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Effect of different drugs and drug combinations on killing stationary phase and biofilms recovered cells of *Bartonella henselae* in vitro

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Abstract

Background: *Bartonella henselae* is a Gram-negative bacterium transmitted to humans by a scratch from cat in the presence of ectoparasites. Humans infected with *B. henselae* can result in various clinical diseases including local lymphadenopathy and more serious systemic disease such as persistent bacteremia and endocarditis. The current treatment of persistent *B. henselae* infections is not very effective and remains a challenge. To find more effective treatments for persistent and biofilm *Bartonella* infections, in this study, we evaluated a panel of drugs and drug combinations based on the current treatment and also promising hits identified from a recent drug screen against stationary phase and biofilm recovered cells of *B. henselae*.

Results: We evaluated 14 antibiotics and 25 antibiotic combinations for activity against stationary phase *B. henselae* (all antibiotics were at 5 μg/ml) and found that ciprofloxacin, gentamicin, and nitrofurantoin were the most active agents, while clofazimine and miconazole had poor activity. Drug combinations azithromycin/ciprofloxacin, azithromycin/ methylene blue, rifampin/ciprofloxacin, and rifampin/methylene blue could rapidly kill stationary phase *B. henselae* with no detectable CFU after 1-day exposure. Methylene blue and rifampin were the most active agents against the biofilm *B. henselae* after 6 days of drug exposure. Antibiotic combinations (azithromycin/ciprofloxacin, azithromycin/methylene blue, rifampin/ciprofloxacin, rifampin/methylene blue) completely eradicated the biofilm *B. henselae* after treatment for 6 days.

Conclusions: These findings may facilitate development of more effective treatment of persistent *Bartonella* infections in the future.

Keywords: *Bartonella henselae*, Stationary phase, Biofilm, Antimicrobial activity, Drug combination

Background

Bartonella species are fastidious, Gram-negative intracellular bacteria [1] that are widely present in various mammals including cats, rodents, ruminants, and humans [2, 3]. They are transmitted mainly by direct contact such as animal scratches and bites, or by some arthropods such as sand flies, lice, fleas, biting flies, and ticks [4]. So far, at least 40 species or subspecies of *Bartonella* have been discovered [5]. At least 13 *Bartonella* species or subspecies are zoonotic [2]. Three species of *Bartonella* including *B. henselae, B. quintana,* and *B. bacilliformis,* are responsible for the great majority of infections in humans [6]. While *B. henselae* is the most common zoonotic *Bartonella* species, and the infection has a worldwide distribution [7] . According to a study from the USA, the incidence of *B. henselae* infection in human is

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about 3.7 per 100,000 [8]. Cat is its native host, and it is transmitted by the cat flea [9] . However, *B. henselae* can infect humans through an infected cat's scratch causing cat scratch disease (CSD), which is a disease characterized by self-limiting lymphadenopathy [10]. In the United States, CSD affects about 24,000 people annually [11]. Humans infected with *B. henselae* may also have other various clinical symptoms, such as fever with bacteremia, bacillary peliosis, bacillary angiomatosis and some infected individuals may get life-threatening blood-culture-negative endocarditis (BCNE) [12, 13]. Because *B. henselae* is capable of growing as aggregates and forming biofilms on infected native and prosthetic heart valves, it is a common cause of blood-culture negative endocarditis [14]. *B. henselae* biofilms have been involved in two distinct parts of the life cycle. First, they colonize and persist in the arthropod vector which increases transmission from the flea to the vertebrate host [15]. Second, *B. henselae* biofilms are an important composition of the heart valve vegetations found in patients with BCNE [16]. Biofilms are characterized by their stability, increased resistance to antibiotics, and chronic bacterial infections. In addition, biofilms protect the bacteria from antibiotics and host immune defenses such as macrophage engulfment [17]. Studies have shown that treatment failures of *Bartonella* infections are a significant problem despite low MICs suggesting a persistence problem [18]. *B. henselae* has a substantial capacity to withstand antimicrobial agents due to bacterial persistence and biofilm formation which pose significant challenge for treatment [19, 20]. Because *B. henselae* is extremely fastidious, it is difficult to isolate and culture in liquid media especially from clinical samples. Therefore, the diagnosis is often combined with clinical features, serology, and PCR instead of culture to confirm [21]. Treatment of systemic *B. henselae* infections has been difficult with poor clinical outcomes despite antibiotic treatment for weeks and months [22].

To identify agents that are useful for treating persistent *B. henselae* infection, in our previous studies, we have used the SYBR Green/PI viability assay for drug screens against stationary phase *B. henselae* successfully [23–26]. In this study, we used the same SYBR Green/PI methodology and evaluated a range of commonly used antibiotics and agent from our recent screen [26] and their combinations. We identified several drug candidates and drug combinations that have much better activity against stationary phase and *B. henselae* biofilms. Azithromycin and rifampin are typically used as the first-line treatment for local manifestations of *Bartonella* infections, and doxycycline and gentamicin are used to treat trench fever, chronic bacteremia and endocarditis [26]. Often, with serious infections, more than one antibiotic is used. Thus, in our study, we also evaluated the

efficiency of azithromycin or rifampin plus other antibiotics against stationary phase and *B. henselae* biofilms. Our study was the first to evaluate drug combinations against *B. henselae* non-growing stationary phase bacteria and biofilms and could provide experimental basis for further clinical evaluation.

Results

Growth behavior of B. henselae in modified Schneider's medium

The *B. henselae* cultures of varying ages (1 day, 2 day, 3 day, 4 day, 5 day and 6 day) were stained with SYBR Green I/PI assay and observed under the microscope (400 × magnification). The initial inoculum size was 1×10^6 CFU/mL. As shown in Fig. 1, *B. henselae* grew to logarithmic growth phase in 1 to 2 days, and then reached stationary phase from 3 to 6 days. This is consistent with the bacterial growth curve reported in our previous study [26]. Based on these, we considered 1 to 2 day old *B. henselae* culture as log phase culture and 3 to 6 day old culture as stationary phase culture.

MICs of candidate drugs

The candidate antibiotics evaluated were based on some antibiotics with good activity against stationary phase *B. henselae* [26] as well as antibiotics commonly used to treat *B. henselae* infections as controls. We used the standard method to determine the MICs of the candidate drugs for *B. henselae* after incubation of 6 days after drug addition as described in our previous study [26]. As shown in Table 1, rifampin was the most active agent which could inhibit *B. henselae* proliferation at the lowest concentration of <0.01 μg/mL. The growth of *B. henselae* was inhibited efficiently by azithromycin, doxycycline and methylene blue at a concentration of 0.08–0.16 μg/mL, by amikacin and nitrofurantoin at 0.31–0.63 μg/mL, by gentamicin at 0.63–1.25 μg/mL, by ciprofloxacin at 1.25–2.5 μg/mL, by cefuroxime, disulfiram, miconazole and SXT at 2.5–5.0 μg/mL. Daptomycin had relatively poor activity against growing *B. henselae* with a high MIC of 10–20 μg/mL. Clofazimine was the least effective agent for inhibiting the growth of *B. henselae*, with MIC higher than 40 μg/mL.

Drug exposure study to assess the activity of candidate drugs or drug combinations against stationary phase B. henselae

To confirm the activity of the drugs and drug combinations in killing stationary phase *B. henselae*, we performed a 24-h drug exposure study against a six-day-old stationary phase *B. henselae* culture as described in our previous study [26]. The concentration of each antibiotic was 5 μg/mL, as it is the average of most antibiotics' Cmax in serum. As shown in Table 2, when used alone,
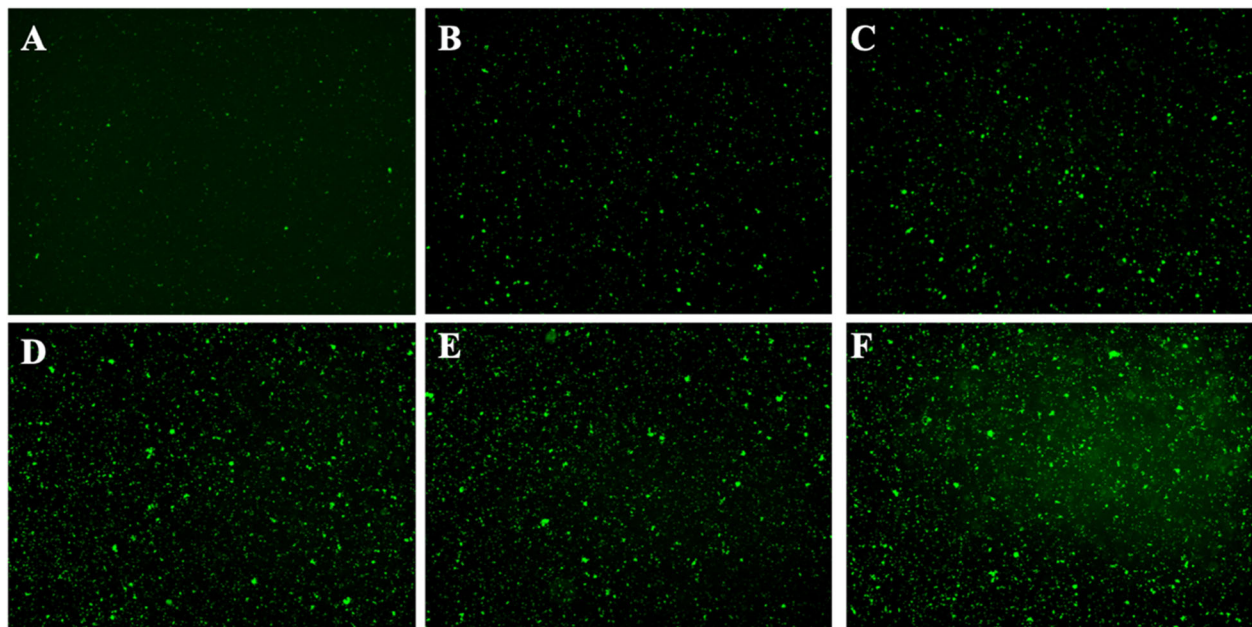
Fig. 1 Representative images of 1 day (A), 2 day (B), 3 day (C), 4 day (D), 5 day (E) and 6 day (F) old *B. henselae* cultures. The *Bartonella henselae* cultures of varying ages were stained with SYBR Green I/PI assay and observed under the fluorescence microscope (400 × magnification). The bacterial cells were stained as green by SYBR Green I

nitrofurantoin, gentamicin, and ciprofloxacin were the most active agents, with 2.8×10^2 CFU/mL, 5×10^2 CFU/mL, and 6×10^2 CFU/mL, surviving, respectively. Methylene blue and amikacin had significant activity with 10^4 CFU/mL remaining. Rifampin, cefuroxime, azithromycin had moderate activity with 10^{5-6} CFU/mL remaining. Doxycycline, disulfiram, SXT had weak activity. In contrast, clofazimine and miconazole had poor activity against stationary phase *B. henselae*, with no obvious

Table 1 MICs of select drug candidates against *B. henselae*

Antibiotics	MIC (μg/mL)
Amikacin	0.31–0.63
Azithromycin	0.08–0.16
Cefuroxime	2.5–5.0
Ciprofloxacin	1.25–2.5
Clofazimine	> 40
Daptomycin	10.0–20.0
Disulfiram	2.5–5.0
Doxycycline	0.08–0.16
Gentamicin	0.63–1.25
Methylene blue	0.08–0.16
Miconazole	2.5–5.0
Nitrofurantoin	0.31–0.63
Rifampin	< 0.01
SXT	2.5–5.0

decrease in CFU compared with the drug-free control. In the two drug combination study, it is worth noting that among the 13 azithromycin drug combinations, only azithromycin/ciprofloxacin and azithromycin/methylene blue combinations were able to completely eradicate all stationary phase *B. henselae*, whereas 11 other azithromycin drug combinations were not able to do so (Table 2). Similarly, among the 12 rifampin two drug combinations, only rifampin/ciprofloxacin and rifampin/methylene blue were found to rapidly kill stationary phase *B. henselae* with no detectable CFU after 1-day exposure.

In order to compare the efficacy of the identified active drug combinations with currently recommended antibiotic therapy (doxycycline/gentamicin) for treating B. henselae endocarditis, we performed a time-kill drug exposure assay of these active hits against a six-day-old stationary phase B. henselae culture. The concentration of each antibiotic was 5 μg/mL. As shown in Fig. 2, doxycycline/gentamicin could eradicate all stationary phase B. henselae cells after a 5-day drug exposure. Gentamicin was highly active even used alone, and methylene blue alone was more active than doxycycline/gentamicin combination. The drug combinations containing methylene blue, including azithromycin/methylene blue and rifampin/methylene blue were the most active ones that could rapidly kill all stationary phase B. henselae cells after a shorter time of 3-day drug exposure (Fig. 2), indicating our new drug combinations are more active

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Table 2 Effect of drugs or drug combinations on survival of stationary phase *B. henselae* [a]

Drugs (5 μg/ml)	CFU per mL after drug exposure
Drug free control	$2.8 \pm 0.4 \times 10^{10}$
Amikacin	$8.0 \pm 0.2 \times 10^{4}$
Azithromycin	$6.5 \pm 0.4 \times 10^{5}$
Cefuroxime	$2.2 \pm 0.2 \times 10^{5}$
Ciprofloxacin	$6.0 \pm 0.1 \times 10^{2}$
Clofazimine	$1.6 \pm 0.2 \times 10^{10}$
Daptomycin	$5.0 \pm 0.2 \times 10^{7}$
Disulfiram	$1.0 \pm 0.2 \times 10^{7}$
Doxycycline	$8.0 \pm 0.3 \times 10^{6}$
Gentamicin	$5.0 \pm 0.2 \times 10^{2}$
Methylene blue	$3.2 \pm 0.4 \times 10^{4}$
Miconazole	$1.5 \pm 0.1 \times 10^{10}$
Nitrofurantoin	$2.8 \pm 0.1 \times 10^{2}$
Rifampin	$6.0 \pm 0.3 \times 10^{5}$
SXT	$3.5 \pm 0.2 \times 10^{6}$
Azithromycin+Amikacin	$4.3 \pm 0.3 \times 10^{5}$
Azithromycin+Rifampin	$2.0 \pm 0.2 \times 10^{5}$
Azithromycin+Cefuroxime	$5.2 \pm 0.2 \times 10^{5}$
Azithromycin+Ciprofloxacin	0
Azithromycin+Clofazimine	$2.2 \pm 0.3 \times 10^{6}$
Azithromycin+Daptomycin	$1.8 \pm 0.1 \times 10^{5}$
Azithromycin+Disulfiram	$1.4 \pm 0.2 \times 10^{5}$
Azithromycin+Doxycycline	$1.2 \pm 0.3 \times 10^{6}$
Azithromycin+Gentamicin	$5.1 \pm 0.3 \times 10^{4}$
Azithromycin+Methylene blue	0
Azithromycin+Miconazole	$1.3 \pm 0.2 \times 10^{5}$
Azithromycin+Nitrofurantoin	$4.0 \pm 0.3 \times 10^{5}$
Azithromycin+SXT	$8.5 \pm 0.1 \times 10^{6}$
Rifampin+ Amikacin	$1.6 \pm 0.1 \times 10^{5}$
Rifampin+Cefuroxime	$2.2 \pm 0.1 \times 10^{4}$
Rifampin+Ciprofloxacin	0
Rifampin+Clofazimine	$2.8 \pm 0.1 \times 10^{5}$
Rifampin+Daptomycin	$1.2 \pm 0.1 \times 10^{6}$
Rifampin+Disulfiram	$8.5 \pm 0.2 \times 10^{5}$
Rifampin+Doxycycline	$1.2 \pm 0.1 \times 10^{6}$
Rifampin+Gentamicin	$1.6 \pm 0.1 \times 10^{4}$
Rifampin+Methylene blue	0
Rifampin+ Miconazole	$8.0 \pm 0.3 \times 10^{5}$
Rifampin+Nitrofurantoin	$1.2 \pm 0.1 \times 10^{5}$
Rifampin+SXT	$1.6 \pm 0.1 \times 10^{5}$

[a]Stationary phase *B. henselae* (6-day old) cells were treated with 5 μg/ml drugs alone or drug combinations for 24 h when the survival of the bacteria was determined by CFU count after wash (see Methods for more details)

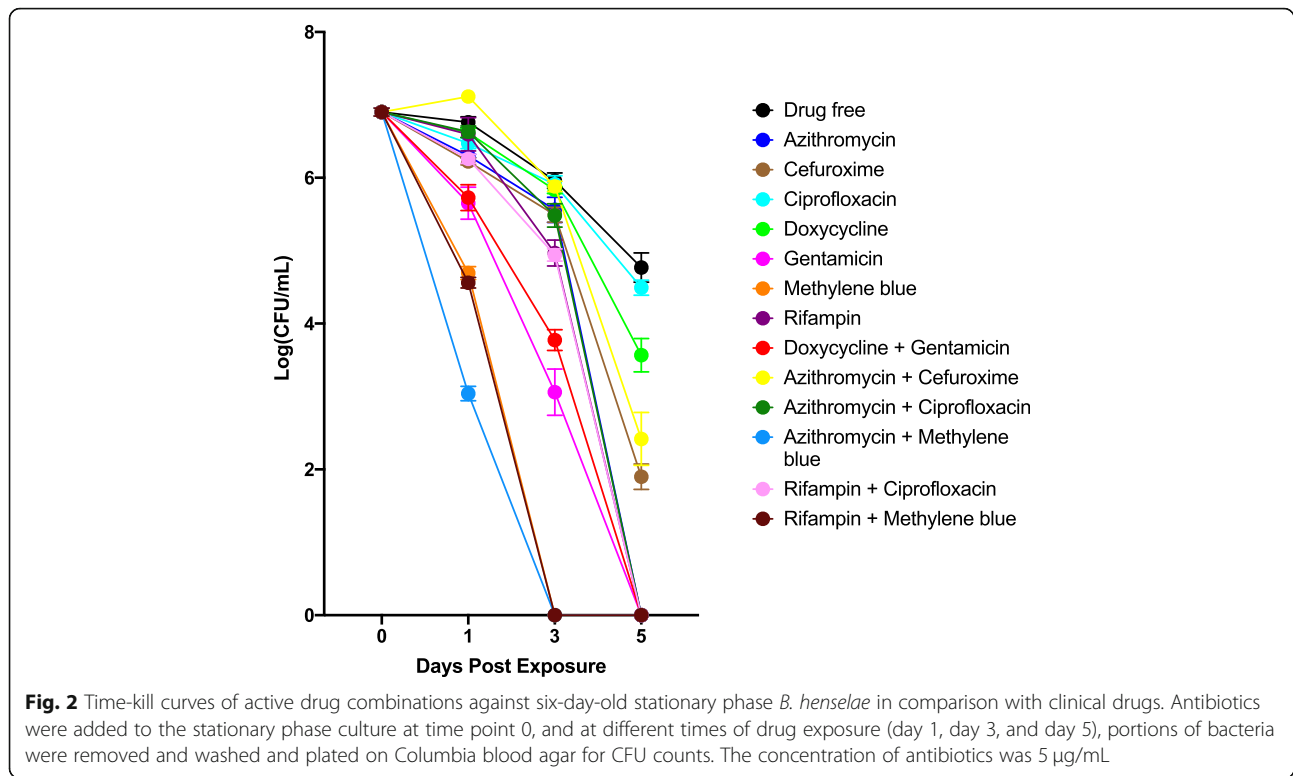than the currently recommended treatment with doxycycline/gentamicin combination.

Biofilm formation in B. henselae culture

B. henselae was cultured in Schneider's liquid medium at 37 °C, 5% CO_2 for 5 days followed by dilution of the culture 1:100 into fresh Schneider's medium for biofilm assays in 96-well plates for 5 days. The supernatant was carefully aspirated to prevent biofilm disruption, and then the biofilm was resuspended in Schneider's medium and scraped up with a pipette tip. We found that compared with the control group, the bottom of the well could be seen to form a thin layer of biofilm with the naked eyes. Further examination under the microscope showed more obvious biofilm, as shown by aggregated structures of *B. henselae* cells (Fig. 3a) compared with negative control (Fig. 3b).

Effect of select candidate drugs and drug combinations against biofilm recovered cells of B. henselae after drug exposure for different times

Since *B. henselae* biofilm contributes to its ability to persist in the host and could cause infective endocarditis that is difficult treat, it is important to eradicate the biofilm *B. henselae*. Based on the above results (Table 2), to further evaluate drug candidates against the biofilm *B. henselae* culture, we tested the efficacy of 12 antibiotics (azithromycin, cefuroxime, ciprofloxacin, daptomycin, disulfiram, doxycycline, gentamicin, methylene blue, miconazole, nitrofurantoin, rifampin, SXT) and the best 4 two-antibiotic combinations (azithromycin/ciprofloxacin, azithromycin/methylene blue, rifampin/ciprofloxacin, rifampin/methylene blue) in the biofilm *B. henselae* model after treatment for different times (2-day, 4-day, 6-day). The results are presented in Table 3. Overall, as expected, the biofilm derived cells of *B. henselae* were more tolerant to different drugs and drug combinations than the stationary phase cells (Table 2). No single drugs could completely eradicate all viable cells in the biofilm after drug treatment for 2 days, 4 days or 6 days (Table 3). Consistent with the results for stationary phase *B. henselae* drug exposure experiment, ciprofloxacin and gentamicin had good activity against biofilm *B. henselae* after 6 days of drug exposure, with 5.1×10^2 CFU/mL and 8.1×10^2 CFU/mL remaining, respectively (Table 3). Methylene blue and rifampin were the most active agents against the biofilm *B. henselae* after 6 days of drug exposure, with 2.3×10^2 CFU/mL and 3.2×10^2 CFU/mL, respectively (Table 3). Although nitrofurantoin was the most active agent against stationary phase *B. henselae*, its ability to kill biofilm *B. henselae* was poor after 6 days of drug exposure, with 2.8×10^9 CFU/mL.

In the drug combination study, the 4 two drug combinations (azithromycin/ciprofloxacin, azithromycin/

Fig. 2 Time-kill curves of active drug combinations against six-day-old stationary phase *B. henselae* in comparison with clinical drugs. Antibiotics were added to the stationary phase culture at time point 0, and at different times of drug exposure (day 1, day 3, and day 5), portions of bacteria were removed and washed and plated on Columbia blood agar for CFU counts. The concentration of antibiotics was 5 μg/mL

methylene blue, rifampin/ciprofloxacin, rifampin/methylene blue) had little activity against the biofilm bacteria after 2 day drug exposure with more than 10^9 bacteria remaining. Interestingly, after 4 day drug exposure, azithromycin/ciprofloxacin, azithromycin/methylene blue were more effective than rifampin/ciprofloxacin, rifampin/methylene blue as the former azithromycin drug combinations had 10^{5-6} bacteria remaining while the rifampin drug combination still had a very high CFU count of 10^9 bacteria. Nevertheless, all the 4 two drug combinations (azithromycin/ciprofloxacin, azithromycin/methylene blue, rifampin/ciprofloxacin, rifampin/methylene blue) completely eradicated all biofilm bacteria with no viable bacteria detected after 6 day drug exposure (Table 3).

Discussion

In this study, to develop more effective treatment for persistent *Bartonella* infections, we mainly focused on evaluating drugs and drug combinations for activity against stationary phase and biofilm *B. henselae*. We found that in single drug treatments, ciprofloxacin, gentamicin, and nitrofurantoin were the most active agents against stationary phase *B. henselae*, and methylene blue and rifampin were the most active agents against the biofilm *B. henselae*. In drug combination studies, none of the two drug combinations were able to completely kill the biofilm bacteria in drug exposure up to 4 days, but the two azithromycin drug combinations (azithromycin/ciprofloxacin, azithromycin/methylene blue) seemed to be more active than rifampin drug combinations (rifampin/ciprofloxacin,
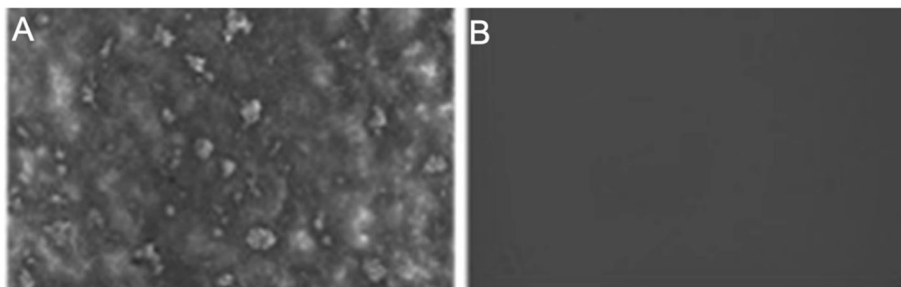

Fig. 3 Representative images of *B. henselae* biofilm. (A) biofilm stained with crystal violet and observed under the microscope (400 × magnification) (B) negative control without *B. henselae* observed under the microscope (400 × magnification)

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Table 3 Evaluation of select drug candidates against *B. henselae* biofilm-recovered cells after drug exposure at different times

Drugs (5 μg/ml)	CFU per mL after drug exposure		
	2 day	4 day	6 day
Drug free control	$1.3 \pm 0.2 \times 10^{10}$	$1.8 \pm 0.2 \times 10^{10}$	$2.6 \pm 0.3 \times 10^{10}$
Azithromycin	$4.5 \pm 0.3 \times 10^{9}$	$5.2 \pm 0.3 \times 10^{9}$	$9.3 \pm 0.2 \times 10^{5}$
Cefuroxime	$5.6 \pm 0.3 \times 10^{9}$	$2.3 \pm 0.2 \times 10^{9}$	$1.7 \pm 0.1 \times 10^{6}$
Ciprofloxacin	$3.2 \pm 0.3 \times 10^{8}$	$2.5 \pm 0.3 \times 10^{8}$	$5.1 \pm 0.3 \times 10^{2}$
Daptomycin	$9.8 \pm 0.2 \times 10^{9}$	$3.4 \pm 0.3 \times 10^{9}$	$2.7 \pm 0.2 \times 10^{6}$
Disulfiram	$6.1 \pm 0.3 \times 10^{9}$	$4.6 \pm 0.3 \times 10^{9}$	$3.8 \pm 0.3 \times 10^{7}$
Doxycycline	$5.3 \pm 0.3 \times 10^{9}$	$3.8 \pm 0.1 \times 10^{9}$	$6.2 \pm 0.3 \times 10^{5}$
Gentamicin	$6.2 \pm 0.3 \times 10^{9}$	$5.8 \pm 0.2 \times 10^{9}$	$8.1 \pm 0.3 \times 10^{2}$
Methylene blue	$8.9 \pm 0.4 \times 10^{9}$	$6.8 \pm 0.2 \times 10^{9}$	$2.3 \pm 0.2 \times 10^{2}$
Miconazole	$9.8 \pm 0.3 \times 10^{9}$	$2.2 \pm 0.1 \times 10^{10}$	$1.6 \pm 0.1 \times 10^{10}$
Nitrofurantoin	$4.3 \pm 0.1 \times 10^{9}$	$3.6 \pm 0.2 \times 10^{9}$	$2.8 \pm 0.2 \times 10^{9}$
Rifampin	$5.6 \pm 0.2 \times 10^{9}$	$4.8 \pm 0.3 \times 10^{9}$	$3.2 \pm 0.2 \times 10^{2}$
SXT	$7.8 \pm 0.3 \times 10^{9}$	$6.4 \pm 0.3 \times 10^{9}$	$2.7 \pm 0.2 \times 10^{9}$
Azithromycin+Ciprofloxacin	$4.1 \pm 0.2 \times 10^{9}$	$5.8 \pm 0.3 \times 10^{6}$	0
Azithromycin+Methylene blue	$6.8 \pm 0.2 \times 10^{9}$	$5.2 \pm 0.3 \times 10^{5}$	0
Rifampin+Ciprofloxacin	$7.6 \pm 0.2 \times 10^{9}$	$4.7 \pm 0.2 \times 10^{9}$	0
Rifampin+Methylene blue	$5.9 \pm 0.1 \times 10^{9}$	$4.8 \pm 0.2 \times 10^{9}$	0

rifampin/methylene blue) at Day 4. Interestingly, all four two drug combinations (azithromycin/ciprofloxacin, azithromycin/methylene blue, rifampin/ciprofloxacin, rifampin/methylene blue) could rapidly kill stationary phase and biofilm *B. henselae*.

Ciprofloxacin is a second-generation fluoroquinolone with a broad spectrum of activity that usually results in the killing of the bacteria. It is active against some Gram-positive and many Gram-negative bacteria including bacterial pathogens responsible for community-acquired pneumonias, bronchitis, urinary tract infections, and gastroenteritis [27]. Ciprofloxacin functions by inhibiting DNA gyrase, a type II topoisomerase, and topoisomerase IV, necessary to separate bacterial DNA, thereby inhibiting cell division [28, 29]. It has been reported that ciprofloxacin can be used in CSD [8, 30].

In our previous study, we found that methylene blue has good activity against stationary phase *B. henselae* [26]. Methylene blue is a medication and dye. As a medication, it is mainly used to treat methemoglobinemia [31]. It is also used as an antimalarial agent and for urinary tract infection (UTIs) treatment [32]. Recent studies found that methylene blue had antifungal effect through redox and membrane disruption [33]. While membrane is a target of persister drugs, our previous finding that methylene blue also had activity against *Borrelia burgdorferi* stationary phase cells is consistent with these [25]. It remains to be determined whether methylene blue could disrupt membranes of *B. henselae* as its basis for killing non-growing stationary phase *B. henselae* in the future.

For persistent and severe infections such as *B. henselae* infections, one drug is not enough, and a drug combination approach is needed [34, 35]. In our study, we evaluated 25 two-antibiotic combinations for activity against stationary phase *B. henselae*. We found four two-antibiotic combinations (azithromycin/ciprofloxacin, azithromycin/methylene blue, rifampin/ciprofloxacin, rifampin/methylene blue) had good activity against stationary phase *B. henselae* with no colony being detected. Furthermore, we evaluated the ability of the 4 two-antibiotic combinations against the biofilm of *B. henselae* and found that in general biofilm bacteria are more difficult to eradicate and it took longer (6 days) for the antibiotic combinations to eradicate the biofilm bacteria (Table 3) than the stationary phase bacteria (1 day) (Table 2). However, it is worth noting that azithromycin/ciprofloxacin and azithromycin/methylene blue were more active than the rifampin/ciprofloxacin and rifampin/methylene blue combinations. For convenience, the drug concentration of 5 μg/mL used in drug combination studies was based on average of Cmax for most antibiotics. The exact Cmax concentrations for each promising drug combination can be used in future drug combination studies.

Because azithromycin and rifampin are the first line drugs for treating *B. henselae* infections, our study only evaluated the efficiency of some two-drug combinations which we found to be more active than single drugs alone. In the future, we could try more three drug combinations using the current drugs used in clinic with the

newly identified drug candidates to kill different bacterial populations for more effective eradication, especially focusing on oral drugs. The biofilm model we used was 5 day old and scraped off the 96-well plate and could be considered young biofilm and may be more easily killed than older and intact biofilms. Future studies are needed to evaluate promising drug combinations on the latter more difficult to kill intact biofilms. It is worth noting that the anti-*Bartonella* activity of these identified drug combinations were obtained from in vitro assay, and further pharmacokinetic studies and in vivo animal efficacy studies are needed. If animal study results are favorable, clinical trials can be conducted to assess the safety and efficacy of the identified active drug combinations. Finally, we need to determine whether our findings derived from one strain *B. henslelae* JK53 are valid for other *B. henselae* strains and other pathogenic *Bartonella* species, such as *B. quintana* and *B. bacilliformis*.

Conclusions
This study identified methylene blue, gentamicin, and nitrofurantoin among 14 antibiotics evaluated to be the most active agents against stationary phase *B. henselae*, and drug combinations azithromycin/ciprofloxacin, azithromycin/methylene blue, rifampin/ciprofloxacin, and rifampin/methylene blue could kill stationary phase and bioifilm *B. henselae* with no detectable CFU. Future studies are needed to confirm the activity of the above active drugs or drug combinations in vivo animal and human studies to assess their utility to improve the treatment of persistent *Bartonella* infections.

Methods
Bacterial strain, culture media and culture conditions
Bartonella henselae strain JK53 was obtained through BEI Resources (ATCC), NIAID, NIH. *B. henselae* was cultured in Schneider's medium supplemented with 10% fetal bovine serum (FBS) as described [26, 36]. Cultures were incubated at 37 °C, 5% CO_2 at all times without shaking. The colony forming unit (CFU) counting was performed after serial dilutions on Columbia sheep blood agar (Becton Dickinson Biosciences, California, USA).

Antibiotics and stocks
Antibiotics including amikacin, azithromycin, cefuroxime, ciprofloxacin, clofazimine, daptomycin, disulfiram, doxycycline, gentamicin, methylene blue, miconazole, nitrofurantoin, rifampin and trimethoprim/sulfamethoxazole (SXT), were purchased from Sigma & Aldrich and were dissolved in appropriate solvents [37] to form stock solutions. All the antibiotic stocks were filter-sterilized by 0.2 μm filter except the DMSO stocks.

Microscopy techniques
The SYBR Green I/propidium iodide (PI) dye was added to *B. henselae* cell suspensions for observing the growth of *B. henselae* as described previously [23, 24]. The strain samples were examined on a BZ-X710 All-in-One fluorescence microscope (KEYENCE, Inc.). The biofilm specimens were stained with 0.1% crystal violet and observed under the microscope (400 × magnification).

Assessing drug activity against stationary phase B. henselae
Based on our previous study [26], we selected 14 antibiotics (amikacin, azithromycin, cefuroxime, ciprofloxacin, clofazimine, daptomycin, disulfiram, doxycycline, gentamicin, methylene blue, miconazole, nitrofurantoin, rifampin, SXT) and 25 two antibiotics combinations (azithromycin/amikacin, azithromycin/rifampin, azithromycin/cefuroxime, azithromycin/ciprofloxacin, azithromycin/clofazimine, azithromycin/daptomycin, azithromycin/disulfiram, azithromycin/doxycycline, azithromycin/gentamicin, azithromycin/methylene blue, azithromycin/miconazole, azithromycin/nitrofurantoin, azithromycin/SXT, rifampin/amikacin, rifampin/cefuroxime, rifampin/ciprofloxacin, rifampin/clofazimine, rifampin/daptomycin, rifampin/disulfiram, rifampin/doxycycline, rifampin/gentamicin, rifampin/methylene blue, rifampin/miconazole, rifampin/nitrofurantoin, rifampin/SXT) for drug screen against stationary phase *B. henselae*. 100 μL *B. henselae* cell suspension from a 6-day old stationary phase culture was added in 96-well plates. Each compound (10 μL, each antibiotic's final concentration was 5 μg/ml, including antibiotic combination) from the pre-diluted plate or pre-diluted stock was added to the cell suspension. Plates were sealed and placed in 37 °C incubator for 5 days. After antibiotic exposure, SYBR Green I/ PI viability assay was used to assess the live and dead cells as described [26]. 10 μL SYBR Green I (100× stock, Invitrogen, Waltham, MA, USA) and propidium iodide (PI, 600 μM, Sigma, St. Louis MO, USA) staining mixture was added to each well and mixed thoroughly. The plates were incubated in the dark for 15 min at room temperature, and then read using microplate reader (HTS 7000 plus Bioassay Reader, Perkin Elmer Inc., Waltham MA, USA). The green/red (535 nm/635 nm) fluorescence ratio of each well was used for calculating the residual viability percentage with least-square fitting analysis as described previously [26]. All tests were run in triplicate.

MIC determination
To determine the minimum inhibitory concentration (MIC) needed to inhibit visible growth of *B. henselae* after 6-day incubation using the standard microdilution method. *B. henselae* cells (1×10^6) from a 6 day old stationary phase culture were inoculated with 90 μL fresh modified Schneider's medium into each well of 96-well microplate. Each diluted drug (10 μL) was then added to

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the culture. The 96-well plates were sealed and incubated at 37 °C with 5% CO_2 for 5 days. After the incubation, cell proliferation was assessed using the SYBR Green I/PI assay and a Petroff-Hausser counting chamber. All experiments were run in triplicate.

Drug exposure assay for stationary phase B. henselae

A six-day-old *B. henselae* stationary phase culture was used for drug exposure experiments. The antibiotic exposure was carried out for 24 h or 5 days at 37 °C without shaking in 1.5 mL Eppendorf tubes. The concentration of each antibiotic was 5 μg/mL. Then the culture was centrifugated to collect the cells, and rinsed with fresh Schneider's medium twice, and then resuspended in 1 mL fresh Schneider's medium. The cell suspension was serially diluted and plated on Columbia blood agar plates for viable bacterial counts (colony forming unit, CFU).

Drug exposure assay for B. henselae biofilm

For biofilm inoculum, *B. henselae* was cultured in Schneider's liquid medium at 37 °C, 5% CO_2 for 5 days. The culture was diluted 1:100 into fresh Schneider's medium for biofilm assays in 96-well plates for 5 days. The supernatant was carefully aspirated to prevent biofilm disruption, and then resuspended in Schneider's medium and scraped up with a pipette tip. The biofilm was stained as described previously [38]. The antibiotic exposure was carried out as described above, except the drug exposure was 2 days, 4 days and 6 days.

Abbreviations
CFU: Colony forming unit; CSD: Cat scratch disease; DMSO: Dimethyl sulfoxide; FBS: Fetal bovine serum; MIC: Minimum inhibitory concentration; PBS: Phosphate-buffered saline; PI: Propidium iodide; SXT: Sulfamethoxazole/Trimethoprim

Authors' contributions
The research was designed by XYZ and YZ. XYZ, XM and WLS performed the experiments. TTL analyzed the data. XYZ, XM and YZ wrote the manuscript. All authors read and approved the final manuscript.

Availability of data and materials
Not applicable.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

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