Characterization of *Coxiella burnetii* strains from ruminants in a *Galleria mellonella* host-based model

A. Selim¹,², E. Yang¹, E. Rousset¹, R. Thiéry¹ and K. Sidi-Boumedine¹

¹) French Agency for Food, Environmental and Occupational Health Safety (ANSES), Sophia Antipolis Laboratory, Animal Q Fever Unit, Sophia Antipolis, France and 2) Animal Medicine Department, Faculty of Veterinary Medicine, Banha University, Banha, Egypt

Abstract

*Coxiella burnetii* is a small Gram-negative intracellular bacterium and is the causative agent of Q fever, which is a zoonotic disease with a worldwide distribution. Domesticated ruminants are the main reservoir of the disease, but the bacterium is able to infect a wide range of hosts, including humans, arthropods and invertebrates. Virulence studies of *Coxiella* strains usually require a suitable animal model. However, mammalian models are costly and are associated with many ethical constraints. An alternative infection model using *Galleria mellonella* has been used to study the virulence of several bacterial as well as fungal pathogens. Moreover, the *G. mellonella* larvae model has been used to identify virulence genes using phase II *C. burnetii* strain Nine Mile mutants. In our study we describe its use for the characterization of *C. burnetii* strains isolated from ruminants.

Keywords: *Coxiella burnetii*, *Galleria mellonella*, Virulence, Characterization, Insect host-based Model

Original Submission: 4 December 2017; Revised Submission: 1 February 2018; Accepted: 6 February 2018

Article published online: 2 March 2018

Introduction

*Coxiella burnetii* is a small Gram-negative intracellular bacterium that causes Q fever, a zoonotic disease with a worldwide distribution. In humans, Q fever presents as influenza-like symptoms, atypical pneumonia and/or hepatitis with spontaneous recovery in its acute stage. However, in its chronic form, Q fever most commonly manifests as an endocarditis, a vascular infection or a chronic infection during pregnancy, although other symptoms may be diagnosed, including chronic fatigue syndrome, osteomyelitis, pericarditis and meningitis [1].

Domesticated ruminants represent the main reservoir of the disease, but a wide range of hosts are responsible for the persistence of the bacterium in a region, namely mammals, birds and arthropods. In domestic ruminants, Q fever is mostly associated with late abortion and reproductive disorders such as premature birth and dead or weak offspring [2]. Infected animals usually shed the agent intermittently in milk, faeces and urine, with no clinical signs of disease, and should be regarded as possible sources of human infection [3].

Several animal models have been used to evaluate in vitro the virulence of phase I *C. burnetii*, such as guinea pigs, mice and primates [4,5]. For instance, the lethality of phase II *C. burnetii* strain Nine Mile (NMII) has been evaluated in severe combined immunodeficiency mice [6,7]. However, animal models are costly and are associated with ethical constraints. Alternative infection models such as insects have several advantages over mammalian ones, as they do not need ethical approval, can be used at a large scale and are less expensive [8].

Recently an alternative infection model using the worm *Galleria mellonella* was used to study the virulence of several bacterial as well as fungal pathogens [9,10]. Moreover, the *G. mellonella* larvae model was used to identify virulence genes using NMII *C. burnetii* mutants [11].

In our study, we describe the use of *G. mellonella* larvae for the characterization of ten *C. burnetii* isolates from ruminants.
The model was implemented, then assessed to follow the replication level of strains as a virulence criterion, and to test the effect of oxytetracycline on the virulence of *Coxiella* strains.

**Material and methods**

**Strains**

Studied *C. burnetii* strains, isolated from ruminants raised in France, are listed in Table 1. Strain Nine Mile (NM) RSA493 was included as reference.

**Susceptibility of *G. mellonella* to *Coxiella* strains**

Insects were obtained from Live Foods, France, and were stored at 8 °C in the dark until use. Tenfold serial dilutions from bacterial concentrations, ranging from 10^4 to 10^5/mL, were prepared in phosphate-buffered saline (PBS). For each studied strain, groups of ten larvae were injected with 10 μL from each dilution. In addition, a group of ten larvae was injected with 10 μL of PBS to ensure that death did not result from trauma during injection.

Larvae were incubated at 37 °C. Survival was monitored every 24 hours; the insects were considered dead when they did not respond to physical manipulation. Larvae were examined for pigmentation, and the deaths were recorded over 96 hours. The 50% lethal dose (LD50) was calculated according to the Reed and Muench method [12].

**Determination of bacterial loads**

Insects were injected with 10 μL of 10^6/mL of NM, E51 and E195 strains. Three larvae were collected at different time after infection (24, 48, 72, 96, 120, 144 and 168 hours). Larvae were infected as described above, then collected at different times after infection (24, 48, 72, 96, 120, 144 and 168 hours). Larvae were chilled for 10 minutes, surface sterilized with 70% alcohol and then dissected. The haemolymph of each larva was collected in a sterile, cold Eppendorf tube containing the same volume of Grace insect medium. Cells and debris were removed by centrifugation at 4000 rpm for 10 minutes at 4 °C, and supernatants were diluted 3:1 with 50 mM PBS.

**Phenoloxidase activity assay**

Larvae were injected as described above, then collected at different times after infection (24, 48, 72, 96, 120, 144 and 168 hours). Larvae were chilled for 10 minutes, surface sterilized with 70% alcohol and then dissected. The haemolymph of each larva was collected in a sterile, cold Eppendorf tube containing the same volume of Grace insect medium. Cells and debris were removed by centrifugation at 4000 rpm for 10 minutes at 4 °C, and supernatants were diluted 3:1 with 50 mM PBS.

The phenoloxidase (PO) activity in the plasma was quantified using a microplate enzyme assay as previously described [13]. Briefly, a reaction mixture containing 115 μL of 50 mM PBS (pH 6.5), 10 μL diluted haemolymph plasma and 2 μL Escherichia coli lipopolysaccharide (5 mg/mL; Sigma) was left at room temperature for 1 hour to allow the activation of the enzyme. The reaction was initiated by addition of 25 μL 20 mM 4-methyl catechol (Sigma), and the final volume was completed to 200 μL with distilled water. The change in absorbance was read at 490 nm every 5 minutes for 1 hour at room temperature. The experiment was performed in triplicate.

**Statistical analysis**

The statistical analysis of obtained results was performed by XLSTAT 2017.1 software (Addinsoft). The results were considered significant at p < 0.05.

**Results**

**Susceptibility of *G. mellonella* to *C. burnetii* infection**

The survival rate was monitored for 96 hours after infection and estimated by observation of macroscopic changes. All strains exhibited high mortality when using inocula of 10^7/mL. However, the mortality tended to decrease with lower infectious doses. The lethality of examined strains differed according to injected doses and strains (Table 2). At 24 hours after infection, 50% of larvae were dead for strains NM and E184 (Fig. 1(A) and (B)), 60% of the larvae were dead for strain E218 (Fig. 1(C)) and 75% of larvae were dead for strain E221 (Fig. 1(D)), suggesting that strains have different levels of virulence. All larvae groups injected with *Coxiella* strains showed a colour change to blackish and no movement with death.

We determined the LD50 for all strains (Table 1). The results indicated that the CBC2, E184, E221, E51 and E195 strains 

---

**TABLE 1. *Coxiella burnetii* strains used in this study**

| Strain    | Host  | Source           | Country | Titer (log LD50) |
|-----------|-------|------------------|---------|------------------|
| Nine Mile | Tick  | None             | USA     | 5.94 ± 0.82      |
| E221      | Cow   | Vaginal mucus    | France  | 5.95 ± 0.82      |
| E51       | Cow   | Placenta         | France  | 5.97 ± 0.82      |
| E235      | Cow   | Vaginal mucus    | France  | 5.97 ± 0.47      |
| CBC2      | Goat  | Milk             | France  | 5.00 ± 0.47      |
| E16       | Goat  | Vaginal mucus    | France  | 4.99 ± 0.94      |
| E184      | Goat  | Placenta         | France  | 5.96 ± 2.9       |
| E189      | Goat  | Vaginal mucus    | France  | 5.93 ± 0.47      |
| E198      | Sheep | Vaginal mucus    | France  | 5.97 ± 0.82      |
| E218      | Sheep | Placenta         | France  | 5.92 ± 0.47      |
| E195      | Sheep | Vaginal mucus    | France  | 4.94 ± 0.94      |

LD50, 50% lethal dose.
had the lowest LD50, which was approximately $10^5$ compared to the LD50 of other strains, which was approximately $10^6$. The LD50 did not statistically differ among strains ($p > 0.05$).

**Effect of tetracycline on C. burnetii virulence**

Tetracycline is a recommended treatment for Q fever [14]. Consequently, the effect of tetracycline on the infectivity of some *Coxiella* strains was evaluated in the *Galleria mellonella* model.

The results showed that the antibiotic treatment increased the mean time to death of infected larvae, with significant differences between all groups. In addition, no significant difference was observed between larvae injected with the low virulent strain E235 and PBS and larvae injected with highly virulent strains and treated by tetracycline or larvae injected with NM strains and PBS.

Moreover, larvae injected with NM strain and PBS showed no significant difference versus the group infected with strain E189 and treated by tetracycline. These results indicated that *C. burnetii* is susceptible to tetracycline and this antibiotic influences the multiplication of bacteria in the *Galleria* model.

**Response of G. mellonella immune system to Coxiella infection**

*Coxiella* infection in *G. mellonella* larvae caused death and a colour change due to increased pigmentation resulting from melanin deposition. This process is indicative of PO activity in the haemolymph. To evaluate the immune response of larvae to *Coxiella* infection, the level of PO activity was evaluated in the haemolymph of larvae infected with strains NM, E221, E51 and E195 besides PBS and its relation with the bacterial load in larvae. The level of PO activity in the group injected with PBS remained stable over the study period. In addition, the PO activity reached a peak at 48 hours, whereas the bacterial multiplication decreased during this time, then increased at 72 hours. The PO activity decreased until the end of the experiment (Figs. 2 and 3).

In addition, the virulence of *C. burnetii* strains was correlated to the level of melanin production, which was high in strains E51 and E195 compared to the NM strain.

| Strain | Standard error | Virulence group |
|--------|----------------|-----------------|
| E184   | 0.151          | A               |
| E235   | 0.156          | A               |
| NM     | 0.151          | A               |
| E189   | 0.151          | A, B            |
| E218   | 0.151          | B               |
| CBC2   | 0.151          | B               |
| E198   | 0.151          | B               |
| E16    | 0.151          | B               |
| E51    | 0.147          | C               |
| E195   | 0.151          | C               |
| E221   | 0.151          | C               |

A, low virulence; B, middle virulence; C, high virulence.

**FIG. 1.** Dose-dependent survival rate of *Galleria mellonella* larvae after infection with *Coxiella* strains for 96 hours.

© 2018 The Authors. Published by Elsevier Ltd. *NMNI*, 24, 8–13

This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
Discussion

An invertebrate infection model has recently been implemented to investigate the pathogenesis of C. burnetii. Galleria mellonella has been used as an infection model to study the pathogenesis of several Gram-positive and Gram-negative bacteria [15,16]. Moreover, the G. mellonella larvae model has been used to identify the virulence genes using NMII C. burnetii mutants [11].

In our study, we describe its use for the characterization of ruminant C. burnetii field strains. The G. mellonella model clearly showed susceptibility and an ability to distinguish between the virulence of Coxiiella strains.

We investigated the virulence of ten Coxiiella strains in G. mellonella at infectious doses ranging from 10^4 to 10^7/mL. The survival rate was recorded every 24 hours for up to 4 days after infection. The results revealed that Coxiiella strains exhibit various levels of pathogenicity for G. mellonella (Table 2).

The severity of Listeria monocytogenes infection in the G. mellonella model has been shown to be dose dependent [17]. In the present study, there were significant virulence differences between the different Coxiiella strains in G. mellonella (Table 3). The obtained results are similar to those obtained with other bacteria such as L. monocytogenes [9], Klebsiella pneumoniae [18] and Burkholderia cepacia [19]. In another hand, the results contrast with those reported by

### Table 3. Newman-Keuls analysis of differences between categories with 95% confidence intervals (survival)

| Contrast | Pr > diff | Significant |
|----------|-----------|-------------|
| Tetracycline vs. E189 + PBS | <0.0001 | Yes |
| Tetracycline vs. E189 + tetracycline | <0.0001 | Yes |
| Tetracycline vs. E235 + PBS | <0.0001 | Yes |
| Tetracycline vs. E235 + tetracycline | <0.0001 | Yes |
| NM + tetracycline vs. E189 + PBS | <0.0001 | Yes |
| NM + tetracycline vs. E189 + tetracycline | <0.0001 | Yes |
| NM + tetracycline vs. NM + PBS | <0.0001 | Yes |
| NM + tetracycline vs. E235 + PBS | <0.0001 | Yes |
| NM + tetracycline vs. E235 + tetracycline | 0.001 | Yes |
| E235 + tetracycline vs. E189 + PBS | <0.0001 | Yes |
| E235 + tetracycline vs. NM + PBS | 0 | Yes |
| E235 + tetracycline vs. E235 + PBS | 0 | Yes |
| E235 + PBS vs. E189 + PBS | <0.0001 | Yes |
| E235 + PBS vs. E189 + tetracycline | 0.209 | No |
| E235 + PBS vs. NM + PBS | <0.0001 | Yes |
| NM + PBS vs. E189 + PBS | <0.0001 | Yes |
| NM + PBS vs. E189 + tetracycline | 0.289 | No |
| E189 + tetracycline vs. E189 + PBS | 0.001 | Yes |

PBS, phosphate-buffered saline.
Norville et al. [11], who did not detect any significant difference between NM phase I and phase II. Herein, the infection of Galleria larvae with ruminant Coxiella strains killed the larvae in a dose-dependent manner, with a LD50 ranging from $10^5$ to $10^6$/mL.

Some antibiotics, like tetracycline, are active on C. burnetii multiplication [14]. Some studies aimed to evaluate the activity of antibiotics against C. burnetii in embryonated eggs or cell cultures [17,20], in animal models [21] and recently in G. mellonella [11]. Here we studied the effects of tetracycline on the multiplication of several C. burnetii strains in G. mellonella. We observed that tetracycline increased the mean time to death and decreased the lethal effect of Coxiella strains.

Taken together, our results emphasize the fact that this insect model is valid to evaluate the virulence of bacteria and the efficacy of antibiotics against C. burnetii.

The production of melanin is a primary humoral immune response to infection in G. mellonella. In order to study this phenomenon, a panel of Coxiella strains with distinct degrees of virulence was selected. The ability of these strains to proliferate in the haemolymph of G. mellonella and to activate the PO/melanin production in the insect was measured. All strains caused variable degrees of melanin production in Galleria larvae compared to the PBS control group. The level of melanin production (PO activity) reached a peak at 48 hours, whilst the proliferation of bacteria was at the lowest at this point. After 48 hours, the proliferation of bacteria increased and melanin production decreased. Overall, melanin production was positively correlated with the virulence of selected strains.

Consequently, the observed immune response of G. mellonella against C. burnetii seems to be induced by C. burnetii, as in Legionella pneumophila [22] and Yersinia pseudotuberculosis [23]. In conclusion, G. mellonella is susceptible to C. burnetii infection, and this model can be used to distinguish the virulence degrees of different strains.

Acknowledgements

A. Selim was supported by a fellowship granted by the French embassy in Egypt (Institut Français d’Egypte) and the Science and Technology Development Fund (STDF). We also would like to thank L. Hébert (ANSES, Dozulé Laboratory for Equine Diseases, Bacteriology and Parasitology Unit) for valuable discussions regarding the Galleria mellonella model establishment.

Conflict of interest

None declared.

References

[1] Bacci S, Villumsen S, Valentiner-Branth P, Smith B, Krogfelt K, Melbak K. Epidemiology and clinical features of human infection with Coxiella burnetii in Denmark during 2006–07. Zoonoses Public Health 2012;59:61–8.
[2] Agerholm JS. Coxiella burnetii associated reproductive disorders in domestic animals—a critical review. Acta Vet Scand 2013;55:13.
Guatteo R, Seegers H, Taurel AF, Joly A, Beaudou F. Prevalence of Coxiella burnetii infection in domestic ruminants: a critical review. Vet Microbiol 2011;149:1–16.

Melenotte C, Lepidi H, Nappez C, Bechah Y, Audoly G, Terras J, et al. Mouse model of Coxiella burnetii aerosolization. Infect Immun 2016;84:2116–23.

Brooke RJ, Kretzschmar ME, Mutters NT, Teunis PF. Human dose response relation for airborne exposure to Coxiella burnetii. BMC Infect Dis 2013;13:488.

Van Schaijk EJ, Case ED, Martinez E, Bonazzi M, Samuel JE. The SCID mouse model for identifying virulence determinants in Coxiella burnetii. Front Cell Infect Microbiol 2017;7:25.

Islam A, Lockhart M, Stenos J, Graves S. The attenuated Nine Mile phase II clone 4/RSA439 strain of Coxiella burnetii is highly virulent for severe combined immunodeficient (SCID) mice. Am J Trop Med Hyg 2013;89:800–3.

Tsai CJY, Loh JMS, Proft T. Galleria mellonella infection models for the study of bacterial diseases and for antimicrobial drug testing. Virulence 2016;7:214–29.

Mukherjee K, Altincicek B, Hain T, Domann E, Vilcinskas A, Chakraborty T. Galleria mellonella as a model system for studying Listeria pathogenesis. Appl Environ Microbiol 2010;76:310–7.

Fuchs BB, O’Brien E, El Khoury JB, Mylonakis E. Methods for using Galleria mellonella as a model host to study fungal pathogenesis. Virulence 2010;1:475–82.

Norville I, Hartley M, Martinez E, Cantet F, Bonazzi M, Atkins T. Galleria mellonella as an alternative model of Coxiella burnetii infection. Microbiology 2014;160:1175–81.

Ramakrishnan MA. Determination of 50% endpoint titer using a simple formula. World J Virol 2016;5:85–6.

Eleftherianos I, Millichap PJ, Reynolds SE. RNAi suppression of recognition protein mediated immune responses in the tobacco hornworm Manduca sexta causes increased susceptibility to the insect pathogen Photobahbus. Dev Comp Immunol 2006;30:1099–107.

Brennan RE, Samuel JE. Evaluation of Coxiella burnetii antibiotic susceptibilities by real-time PCR assay. J Clin Microbiol 2003;41:1869–74.

Joyce SA, Gahan CG. Molecular pathogenesis of Listeria monocytogenes in the alternative model host Galleria mellonella. Microbiology 2010;156:3456–68.

Olsen RJ, Watkins ME, Cantu CC, Beres SB, Musser JM. Virulence of serotype M3 group A Streptococcus strains in wax worms (Galleria mellonella larvae). Virulence 2011;2:111–9.

Maurin M, Raoult D. Q fever. Clin Microbiol Rev 1999 Oct;12(4):518–53.

Wand ME, McCowen JW, Nugent PG, Sutton JM. Complex interactions of Klebsiella pneumoniae with the host immune system in a Galleria mellonella infection model. J Med Microbiol 2013;62:1790–8.

Seed KD, Dennis JJ. Development of Galleria mellonella as an alternative infection model for the Burkholderia cepacia complex. Infect Immun 2008;76:1267–75.

Omsland A, Beare PA, Hill J, Cockrell DC, Howe D, Hansen B, et al. Isolation from animal tissue and genetic transformation of Coxiella burnetii are facilitated by an improved axenic growth medium. App Environ Microbiol 2011;77:3720–5.

Huebner RJ, Hottle GA, Robinson EB. Action of streptomycin in experimental infection with Q fever. Public Health Rep 1896–1970;1948:357–62.

Harding CR, Schroeder GN, Collins JW, Frankel G. Use of Galleria mellonella as a model organism to study Legionella pneumophila infection. J Vis Exp 2013;81. e50964.

Champion OL, Cooper IA, James SL, Ford D, Karlyshev A, Wren BW, et al. Galleria mellonella as an alternative infection model for Yersinia pseudotuberculosis. Microbiology 2009;155:1516–22.