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

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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.
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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 $10^{7/mL}$, 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 and 144 hours). Each larva was homogenized in 2 mL PBS using a tissue sieve, DNA was extracted using chelex and the bacterial load was quantified using real-time PCR targeting the IS1111 repeated element.

Effect of tetracycline on virulence of *Coxiella* strains

Groups of ten larvae were injected with 10^6/mL suspensions of NM, highly virulent strain E189 or low virulent strain E235. At 24 hours after infection, larvae were injected with 50 mg tetracycline. A group of larvae was injected with PBS as an untreated control, and another group was injected with 50 mg tetracycline as an uninfected control group. The experiments were performed in triplicate.

Phenoloxidase activity assay

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.

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 $p \leq 0.05$.

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	4.95 ± 0.82
E51	Cow	Placenta	France	4.97 ± 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.

TABLE 2. Lethal effect of *Coxiella* strains in *Galleria mellonella* larvae

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.

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 *G. 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.
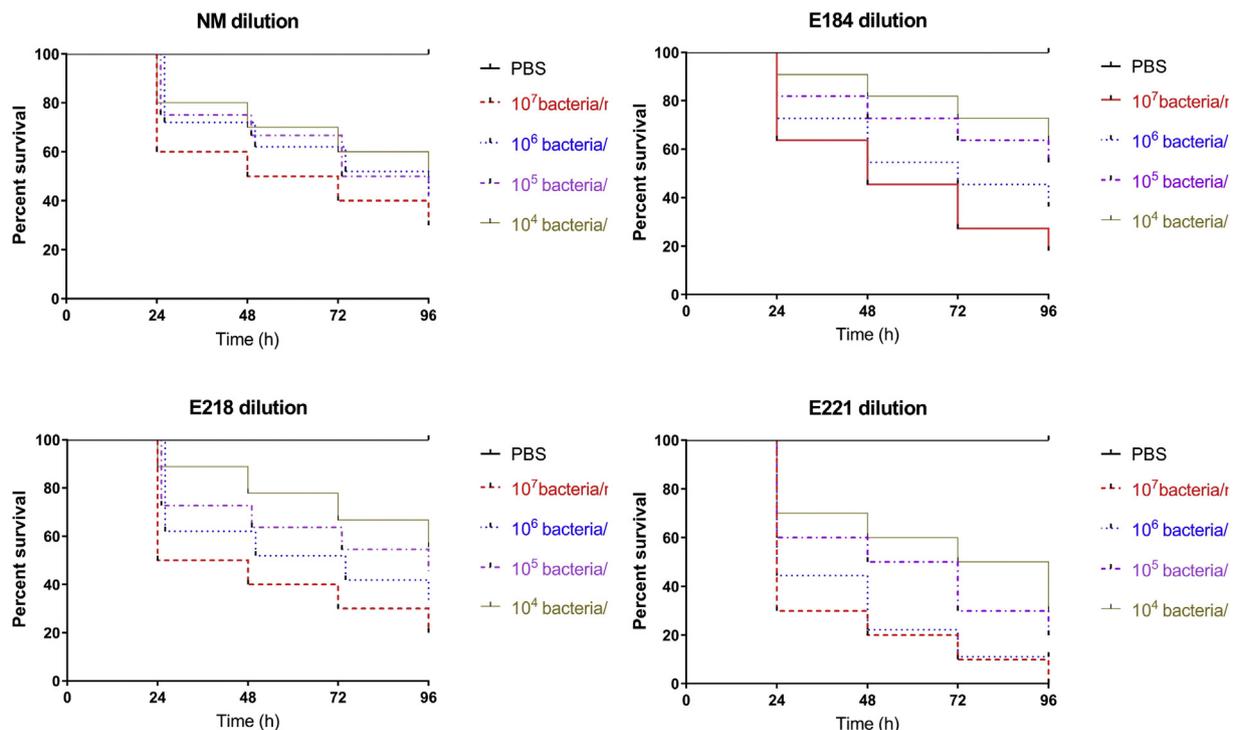
FIG. 1. Dose-dependent survival rate of *Galleria mellonella* larvae after infection with *Coxiella* strains for 96 hours.
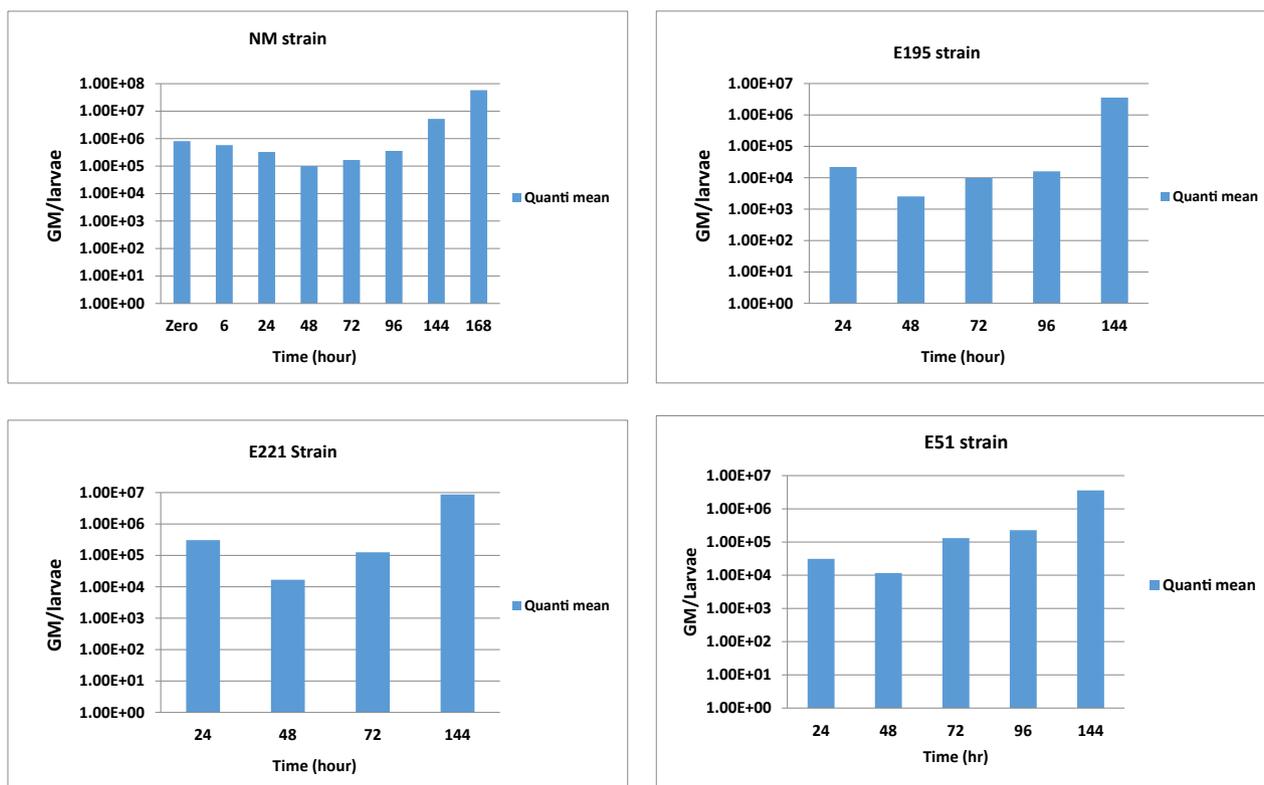
FIG. 2. Proliferation of *Coxiella* strains in *Galleria mellonella*. All strains showed decrease in multiplication in first 48 hours and then started to multiply until end of experiment.

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 *Coxiella* strains.

We investigated the virulence of ten *Coxiella* 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 *Coxiella* 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 *Coxiella* 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. NM + PBS	<0.0001	Yes
Tetracycline vs. E235 + PBS	<0.0001	Yes
Tetracycline vs. E235 + tetracycline	<0.0001	Yes
Tetracycline vs. NM + 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	0.002	Yes
E235 + tetracycline vs. E189 + PBS	<0.0001	Yes
E235 + tetracycline vs. E189 + tetracycline	<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	–	No
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.
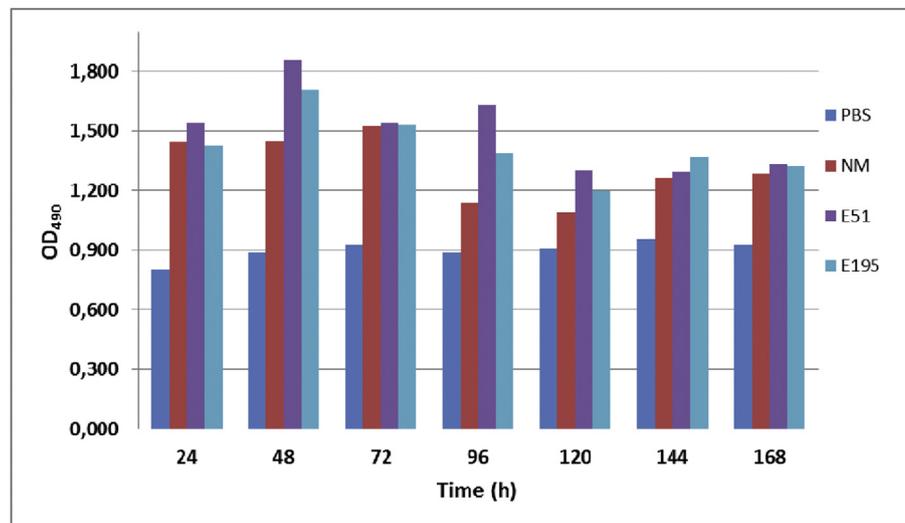
FIG. 3. *Coxiella burnetii* infection of *Galleria mellonella* initiates an immune response. Level of melanin production reached peak at 48 hours and then decreased until end of experiment.

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

Conflict of interest

None declared.

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