

Development of an Assay for Antimicrobial Susceptibility Testing of *Coxiella burnetii*

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# Development of an Assay for Antimicrobial Susceptibility Testing of *Coxiella burnetii*

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## Abstract

*Coxiella burnetii*, a small Gram-negative bacterium, is the causative agent of Q fever, a zoonotic disease with initial flu-like symptoms that lead to both acute and chronic stages of infection. As with many obligate intracellular parasites, *C. burnetii* has been difficult to culture and therefore has been difficult to establish assays that examine a compound's direct effect upon the bacterium. The development of an axenic medium for *C. burnetii* provided a method with which to culture the bacterium in liquid medium and thereby permitted our development of an assay for examining *C. burnetii* sensitivity to various anti-bacterial compounds. In order to establish the assay, the nonpathogenic *C. burnetii* Nine Mile Phase II Clone 4 (CBNMIIC4) strain was cultured (37°C) in acidified citrate cysteine medium (ACCM2) under hypoxic conditions. After 4 days in culture, the OD<sub>600</sub> of the culture was adjusted to 0.10 (corresponding to approximately 6.8 x 10<sup>8</sup> bacteria per mL) and then added to wells in a 96-well plate containing dilutions of various anti-bacterial compounds. The plates were incubated (37°C) under hypobaric conditions for 5 days after which the OD<sub>600</sub> was measured to determine whether the compounds affected CBNMIIC4 growth. Characterization of CBNMIIC4 growth was verified by measuring CBNMIIC4 genomic DNA and colony forming units in treatment cultures compared to the controls. The results of these studies provide a much-needed assay to assess the effects of anti-bacterial compounds upon *C. burnetii* growth.

## Introduction

*Coxiella burnetii* is an obligate intracellular pathogen and the causative agent of Q fever. The bacterium has a large cell variant (LCV) and a small cell form variant (SCV), and under the right conditions, the bacterium may transition between the two forms (Sandoz et al. 2014). The spore-like SCV is highly stable and resistant to many harsh environmental conditions, allowing the bacterium to survive outside host cells which may contribute to the bacterium's highly infectious nature (Omsland 2012b). The LCV is the actively dividing, infectious form (Sandoz et al. 2015). Depending upon the presence of the lipopolysaccharide (LPS) virulence factor on the cell surface, the bacterium exists in two different phases. Bacteria in Phase I have a complete LPS virulence factor and are highly infectious while the attenuated Phase II have a truncated LPS and are considered noninfectious to animals and humans (Islam et al. 2013).

Exposure to *C. burnetii* can lead to acute and chronic infection. As with many other obligate intracellular parasites, *Coxiella* infections can be difficult to treat and, even with treatment, chronic infections may still occur. Moreover, as a zoonotic pathogen, the bacterium has reservoirs in several domesticated animal species, which further increases sources of human infection (Omsland 2012b).

As an obligate intracellular pathogen, *C. burnetii* has been difficult to culture, making the bacterium difficult to study. Specifically, *C. burnetii* replicates in highly acidic phagolysosomes of cells where oxygen concentration is sub-atmospheric (Larson et al. 2016). Recently, however, an axenic medium for *C. burnetii* has improved the ease of study and aided the development of a method to test *C. burnetii* sensitivity to antimicrobial compounds (Omsland et al. 2009; Omsland 2012; Sandoz et al. 2014). An additional problem in the axenic culture conditions is that *C. burnetii* requires an atmosphere of 2.5% oxygen and 5% CO<sub>2</sub>. In developing our assay to test

antimicrobial agents' effect upon *C. burnetii*, we developed a simple method to culture the bacterium. Doxycycline hyclate, kanamycin, and ciprofloxacin were considered in this study because they good treatments for bacterial infections (Gikas et al. 2001; Kersh 2013).

Doxycycline hyclate has previously been used to treat *C. burnetii* infection in humans (Gikas et al. 2001). Moreover, these antibiotics are stable for at least one week in highly acidic (pH 4.75) environments at a temperature of 37°C, conditions used for the axenic culture *C. burnetii* (Doxycycline hyclate, BioChemica; Kanamycin monosulfate, Product Information). Doxycycline hyclate, kanamycin, and ciprofloxacin were used to establish the assay and could potentially be used as controls in screening assays to identify new anti-bacterial compounds.

Using our culture system, we developed a 96-well plate assay in which we tested multiple concentrations of antibiotics using the axenic liquid medium that mimics the bacteria's environmental niche inside infected mammalian cells. We have been able to determine the minimum inhibitory concentrations (MIC) for doxycycline hyclate, kanamycin, and ciprofloxacin and thus provide a mammalian cell free assay that can determine the MIC values of anti-bacterial agents.

## **Materials and Methods**

### *Bacteria*

The *Coxiella burnetii* Nine Mile II C4 (CBNMIIC4) strain kindly provided by Dr. Robert A. Heizen, Rocky Mountain Laboratories, St. Hamilton, MT (NIH/NIAID, RML) was used for this study. The CBNMIIC4 clone has a mutation that results in a truncated lipopolysaccharide (LPS) molecule which renders the bacteria avirulent; because of the mutation, the Phase II bacterium cannot revert to the virulent wild-type bacteria and is thus considered a biosafety level 2(BSL-2) organism (Islam et al. 2013).

### *Acidified Citric Cysteine Medium-2*

Unless otherwise noted, all chemicals were purchased from Sigma Aldrich, St. Louis MO. Bacto Neopeptone was purchased from Becton Dickerson, (Franklin Lakes, NJ); casamino acids from Fisher Scientific (Hampton, NH); reagents purchased from Invitrogen (Carlsbad CA) were RPMI containing Glutamax and pure Agarose. The acidified citrate cysteine medium (ACCM2) was developed by Omsland et al. (2009) and is a complex media that mimics the environment of *C. burnetii*'s intracellular niche. In order to simplify medium preparation, we prepared 250 mL of concentrated stock solutions for the following chemicals: 20X citric acid (2840 mg), 10X sodium citrate (11850 mg), potassium phosphate (6250 mg), 50X magnesium chloride (2500 mg), 50X calcium chloride (165 mg), 10X sodium chloride (18200 mg), 50X iron sulfate (34.75 mg). The concentrated solutions were stored at 4°C. To prepare ACCM2 for culture, stock solutions of reagents were mixed together, and the degradable compounds were weighed and added to the solution (Table 1).

**Table 1.** ACCM2 Component Reagents

REAGENT	FINAL CONCENTRATION	QUANTITY ADDED (200 ML FINAL VOLUME)	
		1X	2X
Citric acid 20x	13.4 mM	10 mL	20 mL
Sodium citrate 10x	16.1 mM	20 mL	40 mL
Potassium phosphate 50x	3.67 mM	4 mL	8 mL
Magnesium chloride 50x	1.0 mM	4 mL	8 mL
Calcium chloride 50x	0.02 mM	4 mL	8 mL
Sodium chloride 10x	125.4 mM	20 mL	40 mL
Iron sulfate 50 x	0.01 mM	4 mL	8 mL
L-cysteine	1.5 g/L	52.6 mg	105.2 mg
Bacto Neopeptone	0.1 g/L	20 mg	40 mg
Casamino acids	2.5 g/L	500 mg	1000 mg
Methyl-b- cyclodextrin	1 g/L	200 mg	400 mg
RPMI with Glutamax	125 mL/L	25 mL	50 mL
Deionized H <sub>2</sub> O		109 mL	18 mL

The pH of the ACCM2 was adjusted to 4.75 using 6N sodium hydroxide. Then ACCM2 was then filter sterilized using a Stericup 0.2-micron filter unit (EMD Millipore, Burlington, MA) and then stored at 4°C. The 1X medium was used for bacterial culture and the 2X was used to prepare agar plates for colony forming unit (CFU) assays.

#### *Preparation of bacterial stocks*

To prepare stock suspensions, 100 µL of the original CBNMIIC4 seed was added to a 75 CM2 tissue culture flask containing 25 ml ACCM2 medium. The flasks were placed into a modular hypoxic chamber (Billups-Rothenberg, Del Mar CA) and sealed according to instructions by the vendor. A gas mixture containing 5% CO<sub>2</sub>, 2.5% O<sub>2</sub>, and 92.5% N<sub>2</sub> (Airgas, Cherry Hill MJ) was introduced into the chamber and the chamber placed on a rotating platform (30 rpm/min) in a non-humidified incubator (37°C). After 5 days, the media was centrifuged at 15.7 G (4°C) for 45 min. Culture fluid was removed, and the bacterial pellet resuspended to an OD<sub>600</sub> = 0.350 in Dulbecco's modified minimal essential medium (DMEM) containing 20% fetal bovine serum and 20% DMSO. The suspension was distributed into cryovials (1 ml/vial) and stored at -80°C until needed.

#### *Colony Forming Unit Assay and Genomic Equivalency*

Weekly cultures of CBNMIIC4 with our method showed that the optical density at 600 nm wavelength light (OD<sub>600</sub>) was consistent and therefore could be used to approximate bacterial numbers. To determine the actual number of bacteria in the suspension, colony forming units (CFU) were assessed using an agarose plate with a bottom layer comprised of equal volumes of 2X ACCM2 and a 2% sterile agarose solution (final 1% agarose concentration). Dilutions of the

bacteria in liquid suspension were prepared and 100  $\mu$ L were spread over the bottom agarose layer. Immediately thereafter, a solution consisting of equal volumes of 2X ACCM2 and 0.6% agarose was overlaid onto the bottom layer (Omsland 2012b). The plates were placed in the hypobaric chamber, infused with the gas mixture and incubated at 37°C. After 7-9 days, the plates were removed from the chamber and the number of colonies counted.

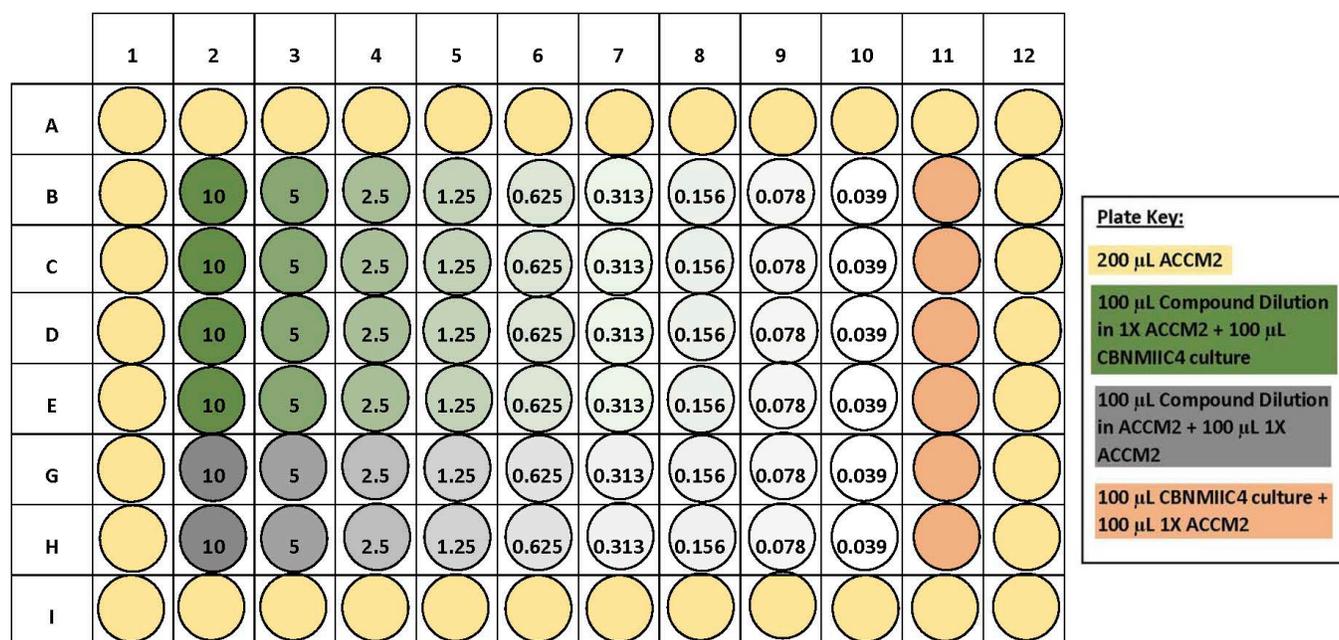
Additional confirmation of the number of bacteria in solution added to the plate was performed by determining the genomic equivalence (GE) of the cultured suspension. One milliliter of bacterial suspension ( $OD_{600}$ = 0.10) was centrifuged for 10 min at 16.1 rcf, then supernatant fluid was removed, and the remaining bacterial pellet had DNA extracted and purified using the QIAmp Mini DNA Kit (Qiagen, Hilden, Germany). DNA was then subjected to real time polymerase chain reaction (rtPCR) using a primer corresponding to the COM1 gene of *C. burnetii* and with PowerUp SYBR Green Master Mix (ThermoFisher Scientific, Waltham, MA) (Howe et al. 2009). The resulting quantified genome equivalents could estimate the number of bacteria, both living and dead, in culture.

#### *Assay to determine the Minimum Inhibitory Concentration*

Antimicrobial susceptibility testing of *C. burnetii* was done by determining the MIC of each compound of interest (Table 2). Varying concentrations of each compound were prepared in 1X ACCM2 and 100  $\mu$ L/well of the varying concentrations were added to a 96-well flat-bottomed microtiter plates. Working suspensions of CBNMIIC4 were prepared by adding 250  $\mu$ L of thawed stock solution to a 75 cm flasks (Sigma) containing 25 ml 1X ACCM2 in. The flasks were placed in the chamber, instilled with the gas mixture and then incubated at 37°C. After 5 days, the bacterial suspension was diluted to an  $OD_{600}$  of 0.10 and 100  $\mu$ L was added to each appropriate well.

In the 36 wells along the outer edge of the 96-well plate were 200 $\mu$ L of 1X ACCM2 to serve as a blank for negative compound control wells. Compound solutions of Doxycycline hyclate and Kanamycin (Sigma Aldrich, St. Louis MO), respectively, were made in sterile 1X ACCM2. Each antimicrobial compound was assessed in triplicate. Each treatment well received 100 $\mu$ L of the compound in 1X ACCM2 solution, then 100 $\mu$ L of CBNMIIC4 culture in 1X ACCM2 was added to the well for a final volume of 200 $\mu$ L. For each concentration of compound treatment there was a blank well that contained 100 $\mu$ L of CBNMIIC4 culture in 1X ACCM2 to which was added 100 $\mu$ L 1X ACCM2.

Positive growth control wells contained 100  $\mu$ L 1X ACCM2 and 100 $\mu$ L of CBNMIIC4 culture in 1X ACCM2. Experimental OD<sub>600</sub> measurements were blanked to ensure that only *C. burnetii* growth with compound was estimated via OD<sub>600</sub> measurements. A sample plate map for a 96-well plate MIC assay with doxycycline hyclate concentrations tested is presented in Figure 1.



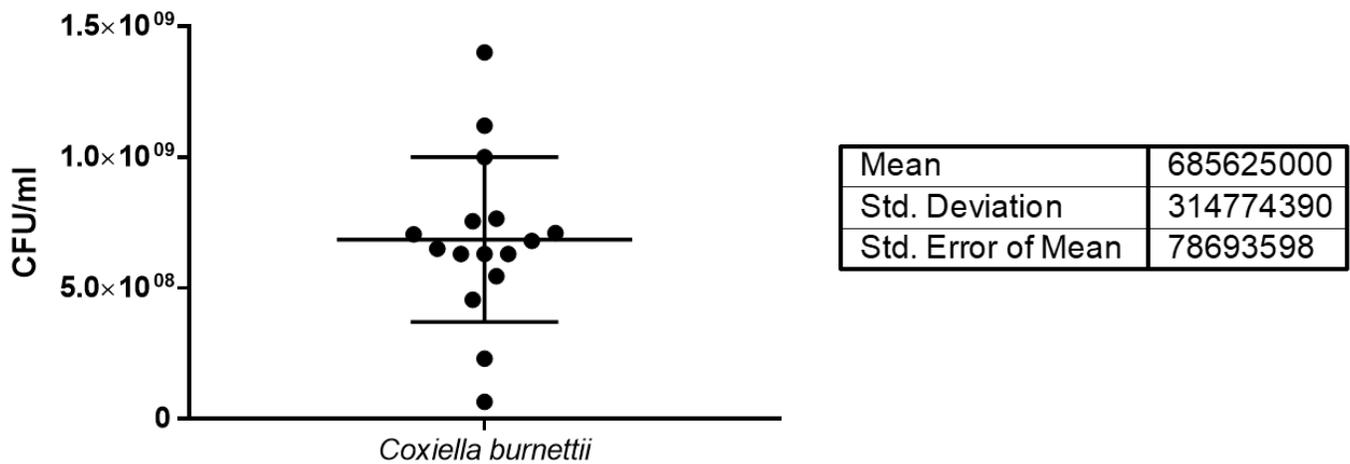
**Figure 1.** 96-well MIC Plate Assay Template with sample Doxycycline Hyclate concentration s ( $\mu$ g/mL) and color code plate key

Plates were placed in a hypoxic chamber, infused with the gas mixture, and incubated for 14 days at 37°C with shaking; chambers were recharged with gas mixture daily. OD<sub>600</sub> measurements were further confirmed with CFU agarose plates.

OD<sub>600</sub> measurements of the 96-well plates were taken immediately after plating, and then periodically (every 3 days) and days 5, 6 and 7 for a total of 14 days. The MIC values were expected to be observed between 5 and 7 days due to the *C. burnetii* growth curve (Coleman et al. 2004) and the stability of compounds in solution. When compared to the negative antibiotic control, the MIC was the minimum concentration at which CBNMIIC4 was inhibited. CBNMIIC4 concentration extrapolated from OD<sub>600</sub> was further confirmed with CFU assays using CBNMIIC4 culture from 96-well plate solutions.

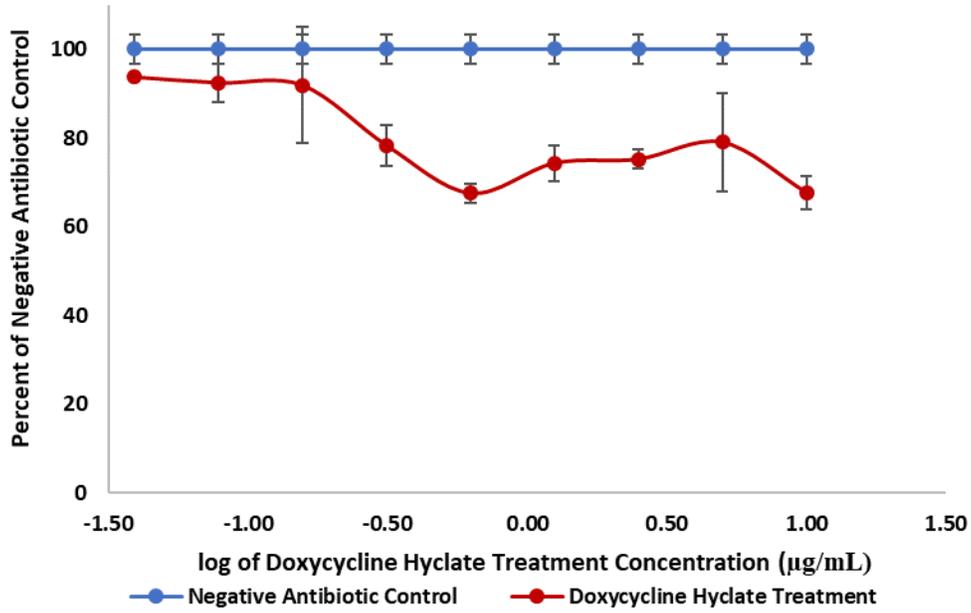
## Results and Discussion

CBNMIIC4 cultured in 1X ACCM2 was assessed by a CFU assay to determine the number of viable bacterial cells in culture. The bacterial concentration in culture was extrapolated based on the number of colonies observed on an agar plate and the assumption that every colony resulted from a single viable bacterium from the CBNMIIC4 culture. The resulting extrapolated CFU concentrations are presented in Figure 2.

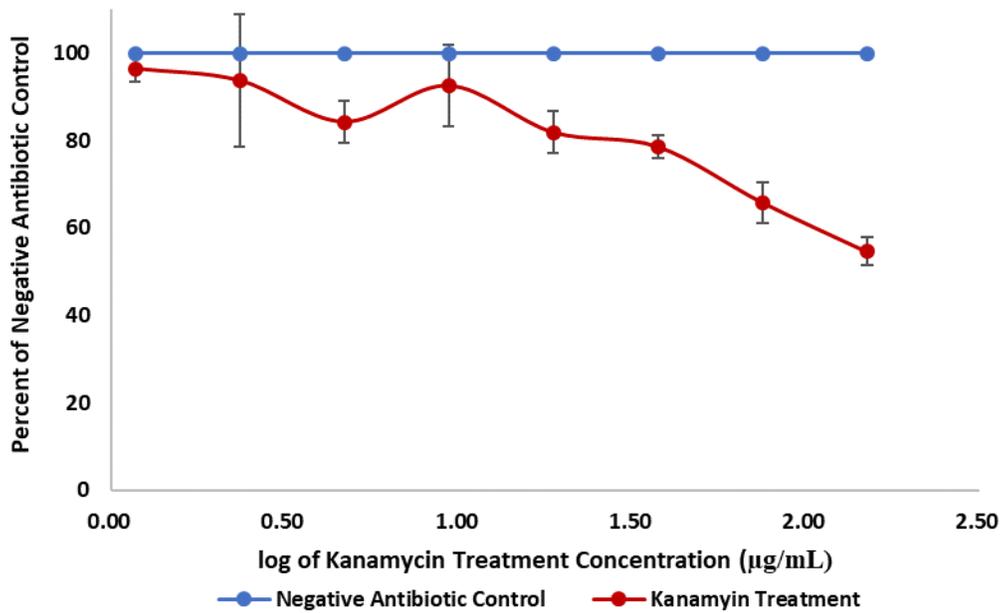


**Figure 2.** Colony Forming Unit Assay for OD<sub>600</sub> and CBNMIIC4 culture concentration correlation

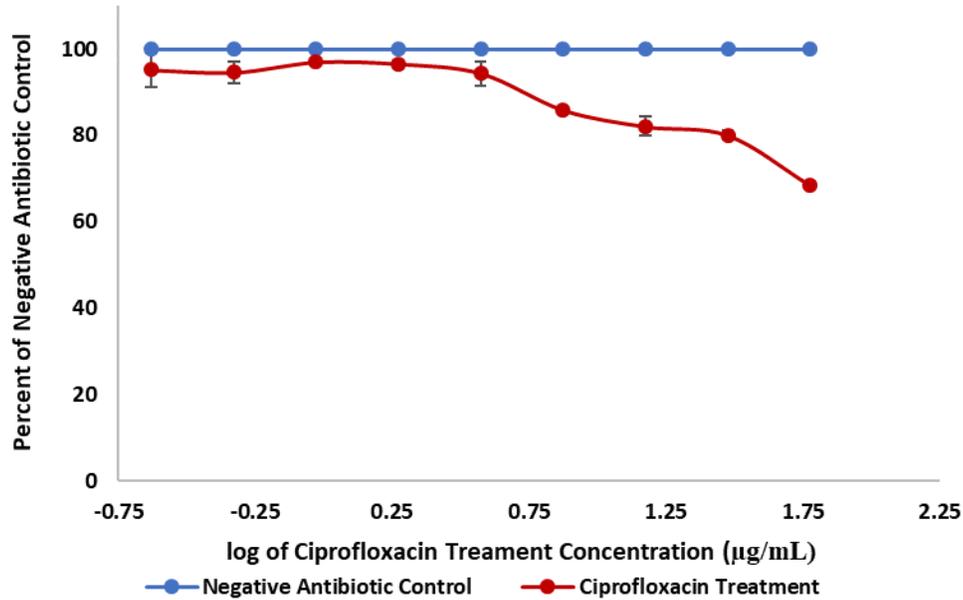
Based on the correlation between CFU and OD<sub>600</sub> values, the change in OD<sub>600</sub> of CBNMIIC4 cultures treated with anti-bacterial compounds were measured. The minimum concentrations of the antibiotics Doxycycline Hyclate (Figure 3) and Kanamycin (Figure 4) that inhibited CBNMIIC4 growth, when compared to the CBNMIIC4 well without antibiotic, were considered the MIC values for those compounds (Table 2).



**Figure 3.** Day 7 OD<sub>600</sub> comparisons of Doxycycline hyclate treated and Negative Antibiotic Control well of 96-well MIC plate assay; error bars represent standard deviation.



**Figure 4.** Day 5 OD<sub>600</sub> comparisons of Kanamycin treated and Negative Antibiotic Control well of 96-well MIC plate assay of CBNMIIC4; error bars represent standard deviation.



**Figure 5.** Day 5 OD<sub>600</sub> comparisons of Ciprofloxacin treated and Negative Antibiotic Control well of 96-well MIC plate assay of CBNMIIC4; error bars represent standard deviation.

The OD<sub>600</sub> measurements in Figures 3-5 are the results from single 96-well plate MIC assays testing doxycycline hyclate, kanamycin, and ciprofloxacin respectively. Growth inhibition of CBNMIIC4 in the treatment wells resulted in wells containing antibiotic concentrations in which there was a significant difference between treatment and positive bacterial control wells. The lowest concentrations for each compound where this inhibition was observed was considered the MIC value for that compound. In the case of both Doxycycline hyclate and Kanamycin, there were several concentrations that showed a comparable inhibition of CBNMIIC4 growth, and the MIC is therefore reported as a range (Table 2).

**Table 2.** Doxycycline Hyclate, Kanamycin, and Ciprofloxacin Minimum Inhibitory Concentrations for CBNMIIC4 from 96-well Assay

Antibiotic Compound	Minimum Inhibitory Concentration ( $\mu\text{g/mL}$ )
Doxycycline Hyclate	0.313-1.25
Kanamycin	2.34- 4.69
Ciprofloxacin	3.75-7.50

The MIC determined from the 96-well assay for Doxycycline hyclate is comparable to the MIC determined using Vero cell culture (Gikas et al. 2001). This suggests that the ACCM2 96-well plate assay method is a reliable alternative to the cell culture method of MIC determination. Furthermore, compared to the 6-well culture method (Gikas et al. 2001) for MIC determination for *C. burnetii*, the 96-well method is more efficient with smaller reagent volumes needed to determine MIC and more potential replicates and possible concentrations tested per plate with compound. In addition, the 96-well plate assay method is more applicable to a high throughput assay to complement more involved mammalian cell-based MIC studies (Hazan et al. 2012).

## Conclusion

There are many methods with which antibacterial compounds target bacteria. A bacterium such as *Coxiella burnetii* is additionally complex, due to its replication inside the highly acidic phagolysosome (Maurin et al. 1992) inside an infected cell. To effectively treat a *C. burnetii* infection, a compound must enter a cell, enter the membrane bound phagolysosome (Maurin et al. 1992), and then retain its antimicrobial properties in the very acidic environment of the phagolysosome (Maurin et al. 1992). As this environment is replicated in ACCM2, the inhibition of bacterial growth in this culture medium suggests that the compounds are still effective in the acidic niche of *C. burnetii*. Furthermore, the ability of the 96-well assay to determine MIC may be applied to test other compounds for anti-bacterial properties and may serve as a MIC method

superior to 6-well plate assays as well as a complement to cell-based studies. This method can also be used to further narrow the MIC range found for kanamycin for more precise MIC values. In addition, this method may be useful for testing antimicrobial susceptibility beyond an MIC, for example the compound concentration needed to reduce bacterial growth by 50 percent. However, the stability of compounds in assay conditions with an organism of interest must be verified before drawing conclusions about MIC values and antimicrobial compound susceptibility in this assay system.

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