

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

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*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 37 °C but had different LD<sub>50</sub> values, ranging from 10<sup>4</sup> to 10<sup>7</sup> c.f.u. per 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. Melanization 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.

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## 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 varies worldwide (Blackall *et al.*, 2002). *A. 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 UK (Dubreuil *et al.*, 2000; O'Neill *et al.*, 2010), and are prevalent in south-eastern 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

Abbreviations: ERIC-PCR, enterobacterial repetitive intergenic consensus PCR; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling.

One supplementary table and one supplementary figure are available with the online Supplementary Material.

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 an 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 micro-organisms) and lacks some of the functional components of the mammalian immune system, such as specialized phagocytic cells (Champion *et al.*, 2010).

Some of the limitations of the *C. elegans* model can be resolved by using insects, such as 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 antimicrobial peptides, complement-like proteins, melanin and products created by proteolytic cascades [e.g. the phenoloxidase pathway], which immobilize 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, such as *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 antimicrobial 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*.

## METHODS

**Micro-organisms, culture conditions and DNA extraction.** *A. pleuropneumoniae* reference strains and clinical isolates from south-eastern Brazil and the 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 a deletion of 220 bp in the ORF, generated by a technique of successive unmarked mutation and chromosomal insertion (Bossé *et al.*, 2014), was also used (Table S1, available in the online Supplementary Material). All *A. pleuropneumoniae* strains from bacterial stocks kept at –80 °C were grown at 37 °C for 16 h in a 5% CO<sub>2</sub> atmosphere in brain-heart infusion (BHI) medium (Becton Dickinson) supplemented with NAD (10 µg ml<sup>-1</sup>) (Sigma-Aldrich). Genomic DNA from *A. pleuropneumoniae* and *G. mellonella* was obtained using the FastDNA Spin kit (MP Biomedicals) according to the manufacturer's instructions.

**Molecular characterization of *A. pleuropneumoniae* isolates using ERIC-PCR.** PCRs were performed in a C1000TM Thermal cycler (Bio-Rad) using 1 U of JumpStart™ *Taq* DNA polymerase (Sigma-Aldrich). The enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) fingerprinting technique was employed to estimate genetic variability among the isolates used. The reaction was performed using the oligonucleotide pair ERIC1R (5'ATGTAAGCTCCTGG-GGATTCAC3') and ERIC2 (5'AAGTAAGTGACTGGGGTGAGCG3') and conducted as described by 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) to reconstruct dendrograms using the unweighted pair group method with arithmetic mean (UPGMA) method. The co-phenetic correlation coefficient (Farris, 1969) was calculated to test the goodness of fit between the similarity shown in the dendrograms and the co-phenetic matrices. ERIC-PCR was standardized and repeated three times independently.

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

***G. mellonella* killing assay.** Insects were 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<sup>2</sup> to 10<sup>8</sup> c.f.u. per larva, which were injected into the first right pro-leg into the haemocoel using insulin syringes (Becton Dickinson). Larvae injected with either *Escherichia coli* K-12 or 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 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 LD<sub>50</sub> value 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 replicate).

**Bacterial *in vivo* growth monitoring.** The *A. pleuropneumoniae* isolates with the most extreme (highest and lowest) LD<sub>50</sub> 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 ( $10^4$  c.f.u. per larva), and bacterial growth was investigated 1, 2, 4 and 24 h 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 of the haemolymph of 10 individual larvae and then 10-fold dilutions of each sample were plated on BHI agar supplemented with NAD, and incubated at 37 °C in 5% CO<sub>2</sub> atmosphere. The concentration of c.f.u. in the haemolymph was calculated as the mean 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 oligonucleotide pair APXIVAF (5'GCCTCCGACCTGAATAAACC3') and APXIVAR (5'CAACCATCTTCTCCACC3'). The reaction and cycle parameters of the PCRs 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 of pH 4.5 (Mead *et al.*, 1986) in microcentrifuge tubes previously siliconized with Sigmacote (Sigma-Aldrich). The total cell number was counted under a Zeiss Primo Star light microscope (Carl Zeiss).

**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) (Sigma-Aldrich) in PBS/Triton 1% for 30 min and washed three times in PBS. The slides were mounted in Mowiol anti-fade medium (Fluka), analysed and photographed using a Zeiss LSM 510 confocal microscope (Carl Zeiss) at the Microscopy and Microanalysis Facilities at the Universidade Federal de Viçosa. For the detection of nuclear DNA damage, PBS-washed haemocytes (from both infected and control larvae) were analysed using the terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) reaction. The fragmented DNA was labelled using an *in situ* cell death detection fluorescein kit (Roche Molecular Biochemicals). 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) at  $1 \mu\text{g} \mu\text{l}^{-1}$ , followed by incubation in the reaction buffer for 45 min at 37 °C, according to the 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-virulence bacteria) were processed as follows: larvae were placed in PBS and dissected under a stereoscope, the abdominal cavity was opened laterally using micro scissors and the visceral organs were 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). Fixed samples were rinsed in PBS, dehydrated in a crescent ethanol series (70–100%) and embedded in historesin (Leica). Samples were sectioned (3–4 µm) using glass knives in a Leica RM2255 microtome. Sections were stained with haematoxylin and eosin, mounted in Eukit (Fluka) mounting medium and photographed under the light microscope.

**Administration of antibiotics.** 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 included ampicillin [ $10 \text{ mg} (\text{kg body weight})^{-1}$ ], enrofloxacin ( $2.5 \text{ mg kg}^{-1}$ ), penicillin ( $24000 \text{ UI kg}^{-1}$ ) and tetracycline ( $6 \text{ mg kg}^{-1}$ ) (all from Sigma). Concentrations were based on those used routinely for swine and by previous tests in *G. mellonella*.

**Statistical analyses.** The LD<sub>50</sub> value of each isolate was calculated by fitting a linear regression using R v.2.13.0 (R Development Core Team). 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 ANOVA 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 characterized using the ERIC-PCR fingerprinting technique. All reactions were reproducible, and the dendrograms were statistically supported by high co-phenetic 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 with resistance to antimicrobial agents.

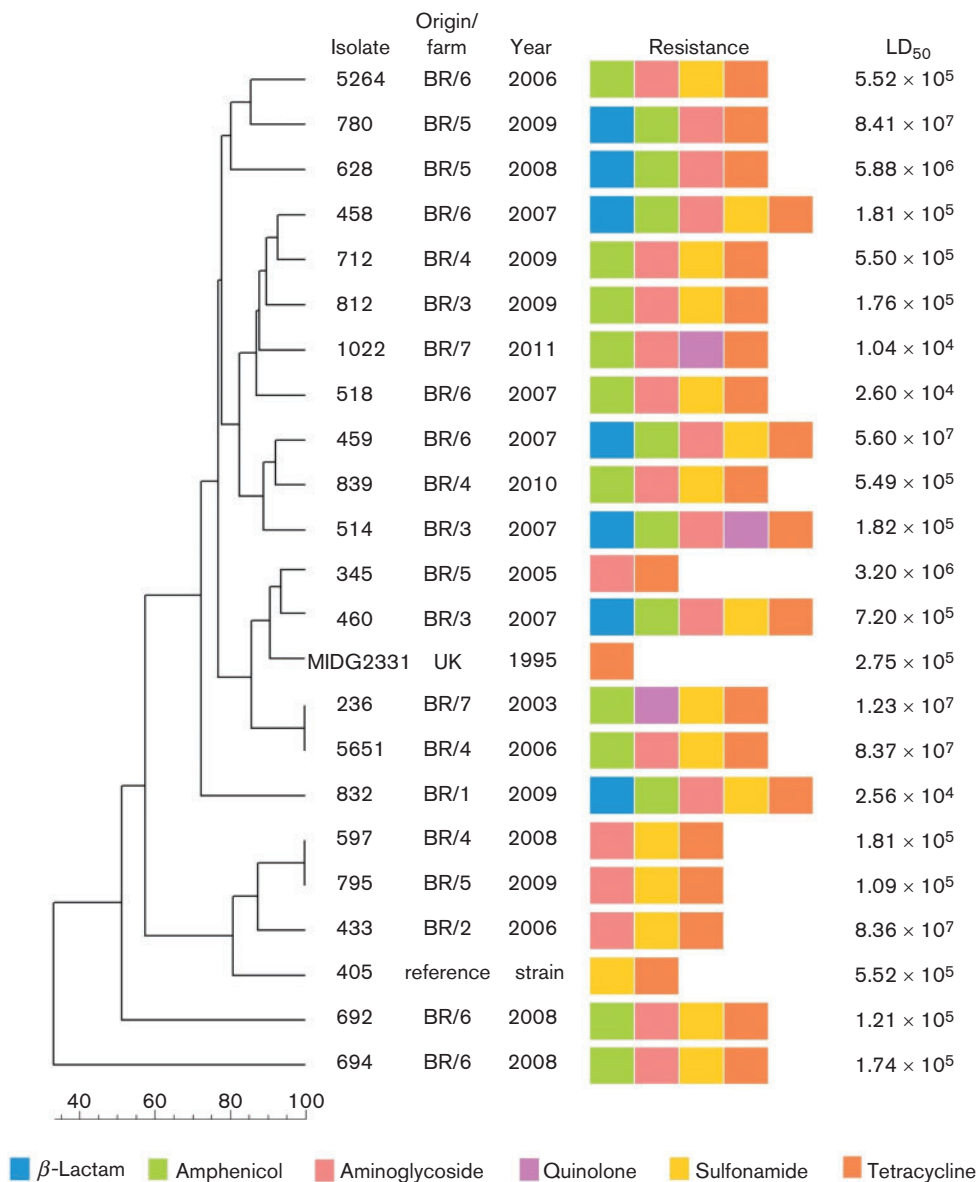
Of the 21 clinical isolates, 1022 was the most virulent, with an LD<sub>50</sub> of  $1.04 \times 10^4$  c.f.u., and 780 was the least virulent, with an LD<sub>50</sub> of  $8.41 \times 10^7$  c.f.u. Further tests were conducted with these two isolates as they have the greatest range in relative virulence. The LD<sub>50</sub> values of 11 of the isolates analysed (52%) were similar (same order of magnitude) to the LD<sub>50</sub> 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. To confirm the efficiency of *G. mellonella* as an alternative host, other reference strains and clinical isolates of 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 toxin 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 with 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.



**Fig. 1.** Genetic and phenotypic variations among serotype 8 *A. pleuropneumoniae* clinical isolates or reference strains from Brazil (BR) and the UK. Genomic differences were observed by ERIC-PCR fingerprinting. Antimicrobial susceptibilities were determined by MIC assay. The LD<sub>50</sub> (c.f.u.) of each isolate was estimated using *G. mellonella* as an infection model. Serotype 8 reference strain 405 was used for comparison.

2). For example, using  $2.8 \times 10^4$  c.f.u. per larvae of isolate 1022 as the inoculum and incubation at 37 °C, 96 % of the *G. mellonella* larvae were killed within 24 h of infection, whereas no significant killing was observed when larvae were inoculated with a 10-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* K-12 strain did not kill *G. mellonella* with an inoculum of up to  $1.0 \times 10^8$  c.f.u. per /larva.

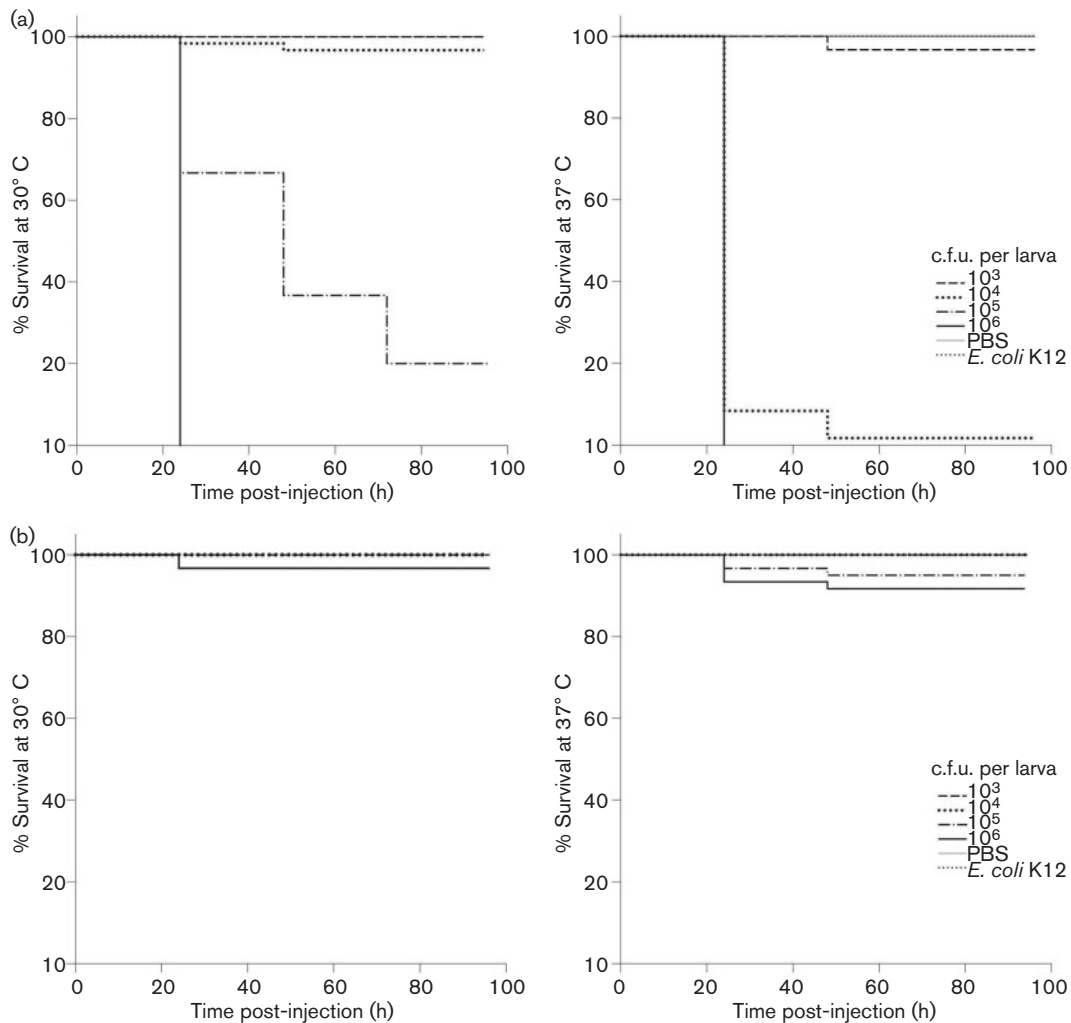
To evaluate the effect of incubation temperature, we analysed the killing of *G. mellonella* at 30 °C (Fig. 2). Similar to our findings at 37 °C, inoculation of *G. mellonella* with *A. pleuropneumoniae* 1022 resulted in larval killing that was dependent on the concentration of bacterial cells injected. However, the killing was more effective at 37 °C, a more appropriate growth temperature for *A. pleuropneumoniae*. In this case, injection of  $2.8 \times 10^4$  c.f.u. per 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 with 96 % mortality 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 no melanization 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).

### ***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



**Fig. 2.** *G. mellonella* killing by *A. pleuropneumoniae* is dependent on inoculum concentration and incubation temperature post-infection. Killing was monitored for the most virulent isolate (1022) (a) 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  $10^4$  c.f.u. per larva at 37 and 30 °C).

the bacteria using both a specific PCR and cell counting after inoculation with doses of  $10^4$  c.f.u. of the high- (1022) and low- (780) virulence isolates. The mean number of bacterial cells from strain 780 recovered from the tested larvae was drastically reduced by four orders of magnitude over the first 4 h of infection, and no bacterial cells were observed in the haemolymph after 24 h of the experiment. In contrast, with isolate 1022, the number of bacteria increased by two orders of magnitude 4 h post-infection, and cells were still present in the larvae 24 h post-infection (Fig. 3a).

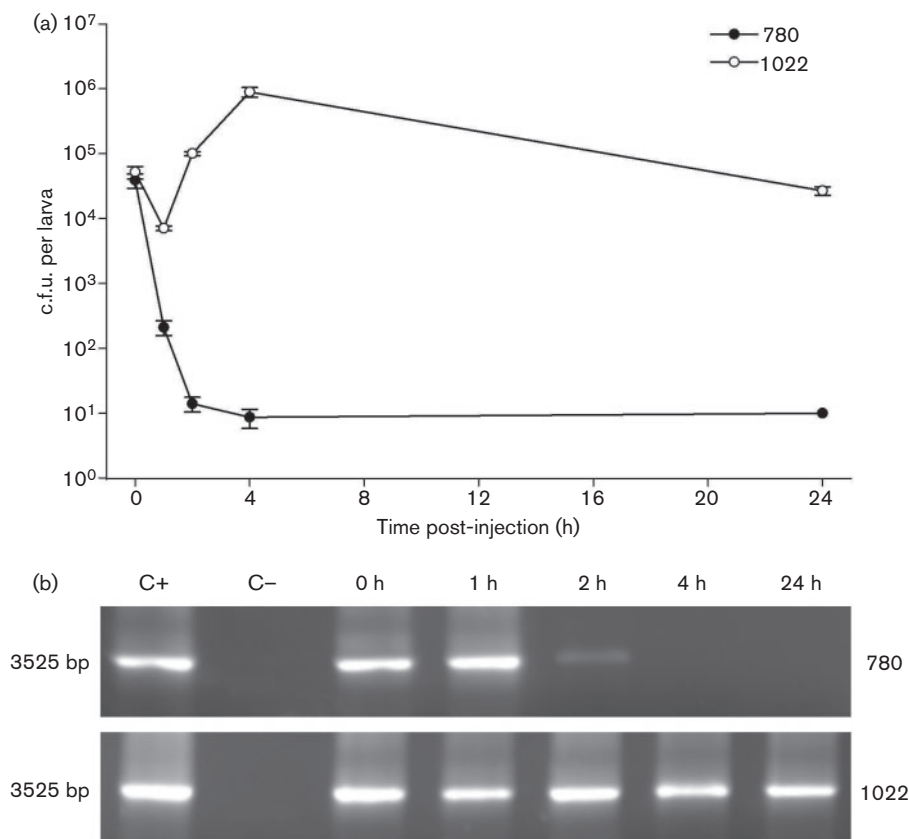
The results were consistent with amplification of the *apxIV* gene, commonly used as a molecular marker to identify this bacterium. Whilst isolate 1022 was detected by PCR at 24 h post-infection, the same was not observed for isolate 780 (Fig. 3b).

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

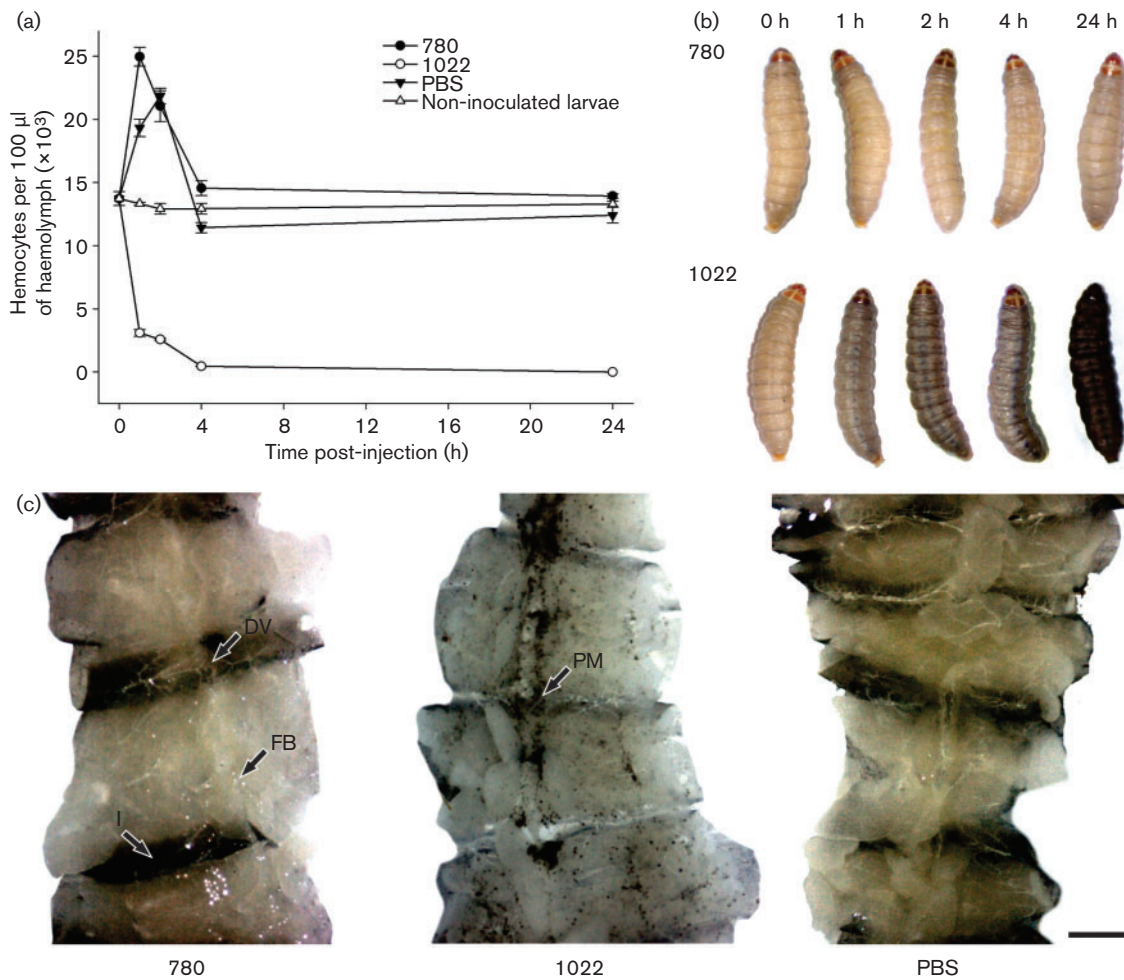
Haemocytes react differently upon exposure to isolates that have different LD<sub>50</sub> (Fig. 4a). The concentration of

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 1 h 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 (melanization) with time, whereas larvae that received isolate 780 showed no signs of darkening during the same period (Fig. 4b). We observed that melanization initially occurs in the dorsal portion of the larva, as clearly observed at 1 and 2 h post-infection with isolate 1022 (Fig. 4b). The differential melanization in the dorsal vessel region of *G. mellonella* was evidenced by the dissection of insects 1 h after inoculation with isolates 780 and 1022 and PBS. The larvae infected with a lethal



**Fig. 3.** *A. pleuropneumoniae* strains display differential abilities to persist and replicate in *G. mellonella*. The larvae were inoculated with  $10^4$  c.f.u. of isolates 780 and 1022. (a) The haemolymph of larvae was collected and plated at 0, 1, 2, 4 and 24 h post-inoculation. Median c.f.u. 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.



**Fig. 4.** *G. 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 melanization. Bar, 0.5 mm.

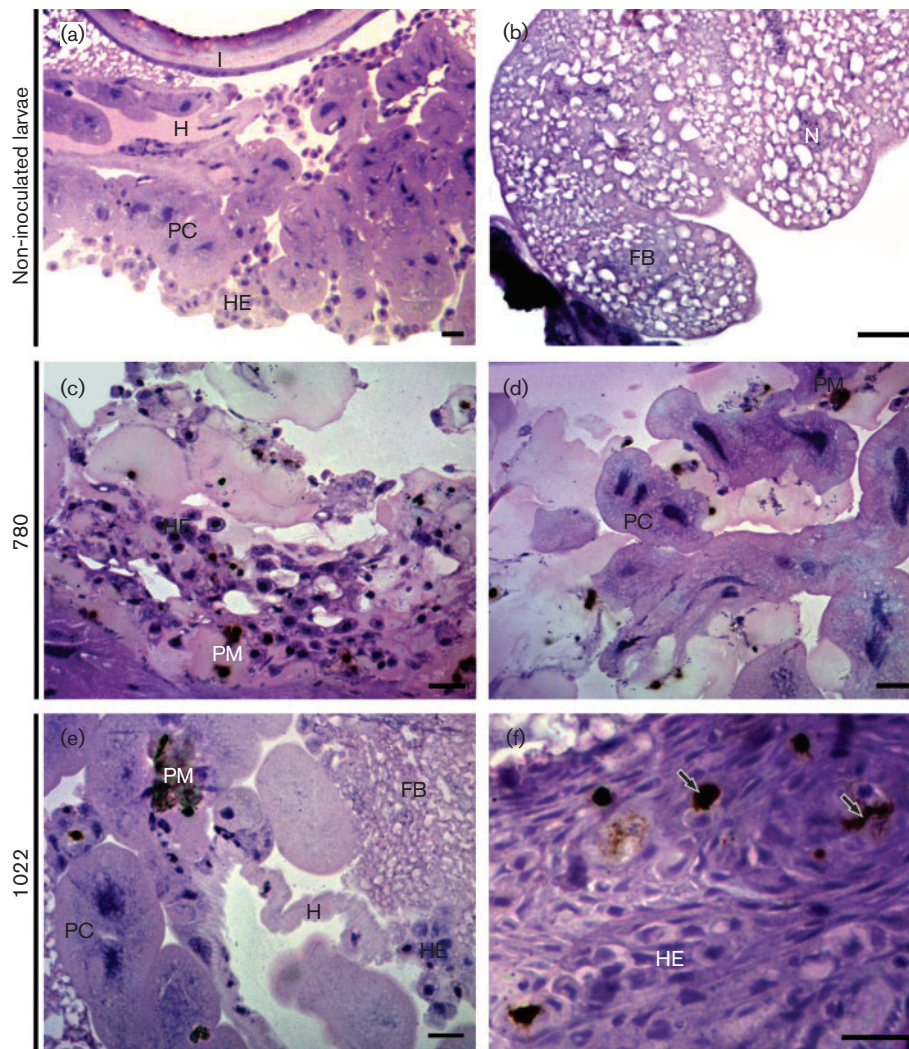
dose of 1022 showed strong dark pigmentation along the dorsal vessel (Fig. 4c).

Histopathological analysis of the dorsal vessels of *G. mellonella* larvae 1 h post-infection revealed pericardial tissues and fat bodies with points of melanization and nodules (Fig. 5c–f); these structures were not observed in the control (Fig. 5a, b). Nodulation and accumulation of haemocytes in the pericardial tissue was more abundant in the larva infected with isolate 1022 (Fig. 5e, f). Points of melanization 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 melanized nodules are observed among circulating haemocytes from caterpillars infected by either isolate 780 or isolate 1022, which was not observed in control (Fig. 6a–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 (Fig. 6d–f). 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 (Fig. 6g–j). 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. 6(g, h).



**Fig. 5.** *A. pleuropneumoniae* infection of *G. mellonella* initiates a robust innate immune response. Histological sections of *G. mellonella* 1 h post-infection with clinical *A. pleuropneumoniae* isolates ( $10^4$  c.f.u. per 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–e) heart and associated tissues of a larvae infected with isolates 780 and 1022 with several points of melanization among pericardial cells (PC) and sessile haemocytes (HE); (f) detail of a nodule with adherent haemocytes (HE) closely associated with melanized matter (arrows) of larvae infected with isolate 1022. Bars, 20  $\mu$ m.

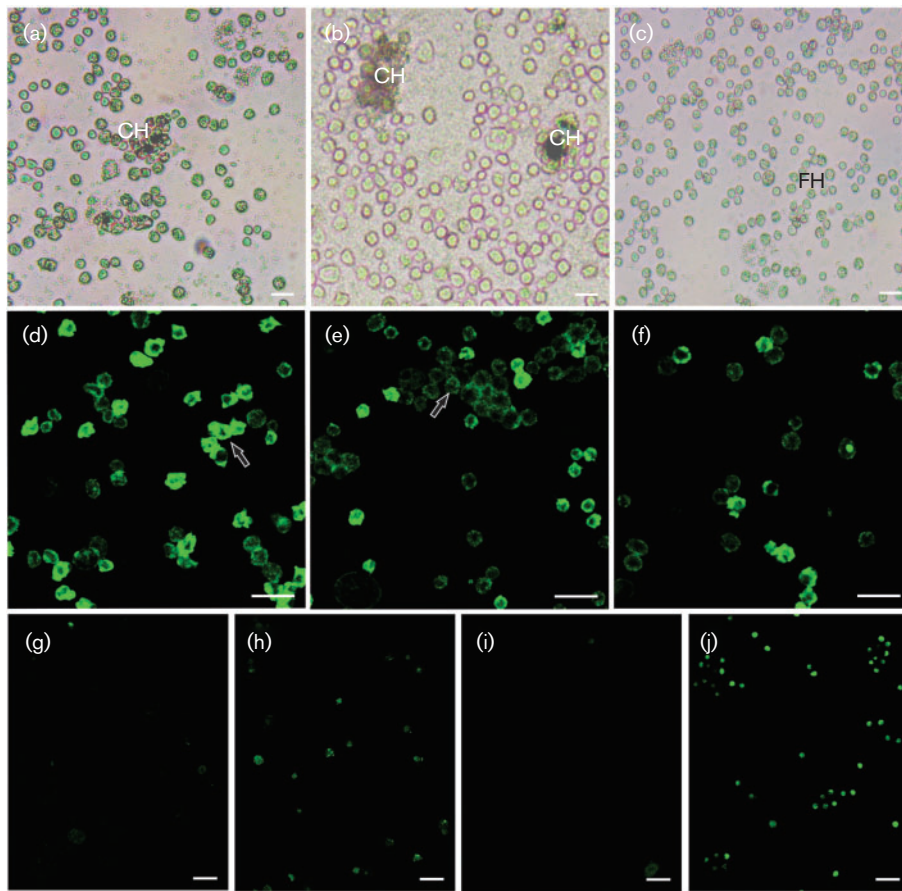
### An *A. pleuropneumoniae* *hfq* mutant is attenuated in the *G. mellonella* infection model

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  $1.0 \times 10^5$  c.f.u. per larva for both strains, we observed a significant decrease in virulence of the *hfq* mutant compared with 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 h of infection. In

contrast, with the wild-type strain, the number of bacteria increased by one order of magnitude at 4 h post-infection, and cells were still present in the larvae at 24 h post-infection (Fig. 7b).

### 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 anti-bacterial agents, we treated *G. mellonella* infected with *A. pleuropneumoniae* with a single injection of several antibiotics. The larvae were infected with a lethal concentration



**Fig. 6.** Cellular immune response of *G. mellonella* against *A. pleuropneumoniae* leads to cell aggregation and DNA damage. Aggregation of *G. mellonella* haemocytes 1 h post-infection with  $10^4$  c.f.u. per 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 isolate 1022 (h) and absent in the PBS control (i). (j) DNase treatment (positive control). CH, clustered haemocytes; FH, free haemocytes. Bars, 20  $\mu$ m.

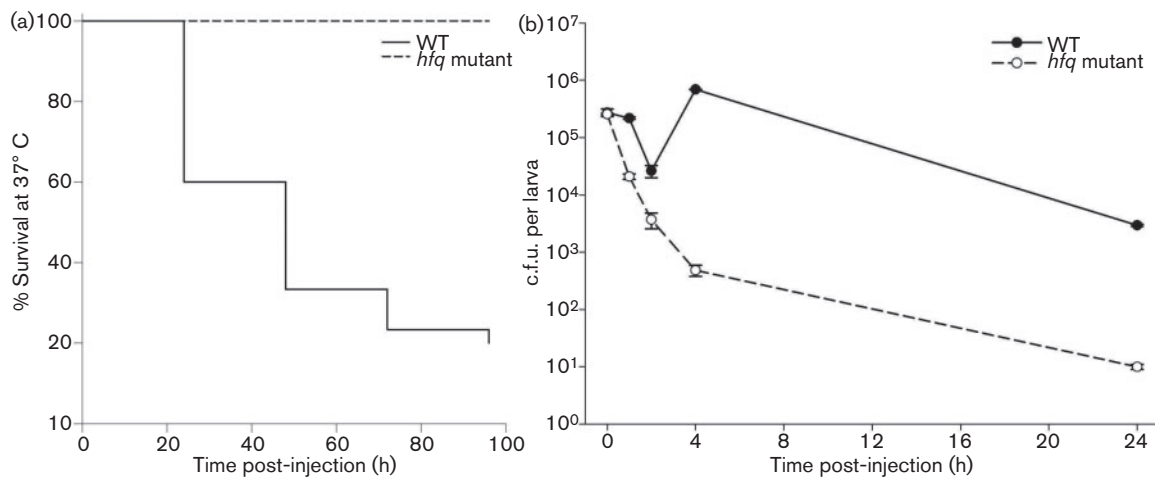
of *A. pleuropneumoniae* 1022 (susceptible to ampicillin, enrofloxacin and penicillin but resistant to tetracycline, Fig. 1). Antibiotic treatment significantly increased the survival rate of *G. mellonella* larvae compared with the control ( $P < 0.018$  for enrofloxacin and  $P < 0.001$  for ampicillin 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.

## DISCUSSION

Using invertebrates for modelling bacterial infection *in vivo* has been investigated for a number of important pathogens. In this study, we describe 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. In parallel with the humoral response, *G. mellonella* induces a cellular response to invading micro-organisms (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 melanization in response to infection and damage to the host.

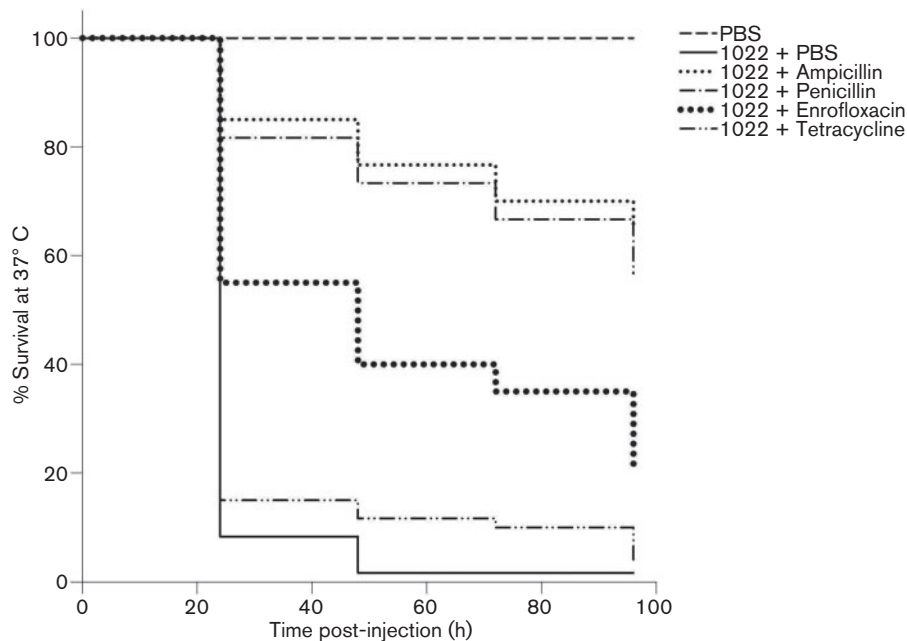
ERIC-PCR fingerprinting showed that the serotype 8 isolates studied are all genetically distinct, independently of their



**Fig. 7.** An *hfq* mutant strain of *A. pleuropneumoniae* displays reduced virulence in *G. mellonella*. (a) Death rate was more pronounced for the wild-type (WT) strain in comparison with the *hfq* mutant ( $P < 0.001$ ) using an inoculum of  $10^5$  c.f.u. per larva. (b) The haemolymph of larvae was collected and plated at 0, 1, 2, 4 and 24 h post-inoculation. Median c.f.u. values for mutant and WT strains reveal differences in growth dynamics.

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



**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 ( $10^4$  c.f.u. per 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 with 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.

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<sup>7</sup> c.f.u. per larva), 100% of the isolates induce death in the *G. mellonella* larvae, whereas a 10<sup>6</sup> c.f.u. per larva inoculum induced death in 22.7% of isolates. This difference is probably 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 probably bound by cellular receptors that recognize bacterial pathogen-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 most virulent *A. pleuropneumoniae* strain led us to investigate whether this effect was related to the secretion of exotoxins, currently considered the most important effectors of clinical signs of swine pleuropneumonia. 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 probably 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 serotype 1 (considered the most virulent, producer of the strongly haemolytic toxin ApxI) was tested, no killing was observed (data not shown). This is not surprising as Apx toxins appear to have specific tropism for porcine cells (Kuhnert *et al.*, 2003). 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, such as 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 highly 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 judged by the lack of lung lesions and mortality, similar to results that were obtained for an *aroA* 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 with the 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 led 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, 2014). 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 with the control in our study.

DNA damage was shown by TUNEL staining in circulating *G. mellonella* haemocytes 1 h 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, 2014; 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 micro-organisms and insects (Brillard *et al.*, 2001; Cho & Kim, 2004; Mizerska-Dudka & Andrejko, 2014).

*A. pleuropneumoniae* infection is also accompanied by humoral defences that lead to melanization of the *G. mellonella* larvae. After recognizing 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 melanization began within 1 h 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 phagocytosing 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 melanization of mature nodules (Browne *et al.*, 2013; Lavine & Strand, 2002). We observed nodule formation with points of melanization 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 *G. mellonella*–*A. pleuropneumoniae* model.

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 the *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, but 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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