MONALESSA FÁBIA PEREIRA

USO DE *Galleria mellonella* COMO MODELO DE INFECÇÃO E ESTUDO DE FATORES RELACIONADOS COM A VIRULÊNCIA DE *Actinobacillus pleuropneumoniae*

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

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SUMÁRIO

RESUMO

PEREIRA, Monalessa Fábia. D.Sc., Universidade Federal de Viçosa, fevereiro de 2015. **Uso de *Galleria mellonella* como modelo de infecção e estudo de fatores relacionados com a virulência de *Actinobacillus* pleuropneumoniae.** Orientadora: Denise Mara Soares Bazzolli. Coorientadores: Elza Fernandes de Araújo, Marisa Vieira de Queiroz e Paul Richard Langford.

Actinobacillus pleuropneumoniae é o agente etiológico da pleuropneumonia suína, uma severa enfermidade que acomete suínos de todas as idades, gerando perdas econômicas significativas para a suinocultura mundial. Embora os 15 sorotipos conhecidos dessa bactéria possam causar a doença, existem diferenças marcantes de virulência entre eles. A virulência de *A. pleuropneumoniae* é multifatorial e está relacionada à composição e estrutura de polissacarídeos da cápsula, LPS, e toxinas da família RTX, além desses fatores, a aderência em forma de biofilme e a resistência a agentes antimicrobianos podem ser determinantes para virulência. Este trabalho estabeleceu um modelo de infecção alternativo para o estudo de *A. pleuropneumoniae*, utilizando larvas de *Galleria mellonella* e, posteriormente esse modelo foi usado para investigar a virulência de isolados clínicos de *A. pleuropneumoniae* sorotipo 8. Os mesmos isolados foram avaliados quanto ao potencial de formação de biofilme e resistência a antimicrobianos comumente empregados em campo. A partir dessas informações, isolados clínicos com diferenças significativas na virulência, no potencial de formação de biofilme e no perfil de resistência foram selecionados para o sequenciamento genômico. Os resultados mostraram que o modelo de infecção *A. pleuropneumoniae – G. mellonella* é capaz de diferenciar níveis de virulência de isolados clínicos de mesmo sorotipo, além de permitir a avaliação da eficiência de agentes antimicrobianos contra este patógeno. O modelo também mostrou eficiência para diferenciar virulência entre linhagens selvagem e mutante da mesma bactéria. Uma análise de correlação entre os dados de virulência, formação de biofilme e resistência a antimicrobianos permitiu que seis isolados fossem selecionados para o sequenciamento. Com a montagem e anotação foi possível verificar que os genomas de *A. pleuropneumoniae* sorotipo 8 apresentam tamanho de 2,2 ± 0,004 Mpb, com o conteúdo GC de 40,33% ± 0,263 e regiões codificadoras com uma média de tamanho de 817,3 ± 6,8 pb. As regiões codificadoras correspondem a 89,05% ± 0,13 do genoma, das quais a maior parte foi anotada como genes funcionais, o que permitirá a realização de estudos comparativos. Estes genomas apresentam em média 79,5 ± 24,05 genes exclusivos, revelando a alta variabilidade genética dessa espécie, que pode estar relacionada com a variação da virulência entre os isolados estudados.

ABSTRACT

PEREIRA, Monalessa Fábia. D.Sc., Universidade Federal de Viçosa, February, 2015. **Use of *Galleria mellonella* as a model of infection and study of factors related to the virulence of *Actinobacillus pleuropneumoniae*.** Adviser: Denise Mara Soares Bazzolli. Co-advisers: Elza Fernandes de Araújo, Marisa Vieira de Queiroz and Paul Richard Langford.

Actinobacillus pleuropneumoniae is the etiological agent of porcine pleuropneumonia, a severe disease that affects pigs of all ages, causing significant economic losses to the swine industry worldwide. Although the 15 serotypes of this bacterium are known to cause the disease, there are marked differences in virulence between them. *A. pleuropneumoniae* virulence is multifactorial and involves capsular polysaccharides, LPS, and toxins of the RTX family. In addition to these factors, the adhesion in biofilm form and resistance to antimicrobial agents may be determinant for virulence. This work has established an alternative infection model for the study of *A. pleuropneumoniae*, using larvae of *Galleria mellonella* and this model was subsequently used to investigate the virulence of clinical isolates of *A. pleuropneumoniae* serotype 8. The same isolates were evaluated for biofilm formation potential and resistance to antimicrobials commonly used in the field. From this information, clinical isolates with significant differences in virulence, biofilm formation potential and resistance profile were selected for genomic sequencing. Results show that the *A. pleuropneumoniae* - *G. mellonella* infection model is capable of differentiating levels of virulence of clinical isolates of the same serotype. Furthermore, it can be used to evaluate the effectiveness of antimicrobial agents against this pathogen. The model also showed efficiency to differentiate the virulence between wild and mutant strains of the same bacteria. A correlation analysis between the virulence data, biofilm formation and antibiotic resistance allowed six isolates to be selected for genome sequencing. With the assembly and annotation, we found that the genomes of *A. pleuropneumoniae* serotype 8 present size of 2.2 ± 0.004 Mpb, with GC content of 40.33% ± 0.263 and coding regions with an average size of 817.3 ± 6.8 bp. The coding regions correspond to 89.05 ± 0.13% of the genome, most of which was recorded as functional genes, enabling the comparative studies with these genomes. These genomes have 79.5 ± 24.05 exclusive proteins, revealing the high genetic variability of the species, which may be related to the variation in virulence between these isolates.

1. INTRODUÇÃO GERAL

A suinocultura mundial vem evoluindo de forma marcante nas últimas décadas. No ano de 2013, a indústria de carne suína alcançou a produção de 108,8 milhões de toneladas e a expectativa para o final do ano de 2015 é um crescimento de aproximadamente 1,6% (USDA, 2014). Em 2013, foram produzidas aproximadamente 3.370 milhões de toneladas de carne suína no Brasil, sendo que aproximadamente 20% dessa produção foi destinada à exportação, o que coloca o país na quarta posição dentre os maiores produtores e exportadores de carne suína do mundo. Pesquisas indicam que o Brasil terá um aumento de 5% na produção e 21% na exportação de carne suína até o final de 2015, mantendo assim, o quarto lugar no *ranking* mundial de produção e exportação de carne suína (USDA, 2014).

O bom desempenho da suinocultura brasileira deve-se aos avanços tecnológicos e organizacionais dos sistemas de produção de suínos. No entanto, a crescente modernização do sistema de criação está associada a um aumento no número de animais confinados em um espaço limitado, o que contribui para o surgimento e propagação de doenças infecciosas do trato respiratório (Barcellos *et al*., 2008).

Entre as doenças respiratórias recorrentes em rebanhos suínos, destaca-se a pleuropneumonia, uma enfermidade associada a significativas perdas econômicas ao produtor, visto que está associada à diminuição da média diária de ganho de massa corporal do animal; perdas por necessidade de abate precoce e gastos com intervenções, como medicação e vacinação (Kim & Lee, 2006; Krejci & Newberry, 2011).

A pleuropneumonia suína é causada pelo cocobacilo encapsulado Gram-negativo *Actinobacillus pleuropneumoniae* (Nicolet *et al*., 1992). Esse patógeno pertencente à família *Pasteurellaceae* pode ser classificado em dois biotipos de acordo com o requerimento de nicotinamida adenina dinucleotídeo (NAD) para o crescimento.

São conhecidos 15 sorotipos de *A. pleuropneumoniae*, definidos com base em propriedades antigênicas dos polissacarídeos da cápsula (Blackall *et al*., 2002).

A distribuição de sorotipos é variável de acordo com regiões geográficas específicas: os sorotipos 2, 9 e 11 são prevalentes na França, os sorotipos 1 e 5 são os mais comumente encontrados na Coreia do Sul, o sorotipo 3 é o mais importante na China, enquanto o sorotipo 8 é predominante no Reino Unido, Estados Unidos da América, Canadá e Sudeste do Brasil (O'Neill *et al*., 2010; Xu *et al*., 2010; Rossi *et al*., 2013; Gottschalk & Lacouture, 2014; Yoo *et al*., 2014).

Apesar de todos os sorotipos causarem a doença, eles se diferem no potencial de virulência e imunogenicidade (Schuchert *et al*., 2004). Os sorotipos 1, 5, 9 e 11 são considerados os mais virulentos, sendo responsáveis por quadros agudos ou crônicos da doença, enquanto isolados dos sorotipos 2, 4, 6, 8 e 15 geralmente causam baixa mortalidade, mas estão relacionados com a alta morbidade da pleuropneumonia suína nos países produtores (Gottschalk & Taylor, 2006; Frey, 2011).

A infecção e a persistência de *A. pleuropneumoniae* no hospedeiro são mediadas por múltiplos fatores de virulência, muitos dos quais já foram descritos previamente (Chiers *et al*., 2010; Li *et al*., 2014). Contudo, a virulência tem sido primeiramente relacionada com as propriedades da cápsula e à combinação da secreção de toxinas da família RTX (*Repeat in toxin*) denominadas ApxI, ApxII, ApxIII e ApxIV (Frey, 2011). Os 15 sorotipos de *A. pleuropneumoniae* secretam diferentes combinações das exotoxinas Apx; os sorotipos 1, 5, 9 e 11 secretam as toxinas ApxI, ApxII e ApxIV; os sorotipos 2, 3, 4, 6, 8 e 15 secretam ApxII, ApxIII e ApxIV; os sorotipos 7, 12, e 13 secretam ApxII e ApxIV, enquanto os sorotipos 10 e 14 produzem ApxI e ApxIV (Chien *et al*., 2009).

A cápsula polissacarídica é um importante fator de virulência para *A. pleuropneumoniae* e, um dos principais componentes que protege a bactéria das defesas do hospedeiro, devido a suas propriedades antifagocíticas (Dubreuil *et al*., 2000). Estudos revelam que os diferentes sorotipos de *A. pleuropneumoniae* apresentam variações na composição química e também na estrutura da cápsula e, que esta característica pode estar relacionada, pelo menos em parte com a variação da virulência na espécie (Jessing *et al*., 2008). Com o auxílio da microscopia eletrônica foi demonstrado que existe uma correlação direta entre a virulência de *A. pleuropneumoniae* e a respectiva espessura da cápsula, o que demonstra que esta é determinante na interação patógeno-hospedeiro e manifestação da doença (Dubreuil *et al*., 2000).

Os lipopolissacarídeos (LPS) também são importantes fatores de virulência para *A. pleuropneumoniae*, pois são componentes estruturais essenciais a todas as bactérias Gram-negativas e, ainda desempenham o papel de endotoxina e adesina para essa espécie (Chiers *et al*., 2010).

A habilidade das bactérias em sobreviver e crescer em ambientes com limitadas concentrações de ferro pode ser considerado um fator de virulência. Esse mineral está envolvido nas vias metabólicas, respiração, transporte de oxigênio e síntese de DNA, sendo crítico para invasão do micro-organismo e estabelecimento da infecção. A relação da virulência de *A. pleuropneumoniae* com sistemas de transporte de ferro, sideróforos e reguladores globais envolvidos na aquisição desse mineral já está bem esclarecida (Beddek *et al*., 2004; Liu *et al*., 2011; Klitgaard *et al*., 2012).

As fimbrias também apresentam importante função na patogênese de bactérias Gram-negativas como *A. pleuropneumoniae*, pois são mediadoras da adesão bacteriana às células epiteliais do hospedeiro e consequentemente são fundamentais para a

colonização e formação de biofilme (Craig *et al*., 2004). A relação das proteínas de adesão com a virulência de *A. pleuropneumoniae* se comprova com o estudo da proteína ApfA, que é altamente expressa quando as bactérias entram em contato com o epitélio respiratório suíno. Além disso, a inativação do gene *apf*A reduz significativamente a habilidade de *A. pleuropneumoniae* colonizar o pulmão de camundongos, sugerindo que *apf*A seja um importante fator de virulência para este patógeno (Zhou *et al*., 2013).

Os membros da família *Pasteurellaceae* são habitantes de superfície mucosas de mamíferos, portanto, a formação de biofilme pode ser crucial para que essas bactérias possam aderir aos órgãos do hospedeiro e resistir ao seu sistema imunológico (Li *et al*., 2008). Isolados clínicos e linhagens de referência mantidos em laboratório podem perder a capacidade de formar biofilme, sugerindo que essa característica esteja relacionada com a patogênese de *A. pleuropneumoniae* em campo (Kaplan & Mulks, 2005; Labrie *et al*., 2010).

A formação de biofilme pode ser uma resposta de *A. pleuropneumoniae* a uma condição de estresse (Bossé *et al*., 2010). Durante a infecção, por exemplo, a produção de biofilme torna-se mais acentuada, e essa característica pode contribuir para a persistência do patógeno nos tecidos danificados do hospedeiro e também nos estágios iniciais da colonização (Li *et al*., 2014). Sendo assim, a formação de biofilme pode ter importância na colonização, patogênese e também na transmissão de *A. pleuropneumoniae* (Labrie *et al*., 2010).

O biofilme pode interferir no tratamento da pleuropneumonia suína e contribuir para a permanência da bactéria no trato respiratório do animal pois, quando as células bacterianas estão formando o biofilme se tornam mais resistentes ao tratamento com antimicrobianos, tais como a ampicilina, florfenicol, tiamulina e tilmicosina. A mesma

resistência não é observada quando o tratamento é realizado com células planctônicas (Tremblay *et al*., 2013).

Os biofilmes fornecem um nicho ideal para a troca de DNA extracromossomal (plasmídeos ou outros elementos móveis). O mecanismo de conjugação ocorre com uma frequência maior nas células que estão formando o biofilme do que entre as células planctônicas. Uma vez que os plasmídeos podem codificar resistência a vários agentes antimicrobianos, a formação de biofilme também fornece um mecanismo para a seleção promovendo a disseminação da resistência bacteriana (Donlan, 2002).

Actinobacillus pleuropneumoniae apresenta vários padrões de resistência a agentes antimicrobianos, codificados por DNA cromossomal ou plasmídeos, que variam de acordo com isolados de diferentes regiões geográficas, sorotipo e também em função do período de isolamento (Chang *et al*., 2002; Archambault *et al*., 2012). A identificação de isolados multirresistentes e a emergência de bactérias resistentes à ampicilina, tetraciclina e outros antibióticos vem sendo associada ao uso incorreto da terapia antimicrobiana (Yoo *et al*., 2014). A alta incidência de resistência à tetraciclina foi observada nos países produtores de carne suína, sendo relacionada com a presença dos genes de resistência *tet*B, *tet*O, *tet*H e *tet*L. Além disso, plasmídeos são mediadores da resistência à tetraciclina e outros agentes antimicrobianos, tais como a sulfonamida, estreptomicina e ampicilina (Archambault *et al*., 2012; Yoo *et al*., 2014).

Estudos relacionados à virulência de *A. pleuropneumoniae* normalmente são realizados em modelos suínos e camundongos (Sheehan *et al*., 2003; Liu *et al*., 2011; Klitgaard *et al*., 2012). Entretanto, alguns fatores associados a esses modelos de infecção, como custo, reprodutibilidade, facilidade de uso e considerações éticas associada com análises em mamíferos têm dificultado os estudos (Klitgaard *et al*., 2012).

Uma alternativa aos modelos de infecção em mamíferos é o uso de hospedeiros invertebrados como os nematoides ou insetos. *Caenorhabditis elegans* tem atraído a atenção como um modelo de infecção para uma ampla gama de patógenos bacterianos (Mylonakis *et al*., 2007; Peleg *et al*., 2009). No entanto, *C. elegans* não pode sobreviver a 37°C e faltam homólogos funcionais de alguns componentes do sistema imune de mamíferos, tais como células fagocíticas especializadas (Mylonakis *et al*., 2007; Glavis-Bloom *et al*., 2012). A utilização de insetos como *Drosophila melanogaster* e *Galleria mellonella* como modelo de infecção oferecem a vantagem do uso a 37°C. Além disso, os insetos possuem células fagocíticas especializadas, também conhecidas como hemócitos, que apresentam muitas propriedades em comum com fagócitos de mamíferos. Essas células são capazes de fagocitar patógenos e matá-los usando peptídeos antimicrobianos, melanina e produtos de cascatas proteolíticas (Eleftherianos & Revenis, 2011). Desta forma, as larvas de *G. mellonella* têm sido utilizadas como modelo de infecção para avaliar a virulência de diversos patógenos humanos e veterinários, como *Campylobacter jejuni* (Senior *et al*., 2011), *Klebsiella pneumoniae* (Insua *et al*., 2013), *Listeria monocytogenes* (Joyce & Gahan, 2010), *Staphylococcus aureus* (Fleming *et al*., 2006) e *Streptococcus pneumoniae* (Evans & Rozen, 2012), mostrando que esse modelo pode ser usado para estudo de virulência em uma série de patógenos até então mal compreendida (Browne *et al*., 2013).

A virulência de *A. pleuropneumoniae* tem sido sistematicamente descrita com base em conhecimentos prévios sobre expressão e função gênica e sequências genômicas depositadas em bancos de dados (Foote *et al*., 2008; Xu *et al*., 2008; Klitgaard *et al*., 2012; Li *et al*., 2014). No entanto, a diversidade molecular dos sorotipos de *A. pleuropneumoniae*, e a sua relação com a virulência ainda não está bem clara devido ao número reduzido de sequências genômicas depositadas nos bancos de dados e a falta de representação de alguns sorotipos (Xu *et al*., 2010). Atualmente

encontram-se disponíveis no Genbank (NCBI) 16 genomas de *A. pleuropneumoniae* completamente sequenciados, sob os números de acesso CP000569 (L20 sorotipo 5b), CP000687 (JL03 sorotipo 3), CP001091 (AP76 sorotipo 7), AACK00000000 (4074 sorotipo 1), ADOD00000000 (4074 sorotipo 1), ADOE00000000 (S1536 sorotipo 2), ADOF00000000 (M62 sorotipo 4), ADOG00000000 (Femo sorotipo 6), ADOI00000000 (CVJ13261 sorotipo 9). ADOJ01000000 (D13039 sorotipo 10), ADOK00000000 (56153 sorotipo 11), ADOL00000000 (1096 sorotipo 12), ADOM00000000 (N273 sorotipo 13), ADXN00000000 (4226 sorotipo 2), ADXO00000000 (Femo sorotipo 6) e ALYN00000000 (S8 sorotipo 7).

Os dados obtidos a partir da genômica comparativa entre 12 sorotipos referência de *A. pleuropneumoniae*, revelam que esse micro-organismo possui um único cromossomo circular com tamanho que varia de 2,19 – 2,33 Mb, com 2096 - 2223 sequências codificadoras e uma média de 41% de conteúdo GC para cada genoma (Xu *et al*., 2010). A variabilidade genômica observada entre múltiplas linhagens de uma mesma espécie demonstra que o genoma de um isolado bacteriano subestima as características biológicas da espécie (Mira *et al*., 2010). Portanto a melhor aproximação do ideal para a descrição das características genéticas de uma espécie bacteriana é a utilização do conceito "pan-genoma", que se refere ao genoma core e acessório da mesma (Medine *et al*., 2005). O pan-genoma de *A. pleuropneumoniae* consiste de 3303 clusters gênicos, os quais contêm 1709 genes pertencentes ao genoma core, 822 genes distribuídos entre alguns sorotipos e 772 genes diferenciais (sorotipo específico). As variações dessas características genômicas são justificadas por transferência horizontal de genes e recombinação (Xu et al., 2010).

Em estudos anteriores, foi possível observar que a maior parte dos genes diferenciais de *A. pleuropneumoniae* estão relacionados a mecanismos de defesa,

replicação e recombinação; sistemas de restrição; além de um grande número de transposases, recombinases e integrases, provenientes de elementos transponíveis e profagos. Além disso, foi observado que algumas proteínas que apresentam distribuição diferencial entre sorotipos de *A. pleuropneumoniae* podem desempenhar um papel importante na diferenciação dos sorotipos e na sua virulência (Lei *et al*., 2009; Xie *et al*., 2010; Xu *et al*., 2010).

Significativas variações podem ser encontradas no genoma acessório de uma espécie (Howell *et al*., 2014). Essas diferenças podem ser cruciais para o entendimento da evolução do genoma e, também pode revelar importantes informações sobre a adaptação da bactéria ao seu hospedeiro e seu sistema imunológico (Joseph *et al*., 2011; Wilson, 2012). Entretanto, essas análises ganham uma maior significado estatístico quando um maior número de genomas de uma mesma espécie é utilizado, permitindo até mesmo o estabelecimento de relações entre genótipo e fenótipo da espécie (Howell *et al*., 2014).

Estudos que buscam associação entre genótipo e fenótipo, tal como a virulência de uma espécie bacteriana podem fornecer importantes informações epidemiológicas, garantir melhorias no diagnóstico e auxiliar na compreensão do patógeno para produção de novos tratamentos da doença (Howell *et al*., 2014). Tendo em vista a importância de estudos sobre a virulência de *A. pleuropneumoniae*, os objetivos deste trabalho foram estabelecer um modelo alternativo de infecção para estudo de *A. pleuropneumoniae*, utilizando *G. mellonella*; investigar os fatores envolvidos com a virulência de *A. pleuropneumoniae* sorotipo 8; selecionar isolados clínicos sorotipo 8 que apresentam diferenças na formação de biofilme, perfil de resistência a antimicrobianos comumente utilizados em campo e virulência no modelo *G. mellonella,* e por fim, sequenciar e anotar os genomas dos isolados selecionados.

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Capítulo 1

Galleria mellonella is an effective model to study *Actinobacillus pleuropneumoniae* infection

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Title: *Galleria mellonella* is an effective model to study *Actinobacillus pleuropneumoniae* infection

Running title: *Actinobacillus pleuropneumoniae-Galleria mellonella* model

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ABSTRACT

Actinobacillus pleuropneumoniae is responsible for swine pleuropneumonia, a respiratory disease that causes significant global economic loss. Its virulence depends on many factors, such as capsular polysaccharides, RTX toxins and iron-acquisition systems. Analysis of virulence may require easy-to-use models that approximate mammalian infection and avoid ethical issues. Here, we investigate the potential use of the wax moth *Galleria mellonella* as an informative model for *A. pleuropneumoniae* infection. Genotypically distinct *A. pleuropneumoniae* clinical isolates were able to kill larvae at 37ºC but had different LD_{50} values, ranging from 10^4-10^7 CFU/larva. The most virulent isolate (1022) was able to persist and replicate within the insect, while the least virulent (780) was rapidly cleared. We observed a decrease in haemocyte concentration, aggregation and DNA damage post-infection with isolate 1022. Melanisation points around bacterial cells were observed in the fat body and pericardial tissues of infected *G. mellonella*, indicating vigorous cell and humoral immune responses close to the larval dorsal vessel. As found in pigs, an *A. pleuropneumoniae hfq* mutant was significantly attenuated for infection in the *G. mellonella* model. Additionally, the model could be used to assess the effectiveness of several antimicrobial agents against A. *pleuropneumoniae in vivo*. *G. mellonella* is a suitable inexpensive alternative infection model that can be used to study the virulence of *A. pleuropneumoniae*, as well as assess the effectiveness of antimicrobial agents against this pathogen.

INTRODUCTION

Actinobacillus pleuropneumoniae is the causative agent of swine pleuropneumonia, a costly, severe and highly contagious infectious respiratory disease. Currently, 15 known serotypes of this bacterium have been identified, and their prevalence vary worldwide (Blackall *et al*., 2002). *Actinobacillus pleuropneumoniae*

virulence is multifactorial and involves capsular polysaccharides, LPS, membrane proteins, iron-acquisition factors and exotoxins (Bossé *et al.*, 2002; Chiers *et al.*, 2010; Frey, 2011). Serotype 8 isolates display high cytotoxic activity and cause high morbidity. They are widespread in swine producing countries including Mexico and the United Kingdom (Dubreuil *et al.*, 2000; O'Neill *et al.*, 2010), and are prevalent in Southeastern Brazil (Rossi *et al.*, 2013).

Studies on virulence and antibacterial resistance of *A. pleuropneumoniae* have been mostly performed on pigs and mice (Klitgaard *et al.*, 2012; Liu *et al.*, 2011; Sheehan *et al.*, 2003). However, research efforts that depend on mammals as host models are limited by ethical concerns, high costs and practical considerations regarding containment and the numbers of animals that can be used. Thus, the use of invertebrates as models for examining mammalian pathogens has recently attracted significant attention, allowing pathogen development studies to be conducted without the use of mammals. Additionally, invertebrates are convenient and easy to handle, ethically acceptable, relatively inexpensive and permissive hosts to study a variety of human and veterinary infectious diseases. The classic invertebrate models include nematodes and insects (Glavis-Bloom *et al.*, 2012; Trevijano-Contador & Zaragoza, 2014). The nematode *Caenorhabditis elegans* has been used as infection model for a diverse range of bacterial and fungal pathogens. However, *C. elegans* cannot survive at 37°C or under a microaerophilic atmosphere (the optimal growth conditions for some microorganisms) and lack some of the functional components of the mammalian immune system, such as specialised phagocytic cells (Champion *et al.*, 2010).

Some of the limitations of the *C. elegans* model can be resolved by using insects, like the wax moth *Galleria mellonella* (Lepidoptera, Pyralidae). The *G. mellonella* larvae can be maintained at 37°C and, thus, are well suited to study mammalian pathogens. Furthermore, larvae tolerate microaerophilic conditions

(Gundogdu *et al.*, 2011; Mylonakis *et al.*, 2007; Peleg *et al.*, 2009). The use of *G. mellonella* larvae also ensures the accurate quantification of inocula, which are injected directly into the larval haemocoel. Moreover, the large size of the moth larvae (12–20 mm) allows easy manipulation and facilitates tissue and haemolymph collection for analysis (Cook & McArthur, 2013; Fallon *et al.*, 2012).

The *G. mellonella* immune system shares a high degree of structural and functional similarity to the vertebrate innate immune system. Because of these similarities, their larvae have been employed to study the virulence of bacterial and fungal pathogens of mammals (Lionakis, 2011; Mukherjee *et al.*, 2013). The *G. mellonella* immune response consists of two tightly interconnected components, the cell-mediated and humoral responses. The insect cell-mediated response is mediated by haemocytes and involves phagocytosis, encapsulation and clotting (Browne *et al.*, 2013; Satyavathi *et al*., 2014). The humoral response involves soluble effector molecules such as anti-microbial peptides, complement-like proteins, melanin, and products created by proteolytic cascades [e.g., phenoloxidase (PO) pathway], which immobilise or kill pathogens (Eleftherianos & Revenis, 2011).

Because of the advantages offered by *G. mellonella* larvae as an infection model, the virulence of several bacteria of both human and veterinary origins, like *Campylobacter jejuni* (Senior *et al*., 2011), *Klebsiella pneumoniae* (Insua *et al.*, 2013), *Listeria monocytogenes* (Joyce & Gahan, 2010), *Staphylococcus aureus* (Purves *et al*., 2010) and *Streptococcus pneumoniae* (Evans & Rozen, 2012), have been successfully evaluated. Additionally, *G. mellonella* has also been exploited to assess the *in vivo* efficacy of antimicrobials agents (Peleg *et al.*, 2009; Thomas *et al.*, 2013). The objectives of this work were to investigate the host-pathogen interaction between *G. mellonella* larvae and *A. pleuropneumoniae*, and to evaluate the possibility of using the model as an alternative for the natural animal host of the bacterium. The results suggest

that a *G. mellonella* infection model can be used to assess the virulence of, and antimicrobial efficacy against *A. pleuropneumoniae*.

MATERIALS AND METHODS

Microorganisms, culture conditions and DNA extraction. *Actinobacillus pleuropneumoniae* reference strains and **c**linical isolates from southeastern Brazil (BR) and United Kingdom (UK) obtained from lungs and tonsils of swine with clinical signs of pleuropneumonia were used in this work. An isogenic *hfq* mutant of isolate MIDG2331 with an deletion of 220 bp in the open reading frame (ORF), generated by a technique of successive unmarked mutation and chromosomal insertion (Bossé *et al.*, *in press*), was also used (Table S1). All *A. pleuropneumoniae* strains from bacterial stocks kept at -80°C were grown at 37°C for 16 h in a 5% CO_2 atmosphere in Brain-Heart Infusion (BHI) medium (Becton Dickinson, USA) supplemented with NAD (10 $\mu g.ml^{-1}$) (Sigma-Aldrich, UK). Genomic DNA from *A. pleuropneumoniae* and *G. mellonella* were obtained using the FastDNA® Spin kit (MP Biomedicals, USA) according to the manufacturer's instructions.

Molecular characterisation of *A. pleuropneumoniae* isolates using ERIC-PCR. PCR reactions were performed in a C1000TM Thermal cycler (BioRad, USA) using 1 U of JumpStartTM *Taq* DNA polymerase (Sigma-Aldrich, UK). The ERIC-PCR fingerprinting technique was employed to estimate genetic variability among the isolates used. The reaction was performed using the oligonucleotides pair ERIC1R (5' ATG TAA GCT CCT GGG GAT TCA C 3') and ERIC2 (5' AAG TAA GTG ACT GGG GTG AGC G 3') and conducted as in a previous study (Mohapatra & Mazumder, 2008). After electrophoretic separation in a 2.0% agarose gel, the fingerprinting patterns were analysed using the BioNumerics 5.0 software (Applied Maths, Belgium) to

construct dendrograms using the UPGMA method. The cophenetic correlation coefficient (Farris, 1969) was calculated to test the goodness of fit between the similarity shown in the dendrograms and the cophenetic matrices. The ERIC-PCR was standardised and repeated three times independently.

Antimicrobial susceptibility testing. The broth microdilution method was performed to determine antimicrobial resistance profile in *A. pleuropneumoniae* clinical isolates using Sensititre Standard Susceptibility MIC Plates BOPO6F (Trek Diagnostic Systems, Thermo Fisher Scientific, Cleveland, Ohio, USA) in accordance with CLSI guidelines (Clinical and Laboratory Standards Institute), document M31-A3, 2008.

***Galleria mellonella* killing assay.** Insects provided by the Universidade Estadual do Norte Fluminense and experiments were conducted according to Ramarao *et* al., (2012). Briefly, last-instar larvae, each weighing 250-300 mg, were used. *A. pleuropneumoniae* cultures in mid-exponential phase were used to infect the *G. mellonella* larvae. Inocula consisted of 10 µl of serially diluted cell suspensions, varying from 10^2-10^8 CFU/larva, which were injected into the first right pro-leg into the haemocoel using insulin syringes (Becton Dickinson, USA). Larvae infected with either *Escherichia coli* K12, phosphate buffered saline (PBS) or uninoculated larvae were used as negative controls. To test whether secreted virulence factors are involved in the virulence of mid-exponential phase *A. pleuropneumoniae*, filtered supernatants from cultures in the logarithmic stage of growth were also injected directly into the larvae and monitored for toxicity. These tests were performed with larvae incubated at either 37°C or 30°C, in the dark. Insects were individually examined for the production of pigmentation and time of death was monitored over 96 h. Larvae that did not move in response to touch were considered to be dead. The dose of bacteria lethal for half (LD_{50} value) of the *G. mellonella* was

calculated for each isolate by linear regression. All the tests involving the inoculation of bacteria in the *G. mellonella* larvae were performed in biological and experimental triplicate (n = 10 larvae per each replica).

Bacterial *in vivo* growth monitoring. The *A. pleuropneumoniae* isolates with the most extreme (highest and lowest) LD_{50} values were selected for the virulence tests, in which the microbial growth and the *G. mellonella* immune response were tested. The isolates were injected into the haemocoel (10^4 CFU/larva), and bacterial growth was investigated 1, 2, 4 and 24 hours post injection. Larvae that were left untreated or injected with PBS were used as negative controls. Before bleeding by cutting near the pseudolegs, the larval body surface was disinfected with 70% ethanol. To recover bacteria from the larvae at each time point, we separated 10 µL the haemolymph of 10 individual larvae and then tenfold dilutions of each sample were plated on BHI agar supplemented with NAD, and incubated at 37°C in 5% CO_2 atmosphere. The concentration of CFU in the heamolymph was calculated as the average between the larvae tested. The presence of *A. pleuropneumoniae* within the larva was also monitored by haemolymph DNA extraction and PCR amplification of the *A. pleuropneumoniae*-specific *apxIV* gene, using the oligonucleotides pair APXIVAF (5' GCC TCC GAC CTG AAT AAA CC 3') and APXIVAR (5' CAA CCA TCT TCT CCA CC 3'). The reaction and cycle parameters of the PCR reactions were conducted according to Jaglic *et al.* (2004), and the amplified fragments were separated by electrophoresis in a 1.0% agarose gel.

Haemocyte quantification during infection. For haemocyte counting, 10 µl of haemolymph, obtained from larva inoculated as above, were transferred to anticoagulant solution pH 4.5 (Mead *et al.*, 1986) in microcentrifuge tubes previously siliconised with

Sigmacote (Sigma-Aldrich, UK). The total cell number was counted under the Zeiss Primo Star light microscope (Carl Zeiss, Germany).

Changes in haemocyte morphology and DNA damage during infection. Haemolymph suspensions from 10 infected larvae and controls were transferred to glass slides and incubated in a humid chamber for 30 min to allow cells to adhere to the glass slide surface. Adherent haemocytes were fixed with 4% paraformaldehyde for 30 min and kept in distilled water at 4°C overnight. To evaluate haemocyte morphology after inoculation with a lethal dose of *A. pleuropneumoniae*, fixed slides were washed three times in PBS and stained with phalloidin-FITC (1:100) (fluorescein isothiocyanate) (Sigma-Aldrich, UK) in PBS/Triton 1% for 30 min and washed three times in PBS. The slides were mounted in Mowiol anti-fade medium (Fluka, Germany), analysed and photographed using a Zeiss LSM 510 confocal microscope (Carl Zeiss, Germany) at the Microscopy and Microanalysis Facilities at the Universidade Federal de Viçosa (NMM-UFV). For the detection of nuclear DNA damage, PBS-washed haemocytes (from both infected and control larvae) were analysed using the TUNEL reaction. The fragmented DNA was labelled using the *In Situ Cell Death Detection Fluorescein kit* (Roche Molecular Biochemicals, Germany). The negative control for the TUNEL reaction was performed on haemocytes obtained from PBS-injected caterpillars. For the positive control, cells from PBS-injected caterpillars were incubated in DNase I (Promega, USA) at 1 $\mu g.\mu L^{-1}$, followed by incubation in the Reaction Buffer for 45 min at 37°C, according to manufacturer's instructions. Slides were mounted and analysed as described above.

Histopathological analysis of *G. mellonella* infected with *A. pleuropneumoniae*. For histopathological analysis, at least 10 individuals from each treatment (injected with

low or high virulent bacteria) were processed as follows: larvae were placed in PBS and dissected under a stereoscope, the abdominal cavity opened laterally using micro scissors, and the visceral organs removed. The dorsal abdomen (including the fat body and dorsal vessel) was separated from the rest of the larval body. Samples were transferred to microcentrifuge tubes containing 4% paraformaldehyde in PBS (0.1 M, pH 7.2) and stored at 4°C. Three fixed carcasses per treatment were transferred to glass slides containing PBS and photographed using a stereoscope microscope coupled with a digital AxioCamERc5s camera (Carl Zeiss, Germany). Fixed samples were rinsed in PBS, dehydrated in a crescent ethanol series (70-100%), and embedded in historesin (Leica, Germany). Samples were sectioned (3-4 µm) using glass knives in a microtome Leica RM2255 (Leica, Germany). Sections were stained with haematoxylin and eosin (HE), mounted in Eukit (Fluka, USA) mounting medium and photographed under the optical microscope.

Administration of antibiotics. The antibiotic tests were conducted according to Peleg *et al.* (2009). *G. mellonella* larvae were infected with a lethal dose of *A. pleuropneumoniae* isolate 1022. After 30 min, antibiotics or PBS were injected into different prolegs. *G. mellonella* larvae injected twice with PBS and larvae that received no injection were used as controls. The antibiotics purchsed from Sigma (Sigma-Aldrich, UK) included ampicillin (10 mg/kg of body weight), enrofloxacin (2.5 mg/kg), penicillin (24000 UI/kg) and tetracycline (6 mg/kg). Concentrations were based on those used routinely for swine and by previous tests in *G. mellonella*.

Statistical analyses. The LD_{50} value of each isolate was calculated by fitting a linear regression using R v.2.13.0 (R Development Core Team, Austria). Survival curves were plotted using the Kaplan-Meier method, and differences in survival were calculated by

using the log-rank test using R v.2.13.0. A *P* value of <0.05 was considered to be statistically significant. Haemocyte quantification tests were performed in biological triplicate, and the values were subjected to analysis of variance and Tukey's test used to compare means using R v.2.13.0.

RESULTS

Differences between strains

In this study, 21 *A. pleuropneumoniae* serotype 8 clinical isolates from different origins and the serotype reference strain 405 were genotypically characterised using the ERIC-PCR fingerprinting technique. All reactions were reproducible, and the dendrograms were statistically supported by high cophenetic correlation values, all greater than 0.85.

Differences in the fragment profiles revealed genetic variability among the isolates, even though they were from the same serotype and from close geographical regions. These differences are consistent with the observation that the virulence of these strains varies greatly in the *G. mellonella* model and resistance to antimicrobial agents.

Of the 21 clinical isolates, 1022 was the most virulent, with an LD_{50} of $1.04x10^4$ CFU, and 780 was the least virulent, with an LD_{50} of $8.41x10^7$ CFU. Further tests were conducted with these two isolates as they have the greatest range in relative virulence. The LD_{50} values of 11 of the isolates analysed (52%) were similar (same order of magnitude) to the LD_{50} of the serotype 8 reference strain used in this study. The differences observed between these isolates indicate that the *G. mellonella* infection model can rapidly and inexpensively quantify differences in the virulence of *A. pleuropneumoniae* clinical isolates. The characteristics of the strains included in the study are shown in Fig. 1.

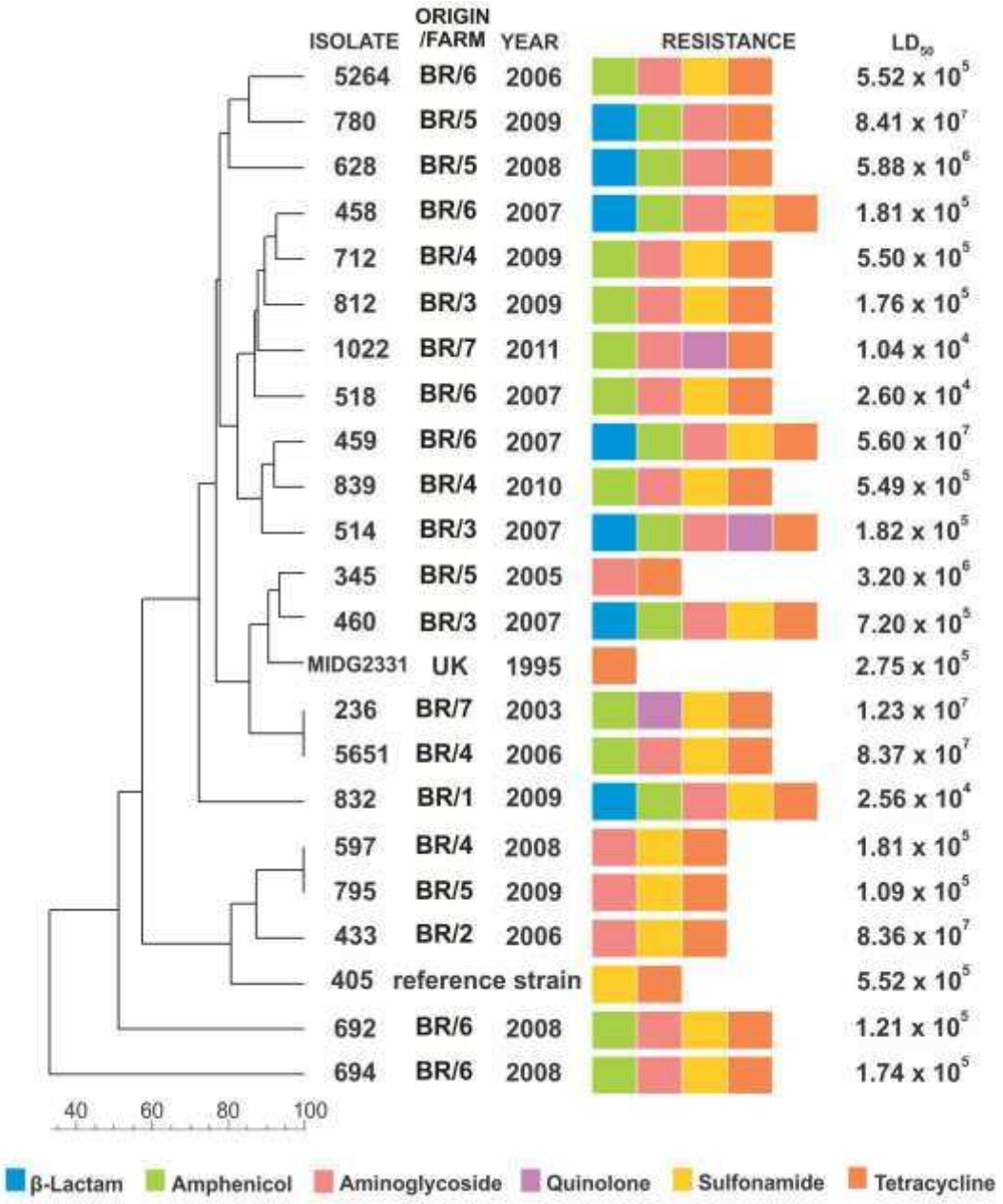

Fig. 1. Genetic and phenotypic variations among serotype 8 *A. pleuropneumoniae* clinical isolates or reference strains from Brazil (BR) and United Kingdom (UK). Genomic differences were observed by ERIC-PCR fingerprinting. Antimicrobial susceptibilities were determined by MIC assay. The LD_{50} of each isolate was estimated using *Galleria mellonella* as an infection model. Serotype 8 reference strain 405 was used for comparison.

To confirm the efficiency of *G. mellonella* as an alternative host, other reference strains and clinical isolates of the serotypes 1 (Apx I, II and IV producer), 2 (Apx II, III and IV producer) and 7 (Apx II and IV producer) were tested. These serotypes can represent all the 15 serotypes because they produce all the Apx toxins combinations found in *A. pleuropneumoniae*. The results showed different *G. mellonella* survival profiles. The *A. pleuropneumoniae* serotype 1, known to be highly virulent in pigs, was more aggressive in the *G. mellonella* model, when compared to less virulent reference strains (Fig. S1).

Killing of *G. mellonella* by *A. pleuropneumoniae* is dependent on inoculum concentration and incubation temperature

Inoculation of *G. mellonella* with *A. pleuropneumoniae* resulted in larval killing in a manner that was dependent on the concentration of bacteria used as the inoculum (Fig. 2). For example, using $2.8x10^4$ CFU/larvae of the 1022 isolate as the inoculum and incubation at 37°C, 96% of the *G. mellonella* larvae were killed within 24 hours of infection, whereas no significant killing was observed when larvae were inoculated with a ten-fold reduced concentration ($P<0.0001$) (Fig. 2a). The least virulent isolate, 780, caused no death when the same amount of cells was injected (Fig. 2b). The non-pathogenic *E. coli* K12 strain does not kill *G. mellonella* with an inoculum of up to $1.0x10^8$ CFU/larva.

To evaluate the effect of incubation temperature, we analysed the killing of *G. mellonella* at 30°C (Fig. 2). Similar to our findings at 37°C, inoculation of *G. mellonella* with *A. pleuropneumoniae* 1022 resulted in larval killing that was dependent on the concentration of bacterial cells injected. However, the killing was more effective at 37°C, a more appropriate growth temperature for *A. pleuropneumoniae*. In this case, injection of $2.8x10^4$ CFU/larva of isolate 1022 resulted in the death of only 2% of the larvae within 24 h of infection when incubated at 30°C, compared to 96% of mortality

observed at 37°C (P<0.0001). Because isolate 780 cannot produce significant killing rates even at high cell concentrations, only a slight variation was observed when the assay temperature was altered (Fig. 2b)

Complete survival and high health index were observed in *G. mellonella* after evaluating the effect of supernatant from exponential phase and media controls. The larvae from the control group showed 100% survival in all tests performed (data not shown).

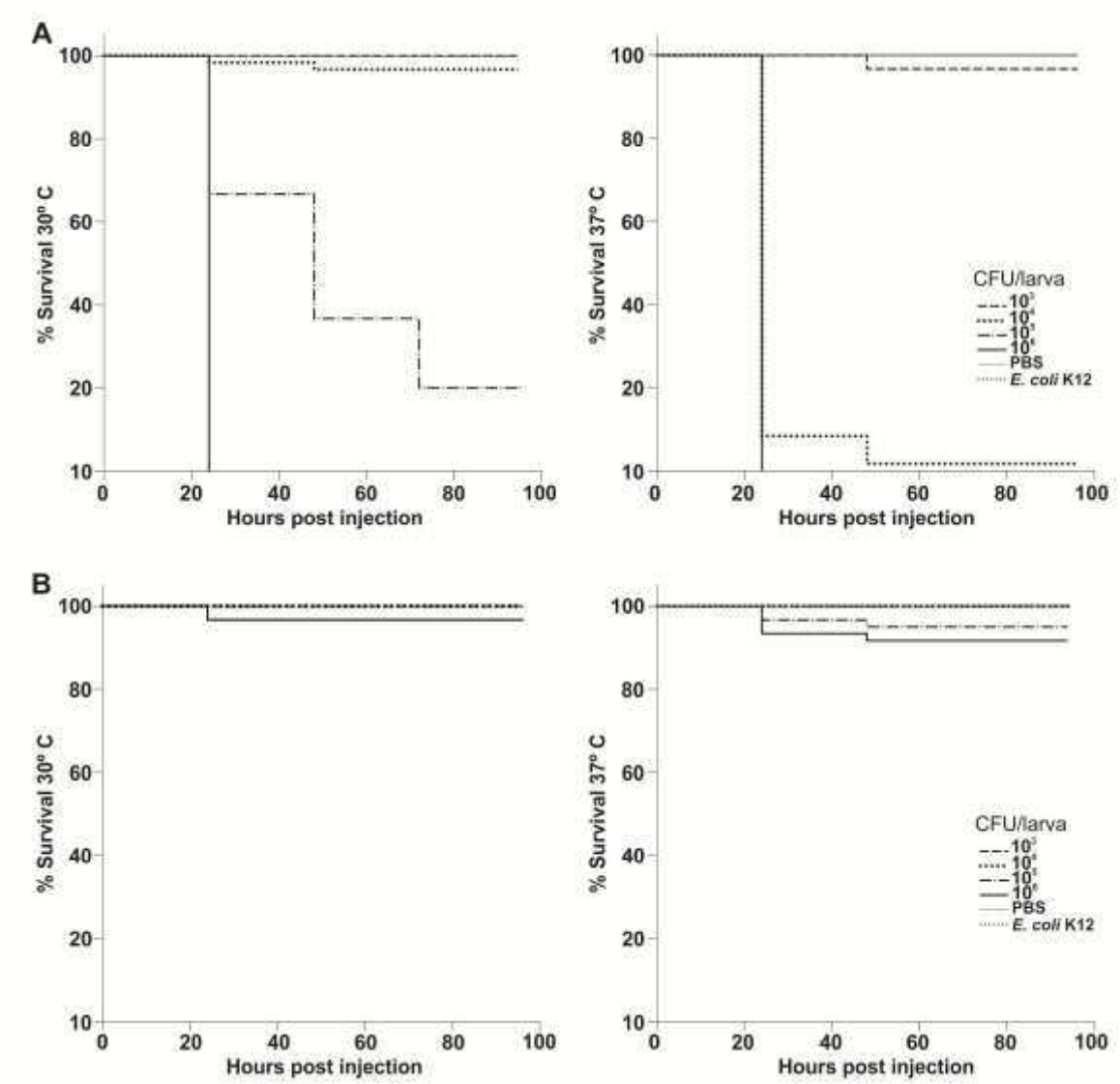

Fig. 2. *Galleria mellonella* killing by *A. pleuropneumoniae* is dependent on inoculum concentration and incubation temperature post-infection. Killing was monitored for (A) the most virulent isolate (1022), and least virulent isolate (780) (B). Death rate was more pronounced at 37°C, for isolate 1022 (P<0.0001 for comparison of an inoculum of 10^4 CFU/larva at 37°C and 30°C).

A. pleuropneumoniae infection of *G. mellonella* is accompanied by bacterial growth

To analyse *A. pleuropneumoniae* growth in *G. mellonella* during the virulence tests, we monitored the presence of the bacteria using both a specific PCR reaction and cell counting after inoculation with doses of 10^4 CFU of the high (1022) and low (780) virulent isolates. The average number of bacterial cells from strain 780 recovered from the tested larvae was drastically reduced by four orders of magnitude over the first 4 hours of infection, and no bacterial cells were observed in the haemolymph after 24 hours of the experiment. In contrast, with isolate 1022, the number of bacteria increased by two orders of magnitude 4 hours post-infection, and cells were still present in the larvae 24 hours post-infection (Fig. 3a).

The results were consistent with the amplification of the *apxIV* gene, commonly used as a molecular marker to identify this bacterium. Whilst isolate 1022 was detected by PCR during 24 hours post-infection, the same was not observed for isolate 780 (Fig. 3b).

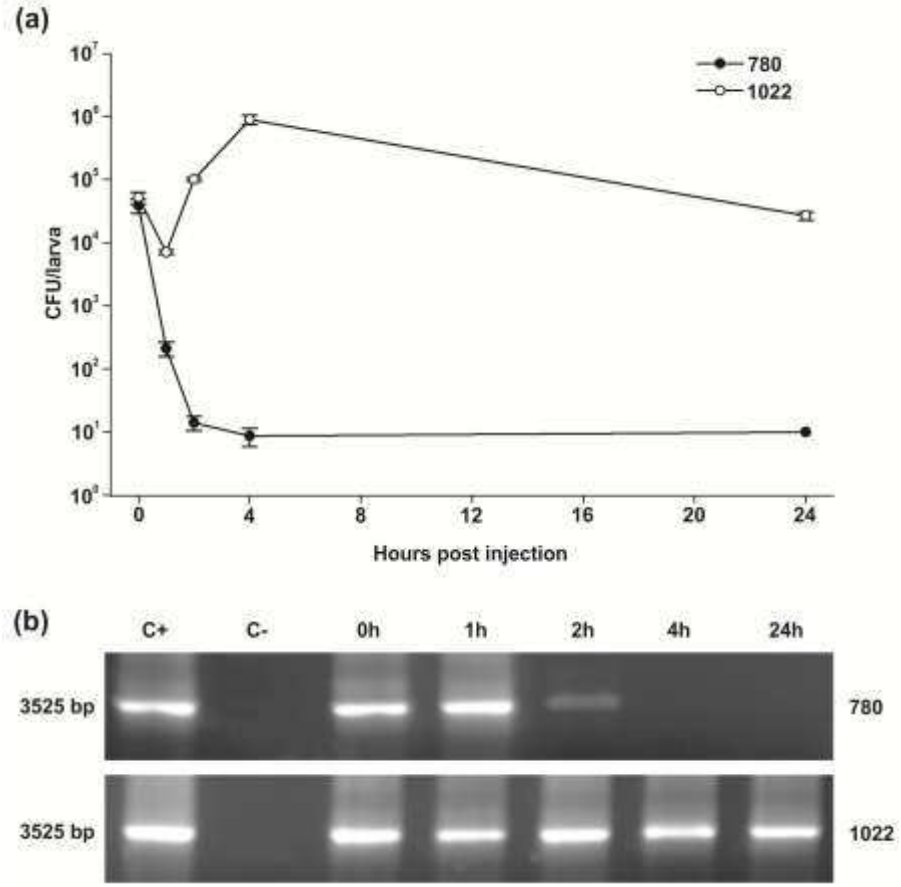

Fig. 3. *Actinobacillus pleuropneumoniae* strains display differential abilities to persist and replicate in *G. mellonella*. The larvae were inoculated with 10^4 CFU of 780 and 1022 isolates. (A) The haemolymph of larvae was collected and plated at 0, 1, 2, 4 and 24 hours post inoculation. Median CFU values for strains 780 and 1022 reveal differences in growth dynamics. (B) The presence of *A. pleuropneumoniae* strains 780 and 1022 was also confirmed by amplification of the *apxIV* gene.

Haemocytes and melanisation are part of the *G. mellonella* response to *A. pleuropneumoniae*

Haemocytes react differently upon exposure to isolates that have different LD_{50} (Fig. 4a). The concentration of circulating haemocytes in the haemolymph of *G. mellonella* following infection with isolates 1022 and 780 showed significant differences. During the first hour, an increase in the number of circulating haemocytes was observed in the haemolymph of larvae infected with isolate 780 and in those inoculated with sterile PBS. However, the number of haemocytes isolated from the larvae infected with isolate 780 was significantly higher (P<0.0012). The concentration of haemocytes in the haemolymph of larvae infected with isolate 1022 decreased

significantly one hour post-infection, when compared with the PBS control and larvae infected with isolate 780 ($P<0.0001$ for 780 and PBS).

Larvae infected with isolate 1022 demonstrated clear signs of progressive dark pigmentation (melanisation) over the time, whereas larvae that received isolate 780 showed no signs of darkening during the same period (Fig. 4b). We observed that melanisation initially occurs in the dorsal portion of the larva, as clearly observed at 1 and 2 hours post-infection with isolate 1022 (Fig. 4b). The differential melanisation in the dorsal vessel region of *G. mellonella* was evidenced by the dissection of insects 1 hour after inoculation with isolates 780 and 1022 and PBS. The larvae infected with a lethal dose of 1022 showed strong dark pigmentation along the dorsal vessel (Fig. 4c).

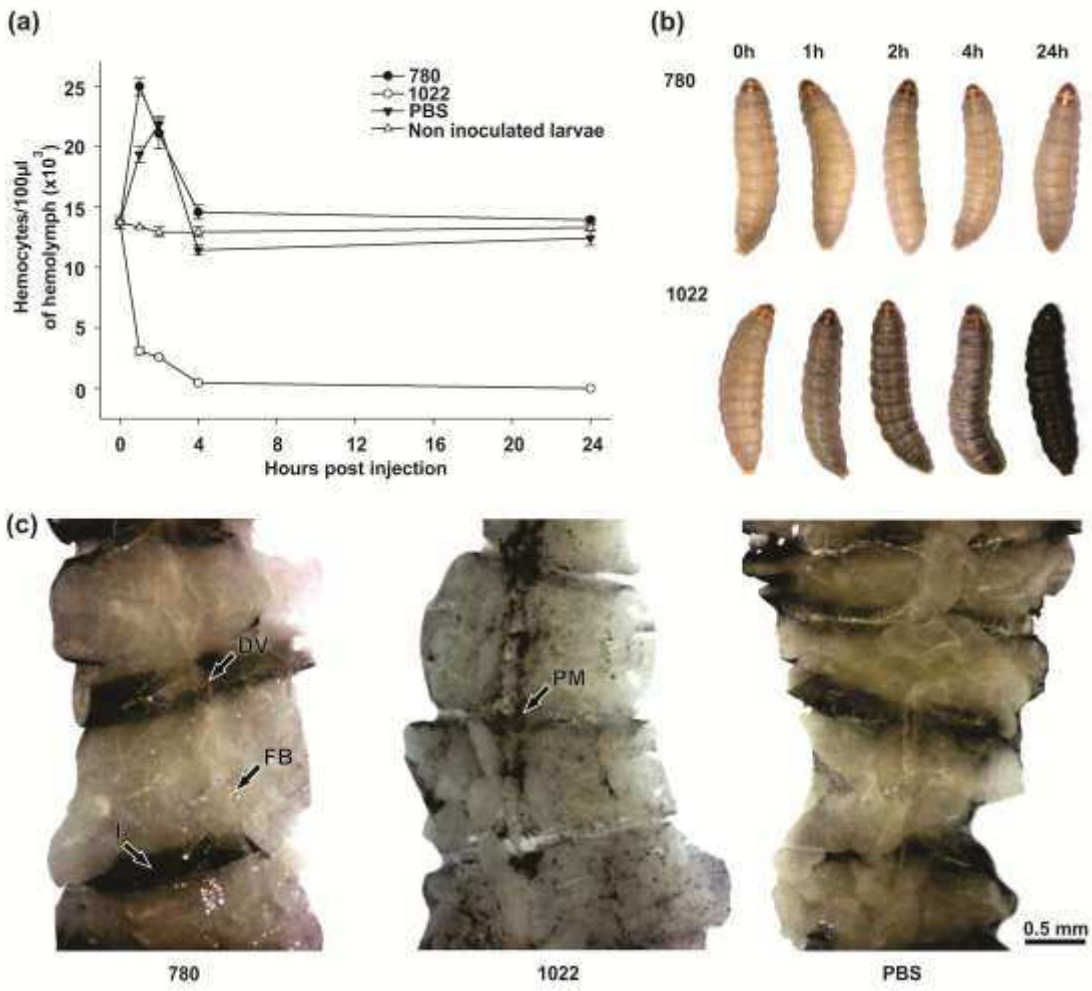

Fig. 4. *Galleria mellonella* responds differently to the presence of low (780) and high (1022) virulence isolates of *A. pleuropneumoniae* and exhibits variations in haemocyte concentration (A) and dark pigmentation observed in the larvae whole body (B) and in the interior of larvae dorsal carcass (C). DV- dorsal vessel; FB- fat body; I- integument; PM- points of melanisation. Bar = 0.5 mm.

The histopathological analysis of the dorsal vessels of *G. mellonella* larvae 1 hour post-infection shows pericardial tissues and fat bodies with points of melanisation and nodules; these structures were not observed in the control (Fig. 5). Nodulation and accumulation of haemocytes in the pericardial tissue was more abundant in the larva infected with isolate 1022 (Fig. 5e and f). Points of melanisation around groups of bacterial cells were also observed in the fat bodies of *G. mellonella* larvae infected with *A. pleuropneumoniae*, showing vigorous cellular and humoral immune responses close to the dorsal vessel of *G. mellonella*.

Clustered haemocytes forming melanised nodules are observed among circulating haemocytes from caterpillars infected by either isolate 780 or 1022, which was not observed in control (Fig. 6a, b and c). The basic feature of haemocytes that enables them to participate in phagocytosis, encapsulation, and nodulation is their ability to adhere to foreign bodies. Our studies show that following infection with *A. pleuropneumoniae*, *G. mellonella* haemocytes form protrusions toward the bacterial agent and aggregate. In both infected and control caterpillars, the haemocytes maintain the same cytoskeletal staining pattern, with the cell periphery strongly stained. However, cytoskeletal staining with phalloidin-FITC allowed a better view of the emission of pseudopods (Fig. 6d) and the formation of cell clusters (i.e., nodules) during the *G. mellonella* response to *A. pleuropneumoniae* (Fig. 6e).

DNA damage in haemocytes was measured 1 h post-infection. Isolate 1022 was more potent than 780 in inducing damage. No stained nuclei were observed in the PBS-treated control. The cytotoxic effects on *G. mellonella* haemocytes after *A. pleuropneumoniae* infection are shown in Fig. 6g and h.

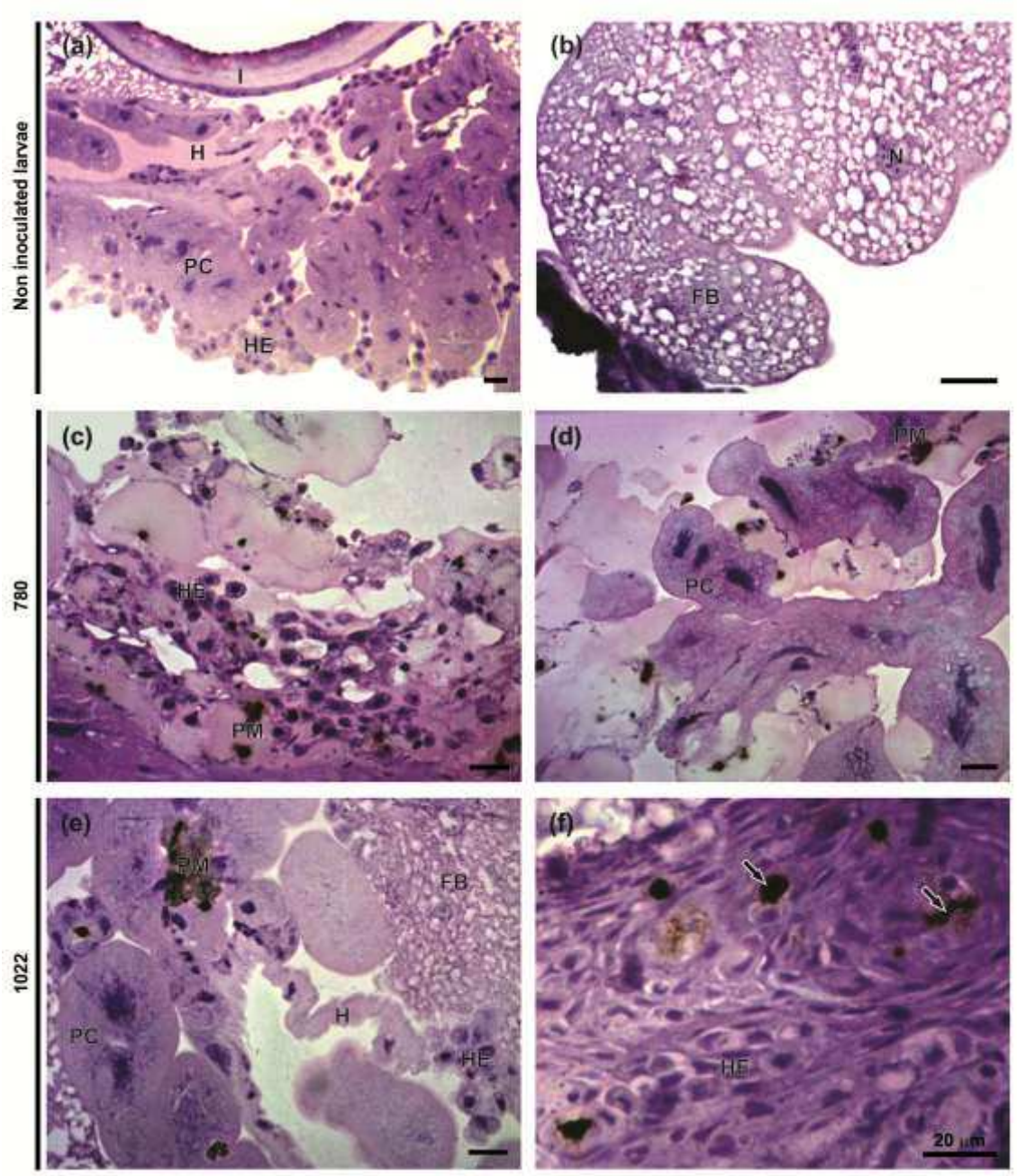

Fig. 5. *Actinobacillus pleuropneumoniae* infection of *G. mellonella* initiates a robust innate immune response. Histological sections of *G. mellonella* 1 hour post-infection with clinical *A. pleuropneumoniae* isolates (10^4 CFU/larva) were stained with haematoxylin and eosin. (A): aspects of the heart (H) and associated tissues and (B) a fat body (FB) lobe of a PBS injected larva; (C, D and E): heart and associated tissues of a larvae infected with isolates 780 and 1022 with several points of melanisation among pericardial cells (PC) and sessile haemocytes (HE); (F): detail of a nodule with adherent haemocytes (HE) closely associated to melanised matter (arrow) of larvae infected with isolate 1022. Bar = 20 µm.

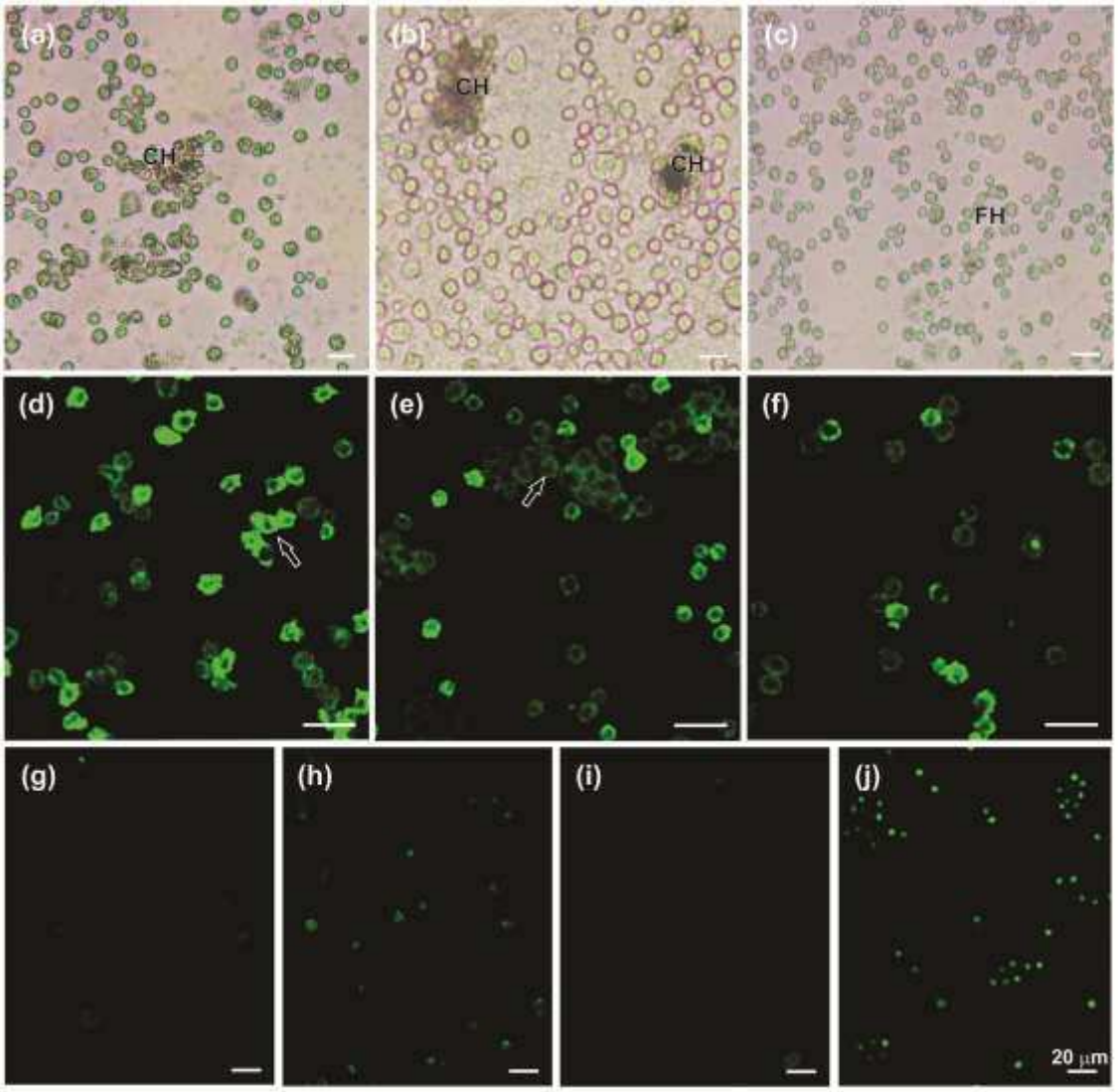

Fig. 6. Cellular immune response of *G. mellonella* against *A. pleuropneumoniae* leads to cell aggregation and DNA damage. Aggregation of *G. mellonella* haemocytes 1 hour post-infection with 10^4 CFU/larva of isolates 780 (A), 1022 (B) and PBS control (C). Cell cytoskeleton staining (green) with phalloidin-FITC shows nodule formation and emission of pseudopods (arrows) in response to infection by isolates 780 (D) and 1022 (E). (F): PBS control. Nuclei positivity (green) for TUNEL reaction reveals that DNA damage is less pronounced in haemocytes of larvae inoculated with isolate 780 (G), more pronounced with that of isolate 1022 (H) and absent in the PBS control (I). (J): DNase treatment (positive control). CH- clustered and FH- free haemocytes. Bar = 20 µm.

An *A. pleuropneumoniae hfq* mutant is attenuated in the *G. mellonella* infection model

To determine whether the *G. mellonella* model would be useful for identifying *A. pleuropneumoniae* mutants with attenuated virulence, we compared the survival of infected larvae after infection with either wild-type or its isogenic *hfq* mutant. Using an inoculum of $1.0x10^5$ CFU/larvae for both strains, we observed a significant decrease in virulence of the *hfq* mutant compared to the wild type ($P<0.001$) (Fig. 7a). During the virulence assay, the number of bacterial cells from the *hfq* mutant that were recovered from the tested larvae was reduced by three orders of magnitude over the first 4 hours of infection. In contrast, with the wild type strain, the number of bacteria increased by one order of magnitude 4 hours post-infection, and cells were still present in the larvae 24 hours post-infection (Fig. 7b).

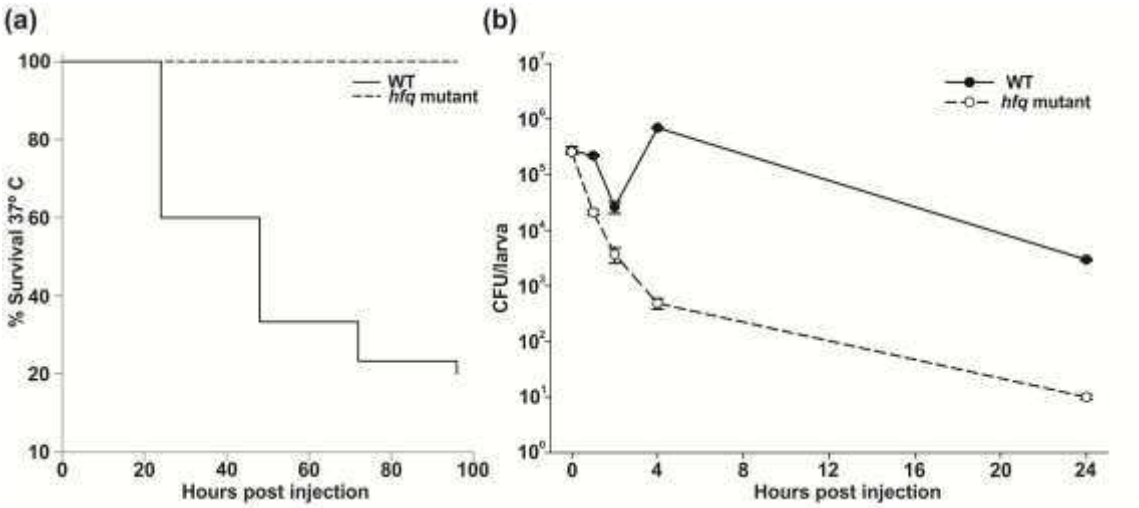

Fig. 7. An *hfq* mutant strain of *A. pleuropneumoniae* displays reduced virulence in *Galleria mellonella*. (A) The death rate was more pronounced for the wild type (WT) strain in comparison to the *hfq* mutant ($P<0.001$) using an inoculum of 10^5 CFU/larva. (B) The haemolymph of larvae was collected and plated at 0, 1, 2, 4 and 24 hours post inoculation. Median CFU values for mutant and wild type strains reveal differences in growth dynamics.

The *G. mellonella* model can be used to study the effectiveness of antimicrobial agents against *A. pleuropneumoniae*

To evaluate whether the *G. mellonella/A. pleuropneumoniae* infection model can be used to study the effect of antibacterial agents, we treated *G. mellonella* infected with *A. pleuropneumoniae* with a single injection of several antibiotics. The larvae were infected with a lethal concentration of *A. pleuropneumoniae* 1022 (susceptible to ampicillin, enrofloxacin and penicillin but resistant to tetracycline, Fig 1). Antibiotic treatment significantly increased the survival rate of *G. mellonella* larvae compared with the control ($P<0.018$ for enrofloxacin and $P<0.001$ for ampicillin and penicillin) (Fig. 8). No significant differences were observed between the group that received the lethal concentration of *A. pleuropneumoniae* and the group treated with tetracycline ($P<0.128$). There were no deaths in the control group.

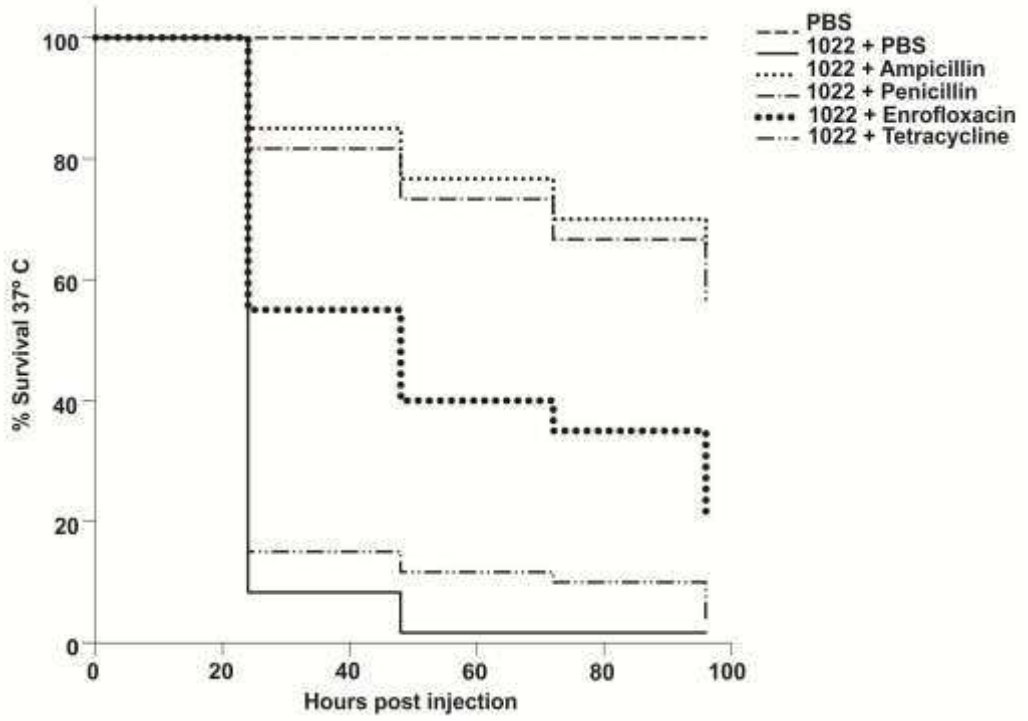

Fig. 8. Antibiotics that are active against *A. pleuropneumoniae* prolong the survival of *A. pleuropneumoniae*-infected *G. mellonella* larva. After infection with a lethal dose of *A. pleuropneumoniae* strain 1022 (10^4 CFU/larva), treatment with antibiotics to which the strain is susceptible (ampicillin, enrofloxacin and penicillin), significantly prolonged survival of *G. mellonella* larvae ($P<0.018$ for enrofloxacin and $P<0.001$ for ampicillin and penicillin compared to untreated larvae). Tetracycline, to which the strain is resistant, causes no differences in killing compared with no antibiotic treatment ($P<0.128$). No deaths were observed in control groups.

DISCUSSION

Using invertebrates for modelling bacterial infection *in vivo* has been investigated for a number of important pathogens. In this study, we described for the first time (to our knowledge), the use of *G. mellonella* larva as an alternative host for studying *A. pleuropneumoniae* virulence. Insects and mammals share common mechanisms in their cellular and humoral innate immune responses to pathogens. *G. mellonella* larvae possess a cuticle that acts as a physical barrier similar to mammalian skin. Additionally, cuticle wounding induces a humoral response in *G. mellonella*, producing soluble factors such as antimicrobial peptides (AMPs). In parallel with the humoral response, *G. mellonella* induces a cellular response to invading microorganisms (Bergin *et al.*, 2003; Kemp & Massey, 2007; Senior *et al.*, 2011). Thus, the *G. mellonella* immune response to *A. pleuropneumoniae* infection is likely to have similarities to that of swine. Because of these similarities, we investigated the response against *A. pleuropneumoniae* infection by various means, such as the bacterial concentration capable of killing *G. mellonella*, infection dynamics, haemocyte numbers, morphology and melanisation in response to infection and damage to the host.

The ERIC-PCR fingerprinting showed that the serotype 8 isolates studied are all genetically distinct, independently of their location or time of isolation. This genetic variability has been suggested to be linked to intrinsic and environmental factors, such as increased homologous recombination given a high number of copies of transposable elements within the genome and the ability of *A. pleuropneumoniae* to transform naturally (Bossé *et al.*, 2004; Liu *et al.*, 2008; Rossi *et al.*, 2013; Xu *et al.*, 2010). Thus, by infecting *G. mellonella* larvae with *A. pleuropneumoniae*, we showed that diverse strains are pathogenic in this model. Additionally, different degrees of pathogenicity can be observed in this host, suggesting that this model can be used for studying clinical isolates from different origins and serotypes. The ability to distinguish the virulence

potential of isolates within the same species using *G. mellonella* has also been evaluated in studies with other bacteria, and some showed a strong correlation with the virulence previously determined in mammalian models (Loh *et al.*, 2013; Mukherjee *et al.*, 2010).

One of the benefits of *G. mellonella* larvae over other invertebrate models is their ability to survive at 37°C, the mammalian body temperature. Larval death caused by *A. pleuropneumoniae* is dependent on the post-infection incubation temperature, as evidenced by the killing rate being lower at 30°C than at 37°C. These results show a limitation of pathogen development at different temperatures from those found in the mammalian body and endorse the *G. mellonella* model for the study of animal pathogens, as observed for other bacteria (Joyce & Gahan, 2010; Loh *et al.*, 2013).

The isolates used herein are all capable of causing disease, as they were obtained from animals with clinical signs of pleuropneumonia. At high inoculum concentrations (up to 10^7 CFU/larva), 100% of the isolates induce death in the *G. mellonella* larvae, whereas a 10^6 CFU/larva inoculum induced death in 22.7% of isolates. This difference is most likely due to a threshold over which processes leading to larval death are induced via the overwhelming activation of the innate immune system. At lower concentrations, bacteria that lack certain virulence factors are most likely bound by cellular receptors that recognise bacterial pathogens-associated molecular patterns (PAMPs), such as the LPS, in particular the lipid A portion, which is a prominent feature of gram-negative bacteria and one of the most potent PAMPs known. The LPS is responsible for the inflammatory response observed during endotoxic shock, leading to the activation of the innate immune system and bacterial clearance (Mogensen, 2009). Non-pathogenic microbial strains of different species do not kill *G. mellonella*, as previously observed for the auxotrophic *E. coli* strain OP50, *Saccharomyces cerevisiae*, some strains of *Aspergillus fumigatus* and here, for the *E. coli* strain K12 (Peleg *et al.*, 2009).

The rapid death of *G. mellonella* infected with the *A. pleuropneumoniae* most virulent strain led us to investigate whether this effect was related to the secretion of exotoxins, currently considered the most important effectors of swine pleuropneumonia clinical signs. The cytotoxic effects of the so-called Apx toxins in porcine leukocytes, neutrophils, and phagocytes have been demonstrated to be relevant in pathogenesis (Frey, 2011). However, we observed that *G. mellonella* mortality is not directly related to the toxicity of culture supernatant, but likely to the presence of other virulence factors that ensure the persistence and survival of the bacteria within the insect, such as the capsule, fimbriae, adhesins and host nutrient acquisition proteins (Chiers *et al.*, 2010). Even when the supernatant of the culture of the serotype 1 (considered the most virulent, producer of the strongly hemolytic toxin ApxI) was tested, no killing was observed (data not shown). This is not surprising since Apx toxins appear to have specific tropism for porcine cells (Kuhnert *et al*., 2002). We would argue this is a strength of the *G. mellonella* model as it allows the identification of other (non-Apx) virulence factors. We envisage that the model can be used as an initial screen to select mutants for testing for virulence in pigs e.g. for evaluation as live attenuated mutants.

Differences in *A. pleuropneumoniae* virulence observed in *G. mellonella* could be due to variations in infection dynamics. Possibly, highly virulent strains are able to grow within the host, while low virulence strains are not, and the mortality of *G. mellonella* is due to an increase of the concentration of bacteria in the larva. Another possibility is that low-virulence strains are killed by the immune system, while those with high virulence are able to evade it, and mortality is due to the persistence of the microbial cells. This is consistent with a preliminary test performed with these isolates killed by UV-light, in which no killing was observed for either the least or the most virulent isolate (data not shown). Our results show that the immune system of *G. mellonella* is able to respond to and rapidly reduce the number of bacteria of the less

virulent isolate, while the virulent isolate had a slight decrease in the number of bacteria during the initial stage of infection, followed by a possible evasion of the immune system and proliferation. Other studies have shown that high pathogen virulence is associated with bacterial proliferation within *G. mellonella*, while low virulence is associated with bacterial clearance (Evans & Rozen, 2012; Joyce & Gahan, 2010; Loh *et al.*, 2013; Norville *et al.*, 2014).

In a pig infection model, as we have described previously (Sheehan *et al*., 2003), the Hfq mutant was attenuated as adjudged by the lack of lung lesions and mortality, similar to results that were obtained for an *aro*A mutant (Garside *et al.*, 2002). Such results are consistent with the low CIs reported for other *A. pleuropneumoniae* Hfq mutants (Subashchandrabose *et al.*, 2013; Zhou *et al.*; 2008). The fact that an *hfq* mutant is, compared to wild-type, similarly attenuated for infection in the *G. mellonella* model, suggests that the latter can be used to screen for *A. pleuropneumoniae* virulence factors, at least in some strains. Thus the situation mirrors other bacteria where *G. mellonella* infection models have been useful in the identification of virulence genes of interest by screening wild-type/mutant pairs (Evans & Rozen, 2012; Purves *et al*., 2010).

The cellular immune response showed different dynamics between infection with the low and high virulence isolates. The difference in the cellular immune response of *G. mellonella* against different *A. pleuropneumoniae* isolates lead us to infer that the presence of different virulence factors can trigger different immune responses, all with the common goal of eliminating the pathogen. The decrease in circulating haemocytes could be a consequence of the bacterial cytotoxic activity on these cells post-infection with virulent strains. The cytotoxic effect of bacterial infection on *G. mellonella* haemocytes could occur by disruption of the actin cytoskeleton, inhibition of DNA synthesis and induction of apoptosis (Mizerska-Dudka & Andrejko, 2013). Although

the cytotoxic effect on the cytoskeleton exerted by proteins secreted by bacteria or structural components has been observed, we found that circulating *G. mellonella* haemocytes show no cytoskeletal alteration when infected with *A. pleuropneumoniae* compared to the control in our study.

DNA damage was shown by TUNEL staining in circulating *G. mellonella* haemocytes 1 hour after *A. pleuropneumoniae* infection. Nevertheless, the amount of TUNEL-positive nuclei was higher in haemocytes from larvae infected with the most virulent *A. pleuropneumoniae* isolate. After infection with isolate 1022, genomic DNA cleavage occurred in most haemocytes. The changes observed in these haemocytes reflect typical changes observed in the haemocytes undergoing apoptosis as an immune defence response to foreign organisms and toxic compounds (Mizerska-Dudka & Andrejko, 2013; Sung *et al.*, 2003). We raised the hypothesis that the cytotoxic factors originating from *A. pleuropneumoniae* 1022 kill circulating haemocytes by activating their own endogenous apoptosis machinery, causing damage to the haemocyte DNA, thereby decreasing the number of these cells in the larva. This effect is one of the mechanisms used by microbial cells to evade the host immune system and is described elsewhere for other infection models with different microorganisms and insects (Brillard *et al.*, 2001; Cho & Kim, 2004; Mizerska-Dudka & Andrejko, 2013).

A. pleuropneumoniae infection is also accompanied by humoral defences that lead to melanisation of the *G. mellonella* larvae. After recognising and phagocytosing the pathogen, melanin is deposited around microbes within the haemolymph and is thought to facilitate bacterial killing (Kavanagh & Reeves, 2004). This response is especially marked after infecting *G. mellonella* with a lethal dose of isolate 1022, in which melanisation began within one hour of infection in the dorsal region of the larva, which contains the heart. Previous studies with other insects show that during infection, pathogens can accumulate on the heart surface. This region has been named as the new

insect immune tissue because it is flanked by haemocytes that sequester the pathogen that are in flux in the haemolymph. These sessile haemocytes perform rapid phagocytosis of pathogens during the course of bacterial infection, and moreover recruit circulating haemocytes to the heart region where they bind to cardiac muscle, and continue phagocyting pathogens (Hillyer *et al.*, 2007; King & Hillyer, 2012).

Sessile *G. mellonella* haemocytes are of great importance during the control of *A. pleuropneumoniae* infection and pathogen recruitment, and circulating haemocytes are essential for the management of infection by the most and least virulent isolates, although the responses were more pronounced in infections with the former. Thus, decreasing the number of circulating haemocytes in the haemolymph of *G. mellonella* after *A. pleuropneumoniae* 1022 infection may be related to the recruitment and accumulation of haemocytes in the heart region and neighbouring organs such as the pericardial cells and fat body, in an attempt to eliminate the pathogen. This fact reinforces the hypothesis that coordinated interactions between the insect's open circulatory system and immune system are essential for effective insect immune responses (King & Hillyer, 2012).

Nodulation occurs when multiple haemocytes stick together, forming an overlapping sheath around the pathogen. Nodulation finishes with prophenoloxidase activation and melanisation of mature nodules (Browne *et al.*, 2013; Lavine & Strand, 2002). We observed nodule formation with points of melanisation in the haemolymph, fat body and pericardial region post-infection with strains of different virulence levels, showing that this is a basic defence mechanism in the model *G. mellonella*/*A. pleuropneumoniae*.

The administration of antimicrobials can successfully prevent lethal infections in *G. mellonella* (Aperis *et al.*, 2007; Peleg *et al.*, 2009; Thomas *et al.*, 2013). Our results show that the *G. mellonella*/*A. pleuropneumoniae* infection model can be used to

evaluate the efficacy of antibacterial agents *in vivo*. Only treatment with tetracycline was not effective, as expected. The result of tetracycline treatment resembles the results obtained in field studies with pigs, as treatment with this drug shows low efficacy due to high incidence of tetracycline resistance in *A. pleuropneumoniae* (Archambault *et al.*, 2012). Thus, this result reinforces the notion that this model can be used to study the efficacy of antibiotic treatments used in the field for the control of porcine pleuropneumonia.

The findings presented herein indicate that *G. mellonella* larva is a suitable infection model for studying *A. pleuropneumoniae* infection. We describe the convenience of this model not only for host-pathogen interactions in *A. pleuropneumoniae* but also to assess the efficacy of antibiotic treatments. A reliable, inexpensive *A. pleuropneumoniae* infection model will reduce the dependence on mammalian infections, although will not replace it. It should also allow several applications for studying this and other veterinary pathogens, facilitating preliminary high throughput laboratorial screening studies with clinical isolates, surveillance of clinical populations, epidemiological studies and the investigation of defined mutants and novel virulence determinants.

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Supplementary material

Table S1. Clinical isolates and reference strains used in this work.

Actinobacillus pleuropneumoniae	
Clinical isolates	Serotype 1: 010; serotype 2: 407; serotype 7: 713; serotype 8: 236, 345, 433, 458, 459, 460, 514, 518, 597, 628, 692, 694, 712, 780, 795, 812, 832, 839, 1022, 5264, 5651, MIDG2331.
Isogenic mutant strain	MIDG2331 Δ*hfq*
Reference strains	Serotype 1: Shope4074; serotype 2: S1536; serotype 7: WF83; serotype 8: 405.
Escherichia coli	K12

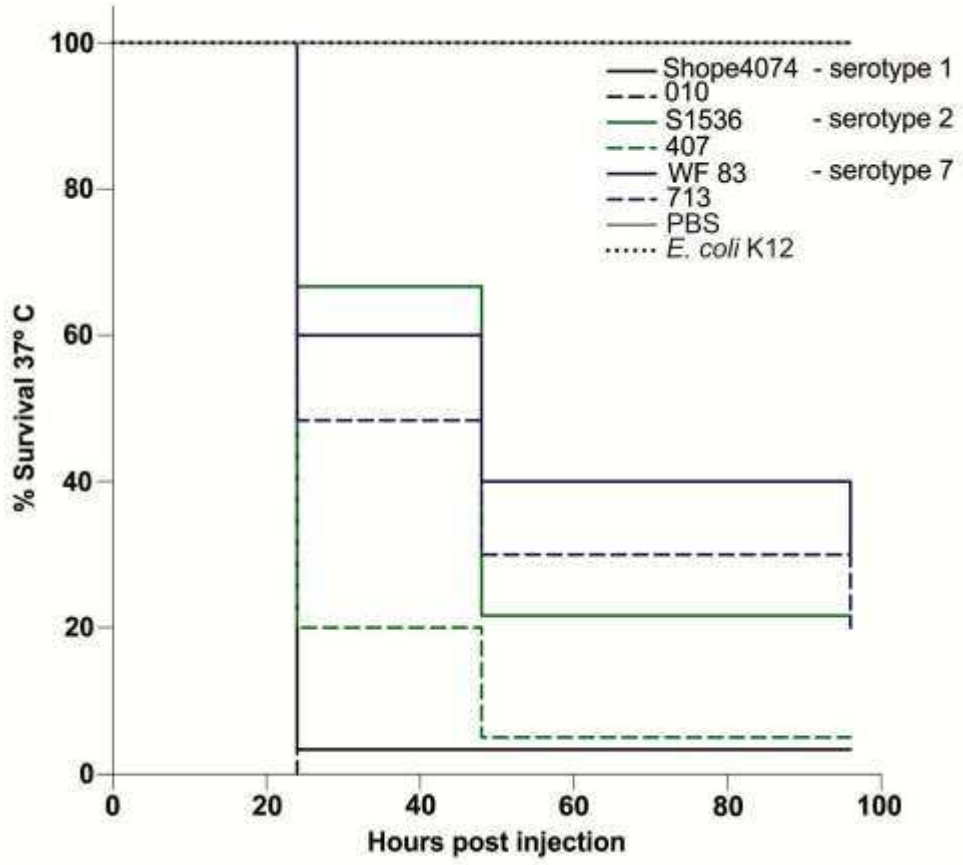

Fig. S1. *Galleria mellonella* killing by different serotype reference strains and clinical isolates of *A. pleuropneumoniae*. Killing was determined for reference strains Shope 4074, S1536 and WF83 and clinical isolates 010, 407 and 713. Shope 4074 expresses the exotoxins ApxI, ApxII and ApxIV, S1536 ApxII, ApxIII and ApxIV, and WF83 ApxII and ApxIV (Beck *et al*., 1994). Larvae were inoculated with 10^5 CFU at 37°C and monitored for 96 h.

Capítulo 2

Virulence complexity of *Actinobacillus pleuropneumoniae* serotype 8 clinical isolates from Brazil

Artigo escrito de acordo com as normas da revista Annals of Microbiology
Section: Short Communications

Title: Virulence complexity of *Actinobacillus pleuropneumoniae* serotype 8 clinical isolates from Brazil

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ABSTRACT

Actinobacillus pleuropneumoniae is the etiological agent of porcine pleuropneumonia, a disease associated with major global economic losses. Infection by *A. pleuropneumoniae* is a multifactorial process and many virulence factors could be involved, as RTX toxins, biofilm and antimicrobial resistance. Here, we evaluated the correlation between the biofilm-forming capacity and antimicrobial resistance phenotypes with virulence of clinical isolates of *A. pleuropneumoniae* serotype 8 in the alternative infection model *Galleria mellonella*. We observed that *A. pleuropneumoniae* virulence is complex even within a single serotype.

Key words: *Pasteurellaceae*, porcine pleuropneumonia, biofilm, antimicrobial resistance, *Galleria mellonella*

Actinobacillus pleuropneumoniae, a member of the family *Pasteurellaceae*, is the causative agent of porcine pleuropneumonia, a costly, severe and highly contagious infectious respiratory disease. Transmission occurs through aerosol from infected animals or asymptomatic carriers and can affect pigs of all ages (Gottschalk & Taylor, 2006). Currently, there are 15 known serotypes of this bacterial pathogen, the prevalence of which varies greatly worldwide. All serotypes are capable of causing disease, although evidences show that the virulence varies in different serotypes (Inzana & Champion, 2007). The virulence of *A. pleuropneumoniae* is influenced by multiple known and putative infection-associated factors (Bossé *et al.*, 2002; Chiers *et al.*, 2010). The best described *A. pleuropneumoniae* virulence factors include: capsular polysaccharide, lipopolysaccharide (LPS), outer membrane proteins, iron-acquisition systems, adhesins, Apx toxins and some anaerobic respiration enzymes (Chiers *et al.*, 2010; Li *et al.*, 2014). In *A. pleuropneumoniae*, the biofilm formation can aid to increase virulence, since this feature allows the permanency of the microorganism in the

host by protecting it from its defense mechanisms and facilitates horizontal gene transfer, thereby contributing to antimicrobial resistance spreading (Labrie *et al.*, 2010).

Various antimicrobial resistance patterns have been observed in several studies among strains from different geographical regions, serovars and also over time. While most isolates of *A. pleuropneumoniae* are reported to be susceptible toward fluoroquinolones, ceftiofur, and florfenicol, resistances toward ampicillin, tetracycline, and other antimicrobials are emerging. A high incidence of tetracycline resistance in *A. pleuropneumoniae* was reported in several producing countries of swine during the last decade (Archambault *et al*., 2012).

Clinical isolates of *A. pleuropneumoniae* serotype 8, the most widespread in southeastern Brazil (Rossi *et al.*, 2013), display high cytotoxic activity and are generally responsible for low mortality but high morbidity. This serotype is also the prevalent in other pig-producing countries, such as Canada, Mexico, the United States of America and the United Kingdom (Blackall *et al.*, 2002; Gottschalk & Lacouture, 2014; O'Neill *et al.*, 2010). This work aimed to verify the correlation among the biofilm-forming capacity, antimicrobial resistance profile and virulence in the alternative infection model *Galleria mellonella* of 21 *A. pleuropneumoniae* serotype 8 clinical isolates obtained from pigs from 2003 to 2011. *Actinobacillus pleuropneumoniae* serotype 8 clinical isolates used in this study were kindly provided by the company MICROVET (Minas Gerais, Brazil). They were obtained from the lungs and tonsils of swine with clinical signs of pleuropneumonia from different farms of Southeastern Brazil and are considered persistent in the respective farms. All isolates from bacterial stocks kept at -80°C were grown at 37 °C for 16 h in a 5% CO_2 atmosphere in Brain-Heart Infusion (BHI) medium (Becton Dickinson, USA) supplemented with NAD (10 $\mu g.ml^{-1}$) (Sigma-Aldrich, UK). Genomic DNA from *A. pleuropneumoniae* isolates was obtained using

the FastDNA® Spin kit (MP Biomedicals, USA) according to the manufacturer's instructions.

Biofilm formation was quantified in 96-well polystyrene microtiter plates (Costar®, USA). Overnight cultures of the bacteria to be tested were resuspended to an OD_{600} of 0.1 in BHI-NAD. A volume of 100 µL aliquots of each dilution was then dispensed into a microtiter plate well. Negative control wells contained only medium and, each isolate was tested in quadruplicate. The adhesion of bacteria were detected following 24 h of incubation at 37 °C, by crystal violet staining as previously described (Stepanovic *et al.*, 2004). The measurement of biofilm formation was done using a Multiskan GO®Microplate Spectrophotometer (Thermo Fisher Scientific, USA).

All tests were carried out in biological replicate. Based on the OD produced by bacterial biofilms, isolates were classified into the following categories; weak, moderate or strong biofilm producer, according to a previously described method (Stepanovic *et al.*, 2004). Briefly, the cutoff OD (ODc) was defined as three standard derivations above the mean OD of negative control. Isolates were classified as follows: OD<ODc = non biofilm producer; ODc<OD<(2.ODc) = weak biofilm producer; (2.ODc)<OD<(4.ODc) = moderate biofilm producer; and (4.ODc)<OD = strong biofilm producer.

The broth microdilution method was performed to determine the antimicrobial resistance profile in *A. pleuropneumoniae* clinical isolates using the Sensititre Standard Susceptibility MIC Plates BOPO6F (Trek Diagnostic Systems, Thermo Fisher Scientific, USA) in accordance with the CLSI guidelines (Clinical and Laboratory Standards Institute), document M31-A3, 2008. The results for biofilm formation test and antimicrobial resistance are shown in Fig. 1A.

Polymerase chain reaction (PCR) amplification of the β-lactam, amphenicol, aminoglycosides, quinolone, sulphonamides and tetracycline resistance genes was performed using Go *Taq* DNA polymerase (Promega, USA) and specific

oligonucleotide primers previously described (Santos, 2013). The PCR products were separated on agarose gel by electrophoresis and visualized using standard techniques.

Virulence of *A. pleuropneumoniae* isolates was determined using larvae of *Galleria mellonella* as previously described (Pereira *et al.*, 2015). The larvae were infected with crescent doses of *A. pleuropneumoniae* suspensions (10^4-10^7 CFU/larva) for establishing the lethal dose of each isolate. Larvae were incubated at 37 °C, and the number of dead animals was scored every 24 h over a 96 h period. The tests involving the inoculation of bacteria in the *G. mellonella* larvae were performed in biological and experimental triplicate (n =10 larvae per each replica). Survival curves were plotted using the Kaplan-Meier method and differences in survival were calculated by using the log-rank test using R v.2.13.0. A *P* value of < 0.05 was considered to be statistically significant.

All the 21 *A. pleuropneumoniae* isolates presented the ability to form biofilm, 23.8% present strong biofilm-forming capacity, while the remaining 76.2% presented moderate capacity. The members of the family *Pasteurellaceae* are inhabitants of mucosal surfaces of mammals, therefore, biofilm formation may be crucial for bacterial adherence to target tissues and resistance to both the host's immune system and to antibacterial treatments (Labrie *et al.*, 2010). Biofilm formation is a stress response in *A. pleuropneumoniae*, and has been suggested to contribute to colonization and persistence of this pathogen not only in damaged tissue of the host during infection but also in other environments such as drinking water of swine farms (Loera-Muro *et al.*, 2013; Li *et al*., 2014;).

Biofilms provide ideal niches for the exchange of extrachromosomal DNA, such as plasmids, which generally code for antibiotic resistance genes (Jacques, 2004). All the 21 clinical isolates analysed were resistant to tetracycline. Whilst the resistance rate to other antibiotics was varied, with 33.33% of the isolates presenting resistance to β-

lactam antibiotics, 81% to amphenicol, 95.23% to aminoglycosides, 14.28% to quinolone and 76.19% to sulfonamides. The biological function of the resistance genes detected by PCR was confirmed by MIC (Minimal Inhibitory Concentration) as described previously.

As observed in this work, resistance to tetracycline is widespread in Brazilian *A. pleuropneumoniae* clinical isolates. This is in accordance to what has already been reported in other countries producing pigs and is related to an indiscriminate management of antimicrobial therapies (Archambault *et al.*, 2012). Studies have also demonstrated the accumulation of tetracycline (*tet*) resistance genes in aquatic biofilms due to periodic loadings from swine lagoons, showing that biofilm formation may promote the persistence of bacteria in the environment. This form of adherence allows the horizontal transfer of resistance genes of these bacteria and disseminates this characteristic for all the flock (Zhang *et al.*, 2009). Thus, the treatment of porcine pleuropneumonia with drugs such as tetracycline shows low efficacy due to high incidence of resistance in *A. pleuropneumoniae*.

Defence mechanisms against bacterial infections are sufficiently similar in invertebrates and mammals to allow the use of invertebrates as infection models for mammalian pathogens (Kamar *et al.*, 2013). In the *G. mellonella* assay, the virulence potential of the isolates showed significant variation: 14.28% of the isolates were significantly more virulent in the insect, while 52.38% showed moderate virulence and 33.33% low virulence. Thus it was possible to rank our clinical isolates in three groups with significant differences in virulence level (Fig. 1B).

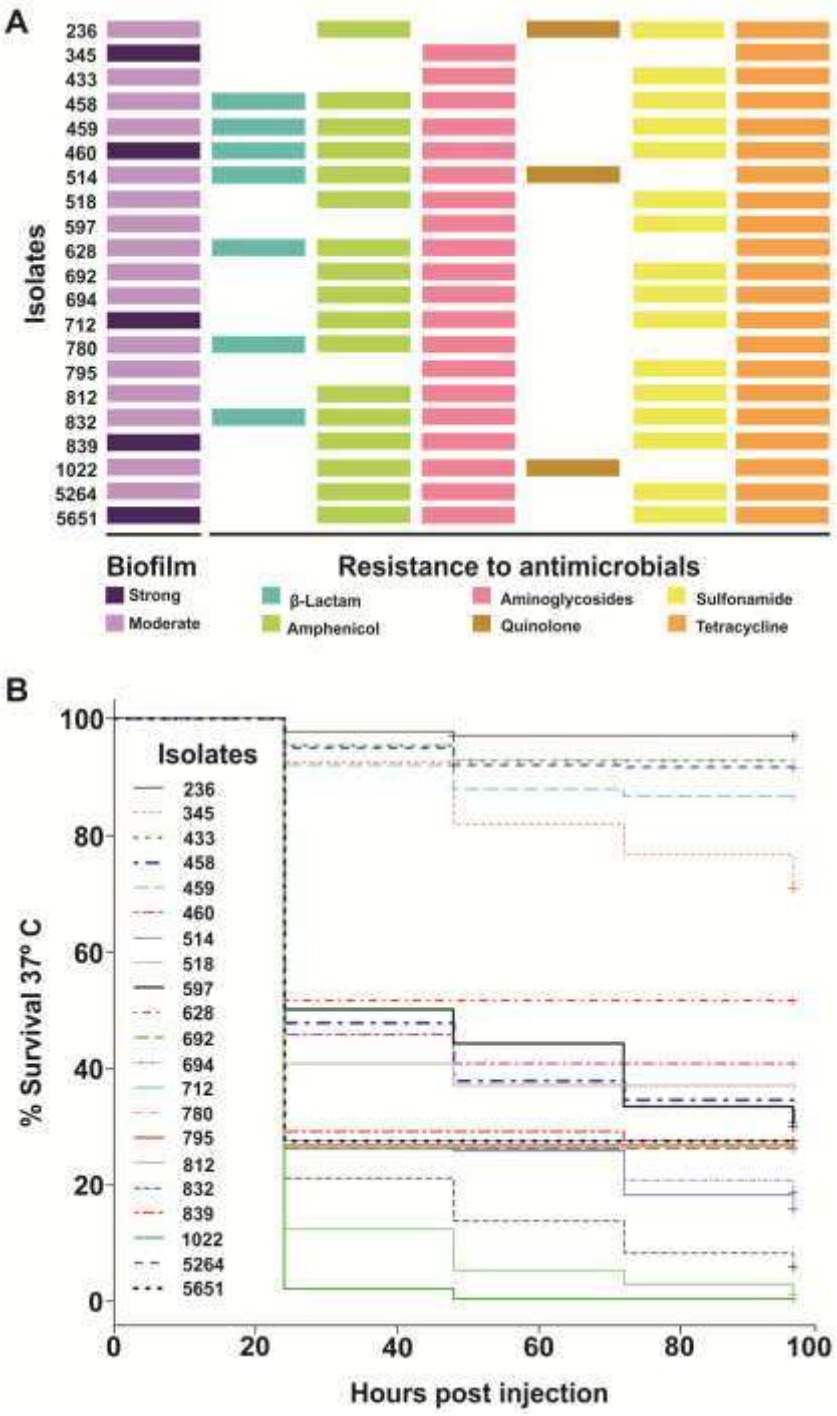

Fig. 1 (A) Biofilm formation potential and resistance profiles among serotype 8 *A. pleuropneumoniae* clinical isolates from Brazil. (B) Different *A. pleuropneumoniae* clinical isolates are able to infect *G. mellonella* to different degrees. Kaplan-Meier survival curves of *G. mellonella* larvae over 96 hours post injection with 1 x 10^5 CFU of *A. pleuropneumoniae* isolates.

Therefore, from the results obtained a pairwise correlation analysis to investigate the correlations between the *A. pleuropneumoniae* phenotypes was used (Fig. 2). The correlation coefficients were calculated together (95% confidence intervals) with the software R3.0.2. The biofilm formation potential in *A. pleuropneumoniae* was positively correlated with the presence of the high number of antimicrobial resistances ($P< 0.05$). This can be explained by the fact that the cells in biofilm dramatically reduced susceptibility to antimicrobial agents and the matrix of biofilm contributes to the dissemination of antimicrobial resistance (Labrie *et al.*, 2010). However, no significant correlation was found between the virulence in *G. mellonella* model and biofilm formation ($P> 0.05$). The ability to form biofilms has been associated with the virulence of *A. pleuropneumoniae* through studies with attenuated mutants. However, the contribution of biofilm development to the virulence of *A. pleuropneumoniae* is still not clear (Bossé *et al.*, 2010). Here it was not possible to observe a correlation between the ability to form biofilm and virulence of our isolates.

No significant result were also found for the correlation between virulence and antimicrobial resistance ($P> 0.05$), suggesting that antimicrobial resistance may not be related to the virulence in *A. pleuropneumoniae,* if considered separately.

The virulence of *A. pleuropneumoniae* is a multifactorial process governed by many factors acting alone or, more often, in combination to establish the pathogen in the porcine host (Jacques, 2004). The variability in the biofilm-forming capacity, lethal doses in the *G. mellonella* model, resistance to antimicrobial and the lack of statistically significance between these virulence aspects analyzed show that *A. pleuropneumoniae* virulence is complex and diverse even within a single serotype.

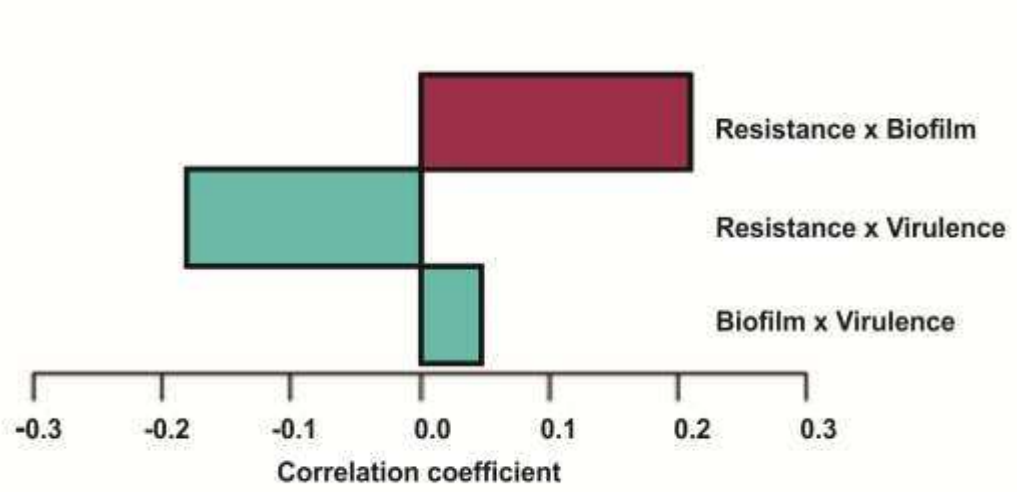

Fig. 2 Pairwise correlation between the virulence and other phenotypes. Each phenotype was compared to each of the others in pairwise tests, and correlation coefficients were determined for each pair of phenotypes, the correlation between biofilm and antimicrobial resistance was significant ($P< 0.05$), and other correlations were not significant ($P> 0.05$).

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Capítulo 3

Draft genome sequences of six *Actinobacillus pleuropneumoniae* serotype 8 Brazilian clinical isolates: insight into new applications

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Title: Draft genome sequences of six *Actinobacillus pleuropneumoniae* serotype 8 Brazilian clinical isolates: insight into new applications

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Running Head: *Actinobacillus pleuroneumoniae* serotype 8 genomes

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ABSTRACT

Actinobacillus pleuropneumoniae is the causative agent of swine pleuropneumonia, a highly contagious disease associated with pigs of all ages that results in severe economic losses to the industry. Here, we report for the first time, six genome sequences of *A. pleuropneumoniae* clinical isolates of the worldwide spread serotype 8.

Actinobacillus pleuropneumoniae is the causative agent of porcine pleuropneumonia, a costly, severe and highly contagious infectious disease (1). There are 15 known serotypes of this microorganism, the prevalence of which varies greatly worldwide (2). Serotype 8 isolates, the most widespread in southeastern Brazil (3), display high cytotoxic activity and are generally responsible for low mortality but high morbidity. This serotype is also dominant in other pig-producing countries, such as Mexico and the United Kingdom (4, 5).

We sequenced six serotype 8 *A. pleuropneumoniae* clinical isolates that are persistent in southeastern Brazil farms and present different virulence degrees (6). Each isolate was grown in culture medium (BHI, BD Biosciences), and total genomic DNA was extracted using the FastDNA® Spin kit (MP Biomedicals). Whole-genome sequencing was performed using an Ion Torrent personal genome machine sequencer (Life Technologies) using 200-bp chemistry with an Ion 318C chip. Assembly for each isolate was performed with Newbler 2.9. Contigs were reordered with Mauve using *A. pleuropneumoniae* JL03 as the reference genome (GenBank accession CP000687). Automatic annotation of genes predicted with the Genemark and Glimmer programs was performed by BLASTp against the NCBI NR, KEGG, UniProt and TCDB databases using 100% query and subject coverage and a minimum of 95% positive as the cutoff. Manual annotation was performed using the System for Automated Bacterial Integrated Annotation (SABIA) (7).

Genome sequencing of *A. pleuropneumoniae* isolates 460, 518, 597, 780, 1022, and 5651 resulted in high-coverage assemblies of the 2.24 ± 0.03 Mbp genomes (between 90 and 123-fold), with 40.33% ± 0.31 GC content and an average coding gene length of 844.97 ± 9.26 bp. The coding regions correspond to 82.41% ± 0.58 of the genome, which should represent most of the functionally annotated genes and allow for comparative studies using these sequences. Approximately 2358.16 ± 42.01 loci were predicted for each genome, and these showed significant hits with databases of functional data: KEGG (90.50%), UniProt (79.40%), NCBI NR (93.03%) and TCDB (23.55%). Gene prediction revealed 48.17 ± 1.94 tRNA genes and four ribosomal RNA operons. According to a Bidirectional Best Hits comparison, the genomes present 2296 ± 33.32 clusters in common with strains JL03 and L20 (GenBank accession CP000569) and 79.5 ± 24.05 non-clustered proteins, revealing high genetic variability of this species, often associated with intrinsic and environmental factors, such as increased horizontal gene transfer frequency, because of the remarkable ability of *A. pleuropneumoniae* to transform naturally (8, 9).

These new six *A. pleuropneumoniae* genome assemblies are the first for serotype 8 to be published in the major sequence databases. Because of the relevance of this serotype in many countries, the public availability of these sequences will be globally helpful for comparative genomic studies, thereby contributing to the discovery and identification of common virulence determinants and molecular markers that could be used for effective diagnosis and vaccine production.

Nucleotide sequence accession numbers. The assembled genomes of the six *A. pleuropneumoniae* isolates have been deposited at the NCBI database under the accessions JSVG00000000, JSVZ00000000, JSVX00000000, JSVV00000000, JSVF00000000 and JSVY00000000.

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3. INFORMAÇÕES ADICIONAIS SOBRE OS GENOMAS SEQUENCIADOS

Genome features of six isolates of *A. pleuropneumoniae* serotype 8						
Features	**Isolates**					
	460	**518**	**597**	**780**	**1022**	**5651**
Length (Mbps)	2.21	2.27	2.21	2.27	2.26	2.26
GC percent	40.12	40.46	40.62	40.70	39.94	40.14
Coding region (% of genome length)	82.31	82.16	81.76	82.95	83.26	82.02
Total locus	2307	2397	2310	2362	2367	2406
tRNA genes	48	51	48	45	48	49
rRNA operons	4	4	4	4	4	4
Genes average length (bps)	846.54	837.96	846.36	852.39	849.4	831.55
Proteins in cluster	2251	2336	2260	2312	2314	2303
Exclusive proteins	85	79	83	57	53	120

4. CONCLUSÕES

Este trabalho demonstra que a infecção por *A. pleuropneumoniae* pode ser estudada no modelo alternativo *G. mellonella*. A condução dos experimentos mostra a conveniência de utilizar esse modelo não só para estudos de interação patógeno-hospedeiro, mas também para avaliar a eficácia de tratamentos com antibióticos e estudo de mutantes. A utilização de um modelo de infecção confiável e de baixo custo como *G. mellonella* é de extrema importância, pois pode reduzir a dependência de mamíferos como modelos de infecção. Além disso, a utilização desse modelo permite várias aplicações para o estudo de *A. pleuropneumoniae* e também outros patógenos. Entre estas possíveis aplicações destacam-se a triagem de isolados clínicos, investigação de linhagens mutantes, vigilância de populações clínicas e estudos epidemiológicos.

A ausência de correlação entre a virulência de isolados clínicos de *A. pleuropneumoniae* sorotipo 8, com a capacidade de formar biofilme e o perfil de resistência à antimicrobianos, mostrou que a virulência de *A. pleuropneumoniae* é complexa mesmo dentro de um único sorotipo.

Com o sequenciamento, montagem e anotação de seis genomas de *A. pleuropneumoniae* sorotipo 8 com diferentes perfis fenotípicos, foi possível observar que embora algumas características genômicas dessa espécie sejam bem conservadas, existem diferenças significativas entre eles e, essas que podem estar relacionadas com a virulência da espécie.

Novos trabalhos devem ser realizados para investigar as características genômicas que estão relacionadas com a diversidade fenotípica de *A. pleuropneumoniae*, principalmente aquelas que possam estar envolvidas com a virulência desse patógeno.