulence factor in several bacteria, but little reported in A. pleuropneumoniae are the outer membrane vesicles (OMVs). OMVs are vesicles generally produced by Gram-negative bacteria, consisting of proteins, lipids and lipopolysaccharides. They can trasnport different molecules like proteins, ions, DNA and RNA, participating in the virulence of several species. In addition, a very important factor for *A. pleuropneumoniae* is the Hfq protein, a chaperone of RNAs that plays a central role in the growth and virulence of this bacterium. Thus, the objective of this work was to characterize OMVs produced by *A. pleuropneumoniae* serotype 8 and their respective *Δhfq* isogenic strain. The proteins profile was differentiated between the cells and the OMVs, however there was no difference between the protein profile found in the OMVs produced by the WT and *Δhfq* strains. It was not possible to identify the Hfq protein in OMVs of *A. pleuropneumoniae*. The microscopic analysis showed that the vesicles have a predominant size between 20-30 nm and can reach up to 100 nm. The virulence assay showed no difference in survival for *Galleria mellonella* larvae infected with WT OMVs compared to those infected with *Δhfq* OMVs. However, larvae infected with OMVs showed faster and more intense melanization in relation to the larvae infected with the cells. In the OMVs it was possible to identify RNAs, including small regulatory RNAs previously studied by our group, but no difference was observed in the composition of these OMVs produced by WT and *Δhfq* stains. From these results it can be concluded that the Hfq protein did not interfere in the virulence, morphology and composition of OMVs produced by *A. pleuoropneumoniae*. This study

was the first to report the presence of regulatory RNAs within OMVs produced by a pathogenic swine bacterium of the family Pasteurellaceae.

## Introdução Geral

### A suinocultura brasileira, pleuropneumonia e *Actinobacillus pleuropneumoniae*

A produção e o consumo da carne suína vêm crescendo consideravelmente nos últimos anos tanto no âmbito nacional como no mundial. Nesse contexto, o Brasil aparece como quarto maior produtor com 3.731 milhões de toneladas, atrás apenas da China, União Europeia e EUA e também quarto maior exportador com 732 milhões de toneladas, atrás apenas da União Europeia, EUA e Canadá (Abipecs, 2017). No Brasil, Minas Gerais éconsiderado o quarto maior estado produtor de carne suína, correspondendo a 11% da produção nacional (Abipecs, 2017).

Apesar de ser um mercado de grande importância e em contínuo crescimento, a suinocultura passa por alguns problemas, sendo um destes, o difícil controle de doenças causadas por microrganismos, que geram perdas significativas no que envolve a comercialização e exportação da carne suína (Abipecs, 2017). Dentre as doenças de suínos causadas por microrganismos, as pneumonias representam perdas econômicas significativas (Fourniéet al., 2015).

A pleuropneumonia suína (PPS) é uma doença de caráter contagioso, sendo uma das mais importantes doenças respiratórias na suinocultura e é descrita em quase todos os países produtores, causando grandes prejuízos econômicos (Gottschalk, 2012.). Essa doença pode ocorrer em suínos de todas as idades (Bossé et al., 2002). A importância da doença não está somente relacionada ao acarretamento da morte do animal, mas também pela redução da produção e custos associados à terapêutica e profilaxia (Taylor, 1999). A pleuropneumonia suína tem como agente etiológico a bactéria *Actinobacillus pleuropneumoniae*, que pode ser transmitida por contato direto ou por aerossol em curtas distâncias (Loera-Muro et al., 2013). *Actinobacillus pleuropneumoniae* pode colonizar a tonsila e o trato respiratório superior do animal, podendo ser isolado da cavidade nasal, tonsilas, cavidade auditiva média e pulmões de animais infectados (Sidibeet al., 1993; Dom et al., 1994; Duff et al., 1996).

A pleuropneumonia suína se caracteriza pelo desenvolvimento de broncopneumonia necrosante e hemorrágica, com exudação de fibrina e pleurite (Vaz e Silva, 2004). As formas aguda e hiperaguda da doença, podem ser caracterizadas por morte súbita, febre, anorexia e frequentemente tosse e/ou vômito. A mortalidade é baixa, mas pode se tornar

elevada em novos surtos de doença e a mortalidade pode chegar aos 100% quando não há tratamento (Coelho et al., 2004). Podem ser observadas também as formas subaguda ou crônica, em que os sinais clínicos são mais brandos: os animais apresentam redução na taxa de ganho de peso e retardo no crescimento (Taylor, 1999).

*Actinobacillus pleuropneumoniae* é um cocobacilo Gram-negativo, anaeróbio facultativo e encapsulado, pertencente à família Pasteurellaceae (Frey, 1995) sendo considerado um patógeno exclusivo do trato respiratório de suínos, uma vez que não infecta outros animais e não é considerado zoonótico (Taylor, 1999). Os isolados de *A. pleuropneumoniae* podem ser divididos em dois biotipos, dependendo do requerimento de NAD (nicotinamida adenina dinocleotídeo), podendo ser NAD dependente, constituindo o biotipo 1 e NAD independente, constituindo o biotipo 2 (Niven e Levesque, 1988). Adicionalmente, são descritos dezoito sorotipos para esta bactéria, que são definidos baseado nas propriedades antigênicas dos polissacarídeos da cápsula (Blackall et al., 2002; Sarkozi, Makrai e Fodor., 2015; Bossé et al., 2018). Dentre estes, o sorotipo 8 o mais difundido em Minas Gerais (Rossi et al., 2013). Todos os sorotipos são capazes de causar a doença, embora alguns sejam mais virulentos que outros (Frey 1995).

A virulência de *A. pleuropneumoniae* é multifatorial, envolvendo fatores como polissacarídeos da cápsula, camada lipopolissacaridica-LPS, proteínas ligantes de transferrina e as exotoxinas RTX (*Repeats-in-toxin*) (ApxI, ApxII, ApxIII e ApxIV) (Chiers et al., 2010; Frey, 2011; Sassu et al., 2017).

As toxinas RTX possuem atividade citotóxica e/ou hemolítica (Frey, 2011) e correspondem ao principal fator de virulência dessa espécie. A toxina ApxI possui forte atividade hemolítica e citotóxica, ApxII possui fraca atividade hemolítica e moderada atividade citotóxica, enquanto ApxIII não possui atividade hemolítica e possui forte atividade citotóxica. Há também outra toxina RTX reportada que é produzida por todos sorotipos de *A. pleuropneumoniae*, como a toxina ApxIV (Shaller et al., 1999), porém, sua participação na patogenia não foi totalmente elucidada. Além destes fatores, outro importante fator que está relacionado com a virulência de *A. pleuropneumoniae* é a proteína Hfq, uma chaperona de RNAs de grande importância.

**Chaperonas de RNA: a proteína Hfq**

A proteína Hfq é um membro da superfamília Sm/Lsm que engloba proteínas envolvidas no metabolismo de RNAs (Wilusz e Wilusz 2005). Hfq é uma proteína hexamérica que foi descoberta em meados de 1960 em um estudo na qual foram analisadas proteínas necessárias para a replicação do bacteriófago Q-beta em *Escherichia coli* (Franze de Fernandez et al., 1968; 1972). O seu papel no metabolismo bacteriano foi apenas descoberta na década de 1990 quando ficou clara a sua importância na regulação da expressão gênica ao interagir com pequenos RNAs. Hfq atua facilitando a interação de sRNAs tipo *trans* com seus respectivos RNAs mensageiro alvos, proporcionando assim a regulação da tradução. Além disso, essa proteína também regula a estabilidade de sRNAs (Storz et al., 2011; Vogel e Luisi, 2011). Essa proteína forma um hexâmero em forma de anel em que exibem duas superfícies para a ligação de moléculas diferentes de RNA, o que permite a interação pequeno RNA-RNA mensageiro (Brennan e Link 2007).

Há diferentes mecanismos na qual Hfq participa da regulação da tradução ou da estabilidade de RNAs (Vogel e Luisi, 2015). A proteína Hfq pode inibir a síntese de uma proteína intermediando a ligação de um pequeno RNA à extremidade 5’ do mRNA alvo, tornando essa região inacessível e impedindo a tradução (Figura 2a). De forma semelhante, Hfq pode induzir a tradução de um mRNA através da ligação de um sRNA à extremidade 5’ do mRNA alvo, rompendo uma estrutura secundária e tornando o mRNA acessível à maquinaria de tradução (Figura 2b). A proteína Hfq pode também proteger os sRNAs de clivagem por ribonucleases (Figura 2c) ou favorecer a degradação de RNAs (Figura 2d). Hfq pode também participar do processo de poliadenilação do mRNA e subsequente degradação exonucleolítica 3' – 5’ (Figura 2e).

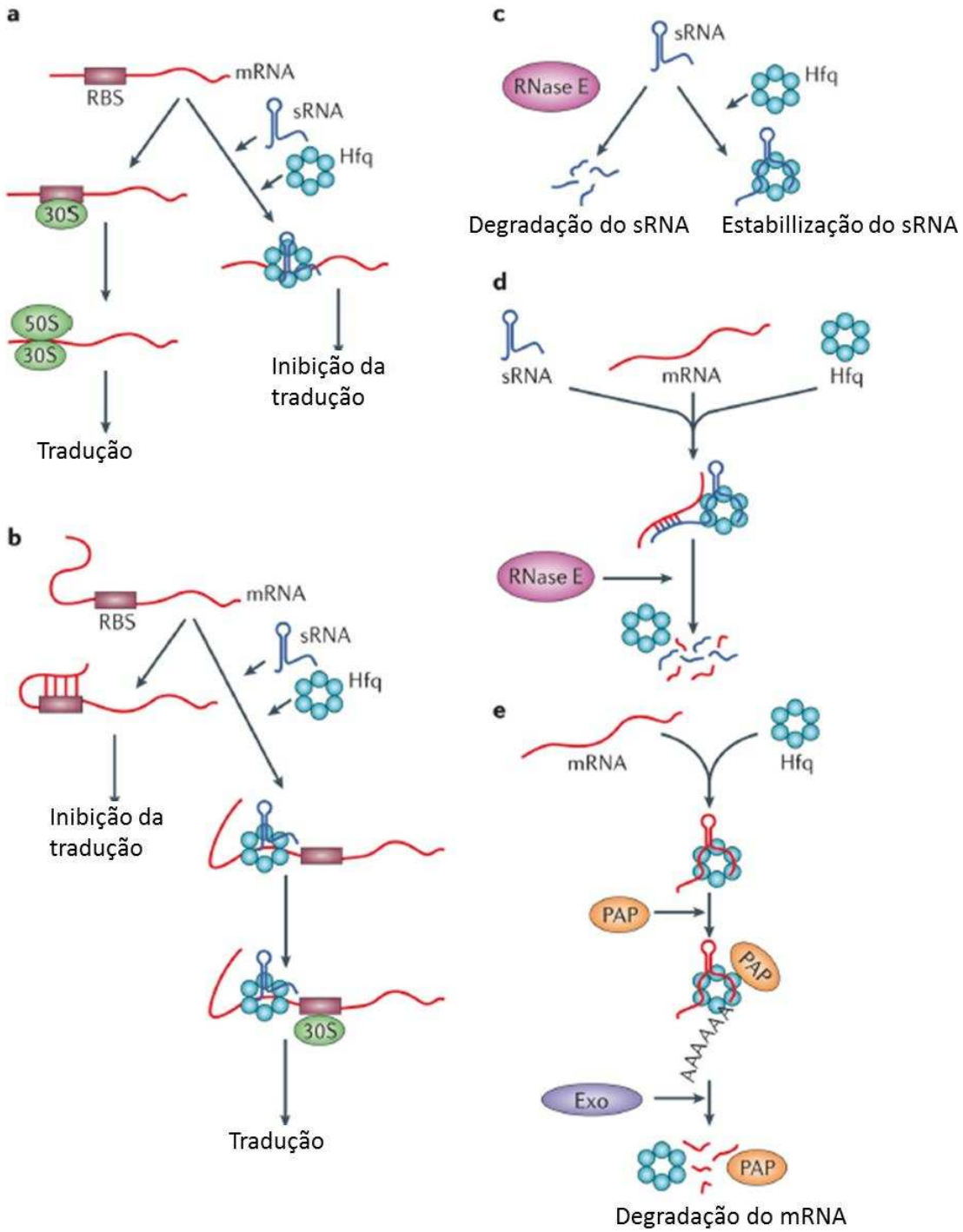


**Figura 2 - Mecanismos de atuação da proteína Hfq. A** - Hfq inibe a síntese de uma proteína intermediando a ligação de um pequeno RNA àextremidade 5' do mRNA alvo. **B** - Hfq induz a tradução de um mRNA através da ligação de um pequeno RNA à extremidade 5' do mRNA alvo. **C** - Hfq protege os pequenos RNAs de clivagem por ribonucleases. **D** - Hfq favorece a degradação de RNAs. **E** - Hfq participa do processo de poliadenilação do mRNA e subsequente degradação exonucleolítica 3' – 5'. Fonte: **Vogel e Luisi, 2015**

A proteína Hfq também está relacionada à atividade de outras proteínas associadas à regulação de mRNAs. Um exemplo disto, é a Poli(A) Polimerase I (PAP I), responsável por grande parte da poliadenilação em *E. coli* (Régnier e Hajnsdorf 2009), em que é sugerido que a proteína Hfq estimula PAP I alterando a poliadenilação de mRNAs (Hajnsdorf e Régnier 2000). Também já foi relatada a interação de Hfq com a polinucleotídeo fosforilase (PNPase) (Mohanty et al. 2004), uma importante exonuclease que está envolvida com a degradação de RNAs (Andrade et al., 2009).

Trabalhos desenvolvidos com diversas espécies mostraram a importância da proteína Hfq na virulência. Estudos feitos com *Yersinia enterocolitica*, *Salmonella typhimurium*, *Burkholderia cepacia*, *Vibrio cholerae, Haemophilus ducreyi* e várias outras espécies mostraram que o mutant hfq é atenuado, apresentando menor virulência em relação ao tipo selvagem (Ding et al., 2004; Sittka et al., 2007; Sousa et al., 2010; Gangaiah et al., 2014; Kakoschke et al., 2016).

Em *A. pleuropneumoniae*, estudos desenvolvidos com mutantes do gene *hfq* para o sotoripo1 e sorotipo 8 mostraram redução da viruência em suínos e em larvas de *Galleria mellonella*, respectivamente. Além disso, mutantes para o gene *hfq* apresentaram maior sensibilidade a condições de estresse, menor tempo de persistência após a infecção e alteração na composição de membrana celular. (Subashchandrabose et al., 2013; Pereira et al 2015).

Existem ainda diferentes fatores de virulência em bactérias pouco estudados em *A. pleuropneumoniae*, como por exemplo, as vesículas de membrana externa (OMVs) que são consideradas fatores de virulência em diferentes patógenos bacterianos, como *Shigella dysenteriae, Vibrio anguillarum, Pseudomonas aeruginosa, Porphyromonas gingivalis* e diversas outras espécies (Ellis e Kuehn, 2010).

**Vesículas de Membrana Externa (OMVs)**

As OMVs possuem estrutura esférica, com membrana constituída de uma bicamada lipídica semelhante à membrana externa de bactérias e um tamanho que pode variar geralmente entre 20–300 nm (Roieret al., 2015; Van der Pol et al. 2015). As OMVs são liberadas espontaneamente a partir da membrana externa durante o crescimento do microrganismo, sendo produzidas geralmente por bactérias Gram-negativas (Van der Polet al., 2015), mas podendo também ser produzidas por bactérias Gram-positivas (Lee et al.,

2009; Rivera et al., 2010), Micobactérias (Prados-Rosales et al., 2011) e Arqueas (Ellen et al., 2009). As OMVs possuem em sua composição fosfolipídios, proteínas da membrana externa e lipopolissacarídeos ou lipooligossacarídeos. Podem possuir também proteínas localizadas no periplasma e componentes da parede celular, os quais podem ser inseridos no lúmem das vesículas durante o processo de formação (Roieret al., 2015). Além disso, as OMVs podem também conter proteínas da membrana interna, proteínas do citoplasma, DNA, RNA, íons, metabólitos e moléculas sinalizadoras (Roieret al., 2015; Pathirana e Kaparakis-Liaskos, 2016).

Inicialmente as OMVs eram consideradas apenas sacos constituídos de material da membrana que eram produzidos durante o crescimento normal de *Vibrio cholerae* (Chatterjee & Das, 1967). Atualmente, vários estudos demonstram a real importância dessas vesículas que estão relacionadas a várias funções (Kim et. al., 2009; Van der Polet al., 2015; Pathirana e Kaparakis-Liaskos, 2016). As OMVs podem estar envolvidas com várias funções como, comunicação intra e interespécies, transferência horizontal de genes, formação de biofilmes, morte de bactérias competidoras, transporte de toxinas e outras biomoléculas, modulação da resposta imune do hospedeiro entre outras funções (Li et al, 1998; Schooling and Beveridge, 2006; Ellis and Kuehn, 2010; Berleman and Auer, 2013; Koeppenet al., 2016). Estudos de proteômica de OMVs produzidas por bactérias Gram-negativas mostram a presença de diferente componentes nas OMVs relacionados com diferentes funções (Figura 1).

Há muitos estudos que demonstram a importâncias dessas vesículas como promissoras candidatas à produção de vacinas, como foi observado em trabalhos que estudaram infecções causadas por *Edwardsiella tarda*, *Haemophilus influenzae, Acinetobacter baumannii, Vibrio cholerae* e várias outras espécies (Schildet al., 2009; Park et al., 2011; Roier et al., 2015; Huang et al., 2016).

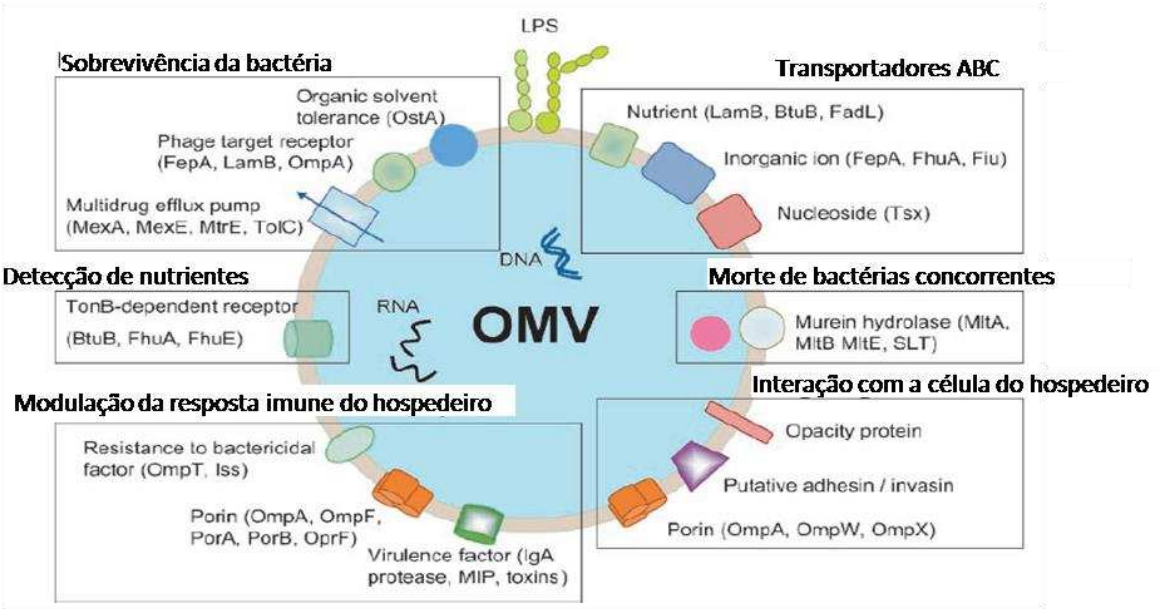


**Figura 1. Funções propostas para OMVs em bactérias Gram-negativas.** As funções de OMVs de bactérias Gram-negativas são preditas com base nos proteomas de OMVs derivados de bactérias patogênicas e não patogênicas. Fonte: Lee et al., 2008

Atualmente, vários autores têm considerado as vesículas de membrana (MVs), como é o caso das OMVs, um tipo de sistema de secreção, chamado de sistema de secreção do tipo 0 (Damm et al., 2014; Kieselbach et al., 2015 Guerrero-Mandujano et al., 2017). O transporte de moléculas por vesículas pode apresentar vantagens em relação a outros mecanismos de secreção de moléculas, justamente pelas moléculas estarem protegidas pela estrutura das vesículas. Associado a isso, a produção dessas vesículas permite maior flexibilidade aos microrganismos que as produzem para responder às mudanças que ocorrem no meio (Lloubeset al., 2013; Kulkarni et al., 2014; Haurat et al., 2015).

Apesar das OMVs possuírem grande importância biológica, até então não há um consenso no mecanismo de biogênese dessas vesículas. Após várias análises genéticas e bioquímicas, alguns mecanismos têm sido propostos para diferentes bactérias (Jan, 2017). Um mecanismo proposto por Burdett e Murray (1974) e Hoekstraet al. (1976) é que a redução das ligações cruzadas entre a membrana externa e a camada de peptideoglicano adjacente resulte em hipervesiculação de OMVs. Outro mecanismo relacionado a produção das OMVs envolve o enriquecimento da membrana externa com LPS, fosfolipídios e outras moléculas, que podem aumentar a curvatura da membrana levando ao aumento da produção de OMVs (Mashburn e Whiteley, 2005). Além disso, outros fatores podem estar

relacionados à produção de OMVs como temperatura ou presença de antibióticos (Kadurugamuwa e Beveridge, 1995; McMahonet al., 2012). Diferentes mecanismos de biogênese de OMVs vêm sendo propostos para diferentes espécies, como para *Pseudomonas aeruginosa* (Turnbullet al., 2016) ou *Salmonella enterica* serovar Typhimurium (Elhenawy et al., 2016). Por mais que vários mecanismos espécie-específicos tenham sido propostos, há um possível mecanismo geral de biogenese de OMVs em Gram-negativas baseado em transportadores de fosfolipídios conservados que vêm sendo reportados (VacJ/Yrb ABC), em que a redução na expressão resulta no aumento da produção de OMVs (Roier et al., 2016).

Muitos trabalhos já descreveram a importância das OMVs no transporte de diferentes moléculas que podem atuar como fatores de virulência, como DNA, RNA (pequenos RNAs) e proteínas/toxinas, como observado em *Pseudomonas aeruginosa* (Koeppenet al., 2016), *Escherichia coli* (Ghosal et al., 2015), *Yersinia pestis* (Eddy et al., 2014), *V. cholera*e (Sjöström et al., 2015) e várias outras espécies.

Na família Pasteurellaceae há alguns estudos sobre OMVs relacionando estas vesículas a fatores de virulência ou relatando seu potencial uso na produção de vacinas, como observado em *Pasteurella multocida* (Fernández-Rojas et al., 2014), *H. influenzae* (Roier et al., 2012; Roier et al., 2015), *Haemophilus parasuis* (McCaig et al., 2016) e em *Mannheimia haemolytica* (Roier et al., 2013).

Em *A. pleuropneumoniae*, há poucos registros sobre estudos envolvendo OMVs, sendo estes descritos por Jacques et al. (1988) e Rosendal e MacInnes (1990) que relataram a produção dessas vesículas por *A. pleuropneumoniae*. A identificação das toxinas Apx I e II em OMVs produzidas por *A. pleuropneumoniae* (Negrete-Abascalet al., 2000). Recentmente Antenucci et al. (2017; 2018) avaliou a utilização de OMVs como imunógeno sorotipo-independente em *A. pleuropneumoniae*, porém sem resultados promissores. Ainda assim, pouco se sabe sobre o conteúdo específico de OMVs produzidas por *A. pleuropneumoniae* e principalamente não há nenhum trabalho relatando a presença de RNAs presentes nestas vesículas.

**Pequenos RNAs regulatórios (sRNAs)**

As OMVs podem transportar diversas moléculas que podem intereferir na resposta do hospedeiro à infecção por microrganismos, como é o caso dos pequenos RNAs

regulatórios (sRNAs). Esses pequenos RNAs estão presentes em todos os domínios da vida e vêm sendo reconhecidos como uma nova e interessante classe de reguladores da expressão gênica em bactérias (Michauxet al., 2014). O controle pós-transcricional feito pelos pequenos sRNAs corresponde a um importante mecanismo de regulação em bactérias (Li et al., 2012). A grande maioria dos sRNAs exerce sua função através do pareamento de bases com sua sequência de RNA alvo podendo afetar a sua tradução, estabilidade e/ou o processamento (Storz et al., 2011, Wagner e Romby, 2015).

Os sRNAs possuem várias funções que envolvem crescimento, adaptação ao estresse, regulação do metabolismo (Michaux et al., 2014). Além disso, apresentam importante papel na patogenicidade de diversas bactérias (Gong et al., 2011; Koo et al., 2011; Westermann et al., 2016). Em *A. pleuropneumoniae,* pouco se sabe sobre pequenos RNAs. Alguns pequenos RNAs já foram identificados em *A. pleuropneumoniae* por Rossi et al (2016) através de análises de bioinformática e confirmados experimentalmente. Além destes, ensaios de Co-imunoprecipitação em *A. pleuropneumoniae* (Rossi, 2015) também identificaram pequenos RNAs para a espécie. Porém, pouco se sabe sobre o papel destes pequenos RNAs na célula.

Os sRNAs são classificados em quatro classes: RNAs moduladores da atividade de proteínas, CRISPRs, pequenos RNAs *cis* e *trans* (Storz et al., 2011).Os pequenos RNAs moduladores da atividade de proteínas não fazem pareamentos de base, eles interagem com proteínas e modificam suas atividades. CRISPR está envolvido na resistência a bacteriófagos e na prevenção da conjugação de plasmídeos. Os pequenos RNAs *cis* são transcritos a partir da fita oposta ao seu RNA alvo e possui complementaridade a esse alvo, os pequenos RNAs *trans* são codificados em localizações diferentes pelo genoma, geralmente apresentando complementariedade parcial aos seus alvos (Melamedet al., 2016). Devido a essa característica apresentada pelos sRNAs *trans,* eles podem possuir uma gama extensa de alvos, e muitos dos quais dependem da ação de chaperonas de RNA, como a proteína Hfq, para mediar a interação destes com os seus respectivos alvos (Wagner e Romby, 2015), ou a proteína ProQ (Chaulk et al., 2011).

Para sobressair durante a infecção, as bactérias precisam se adaptar rapidamente às alterações do ambiente em que se encontram no hospedeiro. Com isso, a presença de pequenos RNAs associados a OMVs é uma interessante estratégia que pode favorecer a

infecção da bactéria no hospedeiro, contribuindo assim para a virulência da bactéria (Koeppen et al., 2016).

**Referências**


Andrade, J.M., Pobre, V., Silva, I.J., Domingues, S., Arraiano, C.M. 2012. The role of 3'-5' exoribonucleases in RNA degradation. Progress in Molecular Biology and Translacional Science 85: 187–229.

Antenucci, F., Fougeroux, C., Bossé, J.T., Magnowska, Z., Roesch, C., Langford, P., Holst, P.J., Bojesen, A.M. 2017. Identifcation and characterization of serovar-independent immunogens in *Actinobacillus pleuropneumoniae.* Veterinary Research 48:74.

Antenucci, F., Fougeroux, C., Deeney, A., Ørskov, C., Rycroft, A., Holst, P.K., Bojesen, A.M. 2018. In vivo testing of novel vaccine prototypes against *Actinobacillus pleuropneumoniae*. Veterinary Research 49:4.

Associação Brasileira da Indústria Produtora e Exportadora da Carne Suína (ABIPECS). http://www.abipecs.org.br/ acessado em 10/01/2018.

Berleman, J., Auer, M. 2013. The role of bacterial outer membrane vesicles for intra- and interspecies delivery. Enviromental Microbiology 15:347–354.

Blackall, P.J., Klaasen, H.B.L.M., Van den Bosch, H., Kuhnert, P., Frey, J. 2002. Proposal of a new serovar of *Actinobacillus pleuropneumoniae*: serovar 15. Veterinary Microbiology 84:47-52.

Bossé, J.T., Janson, H., Sheehan, B.J., Beddek, A.J., Rycroft A.N., Kroll, S., Langford, P.R. 2002. *Actinobacillus pleuropneumoniae*: pathobiology and pathogenesis of infection. Microbes and Infection v.4.p.225-235.

Bossé, J.T., Li, Y. S., Srkzi, R., Fodor, L., Lacouture, S., Gottschalk, M., Amoribieta, M.C., Angen, O., Nedbalcova, K., Holden, M, T, G., Maskell, D. J., Tucker, A. W., Wren, B. W., Rycroft, A. N., Langford, P. R., Proposal of serovars 17 and 18 of *Actinobacillus pleuropneumoniae* based on serological and genotypic analysis, Veterinary Microbiology (2010), https://doi.org/10.1016/j.vetmic.2018.02.019

Brennan, R.G., Link, T.M. 2007. Hfq structure, function and ligand binding. Current Opinion in Microbiology 10: 125–133.

Burdett, I. D., and Murray, R. G. 1974. Electron microscope study of septum formation in *Escherichia coli* strains B and B-r during synchronous growth. Journal of Bacteriology 119, 1039–1056.

Chatterjee, S. N., and Das, J. 1967. Electron microscopic observations on the excretion of cell-wall material by *Vibrio cholerae*. Journal of General Microbiology 49, 1–11.

Chaulk, S. G., Smith-Frieday, M. N., Arthur, D. C., Culham, D. E., Edwards, R. A., Soo, P., Frost, L.S., Keates, R.A., Glover, J.N., Wood, J.M. 2011. ProQ is an RNA chaperone that controls ProP levels in *Escherichia coli*. Biochemistry 50, 3095–3106.

Chiers, K., De Waele, T., Pasmans, F., Ducatelle, R., Haesebrouck, F. 2010. Virulence factors of *Actinobacillus pleuropneumoniae* involved in colonization, persistence and induction of lesions in its porcine host. Veterinary Research 41, 65–80.

Coelho, A. C., Vieira-Brito, F. J., Vieira-Brito, M. G., Rodrigues, J. 2004. Pleuropneumonia suína causada por *Actinobacillus pleuropneumoniae*- diagnostico e estratégias de controlo. Revista Portuguesa de Ciências Veterinárias 99(552): 193-198.

Ding, Y., Davis, B.M., Waldor, M.K. 2004. Hfq is essential for *Vibrio cholerae* virulence and downregulates sigma expression. Molecular Microbiology 53: 345–354.

Dom, P. Haesebrouck, F., Ducatelle, R., Charlier, G. 1994. In vivo association of *Actinobacillus pleuropneumoniae* serotype 2 with the respiratory epithelium of pigs. Infection Immunity 62, 1262–1267.

Dorward, D.W., Garon, C.F., Judd, R.C. 1989. Export and intercellular transfer of DNA via membrane blebs of *Neisseria gonorrhoeae*. Journal of Bacteriology 171:2499–2505.

Duff, J.P., Scott, W.A., Wilkes, M.K., Hunt, B. 1996. Otitis in a weaned pig: a new pathological role for *Actinobacillus (Haemophilus) pleuropneumoniae*, Veterinaty Record 139, 561–563.

Eddy, J. L., Gielda, L. M., Caulfield, A. J., Rangel, S. M. & Lathem, W. W. 2014. Production of outer membrane vesicles by the plague pathogen *Yersinia pestis*. PLoS ONE 9, e107002.

Elhenawy, W., Bording-Jorgensen, M., Valguarnera, E.,Haurat, M. F.,Wine, E., and Feldman, M. F. 2016. LPS remodeling triggers formation of outer membrane vesicles in *Salmonella*. MBio 7:e00940-16.

Ellen, A. F., Albers, S-V., Huibers, W., Pitcher, A. Hobel, C.F., Schwarz, H., Folea, M., Schouten, S., Boekema, E.J., Poolman, B., Driessen, A.J. 2009. Proteomic analysis of secreted membrane vesicles of archaeal *Sulfolobus* species reveals the presence of endosome sorting complex components. Extremophiles13, 67–79.

Ellis, T. N. & Kuehn, M. J. 2010. Virulence and immunomodulatory roles of bacterial outer membrane vesicles. Microbiology and Molecular Biology Reviews 74, 81–94.

Fernandez-Rojas, M.A., Vaca, S., Reyes-Lopez, M., de la Garza, M., Aguilar-Romero, F., Zenteno, E., Soriano-Vargas, E., Negrete-Abascal, E. 2014. Outer membrane vesicles of *Pasteurella multocida* contain virulence factors. Microbiology Open, 3, 711–717.

Fournié, G., Kearsley-Fleet, L., Otte, J., Pfeiffer, D.U. 2015. Spatiotemporal trends in the discovery of new swine infectious agentes. Veterinary Research 46:114.

Franze de Fernandez, M.T., Eoyang, L., August, J.T. 1968. Factor fraction required for the synthesis of bacteriophage Qβ-RNA. Nature 219:588–590.

Franze de Fernandez, M.T., Hayward, W.S., August, J.T. 1972. Bacterial proteins required for replication of phage Q ribonucleic acid. Purification and properties of host factor I, a ribonucleic acid-binding protein. Journal of Biological Chemistry 247:824–831.

Frey, J. 1995. Virulence in *Actinobacillus pleuropneumoniae* and RTX toxins. Trends in Microbiology 3:257–261.

Frey, J. 2011. The role of RTX toxins in host specificity of animal pathogenic *Pasteurellaceae*. Veterinary Microbiology 153, 51–58.

Gangaiah, D., M. Labandeira-Rey, X. Zhang, K. R. Fortney, S. Ellinger, B. Zwickl, B. Baker, Y. Liu, D. M. Janowicz, B. P. Katz, Brautigam, C.A., Munson Jr, R.S., Hansen, E.J., Spinola, S. M. 2014. *Haemophilus ducreyi* Hfq contributes to virulence gene regulation as cells enter stationary phase. MBio 5(1): e01081-13.

Gottschalk, M. 2012. Actinobacillosis. In: L. Karriker, A. Ramirez, K.Schwartz, G. Stevenson & J. Zimmerman (Eds.), Diseases of swine (10th ed., pp. 653–669). Hoboken, NJ: Wiley.

Ghosal, A., Upadhyaya, B.B., Fritz. J.V., Heintz-Buschart, A., Desai, M.S., Yusuf, D., Huang, D., Baumuratov, A., Wang, K., Galas, D., Wilmes, P. 2015. The extracellular RNA complement of *Escherichia coli*. Microbiology Open 4: 252–266.

Gong, H., Vu, G.P., Bai, Y., Chan, E., Wu, R., Yang, E., Liu, F., Lu, S. 2011. A *Salmonella* small non-coding RNA facilitates bacterial invasion and intracellular replication by modulating the expression of virulence factors. PLoSPathogens, 7, e1002120.

Guerrero-Mandujano, A., Hernández-Cortez, C., Antonio Ibarra, J., Castro Escarpulli G. 2017. The Outer Membrane Vesicles: Secretion System Type zero. Traffic Jul;18(7):425-432.

Haurat, M.F., Elhenawy, W., Feldman, M.F. 2015. Prokaryotic membrane vesicles: new insights on biogenesis and biological roles. Biological Chemistry 396(2):95-109.

Hoekstra, D., Van Der Laan, J. W., De Leij, L., Witholt, B. 1976. Release of outer membrane fragments from normally growing *Escherichia coli*. Biochimica et Biophysica Acta 455, 889–899.

Huang, W., Wang, S., Yao, Y., Xia, Y., Yang, X., Li, K., Sun, P., Liu, C., Sun, W., Bai, H., Chu, X., Li, Y., Ma, Y., 2016. Employing *Escherichia coli*-derived outer membrane vesicles as an antigen delivery platform elicits protective immunity against *Acinetobacter baumannii* infection. Scientific Reports. 6, 37242

Jan, A.T., 2017. Outer membrane vesicles (OMVs) of gram-negative bacteria: a perspective update. Frontiers in Microbiology 8, 1053.

Jacques, M., Roy, G. and Mittal, K.R. 1988 Hemagglutinating properties of *Actinobacillus pleuropneumoniae*. Canadian Journal of Microbiology 34, 1046-1049.

Kadurugamuwa, J. L., Beveridge, T. J. 1995. Virulence factors are released from *Pseudomonas aeruginosa* in association with membrane vesicles during normal growth and exposure to gentamicin: A novel mechanism of enzyme secretion. Journal of Bacteriology 177, 3998–4008.

Kakoschke, T.K. Kakoschke, S.C. Zeuzem, C. Bouabe, H. Adler, K. Heesemann, J. Rossier, O. 2016. The RNA Chaperone Hfq Is Essential for Virulence and Modulates the Expression of Four Adhesins in *Yersinia enterocolitica*. Scientific Reports 6, 29275.

Kieselbach, T., Zijnge, V., Granström, E., Oscarsson, J. 2015. Proteomics of *Aggregatibacter actinomycetemcomitans* outer membrane vesicles. PLoS One 10(9):e0138591.

Kim, S.H., Kim, K.S., Lee, S.R., Kim, E., Kim, M.S., Lee, E.Y., Gho, Y.S., Kim, J.W., Bishop, R.E., Chang, K.T. 2009. Structural modifications of outer membrane vesicles to refine them as vaccine delivery vehicles. Biochimica Biophysica Acta 1788, 2150–2159.

Koeppen, K., Hampton, T. H., Jarek, M., Scharfe, M., Gerber, S. A., Mielcarz, D. W., Stanton, B. A. 2016. A novel mechanism of host-pathogen interaction throughs RNA in bacterial outermembrane vesicles. PLoS Pathogens 12, e1005672.

Koo, J. T., Alleyne, T. M., Schiano, C. A., Jafari, N., Lathem, W. W. 2011. Global discovery of small RNAs in *Yersinia pseudotuberculosis* identifies *Yersinia*-specific small, noncoding RNAs required for virulence. Proceedings of the National Academy of Sciences of the United States of America,108, E709–E717.

Kulkarni, H.M., Jagannadham, M.V. 2014. Biogenesis and multifaceted roles of outer membrane vesicles from Gram-negative bacteria. Microbiology 160(10):2109-2121.

Li, L., Huang, D.D., Cheung, M.K., Nong, W.Y., Huang, Q.L, Kwan, H.S. 2013. BSRD: a repository for bacterial small regulatory RNA. Nucleic Acids Research 41:233–238.

Li, Z., Clarke, A.J., Beveridge, T.J. 1998. Gram-negative bacteria produce membrane vesicles which are capable of killing other bacteria. Journal of Bacteriology 180:5478–5483.

Lee, E. Y., Choi, D. Y., Kim, D. K., Kim, J. W., Park, J.O., Kim, S., Kim, S.H., Desiderio, D.M., Kim, Y.K., Kim, K.P., Gho, Y.S. 2009. Gram-positive bacteria produce membrane vesicles: Proteomics-based caracterization of *Staphylococcus aureus*-derived membrane vesicles. Proteomics 9, 5425–5436.

Loera-Muro, V., Jacques, M., Tremblay, Y., Avelar-González, F., Loera-Muro, A., Ramírez-López, E., Medina-Figueroa, A., González-Reynaga, H., Guerrero-Barrera,

A. 2013. Detection of *Actinobacillus pleuropneumoniae* in drinking water from pig farms. Microbiology 159, 536-544.

Lloubes, R., Bernadac, A., Houot, L., Pommier, S. 2013. Non classical secretion systems. Research in Microbiology 164(6):655-663.

Mashburn-Warren, L., McLean, R. J., Whiteley, M. 2008. Gram-negative outer membrane vesicles: beyond the cell surface. Geobiology 6, 214–219.

McCaig, W. D., Loving, C. L., Hughes, H. R., Brockmeier, S. L. 2016. Characterization and vaccine potential of outer membrane vesicles produced by *Haemophilus parasuis*. PloSOne 11, e0149132.

McMahon, K. J., Castelli, M. E., Garcia Vescovi, E., Feldman, M. F. 2012. Biogenesis of outer membrane vesicles in *Serratia marcescens* is thermoregulated and can be induced by activation of the Rcs phosphorelay system. Journal of Bacteriology 194, 3241–3249.

Melamed, S., Peer, A., Faigenbaum-Romm, R., Gatt, Y.E., Reiss, N., Bar, A., Altuvia, Y., Argaman, L., Margalit, H. 2016. Global mapping of small RNA-target interactions in bacteria. Molecular Cell 63: 884–897.

Michaux, C., Verneuil, N., Hartke, A., Giard, J.C. 2014. Physiological roles of small RNA molecules. Microbiology 160, 1007–1019.

Mohanty, B.K., Maples, V.F., Kushner S.R. 2004. The Sm-like protein Hfq regulates polyadenylation dependent mRNA decay in *Escherichia coli*. Molecular Microbiology 54: 905–920.

Negrete-Abascal, E., Garcia, R. M., Reyes, M. E., Godinez, D., de laGarza, M. 2000. Membrane vesicles released by *Actinobacillus pleuropneumoniae* contain proteases and Apx toxins. FEMS Microbiology Letters 191, 109–113.

Niven, D.F., Lévesque, M. 1988. V-factor-dependent growth of *Actinobacillus pleuropneumoniae* biotype 2 (Bertschinger 2008/76). International Journal of Systematic Bacteriology, 38, 319–320.

Park, S.B., Jang, H.B., Nho, S.W., Cha, I.S., Hikima, J., Ohtani, M., Aoki, T., Jung, T.S. 2011 Outer membrane vesicles as a candidate vaccine against edwardsiellosis. PLoS One 6: e17629.

Pathirana, R., Kaparakis-Liaskos, M. 2016. Bacterial membrane vesicles: biogenesis, immuneregulation and pathogenesis. Cell Microbiology 18, 1518–1524.

Pereira, M. F., Rossi, C. C., de Queiroz, M. V., Martins, G. F., Isaac, C., Bosse, J. T.,Li, Y., Wren, B. W., Terra, V. S., Cucuui, J., Langford, P. R., and Bazzolli, D. M. 2015. *Galleria mellonella* is an effective model to study *Actinobacillus pleuropneumoniae* infection. Microbiology 161(2), 387-400.

Prados-Rosales, R., Baena, A., Martinez, L. R., Luque-Garcia, J., Kalscheuer, R., Veeraraghavan, U., Camara, C., Nosanchuk, J.D., Besra, G.S., Chen, B., Jimenez, J., Glatman-Freedman, A., Jacobs, W.R. Jr., Porcelli, S.A., Casadevall, A. 2011. *Mycobacteria* release active membrane vesicles that modulate immune responses in a TLR2-dependent manner in mice. Journal of Clinical Investigation 121, 1471–1483.

Régnier P, Hajnsdorf E. 2009. Poly(A)-assisted RNA decay and modulators of RNA stability. Progress in Molecular Biology and Translacional Science 85: 137–185.

Rivera, J., Cordero, R. J., Nakouzi, A. S., Frases, S., Nicola, A., Casadevall, A. 2010. *Bacillus anthracis* produces membrane-derived vesicles containing biologically active toxins. Proceedings of the National Academy of Sciences of the United States of America 107, 19002– 19007.

Roier, S., Leitner, D.R., Iwashkiw, J., Schild-Prufert, K., Feldman, M.F., Krohne, G., Reidl, J., Schild, S. 2012. Intranasal immunization with nontypeable *Haemophilus influenzae* outer membrane vesicles induces cross-protective immunity in mice. PloSOne, 7, e42664.

Roier, S., Fenninger, J.C., Leitner, D.R., Rechberger, G.N., Reidl, J.,Schild, S. 2013. Immunogenicity of *Pasteurella multocida* and *Mannheimia haemolytica* outer membrane vesicles. International Journal of Medical Microbiology 303, 247–256.

Roier, S., Blume, T., Klug, L., Wagner, G.E., Elhenawy, W., Zangger, K., Prassl, R., Reidl, J., Daum, G., Feldman, M.F., Schild, S. 2015. A basis for vaccine development: Comparative characterization of *Haemophilus influenzae* outer membrane vesicles. International Journal of Medical Microbiology, 305, 298–309.

Roier, S., Zingl, F.G., Cakar, F., Durakovic, S., Kohl, P., Eichmann, T.O., Klug, L., Gadermaier, B., Weinzerl, K.,Prassl, R., Lass, A., Daum, G., Reidl, J., Feldman, M.F., Schild, S. 2016. A novel mechanism for the biogenesis of outer membrane vesicles in Gram-negativebacteria. Nature Communicatios 7:10515

Rosendal, S., MacInnes, J.I. 1990. Characterization of anattenuated strain of *Actinobacillus pleuropneumoniae* serotype 1. American Journal of Veterinary Research, 51, 711-717.

Rossi, C.C., Vicente, A.M., Guimarães, W.V., Araújo, E.F., Queiroz, M.V., Bazzolli, D.M.S. 2013. Face to face with *Actinobacillus pleuropneumoniae*: landscape of the distribution of clinical isolates in southeastern Brazil. African Journal of Microbiology Research 7:2916 –2924.

Rossi, C.C., Bossé, J.T., Li, Y., Witney, A.A., Gould, K.A., Langford, P.R., Bazzolli, D.M.S. 2016. A computational strategy for the search of regulatory small RNAs in *Actinobacillus pleuropneumoniae*. RNA, Sep; 22(9): 1373–1385

Sarkozi, R., Makrai, L., Fodor, L. 2015. Identification of a proposed new serovar of *Actinobacillus pleuropneumoniae*: Serovar 16. Acta Veterinaria Hungarica 63, 444–450.

Sassu, E. L., Bosse, J. T., Tobias, T. J., Gottschalk, M., Langford, P. R., and Hennig-Pauka, I. 2017. Update on *Actinobacillus pleuropneumoniae*-knowledge, gapsand challenges. Transboundary and Emerging Diseases. doi: 10.1111/tbed.12739.

Schaller, A., Kuhn, R., Kuhnert, P., Nicolet, J., Anderson, T.J., MacInnes, J.I., Segers, R.P., Frey, J. 1999. Characterization of apxIVA, a new RTX determinant of *Actinobacillus pleuropneumoniae*. Microbiology 145, 2105–2116.

Schild, S., Nelson, E.J., Bishop, A.L., Camilli, A. 2009. Characterization of *Vibrio cholerae* outer membrane vesicles as a candidate vaccine for cholera. Infection Immunity 77 , 472– 484.

Schooling, S.R., Beveridge, T.J. 2006. Membrane vesicles: an overlooked component of the matrices of biofilms. Journal of Bacteriology 188:5945–5957.

Sidibe, M., Messier, S. Lariviere, S., Gottschalk, M., Mittal, K.R. 1993. Detection *of Actinobacillus pleuropneumoniae* in the porcine upper respiratory tract as a

complement to serological tests. Canadian Journal of Veterinary Research, 57, 204–208.

Sittka, A., Pfeiffer, V., Tedin, K. & Vogel, J. 2007. The RNA chaperone Hfq is essential for the virulence of *Salmonella typhimurium*. Molecular Microbiology 63, 193–217.

Sjöström, A.E., Sandblad, L., Uhlin, B.E., Wai, S.N. 2015. Membrane vesicle-mediated release of bacterial RNA. Scientific Reports 5: 15329.

Sousa, S.A., Ramos, C.G., Moreira, L.M., Leitao, J.H. 2010. The *hfq* gene is required for stress resistance and full virulence of *Burkholderia cepacia* to the nematode *Caenorhabditis elegans. Microbiology* vol. 156 (pg. 896-908)

Storz, G., Vogel, J., Wassarman, K.M. 2011. Regulation by small RNAs in bacteria: expanding frontiers. Molecular Cell 43:880–891.

Subashchandrabose, S., Leveque, R. M., Kirkwood, R. N., Kiupel, M. & Mulks, M. H. 2013. The RNA chaperone Hfq promotes fitness of *Actinobacillus pleuropneumoniae* during porcine pleuropneumonia. Infection Immunity 81, 2952–2961.

Taylor, D. 1999. *Actinobacillu pleuropneumoniae*. In: Diseases of Swine. Editores: B. E. Straw, S. D'Allaire, W.L. Mengeling e D. J. Taylor. Oxford: Blackwell Science, 343- 354.

Thay, B., Damm, A., Kufer, T.A., Wai, S.N., Oscarsson, J. 2014. *Aggregatibacter actinomycetemcomitans* outer membrane vesicles are internalized in human host cells and trigger NOD1-and NOD2-dependent NF-κB activation. Infection Immunity 82(10):4034-4046.

Turnbull, L., Toyofuku, M., Hynen, A.L., Kurosawa, M., Pessi, G., Petty, N.K. Osvath, S.R., Cárcamo-Oyarce, G., Gloag, E.S., Shimoni, R., Omasits, U., Ito, S., Yap, X., Monahan, L.G., Cavaliere, R., Ahrens, C.H., Charles, I.G., Nomura, N., Eberl, L., Whitchurch, C.B. 2016. Explosive cell lysis as a mechanism for the biogenesis of bacterial membrane vesicles and biofilms. Nature Communications 7: 11220.

Van der Pol, L., Stork, M., Van der Ley, P. 2015. Outer membrane vesicles as platform vaccine technology. Biotechnology Journal 10, 1689–1706

Vaz, C.S.L.; Silva, S.C. 2004. Aspectos recentes da patogênese e diagnóstico da pleuropneumonia suína. Ciência Rural, v. 34, n. 2, p. 635-643.

Vogel, J.; Luisi, B.F. 2011. Hfq and its constellation of RNA. Nature Reviews Microbiology 9 pp. 578-589

Wagner, E.G., Romby, P. 2015.Small RNAs in bacteria and archaea: who they are, what they do, and how they do it. Advances in Genetics 90:133–208.

Westermann, A.J., Förstner, K.U., Amman, F., Barquist, L., Chao, Y., Schulte, L.N., Mülle r, L. 2016. Reinhardt, R., Stadler, P.F., Vogel, J. Dual RNA-seq unveils noncoding RNA functions in host-pathogen interactions. Nature 529: 496–501.

Wilusz, C.J., Wilusz, J. 2005. Eukaryotic Lsm proteins: lessons from bacteria. Nature Structural and Molecular Biology 12: 1031–1036.

Z. Su, J. Zhu, Z. Xu, R. Xiao, R. Zhou, L. Li, H. Chen. 2016. A transcriptome map of *Actinobacillus pleuropneumoniae* at single-nucleotide resolution using deep RNA-seq. Plos One, 11 (3) p. e0152363.

## Hipótese

A proteína Hfq afeta a morfologia, virulência e o conteúdo de pequenos RNAs das OMVs em *A. pleuropneumoniae* sorotipo 8

## Objetivos

### Geral

Analisar a produção e o conteúdo das OMVs produzidas por *A. pleuropneumoniae* sorotipo 8 e comparar com um mutante *hfq*.

### Específicos

Isolar OMVs produzidas por *A. pleuropneumoniae* WT e Δ*hfq;*

Analisar o perfil de proteínas e a morfologia das OMVs produzidas por *A. pleuropneumoniae* WT e Δ*hfq;*

Analisar a o efeito das OMVs produzidas por *A. pleuropneumoniae* WT e Δ*hfq* em larvas de *Galleria mellonella.*

Verificar a presença e identificar pequenos RNAs nas OMVs produzidas por *A. pleuropneumoniae* WT e Δ*hfq;*

Verificar a presença da proteína Hfq em OMVs produzidas por *A. pleuropneumoniae.*

**Manuscript**

# OMVs in *Actinobacillus pleuropneumoniae*: the effect of Hfq protein in the production and virulence

Written according to The Veterinary Journal


## Abstract

*Actinobacillus pleuropneumoniae* is the causative agent of porcine pleuropneumonia, an important disease responsible for economic losses to the pig farm worldwide. Its virulence is associated with some factors as capsule, biofilm formation and toxins belonging to the RTX family. Moreover, an important protein to *A. pleuropneumoniae* is the protein Hfq, an chaperone of small RNAs which is related with virulence, stress response and membrane composition. However, others factors such as Outer Membrane Vesicles (OMVs) can also be important in this virulence. OMVs is a virulence factor in many Gram-negative bacteria and can transport different molecule as proteins, DNA and RNA. The aim of this work was to analyse OMVs from *A. pleuropneumoniae* serotype 8 wild type and a Δ*hfq*. Our analyzes did not show differences in the proteins profile and morphology between the vesicles produced by the WT and Δ*hfq* strains. However, the virulence analysis developed using the *Galleria mellonella* showed different survival and melanization results by larvae infected with OMVs in comparison to the larvae infected with the WT or Δ*hfq* strains. No difference was observed to the larvae infected with WT OMVs compared to those infected with Δ*hfq* OMVs. In *A. pleuropneumoniae* serovar 8 WT and Δ*hfq* strains's OMVs there were small RNA and they are protected by RNAse degradation. However, no difference was observed to the content small RNAs analyzed between the vesicles from both strains. Furthermore, the Hfq protein was not identified to be associated to the vesicles in the immunoblotting analysis. These results allowed us to conclude that the Hfq protein did not interfere in the production and composition of OMVs by *A. pleuoropneumoniae* and also this is the first report of the presence of regulatory small RNAs within OMVs produced by *A. pleuropneumoniae*.

## Introduction

*Actinobacillus pleuropneumoniae* is a Gram-negative pathogen belonging to the Pasteurellaceae family and it is the causative agent of porcine pleuropneumonia, an economically respiratory disease responsible for considerable costs in pig farm in the world (Sassu et al., 2017; Chiers et al., 2010). *A. pleuropneumoniae* is considered a pathogen exclusive to the respiratory tract of swine, since it does not infect other animals and is not considered zoonotic (Taylor, 1999). The isolates of *A. pleuropneumoniae* can be divided into two biotypes, depending on the requirement of NAD (nicotinamide adenine dinucleotide). The biotype 1 correspond to the NAD dependent serotypes and biotype 2 correspond to those serotypes that can synthesize NAD from precursors (Niven and Levesque, 1988). Additionally, 18 serotypes are described of this bacterium, which are defined based on the antigenic properties of capsule polysaccharides (Sarkozi et al., 2015; Bossé et al., 2018). The virulence of *A. pleuropneumoniae* is multifactorial, involving factors such as presence of capsule, lipopolysaccharides (LPS), transferrin binding proteins and RTX exotoxins (ApxI, ApxII and ApxIII) (Bossé et al., 2002; Chiers et al., 2010; Frey, 2011). Moreover, previously studies have showed the importance of the Hfq protein in the virulence and stress resistance of *A. pleuropneumoniae*.

The Hfq is a hexameric protein that was discovered in the mid-1960 in a study analyzing proteins required for replication of Q-beta bacteriophage in *Escherichia coli* (Franze de Fernandez et al., 1968, 1972). Its role in the bacterial was only discovery in the 1990s when became clear the importance of this protein in the regulation of gene expression by interacting with small RNAs. Hfq facilitates the interaction of trans-sRNAs and their respective mRNAs targets by facilitating the base-pairing between them and leading up or down regulation of translation or sRNA stability (Vogel and Luisi, 2011; Storz et al., 2011). Previously studies developed with serotypes 8 and 1 showed an attenuated phenotype of Δ*hfq* strains in *in vivo* analysis to the both serotypes (Subashchandrabose et al., 2013; Pereira et al 2015). Moreover, differene in membrane composition and resistance to stress conditions also were observed to this strains (Subashchandrabose et al., 2013). Another virulence factor that can be considered to *A. pleuropneumoniae* are OMVs (Outer Membrane Vesicles).

OMVs are usually 50-250 nm spherical particles produced during normal bacterial growth and consist of proteins, lipids and lipopolysaccharides (LPS) (Kuehn, 2005). Many studies reported that OMVs deliver virulence factors, modulate the host immune, biofilme formation, killing of competing microorganisms and horizontal gene transfer (Li et al., 1998; Schooling and Beveridge, 2006; Ellis and Kuehn, 2010; Berleman and Auer, 2013). Furthermore, the OMVs are reported to be able of transporting a wide variety of molecules that can correspond to virulence factors and deliver it in the host (Ellis and Kuehn, 2010). The composition of the OMVs allows them to activate the pathways of the innate or acquired immune response in the host cells. These components appear to act in the immune response enhancing the virulence, stimulating the clearance of the pathogen, or both (Ellis and Kuehn, 2010). Many of these molecules correspond to enzymes that can act in the host, specific antigens associated with LPS, proteins, toxins, DNA and RNA (Ellis and Kuehn, 2010; Kaparakis-Liaskos and Ferrero, 2015), including small RNAs, an important element of the gene expression regulation (Koeppen et al., 2016; Choi et al., 2017). As many others Gram-negative and some Gram-positive bacteria, *A. pleuropneumoniae* also produces Outer Membrane Vesicles (OMVs) during grown, although there are a few reports in the literature (Jacques et al., 1988; Rosendal and MacInnes, 1990; Negrete-Abascal et al., 2000, Antenucci et al., 2017; 2018). However, nothing is known about the presence of RNA and also the importance of Hfq protein to these vesicles to this specie.

Major studies involving small RNA were focused in endogenous functions as regulation of metabolism, virulence or stress response. Furthermore, the presence of small RNAs in OMVs had recently been reported in *Pseudomonas aeruginosa* (Koeppen et al., 2016), *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, and *Treponema denticola* (Choi et al., 2017). Small RNAs are non-coding RNAs involved in metabolism, virulence, stress response and growth. Some small RNAs, as the *trans*-sRNAs, require chaperones of RNAs, which helps the interaction of them with different targets, as proteins ProQ (ProP expression regulator) (Chaulk et al., 2011) or Hfq (Host factor Q) (Wagner and Romby, 2015).

To *A. pleuropneumoniae*, few studies have reported the presence of small RNAs (Rossi et al., 2016; Su et al., 2016). However, no information is available about small RNAs package in OMVs secreted by *A. pleuropneumoniae*. Here, we reported for the first

time, the presence of small RNAs in OMVs and the virulence effect of OMVs produced by an *hfq* mutant of *A. pleuropneumoniae*.

## Material and Methods

### Bacterial strains and maintenance conditions

The genetically treatable isolate MIDG2331 from United Kingdom which belongs to serotype 8, the Δ*hfq* and *hfq::*3XFlag isogenic strains were utilized in this study. All mutants were obtained as described by Crispim (2014). All strains were stocked at -80 °C and were grown at 37 °C in a 5% $CO_2$ atmosphere in brain-heart infusion (BHI) medium (Becton Dickinson) supplemented with NAD (10 mg $ml^{-1}$ ) (Sigma-Aldrich).

### Growth curve analysis

The strains were plated in BHI-NAD at 37° C and 5% $CO_2$ atmospheric for 18 hours. Cells were then inoculated in 20 ml of broth and the $OD_{600}$ adjusted to 0.01. The cultures were incubated at 37° C, 180 rpm for 24 hours in aerobic condition.

### Isolation and purification of OMVs

The OMVs of WT, Δ*hfq* and *hfq*::3X Flag were purified as previously described by Antenucci et al., 2017, with some modifications. All the strains were plated in BHI-NAD at 37° C and 5% $CO_2$ atmospheric for 24 hours, inoculated in 20 ml of BHI-NAD, $OD_{600}$ adjusted to 0.01 and incubated overnight. An aliquot from an overnight culture of *A. pleuropneumoniae* was inoculated in 900 ml of a fresh medium adjusting the $OD_{600}$ to 0.01 and cultivated until late exponential phase ($OD_{600}$ ~2.5). The culture supernatant obtained after centrifugation (15 minutes at 5000 g and 4°C) was filtered through a 0.22 µm filter (Millipore, Billerica, MA, USA). The filtrated was loaded in the 1000 kDa dyalisis membrane (Biotech CE Tubing - Spectrumlabs) which was encased in a glass column sealed with transparent film and incubated overnight at 4 °C. The membrane was filled with 500 ml of PBS to wash the filtered and incubated overnight at 4°C. The filtered was dialyzed in PBS over night at 4°C to purify the sample. The samples were filtered in a 0.45 µm filter (Cole-Parmer), concentrated with a 10 kDa Amicon Ultra Centrifugal Filter Units (Millipore, Billerica, MA, USA) and stored at -20°C until the analysis.

**SDS-PAGE electrophoresis and Immunoblotting**

To analyze the proteins profile of OMVs from the wide type, Δ*hfq* and *hfq*::3XFlag strains, a sample of OMVs of each was dissolved in lysis buffer (50mM Tris-Cl (pH 6,8); 100 mM dithiotreitol; 2% SDS; 0,1% bromophenol blue; 10% glycerol) and heated 10 minutes at 100°C. Samples of whole cell extract from the wide type, Δ*hfq* and *hfq*::3XFlag strains also were used as controls. The whole cell extracts were prepared as described below. An aliquot of 1 ml of culture was collected of the culture utilized to OMVs extraction just before the first centrifugation. These samples were centrifuged (5 minutes at 12.000 rpm) and the pellet washed twice with PBS. The samples were lised using Precellys, then centrifuged (10 minutes at 10.000 rpm and 4 °C) and the supernatants were collected.

The samples were analyzed in a 12% polyacrylamide gel and stained with coomassie blue (Sambrook et al., 1990). The presence of the protein Hfq associated with OMVs was assayed by western blotting using OMVs from an Hfq::Flag strain. In this assay, we utilized the Monoclonal ANTI-FLAG® M2, Clone M2 antibody produced in mouse (Cat. Number F1804 – Sigma-Aldrich) according to the manufacturing protocol. As negative controls, we utilized OMVs from a Δ*hfq* strain and total proteins from the Δ*hfq* strain, and as positive control we used total proteins from hfq::3XFlag strain.

**Transmission electron microscopy**

OMVs samples prepared from strains WT and Δ*hfq* were placed on a carbon coated grid, stained with 3% uranyl acetate and analyzed by TEM. The visualization was performed in the Zeiss EM 109 Electron Transmission Microscopy, located at the Microscopy and Microanalysis Center at the Universidade Federal de Viçosa.

**Virulence assay in *Galleria mellonella***

To verify the virulence of the OMVs produced by the Δ*hfq* strain compared to the vesicles produced by the WT strain, we used larvae of the Lepidoptera *Galleria mellonella*, a model of infection already utilized to *A. pleuropneumoniae* (Pereira et al., 2015). Last-instar larvae were used of *G. mellonella* were injected into the first right pro-leg into the haemocel using U-100 ultra fine insulin syringes (31 gauge) (Becton Dickinson). For each experimental replicate were utilized 10 larvae which was infected with 10 µg or 5 µg of OMVs or lyzed OMV by heat treatment (100 °C for 10 minutes) using an inoculum of 10

µl. The samples of OMVs were quantified by proteins content using Bradford reagent. As positive controls, larvae were injected with cells of WT and Δ*hfq*. For this, cells were growed until $OD_{600}$ of 2.0, 1 ml of culture were centrifuged at 10.000 rpm and the supernatant containing OMVs was discarded, the pellet were washed once with Phosphate-buffered saline (PBS) 1X and resuspended in 1 ml of PBS 1X. An inoculum of 10 µl of the samples was injected in the larvae. As negative control, PBS was injected in the larvae, BSA (Bovine Serum Albumin - Sigma) also was utilized as negative control at the same concentrations of OMVs samples. The larvae were incubated at 37 °C and observed for 96 hours. Survival curves were plotted using the Kaplan–Meier method, and differences in survival were calculated by using the log-rank test using Sigma Plot 12.0 (Systat Software Inc., Chicago, IL, USA). A p-value of 0.05 was considered to be statistically significant.

**Identification of RNAs in OMVs and RNAse protection assay**

To verify the presence of RNAs in OMVs, total RNA of the vesicles were extracted using the miRNeasy kit (Qiagen),and samples were then treated with 0,5 µg/ml of RNAse A (Qiagen) for 30 minutes at 37 °C. The result was analyzed in a 10% TBE - urea gel (Sambrook et al., 1990). A RNAse protection assay was developed as previously described (Koeppen et al., 2016) to verify if the RNAs were inside the vesicles and therefore protected from RNAse degradation. Intact OMVs were incubated with 0,5 µg/ml RNase A (Qiagen) for 30 minutes at 37°C. As the control, OMVs were incubated for 1 h at 37°C in the absence of RNaseA. The OMVs treated with RNAse were washed with PBS in a 100 kDa Amicon Ultra Centrifµgal Filter Units (Millipore, Billerica, MA, USA) to remove the RNAse and then the small RNAs were extracted. The result was observed in a 0,8% agarose gel. A histogram of the bands was generated using ImageJ (Schneider et al., 2012) to see the difference in the amount of small RNAs.

**Identification of *A. pleuropneumoniae's* small RNAs in OMVs**

Small RNA was extracted from OMVs of WT and Δ*hfq* strains using the miRNeasy kit (Qiagen) and a DNAse treatment was performed with a TURBO DNA-free kit™ (Ambion, Austin, TX). The cDNA was generated using the ImProm-II™ Reverse Transcription System kit (Promega). To identify the small RNAs, specifics primers designed for 27 small RNAs previously identified (Rossi et al., 2016; Rossi, 2015) (Table 1) were used in PCR

reactions. The PCR reactions were performed with 1 U of GoTaq DNA polymerase (Promega) in a final volume of 25 µL of enzyme buffer containing 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, and 0.2 µM of each primer. The samples were initially denatured at 95°C for 2 min, followed by 35 reaction cycles (94°C for 45 seconds, 45 seconds to annealing (Tm° in the Table 1), and 72°C for 30 sec) and a final extension step at 72°C for 5 min. As a negative control, total RNA-treated DNAse from WT and Δ*hfq* strains was used. As positive controls, cDNA prepared from total RNA of WT and Δ*hfq* strains and 50 ng total DNA from the WT and Δ*hfq* strains were used, total DNA was extracted using the FastDNA SPIN Kit (MP Biomedicals). The small RNAs detected in these PCR reactions were analyzed in the intravesicular sRNAs portion after the RNAse protection analysis to verify if these small RNAs were also packaged inside the vesicles.

**Table 1-** ***Actinobacillus pleuropneumoniae*** **sRNAs primers used in this work.**

| sRNA | Primer name | Sequence (5'-3') | Amplicon lenght (bp) | Annealing (°C) | Reference |
|---|---|---|---|---|---|
| Arrc01 | ARRC01_F<br>ARRC01_R | TGTTGTGTTTGCATATTGGTCTAGG<br>TGGACGGTTATAAACCAAAAAGGT | 122 | 55,6<br>54,9 | Rossi et al., (2016) |
| Arrc02 | ARRC02_F<br>ARRC02_R | TGCTGATTTCAAGGTAAAAGCG<br>GGCTTAAAAGACGAGGGCGA | 130 | 54<br>57,5 | Rossi et al., (2016) |
| Arrc04 | ARRC04_F<br>ARRC04_R | CGCAAAAAGTGCTTGCATTGG<br>GCCTTAAAACTGGTTGCGGG | 140 | 56,3<br>57,1 | Rossi et al., (2016) |
| Arrc05 | ARRC05_F<br>ARRC05_R | CGGTGTGTAAGCGGTCTGAT<br>GGATACCGAGCTTGTATGCCT | 103 | 57,2<br>56,7 | Rossi et al., (2016) |
| Arrc07 | ARRC07_F<br>ARRC07_R | AGGTAGCTGGAGAAGAGCGA<br>TTCTCCCCTGTCCTTTTGCC | 182 | 57,5<br>57,5 | Rossi et al., (2016) |
| Arrc08 | ARRC08_F<br>ARRC08_R | AGAGCAAGCTGATGGTGCTT<br>CGCTTGCATCGCAAGTAGC | 160 | 57,1<br>57,3 | Rossi et al., (2016) |
| Arrc09 | ARRC09_F<br>ARRC09_R | GGGAGATTTGGTAACGATG<br>GGGAGATTTGGCAGAAGTA | 62 | 50,5<br>51,7 | Rossi, (2015) |
| Arrc11 | ARRC11_F<br>ARRC11_R | TGTCCAATAAATAGGCTTCCCA<br>AACTATCCAAATAAAAAGTACGGCT | 126 | 53,9<br>52,8 | Rossi et al., (2016) |
| Arrc12 | ARRC12_F<br>ARRC12_R | AAAGCAATCTACAGGCAC<br>GGCTAAAAAGCAAATCAGG | 47 | 50<br>49,5 | Rossi, (2015) |
| Arrc14 | ARRC14_F<br>ARRC14_R | ACGACTATCTCTTCGACTGCT<br>GCATCAATGTGCGGGCAAAG | 103 | 55,1<br>57,9 | Rossi et al., (2016) |
| Arrc16 | ARRC16_F2<br>ARRC16_R2 | CAACTCTTACGCCTCATTC<br>GGTTTAATCGGTTATGGAC | 64 | 50,7<br>48,1 | Rossi, (2015) |
| Arrc17 | ARRC17F<br>ARRC17R | TTCTTTCTTGCAAAGAACCCGC<br>ATGCTGATCTTGAAAAGCCCG | 100 | 56,4<br>55,9 | Rossi et al., (2016) |
| Arrc18 | ARRC18_F<br>ARRC18_R | GATGAGTCGCAAATTCCC<br>AGAGAAACTCCGCACAAC | 120 | 51,1<br>52,5 | Rossi, (2015) |
| Arrc20 | ARRC20_F | GCATTTGACGCTAAAACGGT | 128 | 54,2 | Rossi et |

| | | | | | |
|---|---|---|---|---|---|
| | ARRC20_R | AATTAGTGGCTCCTCCTGCG | | 57,2 | al., (2016) |
| Arrc21 | ARRC21_F<br>ARRC21_R | GACCCTTTAGAAGGCGTTGC<br>CGCAACGTTAAGGGTCGTTAG | 115 | 56,3<br>56,1 | Rossi et al., (2016) |
| RNA01 | RNA01_F<br>RNA01_R | CTAACTGACAGAATTTATGTAAG<br>ACCAAGAAAGCGATGCCG | 72 | 46,9<br>56,1 | Rossi, (2015) |
| RNA02 | RNA02_F<br>RNA02_R | ACTTAATAAAAAGTGTTGTG<br>AAGCCCCTCAACTTAGG | 76 | 43,7<br>51,2 | Rossi, (2015) |
| RNA03 | RNA03_F<br>RNA03_R | GAATATCGTCTGTTTTGCGGC<br>AATGCGGCACTACTTTTAAGA | 48 | 52,5<br>57,8 | Rossi, (2015) |
| RNA04 | RNA04_F<br>RNA04_R | ACAAACCACCCTTGTTATAG<br>TTCTAAGTCATTTGGATG | 48 | 49,8<br>43,6 | Rossi, (2015) |
| RNA06 | RNA06_F<br>RNA06_R | TCATTGGGGTGCTTTACG<br>TCAGATCAGGTTCTACGG | 55 | 52,6<br>50,5 | Rossi, (2015) |
| RNA10 | RNA10_F<br>RNA10_R | CGATTTAATATTCGGGCACTT<br>CAACTCGTATAGGGCGGT | 95 | 50,8<br>54,1 | Rossi, (2015) |
| RNA12 | RNA12_F<br>RNA12_R | GAGTGTCAGGTTGTTTT<br>GTCAGAAGCTCCTTTTCA | 45 | 46,7<br>49,3 | Rossi, (2015) |
| RNA13 | RNA13_F<br>RNA13_R | GCACATCTTGTTGTCTGA<br>ATTAAGTTAAGGTGGATAC | 80 | 49,9<br>43,5 | Rossi, (2015) |
| RNA14 | RNA14_F<br>RNA14_R | CACATTTTGTTGTCTGATTATTG<br>CATTAAGTTAAGGTAGATACAC | 81 | 48,8<br>45,9 | Rossi, (2015) |

## Results

### Growth curve analysis

The strains showed similar growth in the condition analyzed and the same OD was used to extract the OMVs from the three strains drown until the second half of exponential phase. The growth curve of the strains is showed in the Figure 1.

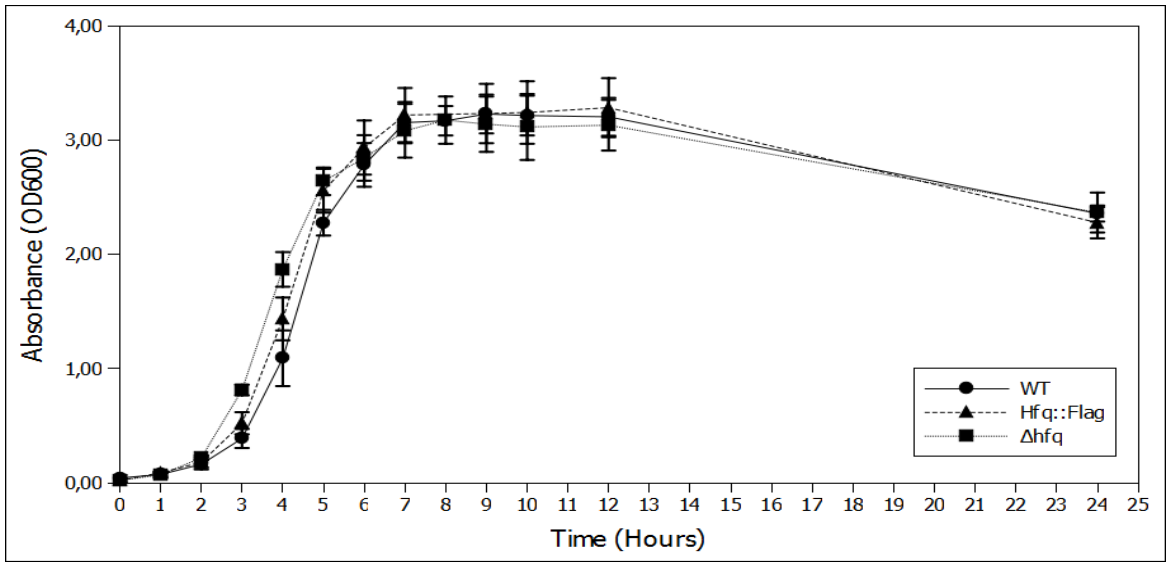


Figure 1– **Growth curve of *A. pleuropneumoniae* strains**. Growth curve of *A. pleuropneumoniae* WT, Δ*hfq* and *hfq*::3XFlag during 24 hours.

**Protein content**

The electrophoretic analysis of OMVs did not show any visible qualitative difference between the proteins profile of the vesicles produced by the different strains (Figure 2). However, is clearly visible that some specifics proteins with approximately 98, 64, 50 and 36 kDa were highly abundant in the vesicles when compared to the whole cell extract.

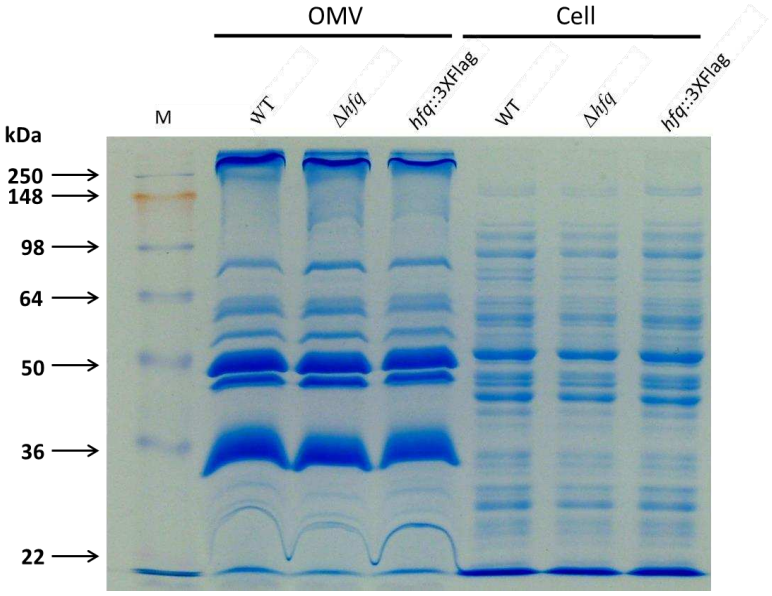


**Figure 2 – Proteins profile of outer membrane vesicles (OMVs) produced by *A. pleuropneumoniae*.** Lanes 1, 2 and 3: OMV samples of WT, Δ*hfq* and *hfq*::3XFlag strains respectively; Lanes 4, 5 and 6: Total proteins of WT, Δ*hfq* and *hfq*::3XFlag strains respectively. M: Ladder See Blue Plus Prestained (Invotrogen)

**Transmission electron microscopy**

The TEM analysis did not show difference among the OMVs produced by WT and Δ*hfq* strains. The most vesicles showed a size of aproximatelly 20-30 nm, but some showed a bigger size, ranging between 50-100 nm to the both strains (Figure 3). The OMVs also showed the same circular morphology to the both strains. Moreover, the vesicles showed some aggregates to the both strains.

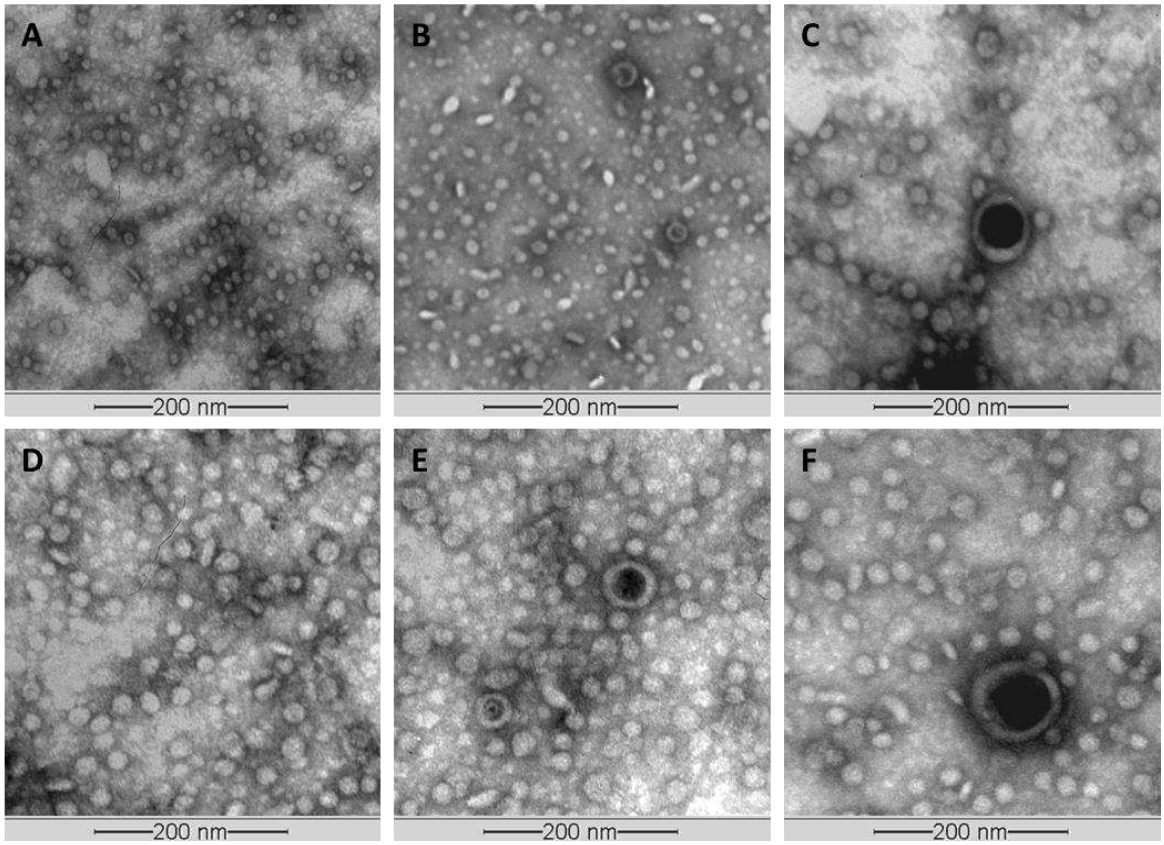


**Figure 3. Transmission electron microscopy (TEM) of OMVs produced by *A. Pleuropneumoniae WT and* Δ*hfq* strains.** A, B and C: WT OMVs; D, E and F: Δ*hfq* OMVs.

**Virulence assay in *Galleria mellonella***

After the infection of the larvae with the intact vesicles or heated vesicles from WT and Δ*hfq*, we did not observe significant difference between these samples (Figure 4). However, we observed a different behavior of the larvae when compared the OMVs and its respective cell. All the larvae infected with the OMVs or heated OMVs from both strains, showed a quick and strong melanization less than 3 hours after injection (Figure 5), but this result was not observed in the larvae infected with the bacteria. Although the OMVs caused

a quickly and strong melanization when compared to the cells, the WT vesicles was not able to kill the larvae as the WT cells did. However the larvae infected with vesicles of the Δ*hfq* did not show a significant difference compared to the larvae infected with Δ*hfq* cell (Figure 4). The larvae infected with the vesicles showed a strong melanization, but almost all showed an active behavior until the last day of assay (96 hours). Different of the larvae infected with the cell, that even those which died, did not show a strong melanization. All the larvae injected with BSA survived and did not show melanization (data not show).

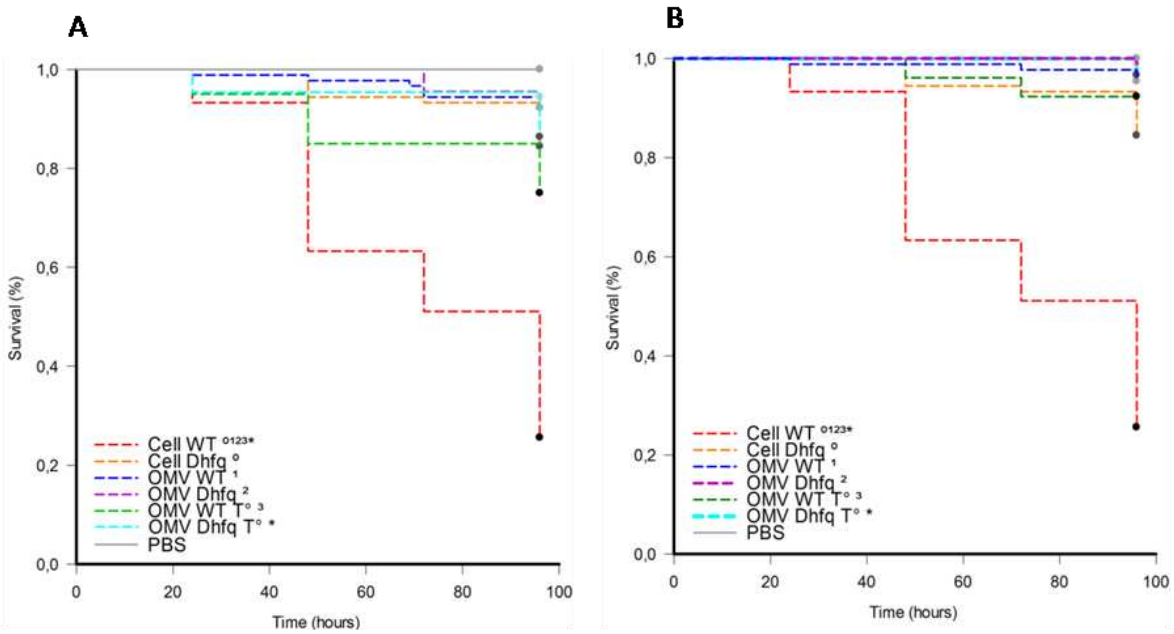


**Figure 4. Survival curve of *Galleria mellonella* infection assay. A**: survival of larvae infected with 10 ug of OMV sample. **B**: survival of larvae infected with 5ug of OMV sample. Cell WT: *A. pleuropneumoniae* WT strain; Cell Dhfq: *A. pleuropneumoniae* Δ*hfq* strain; OMV WT: OMV from *A. pleuropneumoniae* WT strain; OMV Dhfq: OMV from *A. pleuropneumoniae* Δ*hfq* strain; OMV WT T°: heated OMV from *A. pleuropneumoniae* WT strain; OMV Dhfq T°: heated OMV from *A. pleuropneumoniae* Δ*hfq* strain. Significative result is observed only for the WT cell sample in comparison to all of the other samples. Significante is represented by 0,1,2,3 and *.

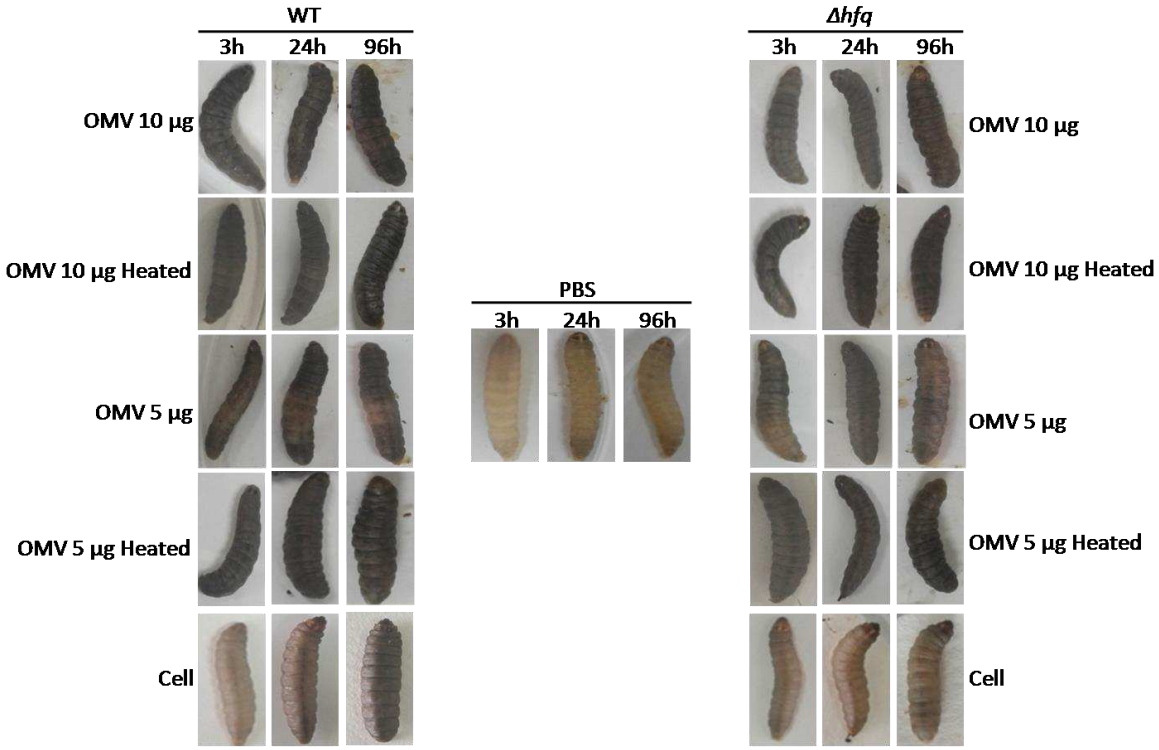


**Figure 5 – Profile of *G. mellonella* larvae melanization during virulence assay using WT and Δ*hfq* OMVs.** Melanization of the larvae infected with OMVs from WT and Δ*hfq* strains, WT and Δ*hfq* strains and PBS in the time of 3 hours, 24 hours and 96 hours.

### Identification of RNAs in OMVs and RNAse protection assay

After the extraction of small RNAs from OMVs, the sample of RNAs showed fragments with a range varying between 20-300 bp. The confirmation of the presence of RNA was proven after the RNAse treatment, in which nothing was observed on the gel (Figure 6A). In the RNAse protection assay, we observed a significant decrease of the amount of small RNAs compared with the non-treated with RNAse sample of OMVs before the RNA extraction (Figure 6B), as the amount of intravesicular sRNAs was to low, we made a histogram to see the result (Figure 6C).

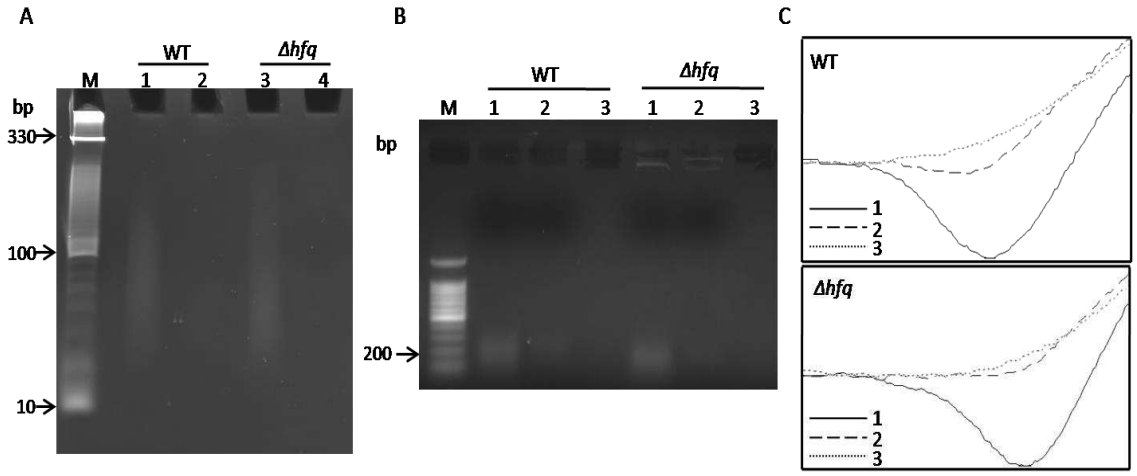


**Figure 6. Identification of small RNAs in OMVs and RNAse protection assay. A**: M: Marker (10 bp DNA Ladder – Thermo Fisher); Lane 1: small RNA extracted from OMV from *A. pleuropneumoniae* WT strain; Lane 2: small RNA treated with RNAse; Lane 3: small RNA extracted from OMV from *A. pleuropneumoniae* Δ*hfq* strain; Lane 4: small RNA treated with RNAse (Polyacrilamide gel 10%). **B**: M: Marker (100 bp DNA Ladder – Promega); Lane 1: small RNA extracted from OMV from *A. pleuropneumoniae* WT strain; Lane 2: small RNA extracted from OMV after RNAse treatment. Lane 3: no sample (Agarose gel 1,5%). **C**: Histogram of the gel B showing an amount of small RNA in the vesicles after the degradation of the outside small RNAs. The histogram image was generated by ImageJ.

**Small RNAs in OMVs**

After synthesis of the complementary strand of the small RNAs present in the OMVs, PCR reactions were conducted with the primers listed in Table 1. All sRNAs with the exception of Arrc02, Arrc16, Arrc18, RNA02, RNA03, RNA04 and RNA13 were identified in all of the biological replicates. So, the primers of these small RNAs identified in the three biological replicates were utilized in new PCR reactions to analyze the presence in the intravesicular sRNAs portion, the results are showed in the Table 2. The same small RNAs were identified in the vesicles of WT or Δ*hfq* strains, either in the total sRNA portion or intravesicular portion, showing no difference in the small RNA presence. With the exception of Arrc09, all of the small RNA identified in the total sRNAs portion of the vesicles also were identified in the intravesicular portion. Although we have identified small RNAs packaged in the vesicles, the western blot analysis did not show the Hfq protein associated to the OMVs (Supplementary Figure S40).

**Table 2 –Identification of small RNAs in OMVs from WT and Δ*hfq* cells.**

| sRNA | Type | Total sRNAs OMVs | | Inside sRNAs OMVs | |
|---|---|---|---|---|---|
| | | WT | Δ*hfq* | WT | Δ*hfq* |
| Arrc01 | *trans* | Yes | Yes | Yes | Yes |
| Arrc02 | *trans* | No | No | NA | NA |
| Arrc04 | *trans* | Yes | Yes | Yes | Yes |
| Arrc05 | *trans* | Yes | Yes | Yes | Yes |
| Arrc07 | *trans* | Yes | Yes | Yes | Yes |
| Arrc08 | *trans* | Yes | Yes | Yes | Yes |
| Arrc09 | *trans* | Yes | Yes | No | No |
| Arrc11 | *trans* | Yes | Yes | Yes | Yes |
| Arrc12 | *trans* | Yes | Yes | Yes | Yes |
| Arrc14 | *trans* | Yes | Yes | Yes | Yes |
| Arrc16 | *trans* | No | No | NA | NA |
| Arrc17 | *trans* | Yes | Yes | Yes | Yes |
| Arrc18 | *trans* | No | No | NA | NA |
| Arrc20 | *trans* | Yes | Yes | Yes | Yes |
| Arrc21 | *trans* | Yes | Yes | Yes | Yes |
| RNA01 | *trans* | Yes | Yes | Yes | Yes |
| RNA02 | *trans* | No | No | NA | NA |
| RNA03 | *trans* | No | No | NA | NA |
| RNA03A | *trans* | Yes | Yes | Yes | Yes |
| RNA04 | *trans* | No | No | NA | NA |
| RNA06 | *trans* | Yes | Yes | Yes | Yes |
| RNA10 | *trans* | Yes | Yes | Yes | Yes |
| RNA12 | *trans* | Yes | Yes | Yes | Yes |
| RNA12A | *trans* | Yes | Yes | Yes | Yes |
| RNA13 | *trans* | No | No | NA | NA |
| RNA14 | *trans* | Yes | Yes | Yes | Yes |
| 5S | *Housekeeping* | Yes | Yes | Yes | Yes |

*The tag for "Yes" is only for those identified in the three biological replicates.
**The small RNAs RNA03A and RNA12A were identified proximal of RNA03 and RNA12 respectively.
NA: not applicable, the small RNAs not identified in the total sRNAs from OMVs were not analysed in the inside portion of small RNAs from OMVs.

## Discussion

In this work, we analyzed the virulence of OMVs and the presence of small RNAs in these vesicles produced by MIDG2331, which belongs to the serotype 8 and, we also compared it to a Δ*hfq* strain. *A. pleuropneumoniae* is recognized for having different virulence factors that causes the disease in the host, but not much is known about the importance of OMVs in this virulence. As Hfq protein correspond an important chaperone that mediates the regulation of many genes by the interaction of a diversity small RNA with mRNAs (Vogel and Luisi, 2011), is possible that some Hfq-binding small RNA in *A. pleuropneumoniae* is a regulator of any gene related with the OMVs production. However, we did not see any difference in the proteins profile and electronic microscopy between the

OMVs produced by the Δ*hfq* strain in comparison to the OMVs produced by the WT. In our analysis, we observed the presence of specific proteins more abundant in the vesicles showing a size approximately of 98, 64, 50 and 36 kDa in comparison to the whole cell, moreover, the vesicles showed a size ranging 20-30 nm in most of the vesicles, with some with a size ranging between 50-100 nm.

Previously studies already showed the importance of Hfq protein in the virulence of this bacterium and resistance in stress conditions. Subashchandrabose et al. (2013) and Pereira et al. (2015) showed and attenuated phenotype of the Δ*hfq* mutant in analysis *in vivo*. The virulence tests in *G. mellonella* showed a strong melanization and some deaths caused by OMVs samples. Antenucci et al. (2018) showed an effect of OMVs in analysis in pigs, which was observed by lesions caused by OMVs from WT strain in the animals.

We also did not see differences between the virulence in *G. mellonella* caused by the OMVs produced from both strains. The same was not observable to the control with bacteria, that the WT strain showed a higher virulence in comparison to the Δ*hfq* strain. The Hfq may be related with many processes, which can explain the reduction of the virulence of the *hfq* mutant in comparison to the WT. Even we did not see any difference in the virulence between the OMVs produced by WT and Δ*hfq*, both samples at the concentrations tested showed a strong and quick melanization in the result of the virulence assay, a product of humoral response of the *G. mellonella* (Eleftherianos and Revenis, 2011, Tsai el al., 2016). However, this strongly and quickly melanization caused by the vesicles was not observed in the larvae infected with the cells for both strains. According to Tsai et al (2016), the complete melanization (black larvae) correlates with death of the larvae soon after, however, it was not observed with the larvae infected with OMVs. Moreover, the larvae infected with the WT strain showed in most larvae just spots or lines of melanization or a weaker total melanization, but killed more larvae than those infected with vesicles. This same degree of melanization was observed to the larvae infected with Δ*hfq* strain, but it did not kill larvae as WT strain did.

The humoral response of *G. mellonella*, besides melanization, involves soluble effector molecules such as complement-like proteins, peptides with antimicrobial activity and products created by proteolytic cascades, which immobilize or kill pathogens (Eleftherianos and Revenis, 2011; Tsai et al., 2016). The melanization response correspond

to a synthesis and deposition of melanin utilized to encapsulate pathogens at the wound site, this process is followed by coagulation opsonization (Tang, 2009), and this formation of melanin is catalyzed by the activity of the phenoloxidase (Soderhall and Cerenius, 1998). This effect could be caused by higher concentration of LPS which is part of the constitution of the vesicles, which can cause a response in *G. mellonella* (Seitz et al., 2003). The LPS correspond to a bacterial pathogen-associated molecular pattern (PAMPs), one of the most important PAMPs known is the lipid A, which is an important feature of Gram-negative bacteria. The LPS causes the inflammatory response, which is observed during endotoxic shock, thereby leading to the activation of the innate response immune system and bacterial clearance (Mogensen, 2009). Moreover, the effect observed in the larvae could be caused by some protein/toxin. Although the presence of an Apx toxins has been identified in OMVs produced by *A. pleuropneumoniae* (Negrete-Abascal et al., 2000), the fact of the heat treated OMV sample showed the same result of the non-heated sample indicates that the strong melanization was not caused by a protein.

The effect of OMVs produced by *A. pleuropneumoniae* in *G. mellonella* is consistent with another studies developed with *Campylobacter jejuni* and *Serratia marcescens* in *G. mellonella* (Elmi et al., 2012; McMahon et al., 2012). Theses studies showed the virulence caused by the OMVs produced by these bacteria. However, the OMVs produced by *A. pleuropneumoniae* did not show a strong virulence observed to *Serratia marcescens*, but, the heat-treated OMVs produced by *A. pleuropneumoniae* showed the same effect observed in the non-heated samples, which was not observed to *Campylobacter jejuni*. An analysis with LPS portion of the OMVs could give us a better understanding about the virulence observed. More analysis needs to be done to give us a better understanding of how the virulence of the OMVs works.

As the OMVs had been described as a secretion system type zero (Guerrero-Mandujano et al., 2017), it is perfectly reasonable the ability of OMVs carries some kinds of molecules as RNA or even small RNAs. According to this, we verified the possibility of OMVs produced by *A. pleuropneumoniae* being able to carry small RNAs and if there were some difference in the presence of the small RNAs in the Δ*hfq* OMVs. To ensure that the presence of small RNA in the vesicles were not by the presence of free small RNAs from cell lysis, we utilized the hydrostatic filtration method in a 1000 kDa tube membrane with

washing step, and then we concentrate the vesicles in a 10 kDa amicon, which ensured us that any free RNA was taken away in this step too. The presence of small RNAs was proven after the treatment of small RNAs extracted with RNAse, in which all the sample was degraded.

We also observed the presence of small RNAs inside the OMVs, in which after the RNAse protection assay, it was possible to observe bands, however, in a visually lower amount in comparison to the small RNAs extracted from OMV untreated with RNAse before sRNA extraction. This reduction of the amount of RNAs may be explained by the presence of some RNAs adhered outside of the vesicles and susceptible to RNAse degradation.

After the PCR reactions with sRNAs of OMVs, of the twenty-seven sRNAs tested, twenty were identified in the total sRNAs from OMVs in the three biological replicates (Table 2). Moreover, nineteen small RNAs were identified in the intravesicular portion of OMVs in the three biological replicates (Table 2). As the results showed, all of the small RNAs analyzed were present in both of OMVs from WT or Δ*hfq* strains, in the total or intravesicular portion of sRNAs. The reason of this can be explained because Hfq protein can protect the small RNA of degradation until this small RNA is necessary to interact to the targets or even target a small RNAs to degradation (Andrade et al., 2012), but in the absence of Hfq protein, these small RNA can be produced and still in the cell. Even with our identification of these small RNAs in the OMVs, is necessary more investigation to know if these small RNAs are packaged randomly or if they are selected packaged in the vesciles to act in the host cell, as observed in *P. aeruginosa* (Koeppen el al., 2016). Besides that, we cannot discard the possibility of some small RNA package in OMVs act as a mechanism of competition with other microbes that inhabit the same ecological niche by regulation of specific targets in these microbes. This idea was proposed by Koeppen el al, (2016) after the prediction of targets of the sRNA52320 (identified in OMVs produced by *P. aeruginosa*) in genomes of bacteria which share the same niche. Even with the need of this information, the possibility of some small RNA interact with the eukaryotic host cell would not be dependent of Hfq protein, as we did not identified this protein in our western blot analysis. We can not discard the participation of another protein in this interaction, even if this protein originates from the bacterium, or from the eukaryotic host cell, since

there are other Sm-like proteins in eukaryotic cells (Wilusz and Wilusz, 2005), but more investigation is needed.

## Conclusions

In summary, no difference was observed in the proteins profile and morphology between OMVs produced by *A. pleuropneumoniae* serotype 8 WT and Δ*hfq*. Although the Hfq protein exerts a strong influence on the virulence of *A. pleuropneumoniae*, its absence did not affect the effect caused by the OMVs, since the virulence of the OMVs from the WT and Δ*hfq* cells showed no difference in the alternative model *G. mellonella*. This is the first report of the existence of sRNAs in vesicles produced by *A. pleuropneumoniae*. OMVs from *A. pleuropneumoniae* contain sRNAs previously identified by our group and showed no difference in the specific content of OMVs from WT and Δ*hfq*. Even with this identification, a complete transcriptome analysis of the OMVs is necessary to provide us a better knowledge of small RNAs and a possible role of it in the virulence of this bacterium.

## Acknowledgments

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**References**


Andrade, J.M., Pobre, V., Silva, I.J., Domingues, S., Arraiano, C.M. 2012. The role of 3'-5' exoribonucleases in RNA degradation. Progress in Molecular Biology and Translacional Science 85: 187–229.

Antenucci, F., Fougeroux, C., Bossé, J.T., Magnowska, Z., Roesch, C., Langford, P., Holst, P.J., Bojesen, A.M. 2017. Identifcation and characterization of serovar-independent immunogens in *Actinobacillus pleuropneumoniae.* Veterinary Research 48:74.

Antenucci, F., Fougeroux, C., Deeney, A., Ørskov, C., Rycroft, A., Holst, P.K., Bojesen, A.M. 2018. In vivo testing of novel vaccine prototypes against *Actinobacillus pleuropneumoniae*. Veterinary Research 49:4.

Berleman, J., Auer, M. 2013. The role of bacterial outer membrane vesicles for intra- and interspecies delivery. Enviromental Microbiology 15:347–354.

Blackall, P.J., Klaasen, H.B.L.M., Van den Bosch, H., Kuhnert, P., Frey, J. 2002. Proposal of a new serovar of *Actinobacillus pleuropneumoniae*: serovar 15. Veterinary Microbiology 84:47-52

Bossé, J.T., Janson, H., Sheehan, B.J., Beddek, A.J., Rycroft A.N., Kroll, S., Langford, P.R. 2002. *Actinobacillus pleuropneumoniae*: pathobiology and pathogenesis of infection. Microbes and Infection v.4.p.225-235.

Bossé, J. T., Soares-Bazzolli, D. M., Li, Y., Wren, B. W., Tucker, A. W., Maskell, D. J., Rycroft, A. N., Langford, P. R. & on behalf of the BRaDP1T Consortium. 2014. The generation of successive unmarked mutations and chromosomal insertion of heterologous genes in *Actinobacillus pleuropneumoniae* using natural transformation. PLoS ONE 9, e111252.

Bossé, J.T., Li, Y. S., Srkzi, R., Fodor, L., Lacouture, S., Gottschalk, M., Amoribieta, M.C., Angen, O., Nedbalcova, K., Holden, M, T, G., Maskell, D. J., Tucker, A. W., Wren, B. W., Rycroft, A. N., Langford, P. R., Proposal of serovars 17 and 18 of *Actinobacillus pleuropneumoniae* based on serological and genotypic analysis, Veterinary Microbiology (2010), https://doi.org/10.1016/j.vetmic.2018.02.019

Chaulk, S. G., Smith-Frieday, M. N., Arthur, D. C., Culham, D. E., Edwards, R. A., Soo, P., Frost, L.S., Keates, R.A., Glover, J.N., Wood, J.M. 2011. ProQ is an RNA

chaperone that controls ProP levels in *Escherichia coli*. Biochemistry 50, 3095–3106.

Choi, J-W, Kim, SC, Hong, S-H, Lee, HJ. 2017. Secretable small RNAs via outer membrane vesicles in periodontal pathogens. Journal of Dental Research 96: 458–66

Crispim, J.S. Envolvimento da chaperona Hfq na virulência de *Actinobacillus pleuropneumoniae*. 2014. 62 p. Dissertation (M.Sc. in Agricultural Microbiology). Universidade Federal de Viçosa, Viçosa.

Eleftherianos, I., Revenis, C. 2011. Role and importance of phenoloxidase in insect hemostasis. Journal of Innate Immunity 3, 28–33.

Ellis, T. N. & Kuehn, M. J. 2010. Virulence and immunomodulatory roles of bacterial outer membrane vesicles. Microbiology and Molecular Biology Reviews 74, 81–94.

Elmi, A., Watson, E., Sandu, P., Gundogdu, O., Mills, D.C., Inglis, N.F., Manson, E., Imrie, L., Bajaj-Elliott, M., Wren, B.W., Smith, D.G.E., Dorrell, N. 2012. *Campylobacter jejuni* outer membrane vesicles play an important role in bacterial interactions with human intestinal epithelial cells. Infection Immunity 80: 4089–4098

Franze de Fernandez, M.T., Eoyang, L., August, J.T. 1968. Factor fraction required for the synthesis of bacteriophage Qβ-RNA. Nature 219:588–590.

Franze de Fernandez, M.T., Hayward, W.S., August, J.T. 1972. Bacterial proteins required for replication of phage Q ribonucleic acid. Purification and properties of host factor I, a ribonucleic acid-binding protein. Journal of Biological Chemistry 247:824–831.

Frey, J. 1995. Virulence in *Actinobacillus pleuropneumoniae* and RTX toxins. Trends in Microbiology 3:257–261.

Ghosal, A., Upadhyaya, B.B., Fritz. J.V., Heintz-Buschart, A., Desai, M.S., Yusuf, D., Huang, D., Baumuratov, A., Wang, K., Galas, D., Wilmes, P. 2015. The extracellular RNA complement of *Escherichia coli*. Microbiology Open 4: 252–266.

Guerrero-Mandujano, A., Hernández-Cortez, C., Antonio Ibarra, J., Castro Escarpulli G. 2017. The Outer Membrane Vesicles: Secretion System Type zero. Traffic Jul;18(7):425-432.

Ho, M.H., Chen, C.H., Goodwin, J.S., Wang, B.Y., Xie, H. 2015. Functional Advantages of *Porphyromonas gingivalis* Vesicles. PLOS ONE 10: e0123448.

Jacques, M., Roy, G. and Mittal, K.R. 1988 Hemagglutinating properties of *Actinobacillus pleuropneumoniae*. Canadian Journal of Microbiology 34, 1046-1049.

Kaparakis-Liaskos, M., & Ferrero, R. L. 2015. Immune modulation by bacterial outer membrane vesicles. Nature Reviews Immunology 15, 375–387.

Koeppen, K., Hampton, T. H., Jarek, M., Scharfe, M., Gerber, S. A., Mielcarz, D. W., Stanton, B. A. 2016. A novel mechanism of host-pathogen interaction throughs RNA in bacterial outermembrane vesicles. PLoS Pathogens 12, e1005672.

Kuehn, M.J.2005. Bacterial outer membrane vesicles and the host-pathogen interaction. Genes and Develpment 9: 2645–2655.

Lee, T. Feig, A.L. 2008. The RNA binding protein Hfq interacts specifically with tRNAs. RNA 14, 514–523.

Li, Z., Clarke, A.J., Beveridge, T.J. 1998. Gram-negative bacteria produce membrane vesicles which are capable of killing other bacteria. Journal of Bacteriology 180:5478–5483.

McMahon, K. J., Castelli, M. E., Garcia Vescovi, E., Feldman, M. F. 2012. Biogenesis of outer membrane vesicles in *Serratia marcescens* is thermoregulated and can be induced by activation of the Rcs phosphorelay system. Journal of Bacteriology 194, 3241–3249.

Mogensen, T. H. 2009. Pathogen recognition and inflammatory signaling in innate immune defenses. Clinical Microbiology Reviews 22, 240–273.

Molecular cloning. 1990. 2ª Edition. Sambrooket al. Cold Spring Harbor Lab. Press.3 Volumes.

Negrete-Abascal, E., Garcia, R. M., Reyes, M. E., Godinez, D., de laGarza, M. 2000. Membrane vesicles released by *Actinobacillus pleuropneumoniae* contain proteases and Apx toxins. FEMS Microbiology Letters 191, 109–113.

Niven, D.F., Lévesque, M. 1988. V-factor-dependent growth of *Actinobacillus pleuropneumoniae* biotype 2 (Bertschinger 2008/76). International Journal of Systematic Bacteriology, 38, 319–320.

Pereira, M. F., Rossi, C. C., de Queiroz, M. V., Martins, G. F., Isaac, C., Bosse, J. T.,Li, Y., Wren, B. W., Terra, V. S., Cucuui, J., Langford, P. R., and Bazzolli, D. M. 2015. *Galleria mellonella* is an effective model to study *Actinobacillus pleuropneumoniae* infection. Microbiology 161(2), 387-400.

Rasmussen, A.A., Johansen, J., Nielsen, J.S., Overgaard, M., Kallipolitis, B., Valentin-Hansen, P. 2009. A conserved small RNA promotes silencing of the outer membrane protein YbfM. Molecular Microbiology 72 566–577.

Roier, S., Blume, T., Klug, L., Wagner, G.E., Elhenawy, W., Zangger, K., Prassl, R., Reidl, J., Daum, G., Feldman, M.F., Schild, S. 2015. A basis for vaccine development: Comparative characterization of Haemophilus influenzae outer membrane vesicles. International Journal of Medical Microbiology, 305, 298–309.

Rosendal, S., MacInnes, J.I. 1990. Characterization of anattenuated strain of *Actinobacillus pleuropneumoniae* serotype 1. American Journal of Veterinary Research, 51, 711-717.

Rossi, C. C. *Identification of small regulatory rnas and development of a molecular marker for the detection of Actinobacillus pleuropneumoniae*. 2015. 116p. Thesis (PhD in Agricultural Microbiology). Universidade Federal de Viçosa, Viçosa.

Rossi, C.C., Bossé, J.T., Li, Y., Witney, A.A., Gould, K.A., Langford, P.R., Bazzolli, D.M.S. 2016. A computational strategy for the search of regulatory small RNAs in *Actinobacillus pleuropneumoniae*. RNA, Sep; 22(9): 1373–1385

Sarkozi, R., Makrai, L., Fodor, L. 2015. Identification of a proposed new serovar of *Actinobacillus pleuropneumoniae*: Serovar 16. Acta Veterinaria Hungarica 63, 444–450.

Sassu, E. L., Bosse, J. T., Tobias, T. J., Gottschalk, M., Langford, P. R., and Hennig-Pauka, I. 2017. Update on *Actinobacillus pleuropneumoniae*-knowledge, gapsand challenges. Transboundary and Emerging Diseases. doi: 10.1111/tbed.12739.

Schneider, C.A., Rasband, W.S., Eliceiri, K.W. 2012. "NIH Image to ImageJ: 25 years of image analysis". Nature Methods 9, 671-675.

Schooling, S.R., Beveridge, T.J. 2006. Membrane vesicles: an overlooked component of the matrices of biofilms. Journal of Bacteriology 188:5945–5957.

Seitz, V., Clermont, A., Wedde, M., Hummel, M., Vilcinskas, A., Schlatterer, K., Podsiadlowski, L. 2003. Identification of immunorelevant genes from greater wax moth (*Galleria mellonella*) by a subtractive hybridization approach. Developmental and Comparative Immunology 27:207–215.

Sjöström, A.E., Sandblad, L., Uhlin, B.E., Wai, S.N. 2015. Membrane vesicle-mediated release of bacterial RNA. Scientific Reports 5: 15329.

Soderhall, K., Cerenius, L. 1998. Role of the prophenol oxidase activating system in invertebrate immunity. Current Opinion in Immunology 10:23-8;

Subashchandrabose, S., Leveque, R. M., Kirkwood, R. N., Kiupel, M. & Mulks, M. H. 2013. The RNA chaperone Hfq promotes fitness of *Actinobacillus pleuropneumoniae* during porcine pleuropneumonia. Infection Immunity 81, 2952–2961.

Storz, G., Vogel, J.,Wassarman, K.M. 2011. Regulation by small RNAs in bacteria: expanding frontiers. Molecular Cell 43:880–891.

Tang H. Regulation and function of the melanization reaction in *Drosophila*. 2009. Fly (Austin) 3:105-11.

Taylor, D. 1999. *Actinobacillus pleuropneumoniae*. In: Diseases of Swine. Editores: B. E. Straw, S. D'Allaire, W.L. Mengeling e D. J. Taylor. Oxford: Blackwell Science, 343- 354.

Tsai, C. J., Loh, J. M., Proft, T. 2016. *Galleria mellonella* infection models for the study of bacterial diseases and for antimicrobial drug testing. Virulence 1–16.

Udekwu, K.I., Darfeuille, F., Vogel, J., Reimegård, J., Holmqvist, E., and Wagner, E.G. 2005. Hfq-dependent regulation of OmpA synthesis is mediated by an antisense RNA. Genes and Development 19: 2355–2366.

Vogel, J.; Luisi, B.F. 2011. Hfq and its constellation of RNA. Nature Reviews Microbiology 9 pp. 578-589

Wagner, E.G., Romby, P. 2015.Small RNAs in bacteria and archaea: who they are, what they do, and how they do it. Advances in Genetics 90:133–208.

Wilusz, C.J., Wilusz, J. 2005. Eukaryotic Lsm proteins: lessons from bacteria. Nature Structural and Molecular Biology 12: 1031–1036.

Z. Su, J. Zhu, Z. Xu, R. Xiao, R. Zhou, L. Li, H. Chen. 2016. A transcriptome map of *Actinobacillus pleuropneumoniae* at single-nucleotide resolution using deep RNA-seq. Plos One, 11 (3) p. e0152363.

**Supplementary material**

**Figures 1- 28**: Results of PCR reactions with the primers to detection of total sRNAs in the total portion of sRNAs from OMVs.

**General subtitle of the Figures 1-27**: 1- cDNA of small RNA sample of OMV from WT cell; 2- cDNA of total small RNA from WT cell; 3- DNA sample from WT cell; 4- small RNA from WT cell treated with DNAse; 5- cDNA of small RNA sample of OMV from Δ*hfq* cell; 6- cDNA of total small RNA from Δ*hfq*cell; 7- DNA sample from Δ*hfq* cell; 8- small RNA from Δ*hfq*celltreated with DNAse; B- Blank. All the PCR reactions of the three replicates were runned in 1,5% agarose gel.

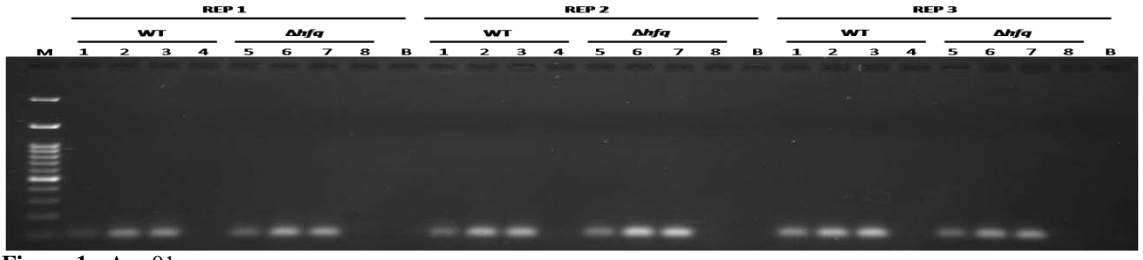



**Figure 1** - Arrc01

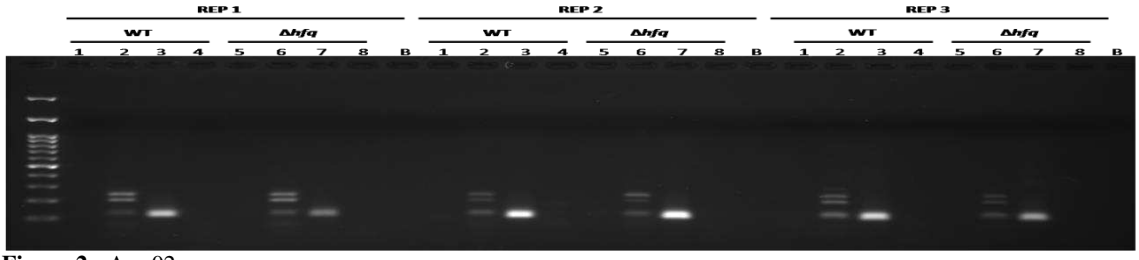



**Figure 2** - Arrc02

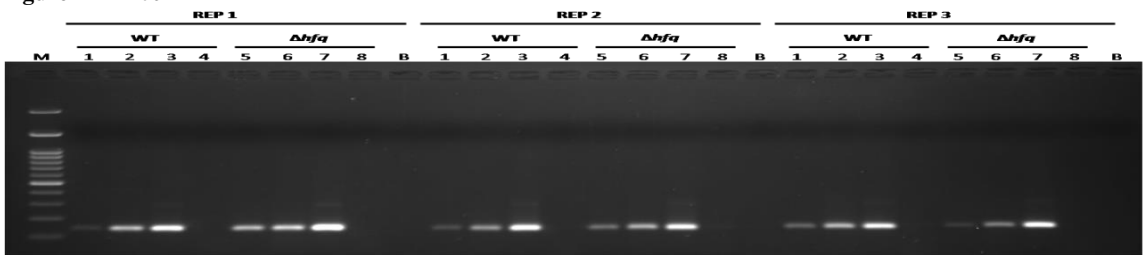



**Figure 3** - Arrc04

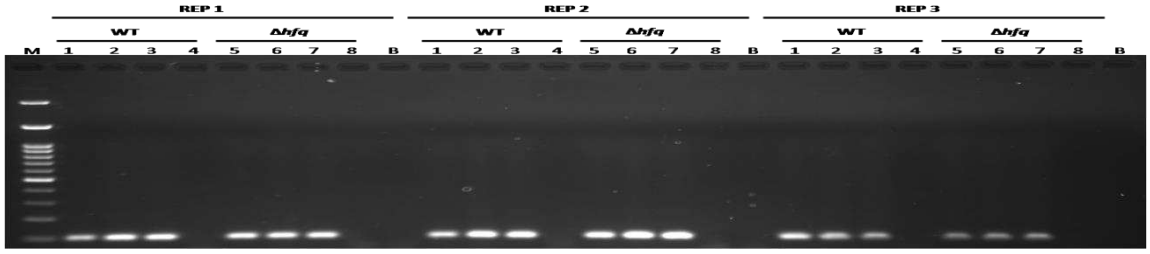



**Figure 4** - Arrc05

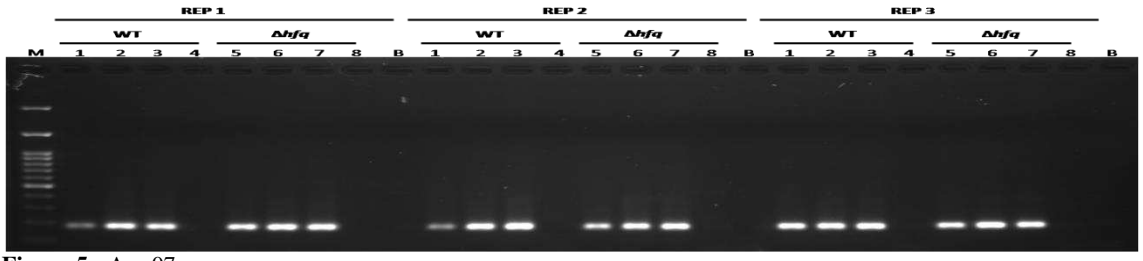



**Figure 5** - Arrc07

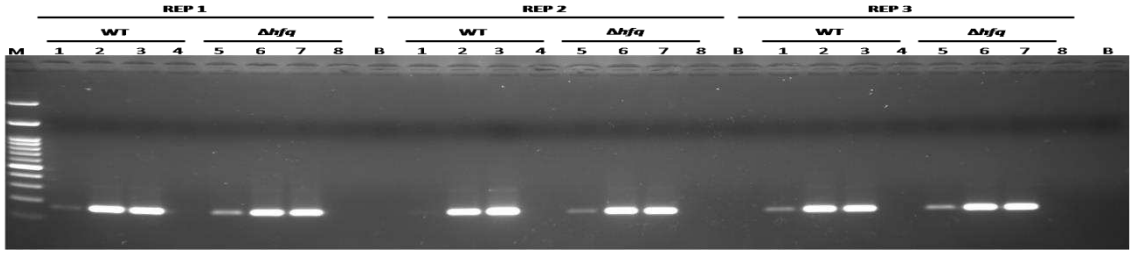



**Figure 6** - Arrc08

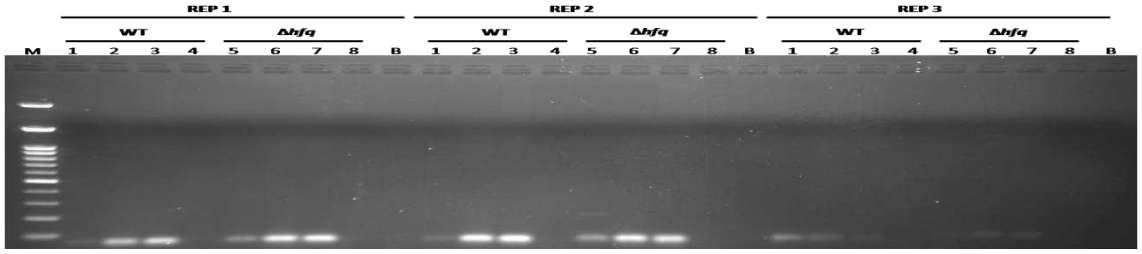



**Figure 7** - Arrc 09

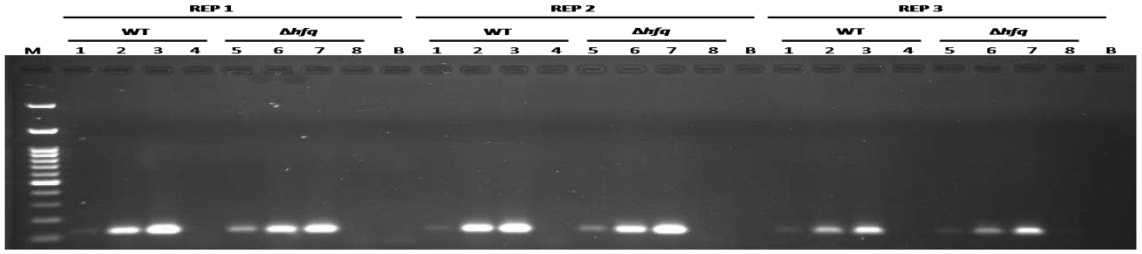



**Figure 8** - Arrc11

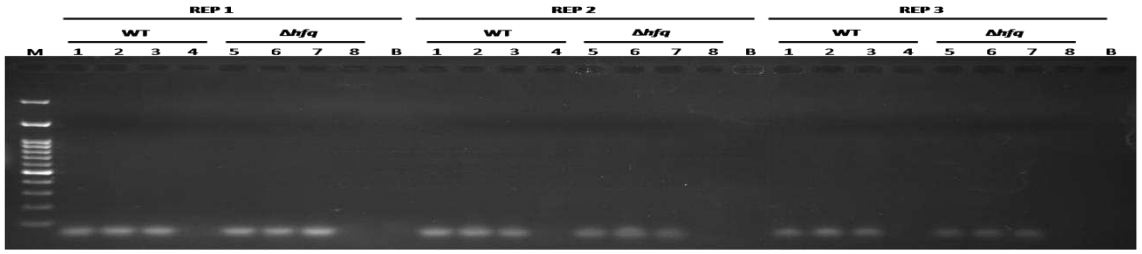



**Figure 9** - Arrc12

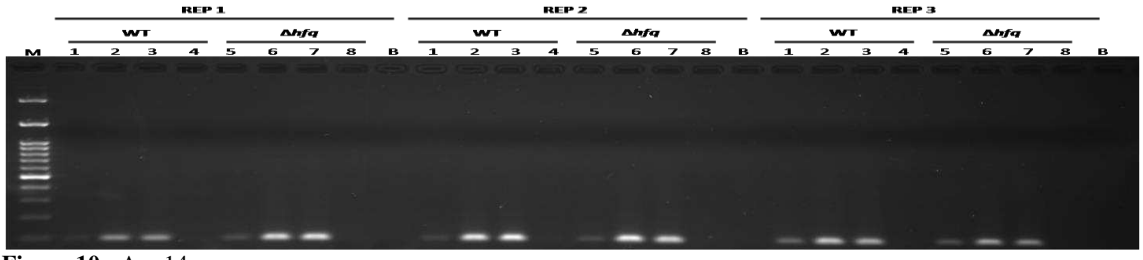



**Figure 10** - Arrc14

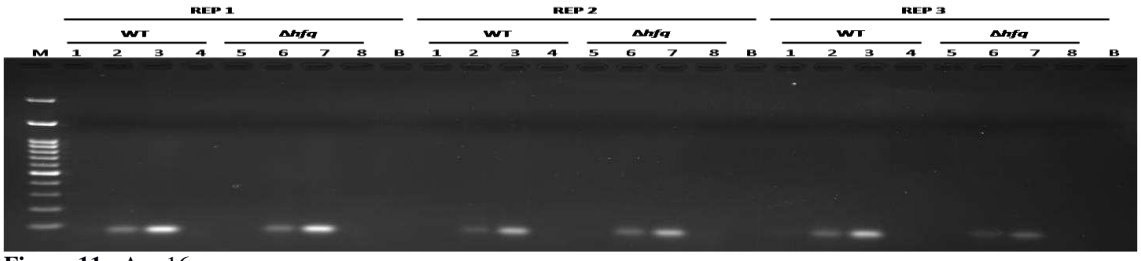



**Figure 11** - Arrc16

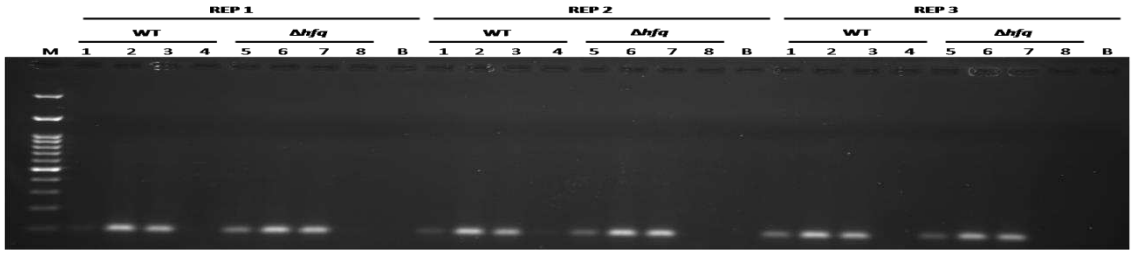



**Figure 12** - Arrc17

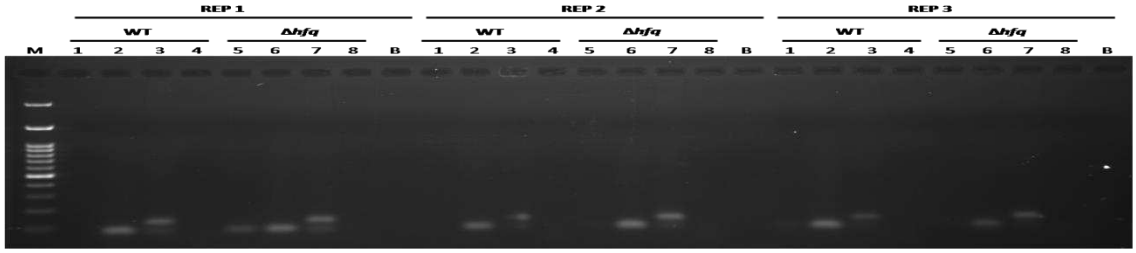



**Figure 13** - Arrc18

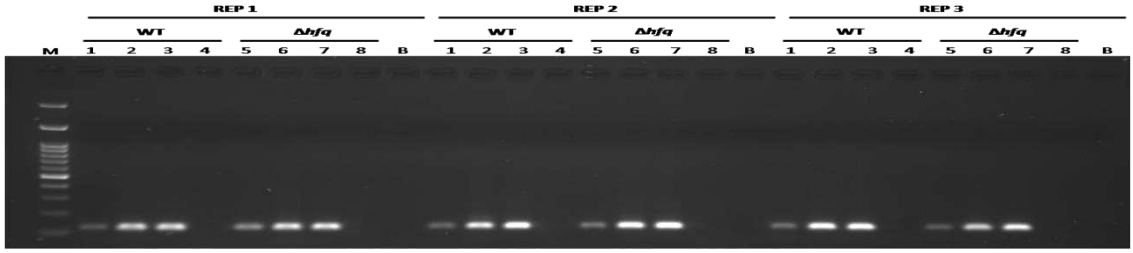



**Figure 14** - Arrc20

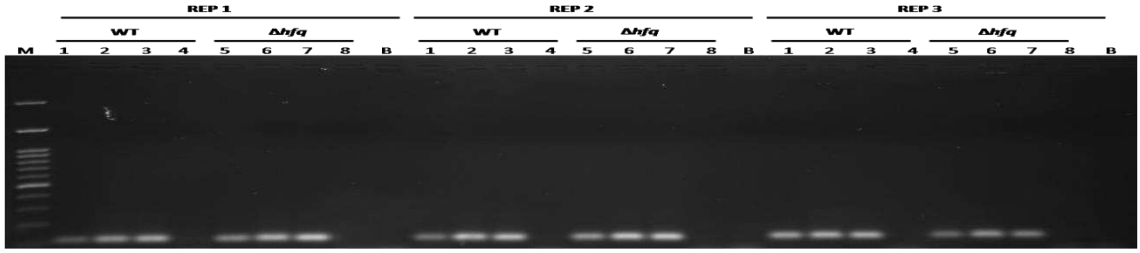



**Figure 15** - Arrc21

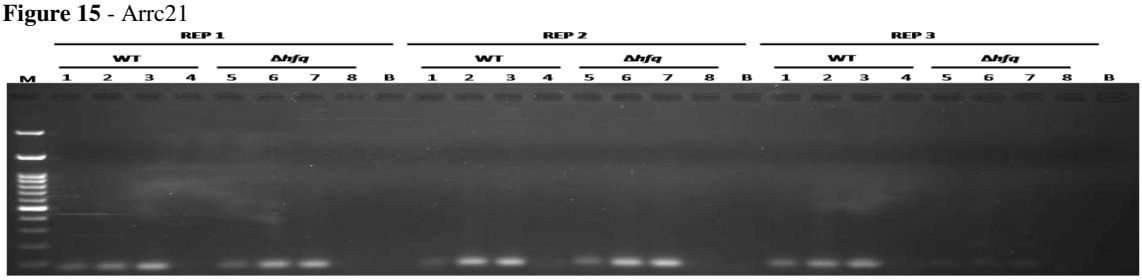



**Figure 16** - RNA01

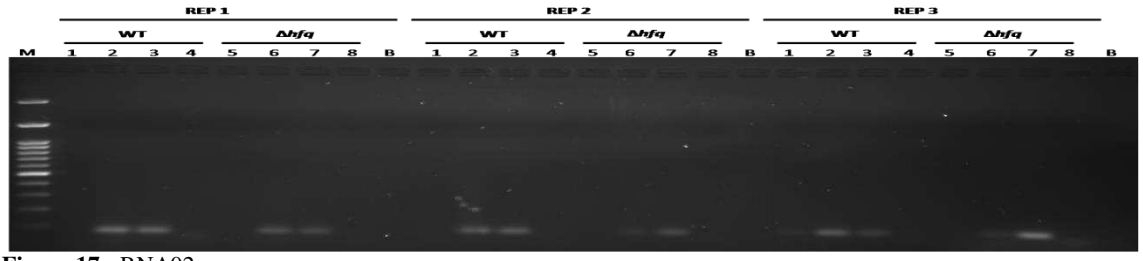



**Figure 17** - RNA02

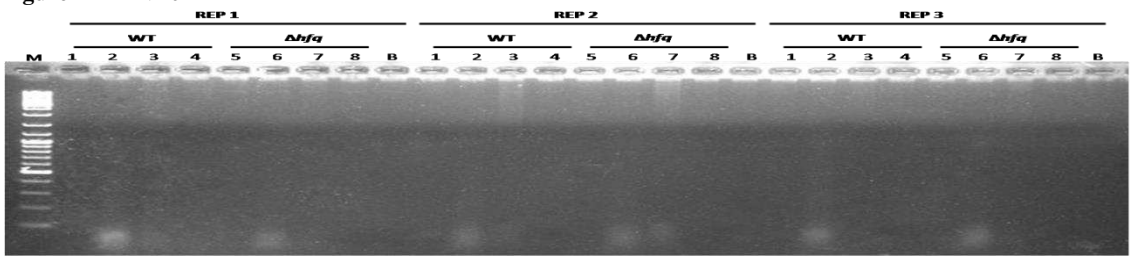



**Figure 18** - RNA03

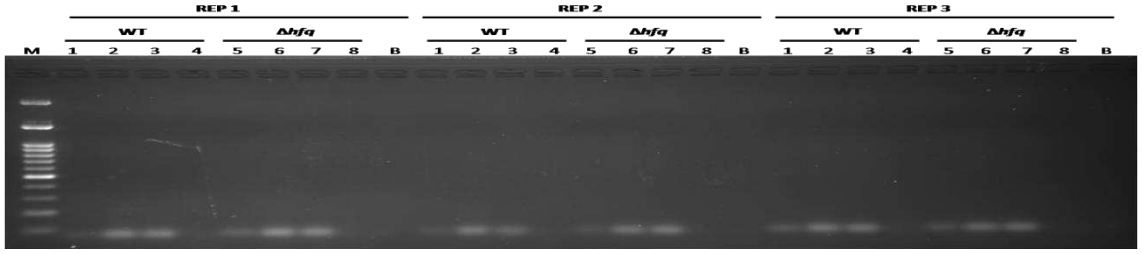



**Figure 19** - RNA03A

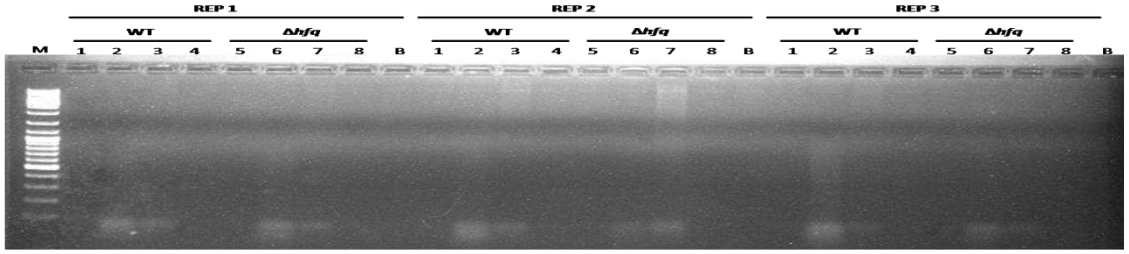



**Figure 20** - RNA04

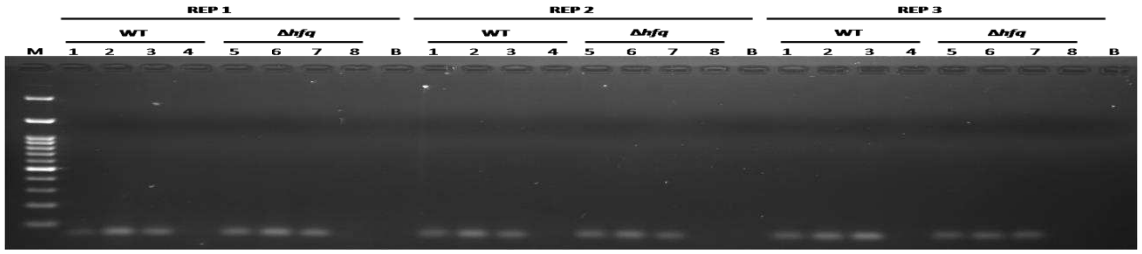



**Figure 21** - RNA06

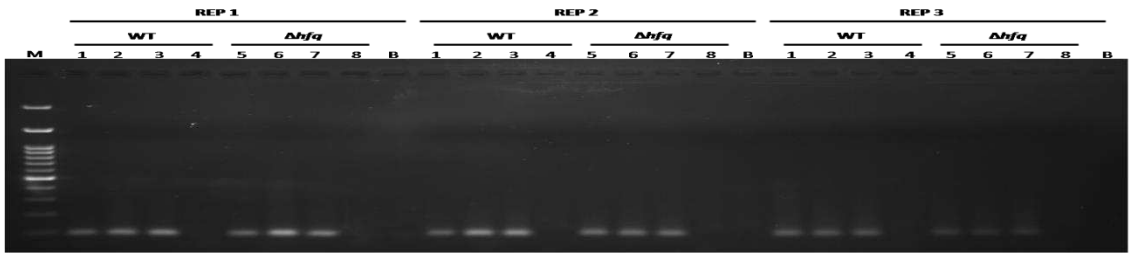



**Figure 22** - RNA10

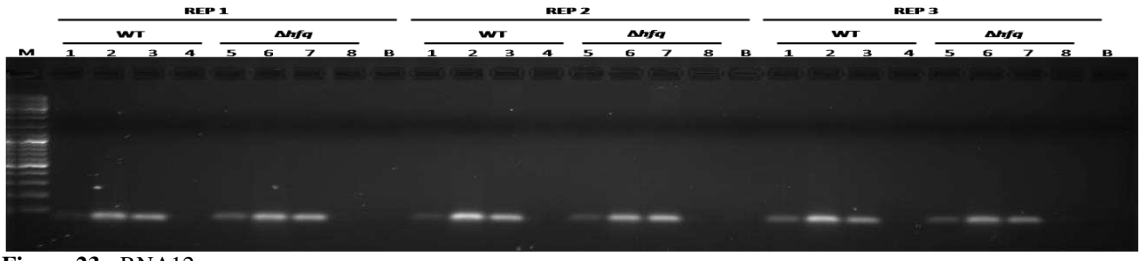



**Figure 23** - RNA12

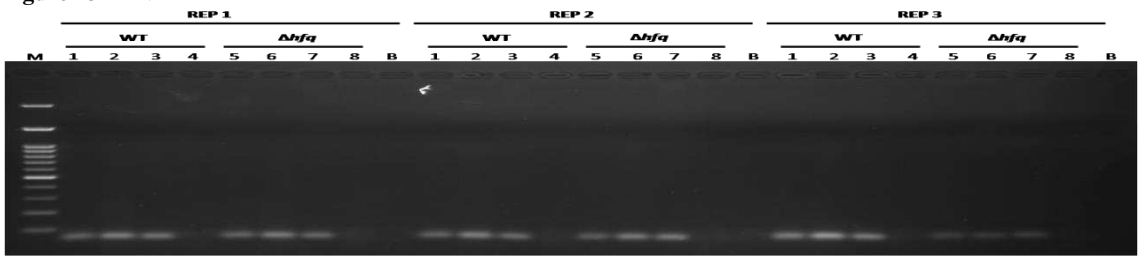



**Figure 24** - RNA12A

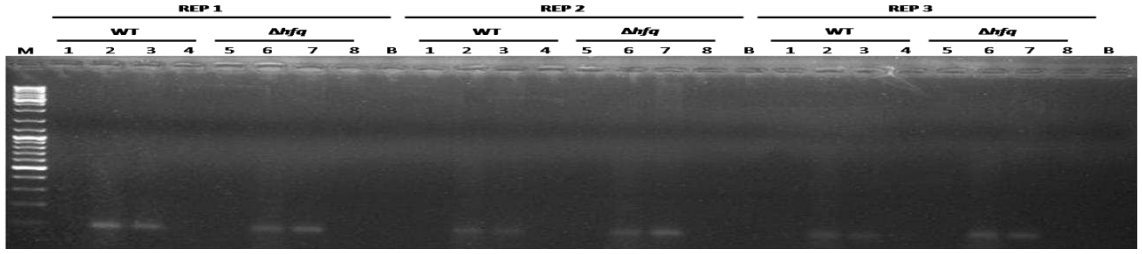



**Figure 25** - RNA13

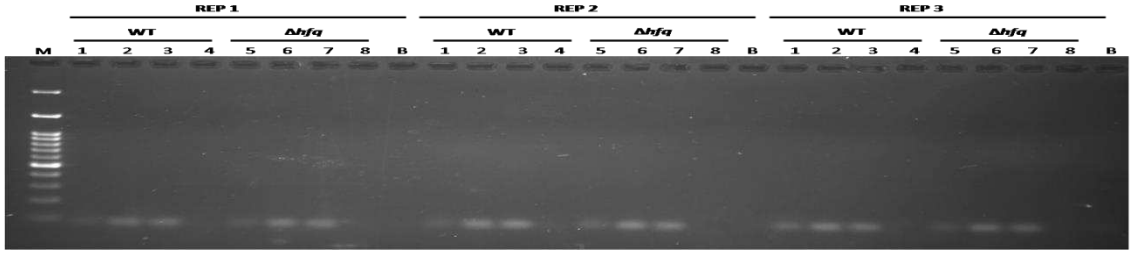



**Figure 26** - RNA14

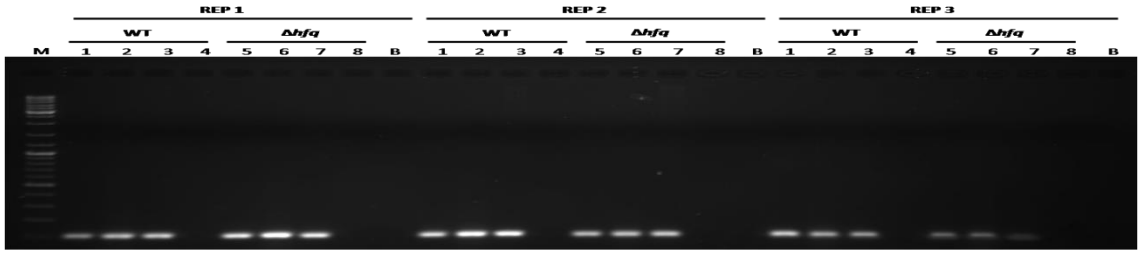
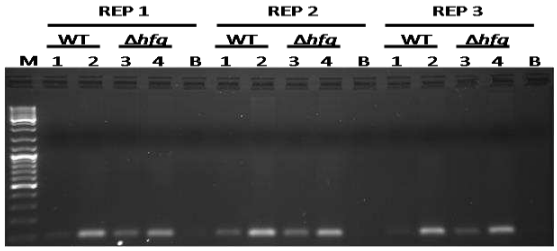



**Figure 27**–RNA 5S

**Figures 28-37**: Results of PCR reactions with the primers to detection of sRNAs in the intravesicular portion of OMVs.

**General subtitle of the Figures 28-38**: 1- cDNA of intravesicular small RNA sample of OMV from WT cell; 2-DNA sample from WT cell; 3- cDNA of intravesicular small RNA sample of OMV from Δ*hfq* cell; 4- DNA sample from Δ*hfq* cell; B- Blank. All the PCR reactions of the three replicates were runned in 1,5% agarose gel.



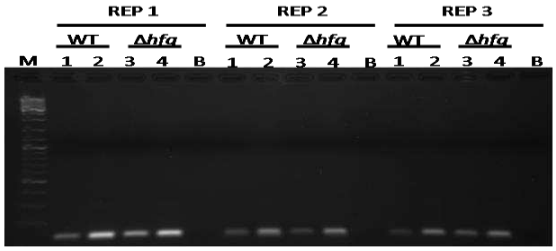
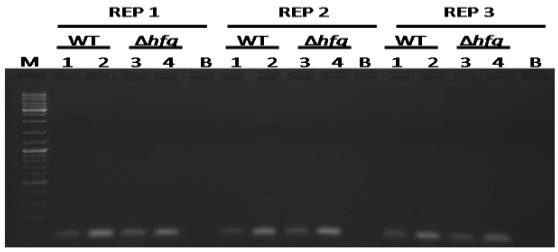



**Figure 28** - Arrc01 (Left) and Arrc04 (Right)



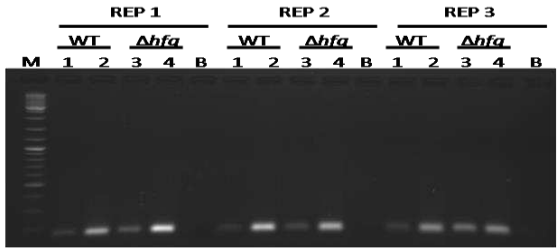
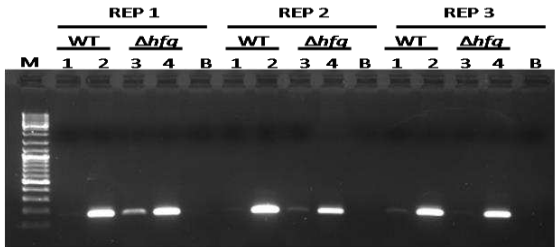



**Figure 29** - Arrc05 (Left) and Arrc07 (Right)



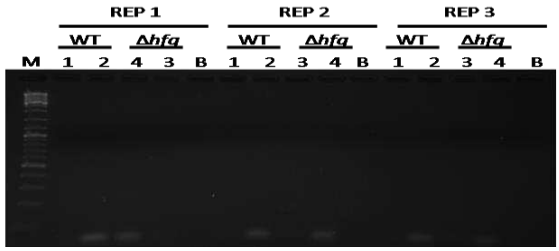
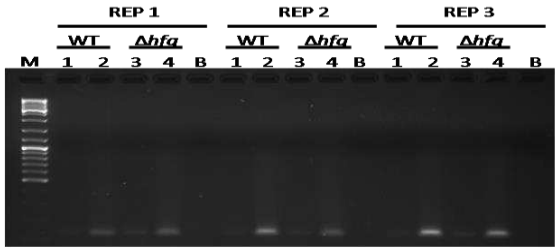



**Figure 30** - Arrc08 (Left)and Arrc09 (Right)



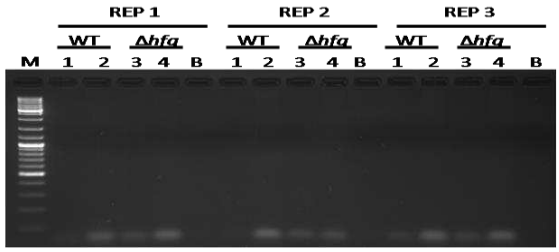
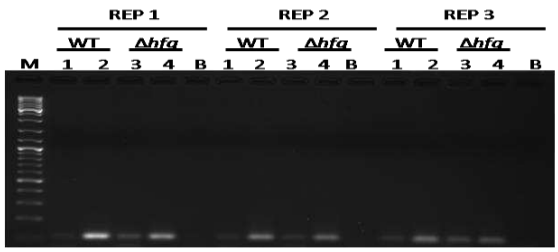



**Figure 31** - Arrc11 (Left) and Arrc12 (Right)



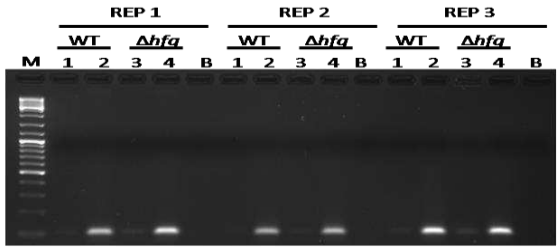
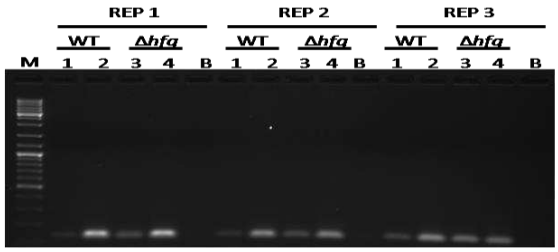



**Figure 32** - Arrc14 (Left) and Arrc17 (Right)



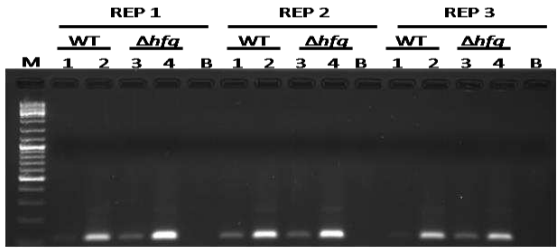



**Figure 33** - Arrc20 (Left) and Arrc21 (Right)

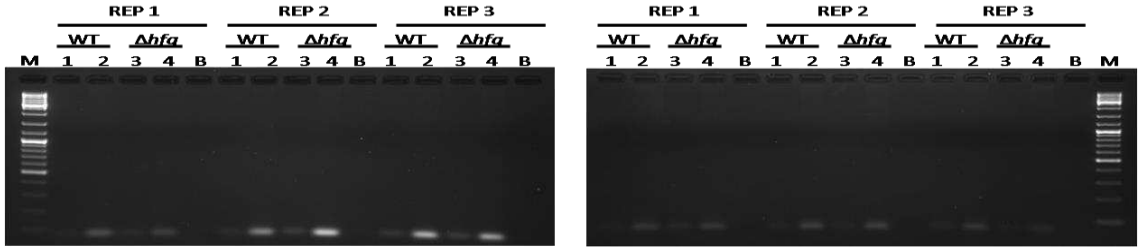



**Figure 34** - RNA01 (Left) and RNA03A (Right)

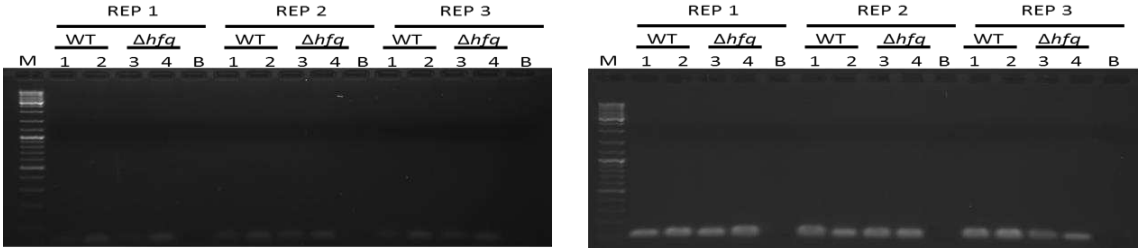



**Figure 35** - RNA06 (Left) and RNA 5S (Right)

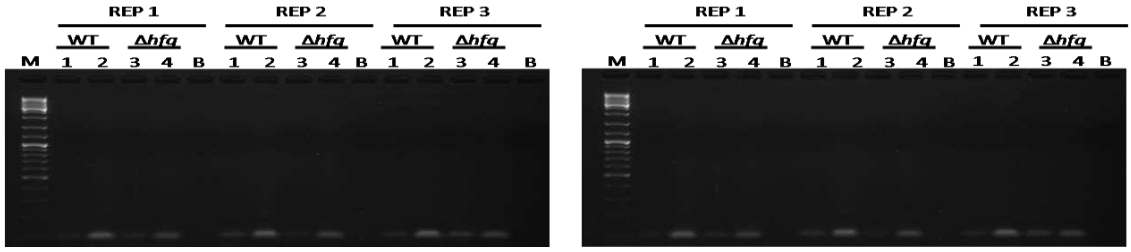



**Figure 36** - RNA10 (Left) and RNA12 (Right)

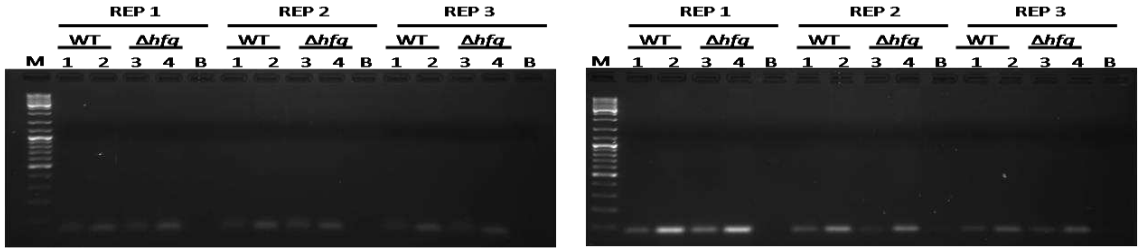



**Figure 37** - RNA12A (Left) and RNA14 (Right)

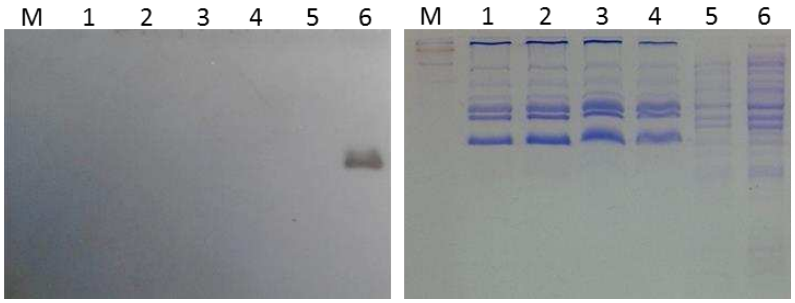



**Figure 38 – Immunoblotting analysis of Hfq protein associated with OMVs.** 1 and 2: OMVs from *A. pleuropneumoniae* Δ*hfq;* 3 and 4: OMVs from *A. pleuropneumoniae hfq*::3XFlag. 5: Total proteins of *A. pleuropneumoniae* Δ*hfq*; 6: Total proteins of *A. pleuropneumoniae hfq*::3XFlag.

Markers utilized:

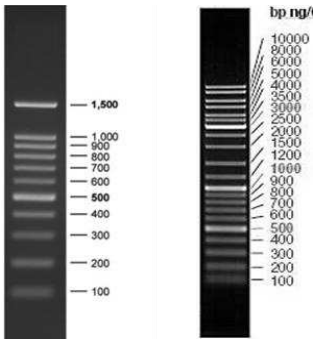



**Figure 39– Ladders utilized in this work**. 100 bp DNA Ladder – Kasvi (Left); O'gene ruler dna ladder mix - Fermentas (Right)