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**Abstract.** The Nine Mile phase II clone 4 (NMIIC4) strain of *Coxiella burnetii* is an attenuated phase II strain that has lost the genes for virulence determinant type 1 lipopolysaccharide. These bacteria were very virulent for severe combined immunodeficient (SCID) mice. The lethal dose 50 (LD<sub>50</sub>) was ~10 bacteria. Infected SCID mice died between Day 28 and Day 53 post-infection. At termination of the experiment (Day 60) only 5 of 24 mice had survived. The degree of splenomegaly was directly related to the bacterial load in the SCID mice spleens. The NMIIC4 was avirulent in immunocompetent wild mice and bacterial DNA copies in splenic tissue were extremely low. The SCID mice that were inoculated with high doses of heat inactivated NMIIC4 *C. burnetii* were all alive at Day 60 and without splenomegaly. It appears that the phase I lipopolysaccharide present in virulent Nine Mile phase I but not in attenuated NMIIC4 is not the only virulence factor for *C. burnetii*.

**BACKGROUND**

*Coxiella burnetii* is the etiologic agent of human Q fever, an infectious disease that was first reported by Derrick in Australia.1 It is a pleomorphic obligate intracellular bacterium that has two antigenic forms, naturally occurring (wild-type) phase I and attenuated (laboratory adapted) phase II. The transition from virulent phase I to non-pathogenic/avirulent phase II is caused by the loss of the methylated sugars virenose and dihydroxyxystreptose2,3 and other sugar components of the “O” antigen side chains of the lipopolysaccharide (LPS) molecule. Such variations in the LPS of phase I and phase II bacteria are linked to shifts in antigenicity and virulence.4–6 The Nine Mile phase II clone 4 (NMIIC4) strain of *C. burnetii* was derived from the virulent Nine Mile phase I (NMI) strain through repeated passage in embryonated eggs and has become antigenically distinct.5 Its genome has a large deletion in an area necessary for O-antigen synthesis.7 The strain RSA541 (also known as “Crazy”) and two other phase II isolates; Nine Mile phase II clone 1 and Nine Mile Baca strain RSA541 (also known as “Crazy”) and two other phase II clones. NMIIC4 and this observation led to speculation that there may be a basis for expression of virulence other than the phase I LPS.

The Nine Mile phase II clone 4 (NMIIC4) strain of *C. burnetii* was grown in a VERO (African green monkey epithelial cell) cell line. The monolayer was scraped following the visible cytopathic effect and 10-fold dilutions of the NMIIC4 cell suspension were prepared. The bacterial load in each dilution was quantified using a quantitative real time polymerase chain reaction (qPCR) assay. A total of eight 10-fold dilutions were used (10<sup>−3</sup> to 10<sup>−8</sup>) ranging from 4.1×10<sup>4</sup> to <1<sub>4</sub> and short infection time in contrast to the current study, which was carried out to determine the dose response of NMIIC4 in SCID mice over a longer experimental period, determining the quantitative bacterial load in spleen tissue and the Lethal Dose 50 (LD<sub>50</sub>) of NMIIC4.

**MATERIALS AND METHODS**

The avirulent NMIIC4 strain of *Coxiella burnetii* was grown in a VERO (African green monkey epithelial cell) cell line. The monolayer was scraped following the visible cytopathic effect and 10-fold dilutions of the NMIIC4 cell suspension were prepared. The bacterial load in each dilution was quantified using a quantitative real time polymerase chain reaction (qPCR) assay. A total of eight 10-fold dilutions were used (10<sup>−3</sup> to 10<sup>−8</sup>) ranging from 4.1×10<sup>4</sup> to <1<sub>4</sub> and short infection time in contrast to the current study, which was carried out to determine the dose response of NMIIC4 in SCID mice over a longer experimental period, determining the quantitative bacterial load in spleen tissue and the Lethal Dose 50 (LD<sub>50</sub>) of NMIIC4.

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performed in a PC3 laboratory at the Department of Microbiology, Pathology North-Hunter, NSW Health Pathology, Newcastle, Australia. Animal use was approved by the “Animal Care and Ethics Committee” of the Australian Rickettsial Reference Laboratory (ARRL).

A total of 30 (27 SCID and 3 wild mice) adult female mice were housed in 10 sterile isolator cages. Eight cages containing 24 SCID mice (3 mice/cage) were allocated to determining the effect of different doses of viable avirulent NMIIC4. The cages were labeled according to the NMIIC4 dilution (10 to 10^8) per inocula (0.1 mL). Mortality of mice Day of mouse death

<table>
<thead>
<tr>
<th>Group</th>
<th>Ten-fold dilution of inocula</th>
<th>C. burnetii numbers per inocula (0.1 mL.)</th>
<th>Mortality of mice</th>
<th>Day of mouse death</th>
<th>Average number of C. burnetii in mouse spleen (0.1 mL.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group A (SCID mice)</td>
<td>10^3 (heat killed)</td>
<td>4.1 x 10^7</td>
<td>0/3</td>
<td>No deaths</td>
<td>1.0 x 10^7</td>
</tr>
<tr>
<td>Control group B (wild mice)</td>
<td>10^-1 (viable)</td>
<td>4.1 x 10^7</td>
<td>0/3</td>
<td>No deaths</td>
<td>7</td>
</tr>
</tbody>
</table>

*SCID = severe combined immunodeficient; NMIIC4 = Nine Mile phase II clone 4.

The Spearman-Kärber method was used to calculate the LD50 of NMIIC4 of C. burnetii in the SCID mice.

RESULTS

In the experimental group, SCID mice infected with eight different doses of viable NMIIC4 bacteria (from 4.1 x 10^7 to < 1) showed varying mortality over time. The first deaths were recorded at Day 28 from the highest doses of NMIIC4 and mortality continued until Day 53 with the lower doses. Out of 24 mice only five mice survived to the termination of the experiment at Day 60 (Table 1). Among these five mice three were inoculated with ~1 bacterium and the other two received 1.2 x 10^3 and 2.4 x 10^3 bacteria, respectively. The time interval between early and late deaths was ~15 days (Figure 1).

No mortality occurred in control groups A and B, i.e., SCID mice inoculated with killed NMIIC4, and wild mice inoculated with viable NMIIC4 (Table 1). Splenomegaly was evident in all dead infected mice. The average weight of the spleens of infected SCID mice was 0.61 g, which was eight times greater than the spleens of uninfected control SCID mice (0.07 g). The qPCR results of infected SCID mice spleen tissue revealed high C. burnetii loads (3.2–6.8 x 10^3) per 0.1 mL spleen suspension (Table 1). However, among the five surviving mice only the mouse that received the 2.4 x 10^3 bacterial inoculum had splenomegaly and a high bacterial load. This mouse was sick and would

![Figure 1](image-url)
likely have died within a few days. No splenomegaly was detected in control mice and the qPCR assay revealed only low copies of *C. burnetii* DNA in spleen tissue (Table 1).

In the *C. burnetii* isolated from the spleens of the SCID mice, all four phase I LPS genes were absent, indicating that these *C. burnetii* cells used in phase II (i.e., NMIIC4) confirming that no switch to virulent phase I had occurred *in vivo.*

**DISCUSSION**

The NMIIC4 resulted in 79% (19 of 24) mortality in the SCID mice with an LD<sub>50</sub> of ~10 *C. burnetii*. Hence, it was very virulent for SCID mice. Our previous study with phase I *C. burnetii* Australian isolates revealed an LD<sub>50</sub> of 1 bacterium for SCID mice. Had we allowed the experiment to have continued for more than 60 days, we may have approached a similar LD<sub>50</sub> for the NMIIC4 strain.

The SCID mouse model, using the NMI strain of *C. burnetii* has been described for lethal Q fever, indicating that the SCID mouse is highly susceptible to *C. burnetii*. The immunodeficiency of the SCID mouse host enhances the severity of Q fever. Phase II attenuated strains have also been used to infect gene knockout mice, SCID, and SCIDbg mice. The NMIIC4 caused a febrile response in gamma interferon knockout mice and toll-like receptor 2 knockout mice. In that study the same SCID model and identical NMIIC4 strain were used, but the study was stopped at Day 28 and a fixed dose (10<sup>4</sup> *C. burnetii*, i/p) was used for infection. Based on our current study, that dose of bacteria needs more than 28 days duration of infection to show an effect. In that study the highest dose (4 x 10<sup>7</sup>) also showed no effect in the first 28 days. Such variations in the dose and duration of infection may be because some SCID mice strains are known to be “leaky” and occasionally produce small numbers of T and B cells. However, we were not able to determine the “leakiness” of our experimental SCID mice.

Until now the full length LPS I of NMI was the only known virulence factor of *C. burnetii*. The avirulent NMIIC4 contains truncated LPS II and has a large chromosomal deletion that eliminates open reading frames involved in the biosynthesis of terminal O antigen sugars, including the rare sugar virenose. This current study confirms that, despite the absence of the O antigen biosynthesis region, the NMIIC4 shows virulence properties of phase I. An earlier study on another Nine Mile variant (RSA 514 “Crazy”), which has a similar large deletion to the NMIIC4 strain, also showed virulence properties of phase I. Both studies support the speculation that there must be an additional basis to phase I virulence.

In conclusion, it can be stated that 1) NMIIC4 is highly virulent for SCID mice (LD<sub>50</sub> = 10) and mortality was related to dose; 2) splenomegaly was evident in all dead infected SCID mice, with high loads of NMIIC4; 3) the NMIIC4 had no effect on immunocompetent wild mice. The NMIIC4 bacteria were lethal to SCID mice despite this bacterium not containing phase I LPS. Other (as yet unidentified) virulence factor(s) of *C. burnetii* are probably involved in the pathogenesis of Q fever and further studies are required to elucidate the full genomic and phenotypic basis of virulence in *C. burnetii*.

**REFERENCES**


