QUANTITATIVE SUSCEPTIBILITY TESTING OF *CHLAMYDIA TRACHOMATIS* AGAINST CLINICALLY RELEVANT DRUGS

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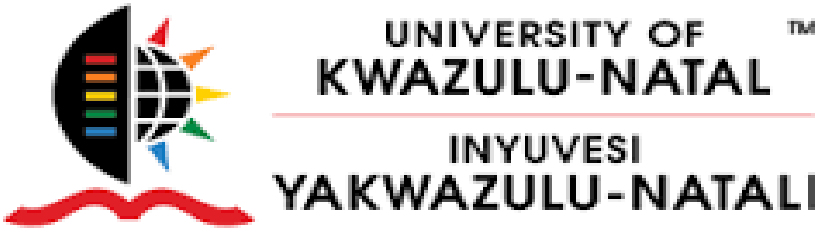

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PREFACE

The experimental work described in this dissertation was carried out in the Infection Prevention and Control Laboratory, Doris Duke Medical Research Institute Building, Nelson R Mandela School of Medicine, University of Kwazulu Natal, South Africa under the supervision of Dr B.C. Joubert and Prof W. Sturm

This study represents the original work by the author and has not otherwise submitted in any form to any other university. Where use of the work of others has been made, it is duly acknowledged in the text.

_____ 29/03/17_____

Riona Matadin (candidate) Date

_____ _____29/03/17_____

(Co-Supervisor) Date

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I, **Riona Matadin** declare as follows:

(i) The research reported in this dissertation, except otherwise indicated, is my original work.

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ETHICAL APPROVAL

This study was approved by the Biomedical Research Ethics Committee of the University of KwaZulu Natal (REF: BE220/13).

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ABSTRACT

Background

Chlamydia trachomatis causes vaginal discharge in females, urethritis in males and lymphogranuloma venereum (LGV) in both males and females. In South Africa *C. trachomatis* infection is treated with a 1g dose of azithromycin as part of the syndromic management regime. Under this regime, patients presenting with a particular set of symptoms are treated using a cocktail of drugs which treat the most common causes of that particular syndrome without performing laboratory diagnosis or susceptibility tests. This includes treatment with 250mg of ceftriaxone and 2g of metroniadazole. For this system to be effective, periodic surveillance needs to take place to determine which organisms are circulating in the population and their susceptibility to the recommended antimicrobial agents. In this study we determined the minimum inhibitory concentration (MIC) of *C. trachomatis* isolates to azithromycin, plus several treatment alternatives.

Methods

Cervical specimens were collected from patients presenting with genital discharge and screened by the BD ProbeTec™ ET assay . Specimens that tested positive were cultured. *C. trachomatis* was grown from four specimens. The MICs of eight antimicrobial agents were determined for a total of 11 *C. trachomatis* isolates using either immunofluorescence or real time PCR. For both methods McCoy cells were infected with *C. trachomatis* with centrifugation. After centrifugation cell culture media was replaced with cell culture media containing different concentrations of each

antimicrobial agent and incubated for 48 hours. After 48 hours, cell culture monolayers were either fixed with 95% ethanol and stained with the MicroTrak *C. trachomatis* Culture Confirmation Kit for inclusion identification using a fluorescent microscope, or lysed with TriSURE for RNA extraction followed by cDNA conversion and detection using real time PCR. The antimicrobial agents used were tetracyclines (tetracycline, doxycycline and minocycline), macrolides (azithromycin, erythromycin and clarithromycin), a fluoroquinolone (ciprofloxacin) and a glycycline (tigecycline). The *C. trachomatis* isolates included 5 isolates collected from discharge patients and 3 isolates collected from LGV patients in South Africa, as well as three LGV reference strains available from the ATCC.

Results

All eight of the antimicrobial agents tested had an inhibitory effect on all *C. trachomatis* isolates used. For the tetracyclines, MICs were ≤ 0.125, 0.031-0.063 and 0.125 mg/L for tetracycline, doxycycline and minocycline respectively . For the macrolides the MICs were 0.125-0.5, 0.125 and ≤ 0.062 mg/L for azithromycin, erythromycin and clarithromycin respectively. The MIC of ciprofloxacin was 0.5 – 1 mg/L for the isolates tested. The MIC of tigecycline ranged from 0.125 to < 0.0625 mg/L for the isolates tested. These MICs are in line with previous published MICs.

Conclusion

All eight of the antimicrobial agents tested had an inhibitory effect on all *C. trachomatis* isolates used, including azithromycin, the current drug of choice in South Africa. However azithromycin had a higher MIC than most antimicrobial agents tested.

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ABBREVIATIONS

°C	degrees Celsius
µl	microlitre
µg	microgram
ATCC	American Type Culture Collection
AZM	azithromycin
CDC	Centers for Diseases Control and Prevention
CIP	Ciprofloxacin
Clar	clarithromycin
CLSI	Clinical Laboratory Standards Institute
CO_2	carbon dioxide
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DOX	doxycycline
e.g	for example
EDTA	Ethylenediaminetetraacetic acid
ERY	Erythromycin
EUCAST	European Union Committee on Antimicrobial Susceptibility
FBS	foetal bovine serum
HIV	human immunodeficiency virus
IMI	intramuscular injection

L	litre
mg	milligram
mM	millimolar
MIC	minimal inhibitory concentration
ml	millilitre
Min	minocycline
NAATs	nucleic acid amplification tests
NICD	National Institute for Communicable Diseases
PCR	polymerase chain reaction
RNA	ribonucleic acid
SA	South Africa
STD	sexually transmitted disease
STI	sexually transmitted infection
TET	tetracycline
TIG	tigecycline
UK	United Kingdom
USA	United States of America
WHO	World Health Organisation

CHAPTER 1 - INTRODUCTION

Sexually transmitted diseases (STDs) are of pandemic proportions globally and prevalence figures are rising (CDC, 2015). Infections caused by the genital biovar of *Chlamydia trachomatis* contribute greatly to this pandemic in all parts of the world (WHO, 2008). In 2012, global prevalence of *C.trachomatis* infections was found to be 4.2% in women and 2.7% in men (Newman *et al*, 2015). The organism causes urethritis and a complication known as epididymitis in men as well as urethritis and cervicitis in women. In women the infection can be complicated by ectopic pregnancies, pelvic inflammatory disease and peri-hepatitis (Karnath, 2009). Lymphogranuloma venereum is an STD caused by a different biovar of C. trachomatis. Several different tests can be applied for the laboratory diagnosis of *C. trachomatis* infections. However nucleic acid amplification tests are the most commonly used, but for many less-resourced settings including South Africa, these commercial NAATs are too expensive for wide use (Papp *et al*, 2014). Use of NAATs are also too demanding in terms of laboratory commitment. In South Africa, STDs are treated syndromically according nationally formulated guidelines (SA Department of Health, 2015).

Amongst the drugs used in syndromic treatment, azithromycin, a macrolide is effective against *C.trachomatis* infections (Department of health 2015). Other drugs can also be used and these include the tetracyclines, other macrolides, fluoroquinolones and glycyclines (Somani *et al*, 2000). Due to extensive antibiotic use, drug resistance is bound to occur (Somani *et al*, 2000). No response to treatment is an indicator of resistance but, as with all STDs, this can also be the result of early reinfection. It is essential to carry out regular susceptibility testing to ensure that the given antibiotic treatment regimens are working correctly. However, being an obligate intra-cellular organism, susceptibility testing of *C. trachomatis* is complicated and requires multiplying

organisms. Therefore, studies on drug susceptibility of *C. trachomatis* are rare. Although infections with the genital and LGV biovar are prevalent in South Africa, susceptibility testing on clinical isolates has not been done.

The aim of this project is to determine the resistance profile of *C. trachomatis* isolates from patients in KwaZulu-Natal, South Africa to azithromycin and other antimicrobial drugs that can potentially be used to treat chlamydia infections.

Susceptibility testing was performed in cell culture and both immunofluorescence and real time PCR were used to establish growth inhibition. *C. trachomatis* L2, strain 434 was used as the reference strain since MIC values of that strain have been published in the literature (Table 1).

Table.1: Published MICs of L2 strain 434 and

Antibiotic	MIC L2	MIC E	Reference
Tetracycline	0.51	0.26	Walsh et al
Minocycline	0.031	0.016	Miyashita et al
Doxycycline	0.031	0.063	Miyashita et al
Tigecycline	0.125	No info	Townsend et al
Clarithromycin	0.031	0.031	Miyashita et al
Azithromycin	0.125	0.125	Miyashita et al
Erythromycin	0.125	0.125	Miyashita et al
Ciprofloxacin	1	1	Miyashita et al

CHAPTER 2 – LITERATURE REVIEW

2.1 Epidemiology

Sexually transmitted diseases (STDs) are a major public health problem globally (WHO, 2001). SomeSTDs, including those caused by *Chlamydia trachomatis*, may increase the transmission of HIV (WHO, 2001). Therefore effective management and prevention of STDs will help to reduce the incidence of HIV (WHO, 2001).

The highest prevalence of STDs occurs in developing countries (WHO, 2001), in particular in the young adult population (WHO, 2001). In 2008, there were 498.9 million new cases of STDs globally and 105.7 million of these cases were caused by *C. trachomatis* (WHO, 2008). High incidence rates of STDs continue to pose a threat to the social and economic status of countries greatly affected by this pandemic (Stephen and Shelagh, 2004). *C. trachomatis* infection is one of the most common STDs in both male and female (Sciara, 1997). Chlamydia prevalence and incidence rates are higher in females than males (WHO, 2005). According to a STD surveillance study carried out in the USA, a notable increase in chlamydia prevalence rates is seen in all regions between 2002 and 2011 (CDC, 2011).

In South Africa, contrary to the WHO report (2005) chlamydia infections are detected in 11% of women who present with vaginal discharge syndrome (Moodley *et al* 2002) and 16% of male patients who present with urethritis (Sturm *et al* 2004). Chlamydia infection accounts for 13.6% of genital ulcers in men (O'Farrell *et al* 2008) and 19% of genital ulcers in women (Sturm *et al* 2005).

2.2 Clinical Manifestations

C. trachomatis is a bacterial pathogen which is usually classified into two biovars, the oculogenital (OG) biovar and the lymphogranuloma venereum (LGV) biovar (Harris *et al*, 2012). However, within the OG biovar, some isolates are oculo-tropic, while others have a higher tropism for genital tissue, indicating that this OG biovar should be further subdivided into two groups which reflect disease tropisms which are based on genetic differences (Harris *et al*, 2012). *C. trachomatis* is also classified into 18 serovars which reflect differences in the Major Outer Membrane Protein (MOMP) (Harris *et al*, 2012). The oculo-tropic strains (serovars A-C) within the OG biovar cause trachoma (Cross et al, 1999) while the genito-tropic strains (serovars D-K) within the OG biovar cause genital discharge disease (Bebear and Barbeyrac, 2008). Strains of the LGV biovar (serovars L1-L3) cause lymphogranuloma venereum (LGV).

As with most STDs, a large percentage of genital chlamydia infections are asymptomatic and go unnoticed (Agrawal *et al* 2009). As a result, the infected person does not seek treatment and becomes a reservoir of infection within the community (Agrawal *et al* 2009). In men who test positive for *C.trachomatis*, symptomatic urethritis occurs in 15 to 55 % but this is considerably lower in older men (Miller, 2006). Untreated infections in men may lead to a complication known as epididymis while in females this can lead to ectopic pregnancy and pelvic inflammatory disease (Agrawal *et al* 2009). Women who develop pelvic inflammatory disease often become infertile

(Miller, 2006). Untreated chlamydia infections may cause Reiter syndrome, which is a reactive arthritis. The prevalence of this manifestation is higher in women than in men (Miller, 2006).

Strains of the LGV biovar, namely L1, L2 and L3 cause a more invasive disease called LGV which begins as a genital ulcer. The chlamydia migrate to the inguinal lymph nodes (Cross *et al*, 1999) resulting in tender inguinal and/or femoral lymphadenopathy (CDC, 2015). The clinical manifestation of LGV which develops in the MSM population and in women due to rectal exposure is called proctocolitis (CDC, 2015) and is accompanied by pelvic lymphadenopathy. Treatment of LGV includes the use of doxycycline, azithromycin or erythromycin (SA Department of Health, 2015).

2.3 Biology of the organism and lifecycle

C. trachomatis is an obligate intracellular bacterial pathogen with a unique biphasic life cycle (Cross *et al*, 1999). *C. trachomatis* exists in two forms, an extracellular form called the elementary body (EB) and an intracellular form called the reticulate body (RB) (Mpiga and Ravaoarinoro, 2006). The EBs are metabolically inactive, osmotically stable and are able to infect cells. The RBs are metabolically active, osmotically fragile and responsible for replication once the organism has entered a susceptible host cell (Mpiga and Ravaoarinoro, 2006). The cell wall of *C. trachomatis* contains inner and outer membranes which is typical of Gram negative bacteria. However no peptidoglycan layer is present (Mpiga and Ravaoarinoro, 2006). Disulphide-cross linked proteins are present, which allows for rigidity of the EB (Mpiga and Ravaoarinoro, 2006).

Once a susceptible host cell has become infected, the EB differentiates into a RB which is internalized in a vacuole formed by the phagosome, and escapes phagolysosomal fusion (Mpiga and Ravaoarinoro, 2006). The RB is metabolically active and divides by binary fission (Mpiga and Ravaoarinoro, 2006). The RB then recondenses to form EB which are released from the cell via exocytosis (Mpiga and Ravaoarinoro, 2006). *C. trachomatis* infections may go unnoticed because the organism is thought to have a metabolically less active state, which it uses to escape from the immune system allowing the bacteria to multiply (Mpiga and Ravaoarinoro, 2006).

2.4 Pathogenesis of infection

Epithelial cells are the primary target cells for *C. trachomatis* infection (Darville and Hiltke, 2010). Upon primary infection of epithelial cells, EBs trigger the secretion of cytokines which recruit neutrophils to the site of infection (Rasmussen *et al*, 1997). Neutrophils are capable of killing extra-cellular EBs (Darville and Hiltke, 2010). T-cells accumulate at a later stage and play a role in clearing the infection (Ramsey and Rank, 1991), but chlamydia-specific immune cells are not able to prevent new infections (Bebear and Barbeyrac, 2008). Instead of having a protective effect they release chemokines at the site of infection, which cause tissue damage brought about by proteases, clotting factors and tissue growth factors (Molano *et al*, 2005). Inflammation and fibrosis occur as a result of infection (Bebear and Barbeyrac, 2008). Persistent forms of *C. trachomatis* may also develop as a result of antibiotic treatment, nutrient depletion and cytokines (Ward, 1999). The cell mediated immune response triggers the release of interferon-γ, resulting in chlamydial Hsp60 expression which is responsible for bringing about a chronic inflammatory

response (Ward, 1999). A marker used to diagnose chronic C. *trachomatis* infection is the presence of anti-Hsp antibodies (Dean *et al*, 2000).

2.5 Laboratory diagnosis

The type of specimen collected varies depending on the laboratory diagnostic technique to be performed (Bebear and Barbeyrac, 2008). When using modern NAATs for diagnosis, specimens such as urethral swabs and cervical swabs are more sensitive than tests done on non-invasive specimens which are usually self-collected, such as vulvovaginal swabs, penile swabs, anal swabs and first void urine specimens (Michel *et al*, 2007). Although these non-invasive specimens are easier to obtain and cause less discomfort to the patient, they contain a high bacterial load with commensal species and are not suitable for cell culture which is required if susceptibility testing is to be performed (Michel *et al*, 2007). Methods of diagnosis include cell culture, antigen-based detection methods, nucleic acid hybridization tests and nucleic acid amplification technologies (Bebear and Barbeyrac, 2008).

Cell culture techniques are laborious but have high specificity (Black, 1997). Cell culture has a long turnaround time and routine use is not recommended due to its lack of sensitivity (Black, 1997). Specimens need to be placed in liquid transport media immediately after collection and same day refrigeration and transport to the laboratory is required (Black, 1997). However, cell culture is highly recommended for antimicrobial susceptibility testing (Bebear and Barbeyrac, 2008). Other methods of detection include direct fluorescent staining and enzyme immunoassay (Michel *et al*, 2006). The fluorescent staining is quick; however it is not practical if large numbers

of specimens need to be processed (Michel *et al*, 2006). Enzyme immunoassay has a higher sensitivity than cell culture, but it can also produce false positives (Michel *et al*, 2006). DNA probing is a molecular test which can be used for diagnosis and is more sensitive than cell culture methods but cannot be used with non-invasive specimens (Schachter *et al*, 2005). Nucleic acid amplification tests (NAATs) are highly sensitive and specific (Leber *et al*, 2006). Non-invasive and invasive specimens can be processed by NAATs (Schachter *et al*, 2006).

2.6 Treatment and prevention

There are several antimicrobial agents which can be used to treat *C. trachomatis* infection and the best choice depends on the type of infection, site of infection, age of the patient, as well as pregnancy status in women (Miller, 2006).

In South Africa STDs are currently managed syndromically (Altini and Coetzee, 2003). Identification of the STD syndrome is based on symptoms and observations during examination (SA Department of Health, 2015). The syndrome is then treated with a combination of drugs which are effective against common causes of the syndrome (Altini and Coetzee, 2003). This enables health care providers to diagnose and treat patients on the same day without waiting for a laboratory diagnosis (Altini and Coetzee, 2003).

In our setting, *C. trachomatis* is a bacterial pathogen which is responsible for the development of genital ulcers with lymphadenopathy (Sturm *et al*, 2005), genital discharge disease (Bebear and Barbeyrac, 2009) and trachoma (Cross et al, 1999) in men and women. Syndromic management of genital discharge disease includes the use of ceftriaxone, azithromycin and metronidazole, while syndromic management of genital ulcers involves the use of benzathine benzyl penicillin,

acyclovir and azithromycin (SA Department of Health, 2015). Amongst these drugs, azithromycin is used to treat chlamydial infections (Frye *et al* 2008).

Tetracyclines, macrolides and fluoroquinolones are effective in treatment of chlamydia infections (Somani *et al*, 2000). Tetracyclines have been used as standard treatment for non-specific genital infections as well as chlamydia infections (Ridgway, 1997). Treatment of chlamydia infection usually consists of a 7-day regimen of doxycycline (Frye *et al* 2008). The macrolides, azithromycin, erythromycin and clarithromycin can also be used as an alternative treatment for chlamydial infections in patients who develop adverse effects to doxycycline but doxycycline has a higher cure rate (Frye *et al* 2008). Azithromycin is a safe drug which can be used during pregnancy, but is more expensive than treatment with doxycycline (Frye *et al* 2008). Fluoroquinolones have a broad spectrum of antimicrobial activity (King *et al*, 2000).

Use of these antibiotics are usually 90-100% effective, however recurring infection is inevitable due to large numbers of asymptomatic infections in the population (Mpiga and Ravaoarinoro, 2006). Antibiotic treatment failure may occur due to poor compliance of patients to their treatment regime (Mpiga and Ravaoarinoro, 2006). Therapy failure may also be due to chlamydial resistance (Somani *et al*, 2000).

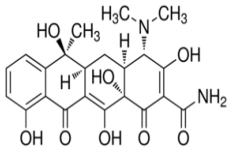
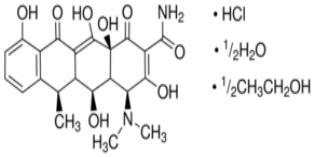
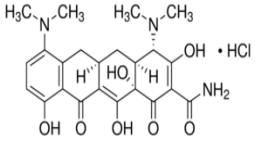
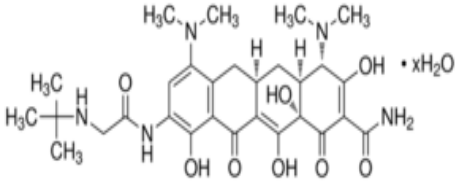
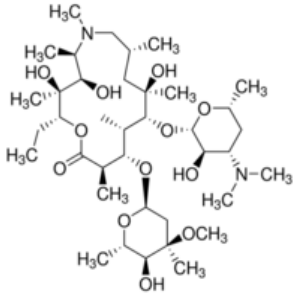
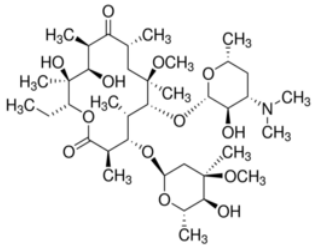
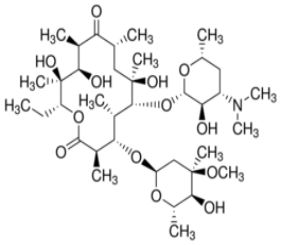
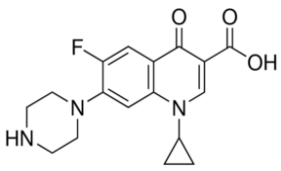
Prevention of STDs is a high priority and the CDC has set guidelines on concepts for prevention (Miller, 2006). These guidelines include education on safe sexual behavior, awareness of symptomatic and asymptomatic infection, availability of diagnosis and treatment facilities, counselling services for both infected persons as well as their partners and also vaccines should be available for those STD treatable by vaccines (Miller, 2006). Vaccines offer a long term advantage

in controlling and reducing prevalence, however no vaccines are available for the prevention of *C. trachomatis* infections (Mpiga and Ravaoarinoro, 2006).

2.7 Antimicrobial agents

Previously macrolides were the drug of choice for treatment of *C.trachomatis* infections, however it has more recently been discovered that tetracyclines are more effective (Handsfield HH, 2011). Quinolones can also be used in treatment as well as the glycycline group of antimicrobial agents (Somani *et al*, 2000). Table 2 below shows the published MIC values for reference strain L2 434 which forms the basis for antibiotic concentrations used in this study.

Table 2: Chemical structure of the antimicrobial agents used in this study

Class	Chemical structure	Reference
Tetracyclines	 Tetracycline  Doxycyline hyclate  Minocycline hydrochloride	Sigma
Glyccycline	 Tigecycline hydrate	Sigma
Macrolides	 Azithromycin  Clarithromycin  Erythromycin	Sigma
Fluoroquinolone	 Ciprofloxacin	Sigma

2.7.1 Tetracyclines

Tetracyclines are broad spectrum antimicrobial agents which are effective against Gram-positive and Gram-negative bacteria, as well as some protozoa (Nelson and Levy, 1999). They are used as growth promotors in animal feeds, for malaria prophylaxis and for treatment of infections (Nelson and Levy, 1999). Tetracyclines are useful because they are low in toxicity, relatively inexpensive and are easily absorbed (Michalova *et al*, 2004).

The structure common to all tetracyclines is a linear fused tetracyclic nucleus (table 2) which serves as a site of attachment for a variety of functional groups (Mitscher, 1978) . Substitutions on the nucleus rings enable variation of tetracyclines to be possible, thus resulting in different tetracyclines for clinical use (Rogalski, 1985). There are 11 different types of tetracyclines that are used clinically (Chopra and Roberts, 2001) however only 3 were used in this study namely; tetracycline, minocycline and doxycyline.

The mechanism of action of tetracylines is inhibition of protein synthesis by preventing the interaction of the aminoacyl tRNAs with ribosomes (Michalova *et al*, 2004). In Gram negatives such as *C. trachomatis*, tetracyclines bind to a positively charged cation and enter the bacterial cell through the pores in the outer membrane (Chopra *et al*, 1992). Once in the periplasm, the tetracycline-cation complex dissociates which allows the tetracycline to enter the lipid bilayers of the inner membrane (Schnappinger and Hilton, 1996). The process of entry of tetracyclines into the cytoplasm is energy dependent and once the tetracycline binds to the ribosome, this binding becomes irreversible resulting in its bacteriostatic effect (Chopra *et al*, 1992).

Emerging resistance to tetracyclines is becoming a hurdle in medical treatment (Chopra and Roberts, 2001). Although clinical practice may have contributed to this resistance, it is thought

that the use of tetracyclines as growth promotors in animal feed may have also contributed to the resistance emerging in human pathogens (Chopra and Roberts, 2001). There are two different types of genes responsible for causing tetracycline resistance through acquisition of plasmids (Levy *et al*, 1999). Efflux pumps are also responsible for resistance to tetracyclines, however these pumps are not effective against macrolides and glycyclines (Chopra *et al*, 1992). Production of ribosomal protection proteins is another mechanism of resistance (Sanchez-Pescador *et al*, 1988) as well as the production of enzymes which inactivate tetracycline (Speer *et al*, 1991).

2.7.2 Glycyclines

Glycyclines are a new class of antimicrobial agent which have been developed due to increasing resistance to tetracyclines (Sum *et al*, 1998). Glycyclines are structurally similar to tetracyclines in that they contain the same central four ring carboxylic skeleton however they differ by the presence of an N-alkyl-glycylamido group on the D ring (table 2) which enables these compounds to have a broader spectrum of activity (Chopra, 2001).

Glycycline antibiotics are known to be effective against Gram positive and Gram negative organisms, including those bacteria resistant to tetracyclines (Sum *et al*, 1998). It has been shown in vitro that tigecycline is effective against many organisms (Jones *et al*, 2004). The use of tigecycline proves very advantageous as it is active against bacteria which are resistant to other antibiotics (Chopra, 2001). Tigecycline is able to overcome de-activation mechanisms common to the tetracyclines. Therefore tetracycline resistance is overcome by use of tigecycline (Bauer *et al*, 2004). The modification in structure of tigecycline enables it to bypass resistance mechanisms to tetracyclines (Chopra, 2001). These resistance mechanisms include active drug efflux and

ribosomal protection (Zhanel *et al*, 2004). These resistance mechanisms are avoided by tigecycline due to steric hindrance as a result of the large substituent at position 9 (Projan, 2000).

2.7.3 Macrolides

Macrolides are a group of antimicrobial agents used commonly to treat Gram-positive bacterial infections as well as some infections caused by Gram negative species such as *Bordetella pertussis* and Campylobacter, Chlamydia, Helicobacter and Legionella species (Gaynor and Mankin, 2003).

Currently there are 5 different natural macrolide antimicrobial agents and 7 semisynthetic macrolides available (Mazzei *et al*, 1993). The macrolides used in this study are erythromycin which is a natural macrolide as well as clarithromycin and azithromycin which are semisynthetic macrolides. The structure common to all macrolides is the lactone ring which usually contains between 12 to 16 atoms (Mazzei *et al*, 1993). Macrolides differ in the number of rings per molecule as well as the different substitution groups present on the atoms which are shown in table 2 (Briskier *et al*, 1986).

Erythromycin was the first macrolide employed for clinical use, however acid instability of the drug led to the production of the newer macrolides clarithromycin and azithromycin (Gaynor and Mankin, 2003). Macrolides inhibit protein synthesis by binding to the ribosome, which leads to inhibition of the formation of peptide bonds (Hansen *et al*, 2002) by releasing peptidyl-tRNA. This disrupts protein synthesis (Menninger JR, 1995) by compromising assembly of the ribosome (Chittum and Champney, 1995).

Resistance to macrolides can occur either by modifying the target site of the drug or excreting the drug from the cell (Weisblum B, 1995).

2.7.4 Fluoroquinolones

Quinolones are recognized by their heterocycles and bicyclic core structure as seen in table 2 (Hu *et al*, 2003). Fluoroquinolones are derivatives of quinolones because of fluoridation of the quinolone molecule at C6 (Paton and Reeves, 1988). Second generation fluoroquinolones are norfloxacin and ciprofloxacin (Paton and Reeves, 1988), third generation fluoroquinolones include levofloxacin, gatifloxacin and moxifloxacin (Barrett, 2000), the fourth generation fluoroquinolone developed isknown as gemifloxacin (Lowe and Lamb, 2000).

Fluoroquinolonesare broad spectrum antibiotics and the most commonly used fluoroquinolone is ciprofloxacin (Fabrega *et al*, 2009). Fluoroquinolones are effective against a wide range of Gram positive and Gram negative bacteria (Hawkey, 2003). The primary target in Gram negative bacteria is DNA gyrase and the primary target in Gram positive bacteria is topoisomerase IV (Drlica and Zhao, 1997). DNA gyrase is responsible for the separation and re-binding of DNA strands during replication (Hawkey, 2003). The activity of topoisomerase IV is similar to that of DNA gyrase, however its primary function is to break the link of newly replicated daughter chromosomes as well as decantation (Kato *et al*, 1990). Quinolones thereby inhibit DNA synthesis by binding to DNA gyrase or DNA topoisomerase complexes (Khodursky and Cozzarelli, 1998). This binding results in a conformational change in the enzyme (Khodursky and Cozzarelli, 1998).

Upon breaking of DNA strands caused by the enzyme, the strands are unable to ligate again since the quinolone has bonded to the DNA enzyme complex, thus inhibiting DNA synthesis (Khodursky and Cozzarelli, 1998). When quinolones bind to gyrase –DNA complexes, replication stops at a fast rate since gyrase binds to DNA ahead of the replication fork (Khodursky and Cozzarelli, 1998). In organisms where quinolones bind to topoisomerase-DNA complexes, DNA synthesis is inhibited at a slower rate since topoisomerase binds to the DNA behind the replication fork (Khodursky and Cozzarelli, 1998). High concentrations of quinolones can be used to kill cells rather than to inhibit growth (Zhao *et al*, 1997). Resistance to quinolones are known to arise and are due to mutations in the genes that encode gyrase and topoisomerase IV (Hoshino *et al*, 1994). Structural changes in the organism are also responsible for resistance, namely changes in the porins, which effects entry into the cell (Nikaido, 1998).

2.8. Susceptibility testing

Because *C. trachomatis* is an obligate intracellular organism, traditional methods of antimicrobial susceptibility testing cannot be used (Cross *et al*, 1999). As such there are no EUCAST or CLSI guidelines for this and no recommended break points. When susceptibility testing of *C. trachomatis* is required, a combination of cell culture and either immunofluorescence or a molecular method is usually used since there is no standardized methodology or interpretation of results available (Suchland et al, 2002). A previous study conducted for antimicrobial susceptibility testing of *C. trachomatis* showed that determining the end point by reverse transcriptase PCR may be useful due to its sensitivity (Cross *et al*, 1999). This study was conducted using 16 different chlamydia isolates against eight different antimicrobial agents (Cross

et al, 1999). Immunofluorescence is rather subjective and a study conducted by Peuchant *et al* showed that using real time PCR for susceptibility testing is more reliable and objective.

2.9 Drug resistance in *Chlamydia trachomatis*

There are two different types of resistance: *in vivo* or clinical resistance and *in vitro* or laboratory resistance. Since determination of in vitro resistance of *C. trachomatis* is hampered by the need for culture and the complicated techniques involved in susceptibility testing of intra-cellular organisms (2.8), reports on *in vitro* resistance are scarce.

Resistance of chlamydia infections to antimicrobial treatment has been reported in Wyoming USA in two patients in which clinical treatment with azithromycin failed as well as one contact of one of these patients. In vitro susceptibility confirmed resistance to azithromycin, doxycycline and ofloxacin in all three isolates (Somani *et al*, 2000).

Tetracycline resistance has been reported in USA as a result of clinical treatment failure (O'Neill *et al*, 2013). These "resistant" strains were further studied and found to be sensitive to tetracycline *in vitro* indicating that the reported treatment failure may have been due to re-infection or poor patient compliance (O'Neill *et al*, 2013). Repeat infections following treatment of *C. trachomatis* is common (Hocking*et al*, 2013).

In vitro tetracycline resistance has also been reported in Indianapolis, USA (Jones *et al*, 1990). This study incorporated five different chlamydia isolates which were reported to have clinical treatment failure (Jones *et al*, 1990). After conducting susceptibility testing on these isolates, it

was found that isolates resistant to tetracyclines were also resistant to doxycycline and erythromycin.

Resistance was also reported in Russia which entailed a study comprising of six chlamydia isolates. These isolates were tested against antimicrobial agents and it was found that four of these isolates were resistant to azithromycin and erythromycin (Misyurina *et al*, 2003).

In bacteria resistance to antimicrobial agents can occur by enzymatic degradation of antibacterial drugs, alteration of bacterial molecules that are antimicrobial targets and changes in membrane permeability (Dever and Dermody, 1991). When presented with adverse circumstances, organisms adapt for survival and can do so via genetic mutations (Woodford and Ellington, 2007).

Although resistance has been reported as mentioned above, a compilation of other studies show azithromycin to be effective against 97% of infections and 98% efficacy for doxycyline (Handsfield HH, 2011).

In the United States of America (USA) and other parts of the world where the prevalence of *C. trachomatis* is high, many patients experience "recurrent" infections (Munday *et al*, 1995). This is either due to re-infection or an infection which persists after treatment (Munday *et al*, 1995). Studies of young women presenting with genital infection in the United Kingdom (UK) (Lamontagne *et al*, 2007) and the USA (Batteiger *et al*, 2010) found that 30% and 34% tested positive for *C. trachomatis* 6 months and 3 months after treatment.

The first line drugs for treatment of chlamydial infections, tetracyclines and azithromycin, are usually effective unless a change in the organism's life cycle brings about persistence (Sandoz and Rockey, 2010). It is this persistence of the organism which is believed to be associated with secondary complications of the infection (Sandoz and Rockey, 2010).

The problem is further compounded by co-infection with other bacteria (Stamm *et al*, 1984). In patients presenting with chlamydia infection, co-infection with *Neisseria gonorrhoea* or *Treponema pallidum* is not uncommon and this complicates treatment (Stamm *et al*, 1984).

2.9.1. Genetics of resistance

C. trachomatis displays heterotypic resistance. This implies that some isolates within the chlamydial population may be susceptible, while others are resistant to the same antimicrobial agents (Somani *et al*, 2000). Chlamydia contain a unique phylogenetic and genetic lineage, with their genomes containing distinct areas with conserved regions (Sandoz and Rockey, 2010).

In most bacteria horizontal gene transfer is an important means to gain resistance genes as a survival mechanism (O'Neill *et al*, 2013). However, the metabolically active form of *C. trachomatis*, the RB, remains inside a susceptible host cell, therefore acquisition of new genes via horizontal gene transfer from other bacteria is unlikely (O'Neill *et al*, 2013). Instead resistance is usually acquired through point mutations and resistant strains are capable of sharing these genes via horizontal gene transfer between each other or homologous recombination, (Sandoz and Rockey, 2010).

Beta-lactam drugs are effective against *T. pallidum* and *N. gonorrhoeae*, however exposure of *C. trachomatis* to these antibiotics *in vitro* causes changes in the structure of the RB (Stamm, 2010). The RB stops cell division, but once the β-lactam drug is removed, cell division continues and EB are formed (Hogan *et al*, 2004). Some patients who produce negative culture results still contain

chlamydia RNA and DNA, which is evident of some type of resistance in chlamydia (Gerard *et al*, 2004). *C. trachomatis* causing secondary infections such as reactive arthritis and chronic prostatitis are known to consist of RBs which differ from the normal RBs (Gerard *et al*, 2009).

Emerging resistance to antimicrobial drugs is a major threat to public health (Somani *et al* 2000). No current standard is available for antimicrobial susceptibility testing of *C. trachomatis* in South Africa. The development and utilization of a test for drug susceptibility of *C. trachomatis* would indicate whether or not drug resistance is emerging in our setting and could pave the way for a change in the treatment modality if drug resistance is present.

CHAPTER 3 - METHODOLOGY

3.1. Study participants and specimen collection

Study participants were recruited from the Boom Street Clinic in Pietermaritzburg from May 2014 to October 2014 and from the Umlazi D clinic from October 2014 to December 2014. Specimens

were collected from females 18 years and older presenting with either vaginal discharge syndrome (VDS) or a genital ulcer and from males 18 years and older presenting with either urethritis syndrome or a genital ulcer. The study was approved by the Biomedical Research Ethics Committee of the University of KwaZulu Natal (BE220/13) as well as the KZN Department of Health and management at the clinics.

Specimens were collected by a study nurse. In females presenting with vaginal discharge syndrome, the cervix was visualised with the aid of a speculum, excess mucus was wiped off the cervical os and an endocervical specimen was collected using a Dacron swab. The swab was inserted for approximately 3 cm and withdrawn under gentle rotation to collect discharge and epithelial cells. In males presenting with urethritis syndrome, urethral specimens were collected. The urethral opening was cleaned using gauze and saline. A urethral Dacron swab on a flexible wire shaft was inserted approximately 3 cm into the urethra and withdrawn while rotating. Genital ulcer specimens were collected the same way for males and females. The ulcer was cleaned with a dry gauze to remove debris. A Dacron swab was rolled over the base and under the edges of the ulcer to collect exudate.

Two swabs were collected from each patient, one for *Chlamydia trachomatis* screening by PCR, and one for *C. trachomatis* culture. The swab that was used for culture was submerged in 1 ml chlamydia transport medium, placed on ice and transported to the laboratory for processing on the same day. The swab that was used for screening was placed in PBS and kept in the fridge at 2-8°C until processing.

3.2 Screening for Chlamydia trachomatis

Cervical and urethral specimens were screened for chlamydia on the day of collection using the BD ProbeTec™ ET *C. trachomatis* NA amplification assay (NAAT) processed on the BD Viper™ System. This system detects chlamydia positive reactions by using strand displacement amplification and fluorescent energy transfer. The L2 434 (ATCC® VR-902B™) reference strain was used as a positive control.

Ulcer specimens were screened for chlamydia using an in house strand displacement amplification method(Sturm *et al*, 2005). These specimens were batched and processed within 1 week. All ulcer specimens were cultured on the day of collection without waiting for PCR results.

3.3 Cell culture

HeLa cells, used at the start of the study, were grown in EMEM supplemented with 10% heat inactivated fetal bovine serum (FBS). Cells were incubated at 37°C in 5% $C0_2$ and grown until 90% confluency. The cells were then trypsinised and stored in freezing fluid at -80°C. When needed, cells were retrieved from the freezer and grown in EMEM supplemented with 10% FBS until 80-90% confluent after which they were trypsinised and split into multiple flasks to obtain a higher yield of cells sufficient for carrying out experiments. A total of 11 NAAT positive specimens were inoculated and incubated in the HeLa cells of which none grew. Since the NAAT positive clinical isolates failed to grow in the HeLa cell line, McCoy cells were used as an alternative to process and culture clinical isolates. McCoy cells were grown in the same manner as the HeLa cells.

3.4 Chlamydia trachomatis

3.4.1 Isolation of *Chlamydia trachomatis* from clinical specimens

In order to grow *C. trachomatis* from clinical specimens, HeLa and later McCoy cells were seeded into Trac vials containing glass coverslips. The seeding density was adjusted to ensure that there were Trac vials with 80 % confluent cells ready for infection on each day of specimen collection. Before inoculation, the cell culture media was removed and replaced with 0.5 ml chlamydia growth media (CGM). CGM consists of EMEM (BioWhittaker^{TM}) with Earle's Balanced Salt Solution (EBSS), nonessential amino acids (NEAA) and sodium pyruvate. CGM was further enriched to yield final concentrations of the following: 10 mM HEPES, 2 mM L-glutamine, amikacin (25 µg / ml), amphotericin B (5 µg / ml), gentamycin (5µg / ml), glucose (5.4 mg / ml) and 10% FBS.

Three sterile glass beads were added to the tubes containing chlamydia transport medium and the swab. This was vortexed for 2 minutes with the swab still in place to release the specimen from the Dacron. Sterile forceps were used to express fluid from the swab by pressing it against the side of the tube thereafter the swab was discarded.

Specimens were inoculated onto 80% confluent McCoy cell monolayers grown in Trak vials. Two Trak vials were inoculated with each specimen: 300 µl each. The Trak vials were then centrifuged at 2500 × g at 30°C for one hour and incubated at 37°C for 2 hours. After this incubation step, the media was removed and replaced with 2 ml fresh CGM and incubated at 37°C for 4 days. A negative control consisting of a Trak vial inoculated with 300 µl sterile chlamydia transport media

as well as a positive control consisting of a Trak vial inoculated with serovar L2 strain 434 (ATCC®VR-902B™) with MOI = 1 was processed with each batch.

After the 4 day incubation step, one Trak vial per specimen, as well as of the negative and positive controls were fixed and stained using the MicroTrak®*Chlamydia trachomatis* Culture Confirmation Test kit (Trinity Biotech) according to manufacturer's instructions (3.4.2). Slides were examined using a fluorescent microscope with a filter system for fluorescein isothiocyanate (FITC) to confirm the presence of chlamydial inclusions.

If a monolayer infected with a particular specimen was found to be positive for *C. trachomatis*, EBs were harvested from the second Trak vial seeded with that specimen. This was done by removing the CGM, adding 1 ml cold sucrose-phosphate-glutamate (SPG) buffer supplemented with 10% FBS and 5 sterile glass beads. This vial was shaken on a vortex mixer for 1 minute to lyse host cells. The resultant chlamydial suspension was collected and inoculated on additional McCoy cell monolayers. Each clinic isolate was sub cultured approximately 3 times until a sufficient titre was obtained for use in experiments. When this was achieved, EB were harvested into cold SPG buffer with 10% FBS, cellular debris was pelleted by centrifugation at 200 × g for 10 minutes at 4°C, and the supernatant containing the organism dispensed into 100µl aliquots which were frozen at -80°C until use.

3.4.2 Immunofluorescence

The Trinity Biotech MicroTrak® *Chlamydia trachomatis* Culture Confirmation test was used for detection of chlamydia. The principle of this test is the detection of the major outer membrane

protein (MOMP) which is present in all human serovars of *C. trachomatis*. The Microtrak® *C. trachomatis* reagent contains monoclonal antibodies against MOMP labelled with fluorescein isothiocyanate. Once this antibody conjugate binds to the MOMP antigen, *C. trachomatis* inclusion bodies become visible as apple-green fluorescing bodies against the red background of the cells counterstained with Evans Blue.

The test was performed according to the manufacturer's instructions. In brief, excess media was removed. Monolayers were fixed by addition of 1 ml 95% ethanol in distilled water for 10 minutes. Following this, the ethanol was removed and the monolayers were rinsed with PB Then 15 µl staining solution was added to each well. This was incubated at 37°C in a humidified chamber for 30 minutes. Excess stain was removed and the monolayers rinsed in distilled water with agitation for 10 seconds. The water was removed and the monolayers were allowed to dry.

3.4.3 Determining the infectious titre

One vial of EB suspension of each isolate was thawed and used to prepare10-fold serial dilutions in SPG buffer. These dilutions were used to infect 80-90% confluent McCoy cell monolayers grown in a 96-well microtiter plate. Media was removed from the wells, washed with PBS and replaced with 50 µl CGM and 50 µl of chlamydia suspension from each dilution series. This was done in triplicate for each dilution series of chlamydia. Sterile SPG buffer was used as the negative control. The plates were centrifuged at 2500 ×g for 60 minutes at 4°C and then incubated for 1 hour at 37°C. Following incubation, media was removed from each well and replaced with 100 µl CGM. This was incubated for 2 days at 37°C, after which the monolayers were fixed, stained

with the Microtrak® *C. trachomatis* culture confirmation kit reagents and viewed under a fluorescent microscope.

The number of inclusions per field of view was enumerated at 100 x magnification and the average number of inclusions per field of view was calculated. Three fields of view were counted for each well and three wells were used for the total calculation. The dilution at which the inclusions were counted was the dilution which resulted in 5 - 40 inclusions per field of view.

The infectious titre was then calculated using the following formula:

$$\text{Concentration (IFU/ ml)} = \frac{\text{inclusions}}{n} \times \frac{1'000\mu l}{V} \times C \times D$$

Where: n = number of fields counted

V = volume of inoculum (µl)

C = objective lens conversion factor

D = dilution factor

3.5. Susceptibility testing

The mimimum inhibitory concentration (MIC) of the chlamydia isolates was determined for each antimicrobial agent using two different methods. Method 1 utilized a microbroth dilution assay to

determine the MIC of *C. trachomatis* to five concentrations of eight antimicrobial agents. For method 2, the MIC was determined by means of quantitative rPCR detecting 16s rRNA. Both methods were performed three times in triplicate.

3.5.1Antimicrobial agents

The following antimicrobial agents were used:

- Tetracyclines: minocycline, tetracycline and doxycycline
- Glycycline: tigecycline
- Macrolides: azithromycin, clarithromycin and erythromycin
- Fluoroquinolone: ciprofloxacin

The range of concentrations of each antimicrobial agent tested was determined based on the published MICs for L2 strain 434 which was used as the susceptible control. We included the MIC plus two 2-fold dilutions above and two 2-fold dilutions below the published MIC. The concentrations of each drug used are listed in Table 3.

Table 3: Antibiotic concentrations used based on the known MIC for L2

Antibiotic	Concentration					References
Tetracycline	2	1	0.5*	0.25	0.125	Walsh *et al*, 1991
Minocycline	0.125	0.0625	0.03125*	0.0156	0.0078	Miyashita *et al*,1996
Doxycycline	0.125	0.0625	0.03125*	0.0156	0.0078	Miyashita *et al*, 1996
Tigecycline	0.5	0.25	0.125*	0.0625	0.03125	Townsend *et al*, 2011
Clarithromycin	0.125	0.0625	0.03125*	0.0156	0.0078	Miyashita *et a*l, 1996
Azithromycin	0.5	0.25	0.125*	0.0625	0.03125	Miyashita *et al*, 1996
Erythromycin	0.5	0.25	0.125*	0.0625	0.03125	Miyashita *et al*, 1996
Ciprofloxacin	4	2	1*	0.5	0.25	Miyashita *et al*, 1996

*** Published MIC value for L2 strain 434**

3.5.2 MIC determination by microbroth dilution assay with immunofluorescence

McCoy cells (8×10^5 cells) suspended in 100 µl cell culture medium without antibiotics were seeded into the wells of flat bottom 96-well tissue culture microtitre plates. These plates were incubated at 37°C for 48 hours to yield an 80% confluent monolayer.

The 80% confluent cells in three wells of each 96-well plate were trypsinized and a cell count was done to determine the number of cells per well. This was used to calculate the appropriate dilution

of the chlamydial isolates to produce inoculum with an MOI of 1. Before infection, cell culture medium was aspirated from the wells and replaced with 100 µl CGM without antibiotics. The 50 µl of the inoculum or the negative control (sterile SPG buffer) was added and the plates centrifuged at 2500 x g for 1 h and incubated at 37°C for 1 h. The antibiotic free media was removed and replaced with 150 µl fresh CGM containing appropriate concentrations of the antimicrobial agent to be tested (Table 1). The plates were incubated at 37°C for 2 days. After this time, the monolayers were fixed and stained using the Microtrak® *Chlamydia trachomatis* culture confirmation test as per manufacturer's instructions (3.4.2).

Stained monolayers were visualised at 100× magnification using a fluorescent microscope with a filter system for FITC (λ = 520 nm). The lowest concentration of antimicrobial agent which resulted in no visible chlamydia inclusions was taken as the MIC.

3.5.3MIC determination by detection of chlamydial 16srRNA

3.5.3.1 Cell culture

McCoy cells were seeded into the wells of 12-well tissue culture plates at a concentration of 8 x 10^5cells per well. After 24 hours of incubation at 37^{o}C , the cells in three wells were trypsinized and a cell count was done to calculate the dilution of the chlamydial isolates to add to each well in order to achieve a MOI of 1. Before inoculation, cell culture medium was aspirated from the wells and replaced with 1 ml CGM without antibiotics. Cells were infected in duplicate with *C. trachomatis* US151 suspended in 100 µl SPG. One hundred microlitres sterile SPG was used as the negative control.

The plates were centrifuged at 2500 × g for 1 h and incubated at 37°C for 1 h. Following this, the inoculum was removed and replaced with 1ml fresh CGM containing appropriate concentrations of each antimicrobial agent. These cultures were incubated at 37°C for 2 days.

3.5.3.2 RNA isolation

After the 48 hour incubation period the CGM with antibiotics was replaced with 2 ml 5M GTC lysis buffer per well. The buffer was pipetted up and down five times in order to lyse the cells. The cell lysate was transferred to a 15ml centrifuge tube and mixed using a vortex mixer for 2 minutes. This was followed by centrifugation at 3000 ×g for 30 mins. The supernatant was removed and the pellet was resuspended in 200µl of GTC lysis solution as mentioned above. This suspension was transferred to a micro-centrifuge tube and centrifuged at 15000 × g for 20 seconds. The supernatant was discarded and the pellet re-suspended in 200µl Trisure (Bioline) and transferred to another micro-centrifuge tube with silicon microbeads. This suspension was subjected to 4 cycles of the following: mix on a vortex mixer for 2 minutes; incubate on ice for 1 minute. The suspensions were stored at -70°C until RNA isolation could be completed.

To complete RNA isolation, the Trisure suspensions were retrieved from the freezer and mixed briefly on a vortex mixer. This was followed by centrifugation at 12,000 × g for 10 min at 4°C. The supernatant was transferred to a 2 ml micro centrifuge tube and incubated at room temperature for 10 min. Forty µl of cold chloroform (Sigma) was added and the tube was mixed by shaking vigorously by hand for 30seconds. The tube was then incubated at room temperature for 10 minutes and centrifuged at 12,000 × g for 15 min at 4°C. The upper aqueous phase containing

RNA was transferred to another 2 ml micro-centrifuge tube and 100 µl of cold isopropanol (Sigma) was added and mixed by inverting the tube three times. This mixture was incubated at room temperature for 15min and then centrifuged at 12,000 × g for 15 min at 4°C. The supernatant was carefully decanted without disturbing the RNA pellet, which was then washed by re-suspending the pellet in 200µl 75% ethanol (Merck) and centrifugation at 7500 × g for 5min at 4°C. The supernatant was again decanted and the pellet was air-dried for 10 minutes then re-suspended in 14 µl of RNA-secure (Sigma) by 10 cycles of aspiration and release using a pipette. This was incubated for 10 min at 60°C and placed on ice. A nanodrop spectrophotometer (Nanodrop 2000c Spectrometer – Thermofisher) was used to quantify RNA concentration and determine the purity. Isolated RNA was stored at -70°C until cDNA could be synthesised.

3.5.3.3 cDNA Synthesis

cDNA synthesis was carried out using the ABI high capacity cDNA synthesis kit according to manufacturer's instructions. Individual PCR tubes were used, to which 10µl of 2 × RT mix was added together with 2000 ng of RNA which was then suspended in 14µl RNA-secure. Tubes were sealed and briefly centrifuged to spin down the contents and eliminate air bubbles. The tubes were then loaded into the thermal cycler (Gene amp 9700 Life Technologies) and subjected to the following cycle conditions:

Step 1: 25°C for 10 min

Step 2: 37°C for 120min

Step 3: 85°C for 5sec

Step 4: 4°C for ∞

Tubes were stored at 2-6°C.

3.5.3.4 Real Time PCR

Real time PCR was carried out using the Taqman gene expression master mix. This was made up according to Goldschmidt *et al*, 2006. The following primers and probes were used:

Forward primer: (5′TCGAGAATCTTTCGCAATCGAC)

Reverse primer: (5′CGCCCTTTACGCCCAATAAA)

Probe: (FAM-AAGTCTGACGAAGCGACGCCGC)

A total of 22.5µl of the mastermix consisting of AmpliTaq Gold® DNA Polymerase (Ultra Pure), Uracil-DNA glycosylase, dNTPs (with dUTP), ROX™ Passive Reference, and optimized buffer components was pipetted into each well, followed by the addition of 2.5µl cDNA (diluted sample) or 2.5µl nuclease free water for the template free control. Plates were centrifuged at 250 × rpm for 20 minutes at 25°C before loaded them into the real time machine (Applied Biosystems 7500 Real time PCR system) and subjected to the following cycle conditions:

2 minutes at 50°C

10 minutes at 95°C

50 two-step cycles of 10 sec at 95°C and 65 sec at 60°C.

Interpretation of PCR results were achieved using the Comparitive C_T Method. This method employs the use of arithmetic formulas to achieve the result for relative quantitation.

CHAPTER 4 – RESULTS

Specimens were collected from 967 patients that attended one of the two clinics (3.1). Of these, 622 (64.3%) were seen at the Boom Street Clinic in Pietermaritzburg and 345 (35.7%) at the Umlazi D Clinic in Durban. Male urethritis syndrome was diagnosed in 531 (54.9%) patients and 411 (42.5%) had female discharge syndrome. All specimens were screened for *C. trachomatis* using the BD ProbeTec^{TM} ET CT/GC DNA Amplification Assay and *C. trachomatis* DNA was detected in 65 (6.7%) specimens. These 65 specimens were cultured, but only four (6%) grew. These four *C. trachomatis* isolates, designated SAR28, SAR90, SAR175 and SAR286, were sub-cultured until a sufficient titre had been reached for use in susceptibility tests. The number of subcultures to achieve this varied between 3 and 5. All four were collected from patients who presented at the Boom Street Clinic in Pietermaritzburg. Three of these patients were female and one was male.

4.1 Minimum Inhibitory Concentration (MIC) determination by immunofluorescence

MICs were determined for total of 11 *C. trachomatis* isolates using clinically relevant antimicrobial agents. Clinical isolates 28, 90, 175 and 286 were obtained from male and female participants presenting at the Pietermaritzburg Boom Street Clinic. These strains were isolated during the collection period of May 2014 – October 2014.

Clinical isolate E was also used which was isolated in 1996 by Maleka and coworkers. This strain belongs to the OG biovar and was obtained from a male patient presenting with urethritis at Prince Cyril Zulu Communicable Diseases Clinic in Durban.

Three additional chlamydia isolates, US151, US162 and US197 were also used in this study. These isolates were grown by Joubert BC, 2009 from ulcer specimens retrieved from patients at the Prince Cyril Zulu Communicable Diseases Clinic in Durban. All three were L2 serovars.

The remaining three organisms used were LGV strains from the ATCC collection. These were L1 440 (ATCC® VR-901B®), L2 434 (ATCC® VR-902B™) and L3 404 (ATCC® VR-903B™). These LGV strains were isolated by Schachter *et al*, 1969 from military servicemen and seamen who presented with typical LGV symptoms. L2 434 (ATCC® VR-902B™) served as the reference strain for MIC determination because its MIC values have been published by others (Miyashita *et al*, 1996, Walsh *et al*, 1991 and Townsend *et al*, 2011)

Table 4 summarises the results. All 11 *C. trachomatis* isolates were inhibited by all the antimicrobial agents tested. For tetracycline, minocycline and erythromycin the MIC was the same for all isolates tested. These were ≤ 0.125 mg/L, 0.125 mg/L and 0.125 mg/L respectively. There was little variation in the MICs of the other antimicrobial agents. For doxycycline all isolates had an MIC of 0.0625 mg/L with the exception of SAR175 which had an MIC of 0.03125 mg/L. For clarithromycin all isolates had an MIC of ≤ 0.0078 mg/L except L2 strain 434 and SAR 90 which both had an MIC of 0.0156 mg/L. For azithromycin all isolates had an MIC of 0.25 mg/L except US197, SAR175 and the serovar E clinical isolate. These were 0.125, 0.125 and 0.5 mg/L respectively. For ciprofloxacin all isolates had an MIC of 1 mg/L except US197 which had an MIC of 0.5 mg/L. The most variation in MICs occurred with tigecycline. The three LGV reference

strains, the serovar E clinical isolate, and SAR175 all had an MIC ≤ 0.03125 mg/L. US151, US162, SAR28 and SAR90 had an MIC of 0.0625 mg/L, while US197 and SAR286 had an MIC of 0.125 mg/L.

Table 4: Minimum Inhibitory Concentrations (MIC) of 11 *Chlamydia trachomatis* isolates

	Tetracyclines			Glycycline	Macrolides			Fluoroquin-olone
Isolate	**Tet**	**Min**	**Dox**	**Tige**	**Clarith**	**Azith**	**Eryth**	**Cipro**
SAR28	≤ 0.125	0.125	0.0625	0.0625	≤ 0.0078	0.25	0.125	1
SAR90	≤ 0.125	0.125	0.0625	0.0625	0.0156	0.25	0.125	1
SAR175	≤ 0.125	0.125	0.03125	≤0.03125	≤ 0.0078	0.125	0.125	1
SAR286	≤ 0.125	0.125	0.0625	0.125	≤ 0.0078	0.25	0.125	1
E	≤ 0.125	0.125	0.0625	≤0.03125	≤ 0.0078	0.5	0.125	1
US151	≤ 0.125	0.125	0.0625	0.0625	≤ 0.0078	0.25	0.125	1
US162	≤ 0.125	0.125	0.0625	0.0625	≤ 0.0078	0.25	0.125	1
US197	≤ 0.125	0.125	0.0625	0.125	≤ 0.0078	0.125	0.125	0.5
L1 440	≤ 0.125	0.125	0.0625	≤0.03125	≤ 0.0078	0.25	0.125	1
L2 434	≤ 0.125	0.125	0.0625	≤0.03125	0.0156	0.25	0.125	1
L3 404	≤ 0.125	0.125	0.0625	≤0.03125	≤ 0.0078	0.25	0.125	1

Tet, tetracycline; Min, minocycline; Dox, doxycycline; Tige, tigecycline; Clarith, clarithromycin; Azith, azithromycin; Eryth, erythromycin; Cipro, ciprofloxacin

The highest magnification at which the stained monolayers could be read with the fluorescent microscope at the bottom of the wells in the 96-well plates was 200 ×. This was because we stained and visualised the monolayer directly in the 96-well plates and the hight of the plates left no space for the objective lenses with higher magnification. To confirm whether or not the antimicrobial agents used had merely slowed the growth of the organism resulting in the formation of tiny inclusions which could not be visualised at a low magnification, we selected one isolate (US151) and repeated each susceptibility test on McCoy cell monolayers grown on glass coverslips inserted in a 24-well plate. The cell monolayers were stained directly in the wells, thereafter the glass cover slips were removed and mounted onto glass slides. This enabled the cell monolayers to be viewed at 1000 × magnification for visualisation of tiny inclusions which may not have been seen at the lower magnification.

Figures 1 A-D compare the images captured at 200 × magnification with those captured at 1000 × magnification for US151 when exposed to each antimicrobial agent. When comparing these images, no inclusions were detected at any of the two magnifications for any of the antimicrobial agents. Because viewing the cells under a higher magnification yielded the same results as the lower magnification, visualisation and interpretation using 200 × magnification is deemed adequate.

Figure 1A-D (pages 39 to 42overleaf). Fluorescent micrographs of McCoy cell monolayers. Cells were infected with *Chlamydia trachomatis* isolate number US 151 by centrifugation, treated with different concentrations of clinically relevant antimicrobial agents and stained with the MicroTrak *C. trachomatis* culture confirmation kit for the detection of green chlamydial inclusions which indicate growth of the organism. Images were captured at 200 × and 1000 × magnification. Antimicrobial agents included (A) tetracyclines (tetracycline, minocycline, doxycycline), (B) a glycycline (tigecycline), (C) a fluoroquinolone (ciprofloxacin) and (D) macrolides (erythromycin, clarithromycin, azithromycin).

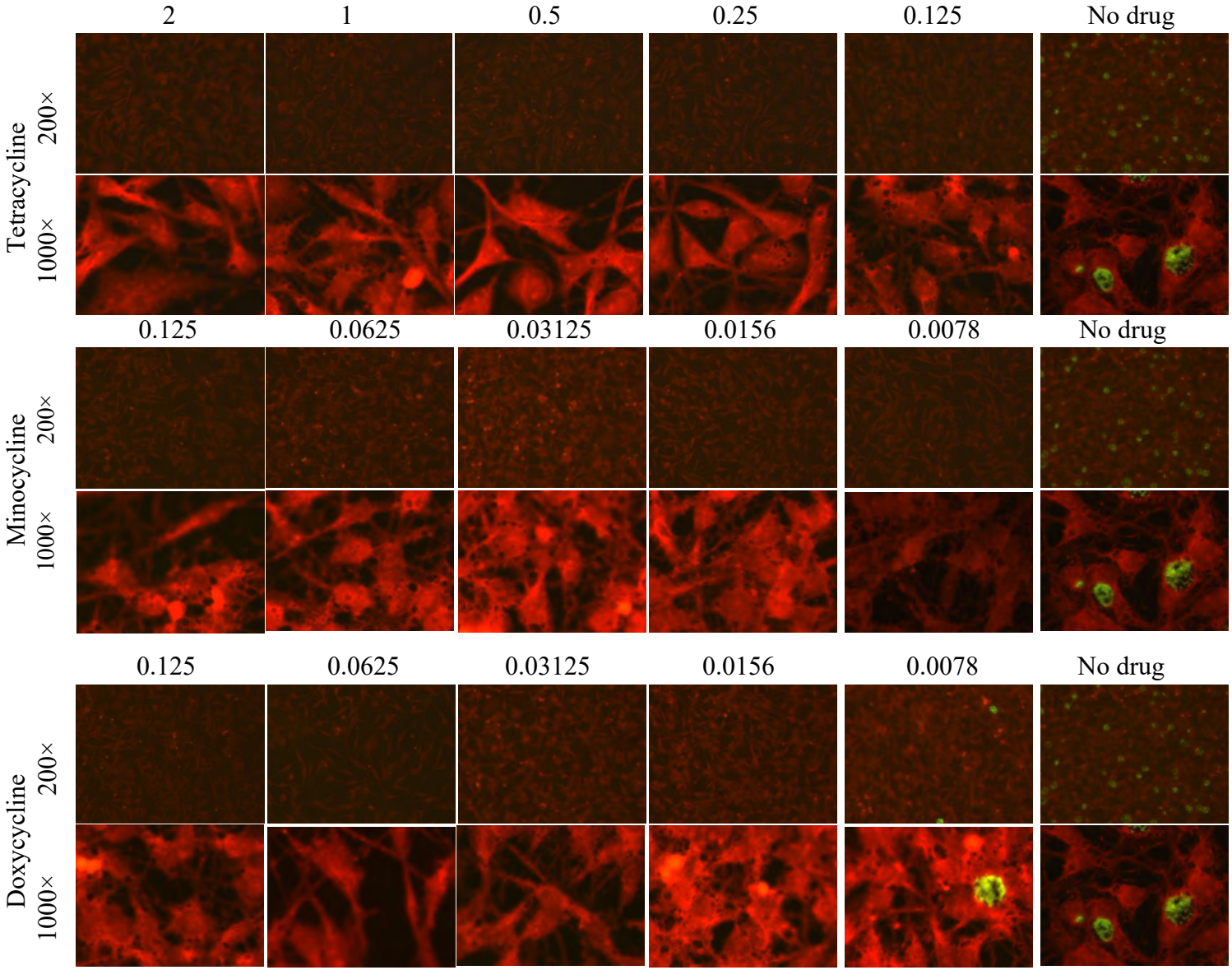

2
1
0.5
0.25
0.125
No drug
Tetracycline
200×
1000×
0.125
0.0625
0.03125
0.0156
0.0078
No drug
Minocycline
200×
1000×
0.125
0.0625
0.03125
0.0156
0.0078
No drug
Doxycycline
200×
1000×

Figure 1A

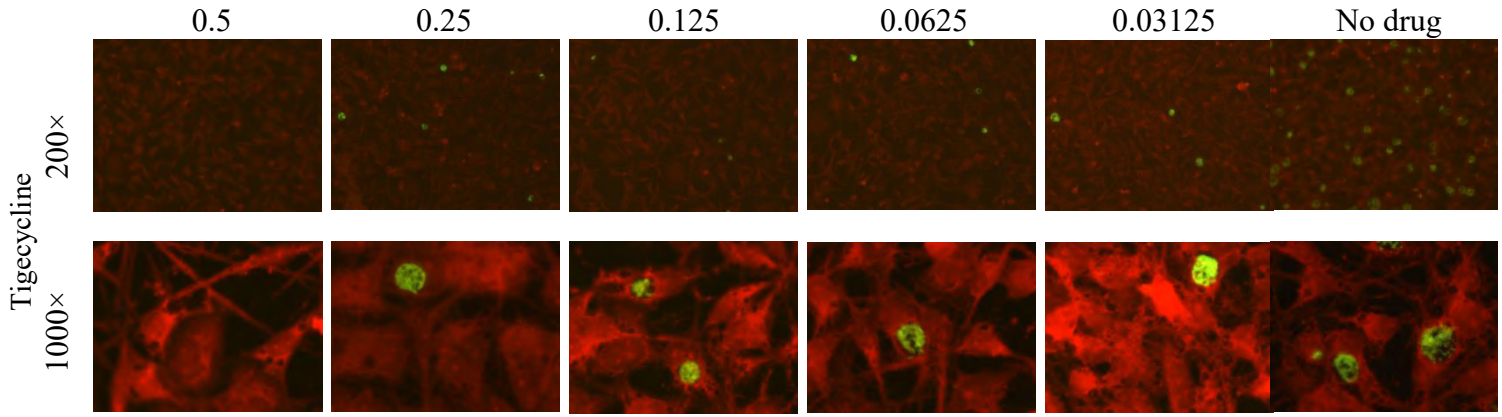


Figure 1B

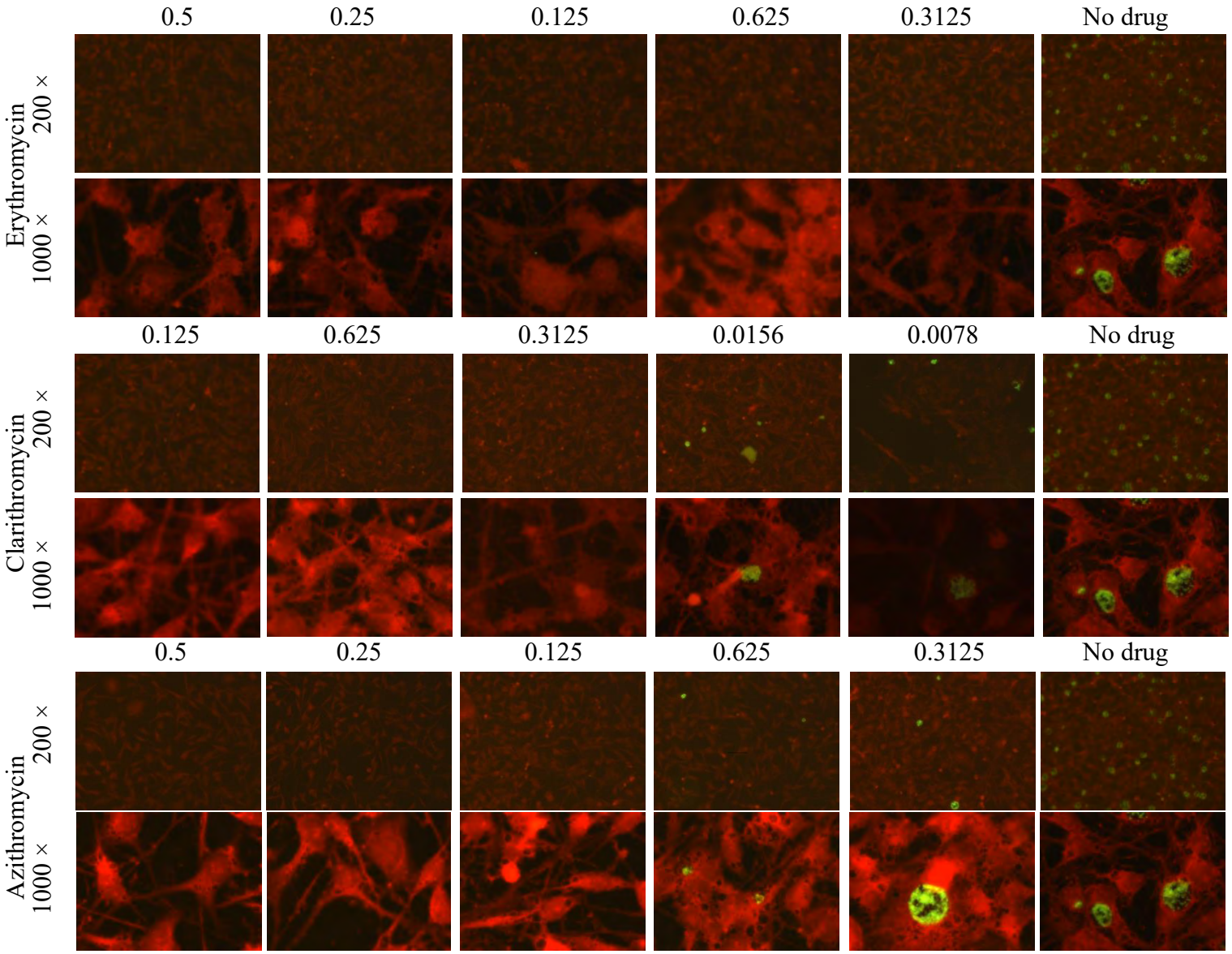

0.5
0.25
0.125
0.625
0.3125
No drug
Erythromycin
200 ×
1000 ×
0.125
0.625
0.3125
0.0156
0.0078
No drug
Clarithromycin
200 ×
1000 ×
0.5
0.25
0.125
0.625
0.3125
No drug
Azithromycin
200 ×
1000 ×

Figure 1C

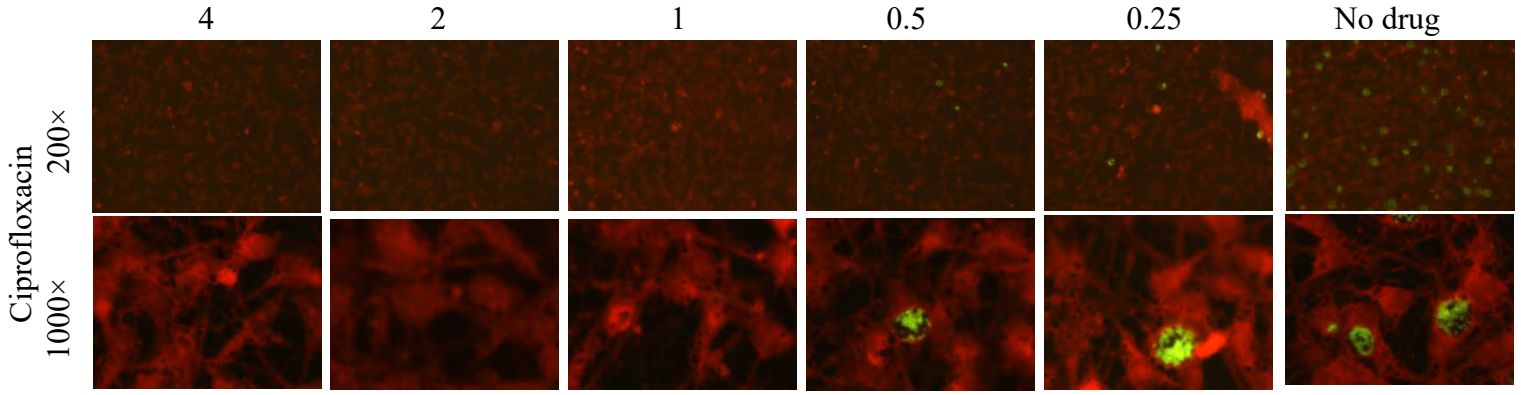


Figure 1D

4.2 MIC determination by real time PCR

The absence of resistance in the IF experiments informed the decision to test only one isolate by real time PCR to develop and assess the methodology and to show proof of principle.

A total of two runs were completed and the results summarized in Table 5. "Detected" (D) represents more than three wells with a positive reaction by real time PCR. "Not detected" (ND) represents less than 3 positive wells by real time PCR. "Undetermined" (U) represents samples which produced 3 positive and 3 negative wells by real time PCR. The minimum inhibitory concentration was taken as the lowest concentration of an antimicrobial agent with the result "ND".

For erythromycin, clarithromycin, azithromycin, ciprofloxacin and tigecycline the MIC was 0.125, 0.0156, 0.125, 1 and 0.5 mg/L respectively. For minocycline chlamydial cDNA was detected after 48 hour of exposure to all concentrations of the antimicrobial agent used in this study. For tetracycline and doxycycline chlamydial cDNA was not detected after 48 hours exposure to any concentration of the antimicrobial agent used.

Table 5: Detection of *C. trachomatis* cDNA (isolate US151) using real time PCR following culture of the organism in the presence of antimicrobial agents

	Concentration of antimicrobial (mg/L)				
Tetracyclines	Tetracycline				
	2	1	0.5	0.25	0.125
	ND	ND	ND	ND	ND
	Minocycline				
	0.125	0.0625	0.03125	0.0156	0.0078
	D	D	D	D	D
	Doxycycline				
	0.125	0.0625	0.031	0.0126	0.0078
	ND	ND	ND	ND	ND
Glycycline	Tigecycline				
	0.5	0.25	0.125	0.0625	0.03125
	ND	D	D	D	D
Macrolides	Clarithromycin				
	0.125	0.0625	0.03125	0.0156	0.0078
	ND	ND	U	ND	U
	Azithromycin				
	0.5	0.25	0.125	0.062	0.03125
	ND	ND	ND	U	U
	Erythromycin				
	0.5	0.25	0.125	0.0625	0.031
	ND	ND	ND	U	U
Fluoroquinolon	Ciprofloxacin				
	4	2	1	0.5	0.25
	ND	ND	ND	U	D

ND, not detected; D, detected; U, undetermined

MICs were determined by PCR twice in triplicate. After these two runs it was evident that the MIC determination by immunofluorescence was more reproducible than that by real time PCR so the third planned run was not performed. The MICs of US151 as determined by immunofluorescence are compared with those determined by real time PCR in Table 6.

The real time PCR results show different MICs for all of the antimicrobials used when compared to immunofluorescence. The discrepancies were no more than two 2-fold dilutions different for six of the antimicrobial agents. The exceptions are doxycycline and tigecycline. The MIC for doxycycline against this isolate was at least 4-fold lower when determined by real time PCR. The

MIC for tigecycline was 3-fold higher when determine by real time PCR instead of immunofluorescence.

Table 6. Minimum Inhibitory Concentrations (MIC) of *Chlamydia trachomatis* isolate number US151 as determine by immunofluorescence and real time PCR

	Tetracyclines			Macrolides			Fluoro-quinolone	Glycy-cline
Method	**Tet**	**Min**	**Dox**	**Eryth**	**Clarith**	**Azith**	**Cipro**	**Tige**
IF	0.125	0.125	0.0625	0.125	≤0.0078	0.25	1	0.0625
PCR	≤0.125	>0.125	≤0.0078	0.0625	0.03125	0.0625	0.5	0.5

Tet, tetracycline; Min, minocycline; Dox, doxycycline; Eryth, erythromycin; Clarith, clarithromycin; Azith, azithromycin; Cipro, ciprofloxacin; Tige, tigecycline; IF, immunofluorescence; PCR, real time polymerase chain reaction

CHAPTER 5– DISCUSSION

Resistance to anti-microbial drugs has become a major problem worldwide and affects pathogens that cause infections in all parts of the human body. The organisms that cause sexually transmitted infections (STIs) are by nature fastidious and therefore, drug resistance profiles are only available for those bacterial species that can be grown in cell-free culture media. This includes *Neisseria gonorrhoeae* and *Haemophilus ducreyi*. Information on drug resistance in organisms that cannot readily be grown is scanty at most. These include *Treponema pallidum*, *Klebsiella granulomatis* and *Chlamydia trachomatis*.

The development of bacterial resistance to antimicrobial agents included in the syndromic management regime is a major concern for the treatment of STDs (Mundayet al, 1995). If a particular pathogen in a given population develops resistance to the antimicrobial agent which covers that pathogen in the syndromic management regime, then that antimicrobial agent needs to be substituted for another suitable antimicrobial agent. The antimicrobial agent being used must cover at least 95% of isolates of that species in the geographical area concerned (WHO, 2003). Therefore periodic susceptibility testing of the pathogens which cause STDs is required in order for syndromic management to be effective. The aim of this study was to determine if *C. trachomatis* isolates from patients in KwaZulu-Natal province of South Africa have developed resistance to the drugs which cover this organism in the syndromic management regime, and if so, to determine which antimicrobial agent would be a suitable alternative. The antimicrobial agents included in this study were three tetracyclines, the closely related tigecycline, three macrolides and one fluoro-quinolone.

Specimens were collected from two different clinics in two major cities in the province, the Boom Street Clinic in Pietermaritzburg and the Umlazi D Clinic in Durban. In an initial trial run, the HeLa cell line was used, but no organisms were isolated using this cell line. This may have been due to shorter incubation times used. We then substituted this for the McCoy cell line and increased the incubation time from 48 to 96 hours. Using this approach, from the 967 specimens collected across both sites, 65 were positive in the *C. trachomatis* NAAT but only 4 isolates could be cultured. Since this is a low success rate, these cultured strains are not very representative. Various factors such as pH, temperature and polarity of the cell types can influence growth of an organism (Suchland *et al*, 2003). McCoy cells are known to produce a higher number of chlamydial inclusions at temperatures of 33-35ºC as compared to lower temperatures of 20-25 ºC during centrifugation (Rota, 1980).

The susceptibility tests were performed with local 8 clinical isolates and 3 strains from the ATCC collection. One of the latter (L2 434 ATCC® VR-902B™) was used as control since MICs for this strain have been published (Miyashita *et al*, 1997 and Walsh *et al*, 1987). The other two LGV serovars used were L1 strain 440 (ATCC® VR-901B® and L3 strain 404 (ATCC® VR-903B™). These LGV strains were isolated by Schachter *et al*, 1969 from military servicemen and seamen who presented with typical LGV symptoms. None of the current isolates nor the LGV clinical isolates or the discharge isolate from 1996 were resistant to any of the antibiotics tested. All MICs were below the breakpoints for resistance independent of whether the EUCAST or CLSI reference values for other tissue infections were applied.

Due to its obligate intracellular lifecycle, susceptibility testing of *C. trachomatis* is technically challenging and there is no single standardized technique. All methods described to date involve culture of the organism in an appropriate cell line with exposure to the antimicrobial agent of

interest at a suitable concentration, followed by a method to determine whether or not the bacterium multiplied in the presence of the antimicrobial agent. This could include immunofluorescence or a quantitative nucleic acid amplification test, however NAATs are required for screening and diagnosis rather than susceptibility testing (Cross *et al*, 1999). Immunofluorescence is an antibody detection based assay and there are two types namely, direct and indirect immunofluorescence (Magro *et al*, 2003). Direct immunofluorescence involves the use of an antibody conjugated with a fluorochrome and directed against the antigen. Indirect immunofluorescence involves the use of an additional antibody conjugated with a fluorochrome which binds to the Fc portion of the first antibody (Magro *et al*, 2003). Immunofluorescence based detection methods detect the presence of chlamydia inclusion bodies by visualizing these on stained slides with a fluorescent microscope (Stamm, 2000).

This study used a commercially available direct immunofluorescence kit in the 96 well plates used and the presence or absence of inclusions was used as an indicator for growth of the organism. To show proof of principle, real time PCR was applied on one isolate. We did not perform this technique on the remaining 10 organisms because of cost. Real time reversed transcriptase PCR was used since it detects RNA of viable organisms only.

Immunoflourescent experiments have shown a decrease in the size and number of chlamydial inclusions with increasing concentration of antimicrobial agent. For some strains of chlamydia, the lowest concentration of antimicrobial agents used was effective in preventing growth of the organism. This is true for tetracycline, clarithromycin and tigecycline. Tetracycline-HCl and

minocycline shared the same MIC for all chlamydia strains, whereas doxycycline MIC (0.0625 and 0.03125) was one dilution lower than tetracycline and minocycline.

The MICs for macrolides were all different. Clarithromycin has the lowest MICs whereas the MICs for azithromycin and erythromycin are slightly higher for all chlamdia isolates but in the susceptible range. The MIC for all strains exposed to ciprofloxacin was the same except for one ulcer sample US197 (0.5), therefore this antimicrobial agent has the same effect on all the chlamydia isolates tested since the MIC of US197 was lower than the others. Ciprofloxacin has the highest MIC values as compared to all other antibiotics used. The use of tigecycyline showed a variety of MIC values. The historical LGV isolates have the lowest MIC for tigecycline, while the local ulcer samples and discharge isolates have a higher MIC. Tigecycline has the widest range of MIC values when compared to all other antibiotics tested.

When comparing the reference strain L2 434 to the MIC values obtained, the values do not differ significantly and are either a dilution series higher or below the published MICs. There are not enough published data on MICs for the different serovars of chlamydia. Data was available for serovar E, and when comparing the published data to the values obtained, azithromycin has a twofold higher MIC and a threefold higher MIC with regards to Minocycline as compared to the published MIC. Other MICs published for serovar E show no significant difference from the data obtained in this study.

The additional immunoflourescence experiment which was visualized at 1000 × magnification (US151 only) (Figure 1A-D) confirmed that the MIC values determined at 200 × magnification were correct. This experiment was performed in the same manner as the other immunofluorescence experiments with the only differences being that it was scaled up to utilize a

24 well plate instead of a 96 well plate, the cells were grown on glass coverslips which were stained in the same manner as the 96 well plate experiment but the glass slides were removed from the 24 well plate, mounted on glass microscope slides and viewed under 1000 × magnification instead of 200 × magnification. The reason for this confirmation experiment was to rule out the possibility of the antimicrobial agents slowing the growth of *C. trachomatis* to a point where inclusions were still present, but too small to be identified at 200 × magnification. Since the MIC values for US151 were the same in both the immunofluorescence susceptibility test visualized at high magnification and that visualized at low magnification. The MIC values obtained at low magnification for the other isolates were taken to be correct.

MICs of the seven antimicrobial agents were also determined for one clinical isolate (US151) using real time PCR. For this assay RNA was extracted, converted to cDNA and detected using a real time PCR assay. However, in our hands the real time PCR was not well reproducible between the two duplicate runs and there were numerous discrepancies for the values obtained with immunofluorescence. For all seven of the antimicrobial agents tested, the MIC value was different for the two methods. In the case of minocycline, clarithromycin and tigecycline the MICs were lower when determined by immunofluorescence than real time PCR, but with all other antimicrobial agents the MIC was higher when determined by immunofluorescence. However, the difference between the MIC values determined with either method only exceed two dilution factors for two antimicrobial agents: doxycycline and tigecycline. The MIC for doxycycline against US151 was more than three-fold higher when determined by immunofluorescence instead of real time PCR, but the MIC for tigecycline was three-fold lower when determined by immunofluorescence instead of real time PCR. Immunoflourescence is a rather subjective assay and has no standardized method for reading results. It is difficult for defining the MIC due the

difficulty in reading the end point. The organism may be present as elementary bodies which are difficult to see under the microscope, however real time PCR is capable of detecting these organisms via RNA detection.

In a previous study Cross *et al* (1999) performed susceptibility tests on *C. trachomatis* and compared the results obtained using immunofluorescence with PCR-based methods (Cross *et al*, 1999). These authors utilized *16 C. trachomatis* isolates, exposed to 8 antimicrobial agents in triplicate. They reported a discrepancy in the MIC values obtained by immunofluorescence versus those obtained by PCR, but the MIC values determined using PCR were consistently higher than those obtained using immunofluorescence ranging from 1.6-fold higher to ≥195-fold higher.

In our hands immunofluorescence was more reproducible than real time PCR and clear MIC values were determined using this method. MIC determination using real time PCR was planned to be performed in triplicate. Due to significant inter-test and intra-test variation, only two runs were performed. A possible reason for the poor reproducibility may be the low copy number of chlamydial RNA as a result of the fragile RNA being easily degraded. When isolating RNA, both host RNA and chlamydial RNA were isolated simultaneously due to the obligate intracellular nature of the bacterium. The starting concentration of RNA was determined using a Nanodrop spectrophotometer and a standardized amount was used for the cDNA conversion, however, this amount would have included both host and bacterial RNA. Although a *C. trachomatis* specific RNA sequence was used for detection in the PCR-based assay, we are uncertain how much chlamydia RNA was used for the cDNA conversion and this would have likely varied depending on the growth rate of the bacteria under the different conditions.

A limitation of this study is the small sample size. Only four fresh clinical isolates were isolated as part of this study. Four stored clinical isolates which were collected as part of previous studies in KZN plus three commercially available ATCC strains were included to increase the sample size. If more clinical isolates had been cultured, we would have had a greater chance of detecting resistance to any of the antimicrobial agents included if there were resistant organisms circulating in the population. The collection and processing of clinical specimens is a labour intensive and time consuming process with a low culture positivity rate in our hands making it financially not feasible to increase the duration of specimen collection to increase the sample size.

In conclusion all the *C. trachomatis* isolates and historical ATCC strains tested in this study are sensitive to all the antimicrobial agents used, including azithromycin which is currently included in the syndromic management regime for the treatment of *C. trachomatis*. The most effective antimicrobial with the lowest MIC value is clarithromycin, followed by tigecycline which has the second lowest MIC. The tetracycline group of antibiotics also have MIC values lower than azithromycin and can be considered as a treatment options as well.

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APPENDICES

APPENDIX A – Antimicrobial agents and Media Preparation

Antimicrobial Agent Preparation:

Antimicrobial agents were prepared according to table A1 below. These were stored in 1ml aliquots until use. When needed, a vial of stock solution was used and 1:2 serial dilutions were prepared in CGM to acquire the desired concentration needed for each antimicrobial agent according to published MICs for reference strain L2 434 (Table 1).

Table A1: Preparation of drug stock

Antimicrobial Agent	Solvent	HPLC (%)	Amount (g)	Volume (ml)	Conc (mg/L)	Storage Temp (°C)
Tetracycline	95% ethanol	98	0.0103	10	1	-20
Minocycline	dH_20	100	0.01	10	1	-20
Doxycycline	dH_20	98	0.0103	10	1	-20
Tigecycline	DMSO	98	0.0103	10	1	-20
Azithromycin	DMSO	95	0.01075	10	1	-20
Clarithromycin	DMSO	98	0.0103	10	1	-20
Erythromycin	dH_20	98	0.0205	10	2	-20
Ciprofloxacin	Acetic Acid	98	0.0103	10	1	-20

Media Preparation

CGM for infection:

102 ml EMEM with NEAA

12 ml FBS

6 ml 20 × glucose

1.2 ml Hepes

12 µl cyclohexamide

Cyclohexamide

Dissolved 0.0196 g cyclohexamide (98%) in 19.2 ml distilled water.

Stored in 1 ml aliquots at -20°C.

Chlamydia Transport Medium

50ml RPMI

5ml FBS

0.5ml HEPES

50µl vancomycin

50 µl gentamycin

50 µl amphotericin B

20 × glucose

Dissolved 5.28g glucose (anhydrous) in 60 ml EMEM

Filter sterilized, and stored in 20 ml aliquots at 2-8°C.

Freezing fluid:

60 ml EMEM

20 ml FBS

20 ml DMSO

Filter sterilized solution. Stored in 10 ml aliquots at -20°C.

GTC Lysing Solution (100ml):

60g 5M guanidinum thiocyanate

0.5g 0.5% sodium-N-lauryl sarcosine

1g 26mM tri-sodium citrate

0.7ml 0.1M 2-mercaptoethanol

Sucrose Phosphate Glutamate buffer (SPG)

30g glucose

0.2075g KH_2PO_4

0.488g Na_2HPO_4

0.369g $C_5H_8NO_4Na.H_2O$

Dissolved the above reagents in 400 ml autoclaved distilled water

Adjusted pH using 4M NaOH (7.4-7.6)

Brought volume up to 500 ml using autoclaved distilled water

Filter sterilized through 0.22 µm filter

Stored in 20 ml aliquots at -20°C

APPENDIX B – Raw data tables

Immunofluorescence

Tables B1-9 below show results from immunoflourescence experiments. Results are shown for each Chlamydia isolate exposed to the different antimicrobials tested.

Real Time PCR

Results from PCR experiments can be seen in tables B10-13 below. The tables represent Chlamydia isolate US151 which was exposed to the antimicrobial agents for PCR experiments. Both PCR runs are recorded on these tables.

Table B1: Summary of MIC for all antimicrobials tested

	Azith	Eryth	Clarith	Tet	Doxy	Mino	Cipro	Tige
E	0.25	0.125	MIC ≤conc used	MIC≤conc used	0.0625	0.125	1	0.03125
L1	0.25	0.125	MIC ≤conc used	MIC≤conc used	0.0625	0.125	1	0.03125
L2	0.25	0.125	0.0625	MIC≤conc used	0.0625	0.125	1	0.03125
L3	0.25	0.125	MIC≤conc used	MIC≤conc used	0.0625	0.125	1	0.03125
US151	0.25	0.125	MIC≤conc used	MIC≤conc used	0.0625	0.125	1	0.0625
US162	0.25	0.125	MIC≤conc used	MIC≤conc used	0.0625	0.125	1	0.0625
US197	0.125	0.125	MIC≤conc used	MIC≤conc used	0.0625	0.125	0.5	0.125
28	0.25	0.125	MIC≤conc used	MIC≤conc used	0.0625	0.125	1	0.0625
90	0.25	0.125	0.0625	MIC≤conc used	0.0625	0.125	1	0.0625
175	0.125	0.125	MIC≤conc used	MIC≤conc used	0.03125	0.125	1	MIC≤conc used
286	0.25	0.125	MIC≤conc used	MIC≤conc used	0.0625	0.125	1	0.125

Table B2: Tetracycline susceptibility

	2	1	0.5	0.25	0.125	No Antibiotic
E	No Growth	No Growth	No Growth	No Growth	No Growth	Growth
L1	No Growth	No Growth	No Growth	No Growth	No Growth	Growth
L2	No Growth	No Growth	No Growth	No Growth	No Growth	Growth
L3	No Growth	No Growth	No Growth	No Growth	No Growth	Growth
US151	No Growth	No Growth	No Growth	No Growth	No Growth	Growth
US162	No Growth	No Growth	No Growth	No Growth	No Growth	Growth
US197	No Growth	No Growth	No Growth	No Growth	No Growth	Growth
28	No Growth	No Growth	No Growth	No Growth	No Growth	Growth
90	No Growth	No Growth	No Growth	No Growth	No Growth	Growth
175	No Growth	No Growth	No Growth	No Growth	No Growth	Growth
286	No Growth	No Growth	No Growth	No Growth	No Growth	Growth

Table B3: Minocycline Susceptibility

	0.125	0.0625	0.03125	0.0156	0.0078	No Antibiotic
E	No Growth	Growth	Growth	Growth	Growth	Growth
L1	No Growth	Growth	Growth	Growth	Growth	Growth
L2	No Growth	Growth	Growth	Growth	Growth	Growth
L3	No Growth	Growth	Growth	Growth	Growth	Growth
US151	No Growth	Growth	Growth	Growth	Growth	Growth
US162	No Growth	Growth	Growth	Growth	Growth	Growth
US197	No Growth	Growth	Growth	Growth	Growth	Growth
28	No Growth	Growth	Growth	Growth	Growth	Growth
90	No Growth	Growth	Growth	Growth	Growth	Growth
175	No Growth	Growth	Growth	Growth	Growth	Growth
286	No Growth	Growth	Growth	Growth	Growth	Growth

Table B4: Doxycycline Susceptibility

	0.125	0.0625	0.03125	0.0156	0.0078	No Antibiotic
E	No Growth	No Growth	Growth	Growth	Growth	Growth
L1	No Growth	No Growth	Growth	Growth	Growth	Growth
L2	No Growth	No Growth	Growth	Growth	Growth	Growth
L3	No Growth	No Growth	Growth	Growth	Growth	Growth
US151	No Growth	No Growth	Growth	Growth	Growth	Growth
US162	No Growth	No Growth	Growth	Growth	Growth	Growth
US197	No Growth	No Growth	Growth	Growth	Growth	Growth
28	No Growth	No Growth	Growth	Growth	Growth	Growth
90	No Growth	No Growth	Growth	Growth	Growth	Growth
175	No Growth	No Growth	No Growth	Growth	Growth	Growth
286	No Growth	No Growth	Growth	Growth	Growth	Growth

Table B5: Tigecycline Susceptibility

	0.5	0.25	0.125	0.0625	0.03125	No Antibiotic
E	No Growth	No Growth	No Growth	No Growth	No Growth	Growth
L1	No Growth	No Growth	No Growth	No Growth	No Growth	Growth
L2	No Growth	No Growth	No Growth	No Growth	No Growth	Growth
L3	No Growth	No Growth	No Growth	No Growth	No Growth	Growth
US151	No Growth	No Growth	No Growth	No Growth	Growth	Growth
US162	No Growth	No Growth	No Growth	No Growth	Growth	Growth
US197	No Growth	No Growth	No Growth	Growth	Growth	Growth
28	No Growth	No Growth	No Growth	No Growth	Growth	Growth
90	No Growth	No Growth	No Growth	No Growth	Growth	Growth
175	No Growth	No Growth	No Growth	No Growth	No Growth	Growth
286	No Growth	No Growth	No Growth	Growth	Growth	Growth

Table B6: Clarithromycin Susceptibility

	0.125	0.0625	0.03125	0.0156	0.0078	No Antibiotic
E	No Growth	No Growth	No Growth	No Growth	No Growth	Growth
L1	No Growth	No Growth	No Growth	No Growth	No Growth	Growth
L2	No Growth	No Growth	No Growth	No Growth	Growth	Growth
L3	No Growth	No Growth	No Growth	No Growth	No Growth	Growth
US151	No Growth	No Growth	No Growth	No Growth	No Growth	Growth
US162	No Growth	No Growth	No Growth	No Growth	No Growth	Growth
US197	No Growth	No Growth	No Growth	No Growth	No Growth	Growth
28	No Growth	No Growth	No Growth	No Growth	No Growth	Growth
90	No Growth	No Growth	No Growth	No Growth	Growth	Growth
175	No Growth	No Growth	No Growth	No Growth	No Growth	Growth
286	No Growth	No Growth	No Growth	No Growth	No Growth	Growth

Table B7: Azithromycin Susceptibility

	0.5	0.25	0.125	0.0625	0.03125	No Antibiotic
E	No Growth	Growth	Growth	Growth	Growth	Growth
L1	No Growth	No Growth	Growth	Growth	Growth	Growth
L2	No Growth	No Growth	Growth	Growth	Growth	Growth
L3	No Growth	No Growth	Growth	Growth	Growth	Growth
US151	No Growth	No Growth	Growth	Growth	Growth	Growth
US162	No Growth	No Growth	Growth	Growth	Growth	Growth
US197	No Growth	No Growth	No Growth	Growth	Growth	Growth
28	No Growth	No Growth	Growth	Growth	Growth	Growth
90	No Growth	No Growth	Growth	Growth	Growth	Growth
175	No Growth	No Growth	No Growth	Growth	Growth	Growth
286	No Growth	No Growth	Growth	Growth	Growth	Growth

Table B8: Erythromycin Susceptibility

	0.5	0.25	0.125	0.0625	0.03125	No Antibiotic
E	No Growth	No Growth	No Growth	Growth	Growth	Growth
L1	No Growth	No Growth	No Growth	Growth	Growth	Growth
L2	No Growth	No Growth	No Growth	Growth	Growth	Growth
L3	No Growth	No Growth	No Growth	Growth	Growth	Growth
US151	No Growth	No Growth	No Growth	Growth	Growth	Growth
US162	No Growth	No Growth	No Growth	Growth	Growth	Growth
US197	No Growth	No Growth	No Growth	Growth	Growth	Growth
28	No Growth	No Growth	No Growth	Growth	Growth	Growth
90	No Growth	No Growth	No Growth	Growth	Growth	Growth
175	No Growth	No Growth	No Growth	Growth	Growth	Growth
286	No Growth	No Growth	No Growth	Growth	Growth	Growth

Table B9: Ciprofloxacin Susceptibility

	4	2	1	0.5	0.25	No Antibiotic
E	No Growth	No Growth	No Growth	Growth	Growth	Growth
L1	No Growth	No Growth	No Growth	Growth	Growth	Growth
L2	No Growth	No Growth	No Growth	Growth	Growth	Growth
L3	No Growth	No Growth	No Growth	Growth	Growth	Growth
US151	No Growth	No Growth	No Growth	Growth	Growth	Growth
US162	No Growth	No Growth	No Growth	Growth	Growth	Growth
US197	No Growth	No Growth	No Growth	No Growth	Growth	Growth
28	No Growth	No Growth	No Growth	Growth	Growth	Growth
90	No Growth	No Growth	No Growth	Growth	Growth	Growth
175	No Growth	No Growth	No Growth	Growth	Growth	Growth
286	No Growth	No Growth	No Growth	Growth	Growth	Growth

Table B10: Real time PCR results for US151 exposed to tetracyclines

	Tetracycline					Minocycline					Doxycycline				
	2	**1**	**0.5**	**0.25**	**0.125**	**0.125**	**0.062**	**0.031**	**0.0156**	**0.0078**	**0.125**	**0.062**	**0.031**	**0.0156**	**0.0078**
Run 1	ND	ND	ND	ND	ND	38.7	41.23	ND	38.25	39.94	ND	ND	39.96	ND	41.78
Run 1	ND	ND	ND	ND	ND	40.53	41.31	41.29	38.18	39.08	ND	ND	ND	39.95	ND
Run 1	ND	ND	ND	39.59	ND	41.58	45.92	42.23	37.97	38.61	ND	ND	ND	ND	41.63
Run 2	36.52	30.7	ND	ND	ND	11.72	37.01	ND	35.76	34.84	12.6	ND	ND	ND	ND
Run 2	ND	37.34	ND	ND	36.88	ND	35.84	36.83	ND	36.53	ND	ND	ND	ND	ND
Run 2	ND	ND	36.33	41.88	ND	ND	34.61	35.05	ND	36.21	ND	ND	ND	ND	ND

Table B11: Real time PCR results for US151 exposed to tigecycline

	Concentration (µg/ml)				
	0.5	**0.25**	**0.125**	**0.0625**	**0.03125**
Run 1	40.87	37.79	37.72	40.54	38.47
Run 1	ND	40.27	37.63	39.19	40.22
Run 1	ND	40.8	38.11	44.26	40.72
Run 2	ND	ND	37.53	36.81	37.15
Run 2	ND	ND	35.18	36.31	ND
Run 2	36.62	36.34	35.99	ND	37.16

Table B12: Real time PCR results for US151 exposed to macrolides

	Erythromycin					Clarithromycin					Azithromycin				
	0.5	**0.25**	**0.125**	**0.062**	**0.031**	**0.125**	**0.062**	**0.031**	**0.0156**	**0.0078**	**0.5**	**0.25**	**0.125**	**0.062**	**0.031**
Run 1	ND	ND	ND	41.22	ND	ND	ND	ND	ND	ND	39.99	ND	ND	ND	ND
Run 1	ND	42.51	ND	ND	45.31	40.94	ND	40.45	ND	ND	ND	ND	ND	ND	ND
Run 1	ND	ND	ND	ND	ND	ND	ND	40.66	ND	40.47	ND	ND	ND	ND	ND
Run 2	34.36	ND	ND	36.15	ND	ND	ND	ND	ND	47.5	ND	ND	ND	36.02	37.44
Run 2	13.35	ND	ND	ND	9.9	13.05	ND	ND	ND	ND	ND	ND	34.9	34.27	37.91
Run 2	ND	ND	36.53	43.35	36.71	ND	ND	10.54	ND	6.86	ND	ND	ND	34.01	35.81

Table B13: Real time PCR results for US151 exposed to ciprofloxacin

	Concentration (µg/ml)				
	4	**2**	**1**	**0.5**	**0.25**
Run 1	ND	ND	ND	40.95	36.59
Run 1	ND	ND	40.42	40.23	37.88
Run 1	ND	ND	39.89	39.92	38.35
Run 2	15.56	ND	ND	ND	37.79
Run 2	6.61	6.77	ND	ND	ND
Run 2	ND	ND	ND	ND	ND

APPENDIX C – Immunofluorescent Images

Figure C1-24 (pages 84 to 107 overleaf). Fluorescent micrographs of McCoy cell monolayers. Cells were infected with *Chlamydia trachomatis* isolates by centrifugation, treated with different concentrations of clinically relevant antimicrobial agents and stained with the MicroTrak *C. trachomatis* culture confirmation kit for the detection of green chlamydial inclusions which indicate growth of the organism. Images were captured at 100 × magnification. Antimicrobial agents included (C1-9) tetracyclines (tetracycline, minocycline, doxycycline), (C10-12) a glycycline (tigecycline), (C13-21) macrolides (clarithromycin, aziithromycin, erythromycin), and (C22-24) a fluoroquinolone (ciprofloxacin).

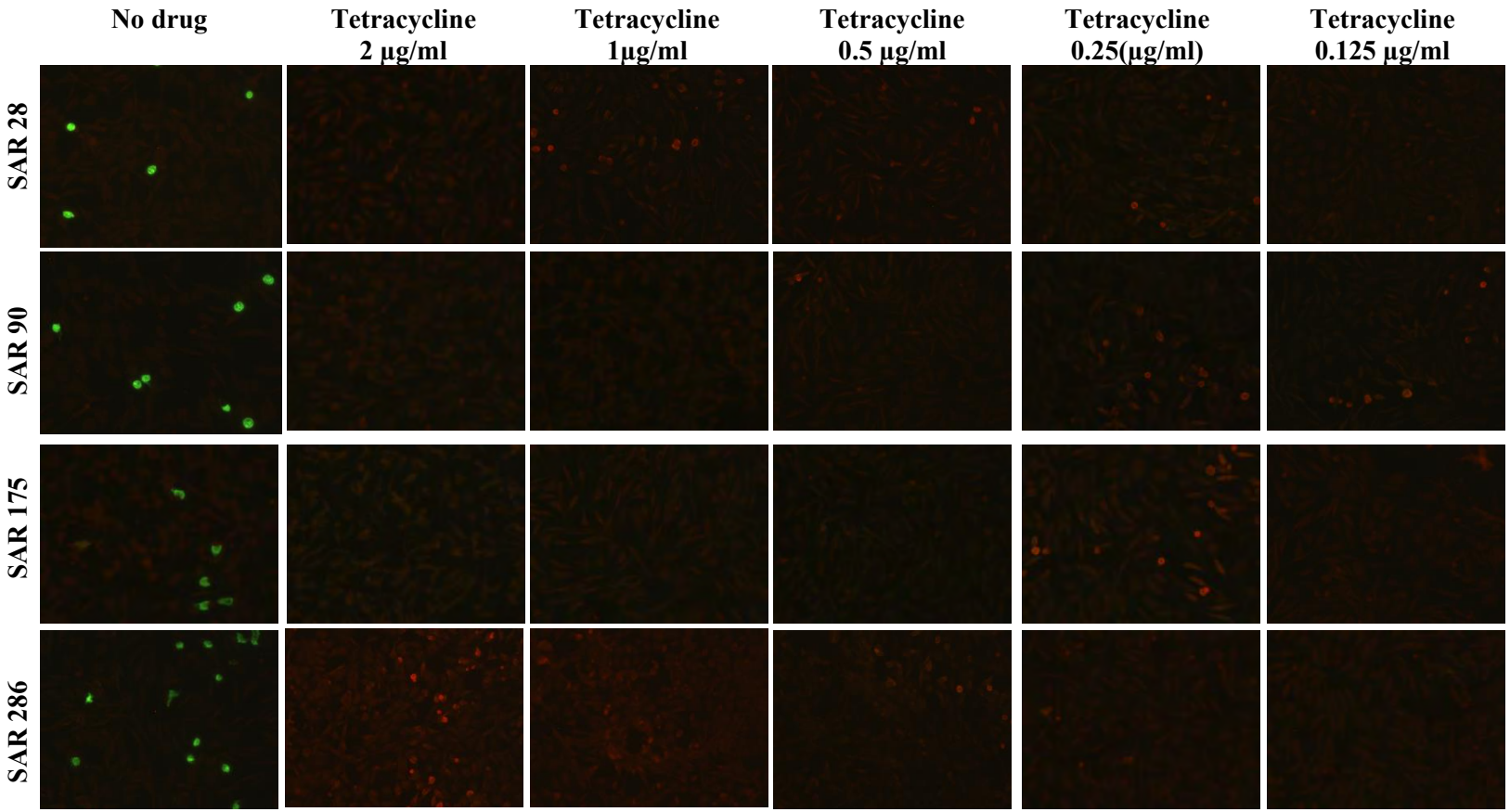

No drug
Tetracycline 2 µg/ml
Tetracycline 1µg/ml
Tetracycline 0.5 µg/ml
Tetracycline 0.25(µg/ml)
Tetracycline 0.125 µg/ml
SAR 28
SAR 90
SAR 175
SAR 286

Figure C1

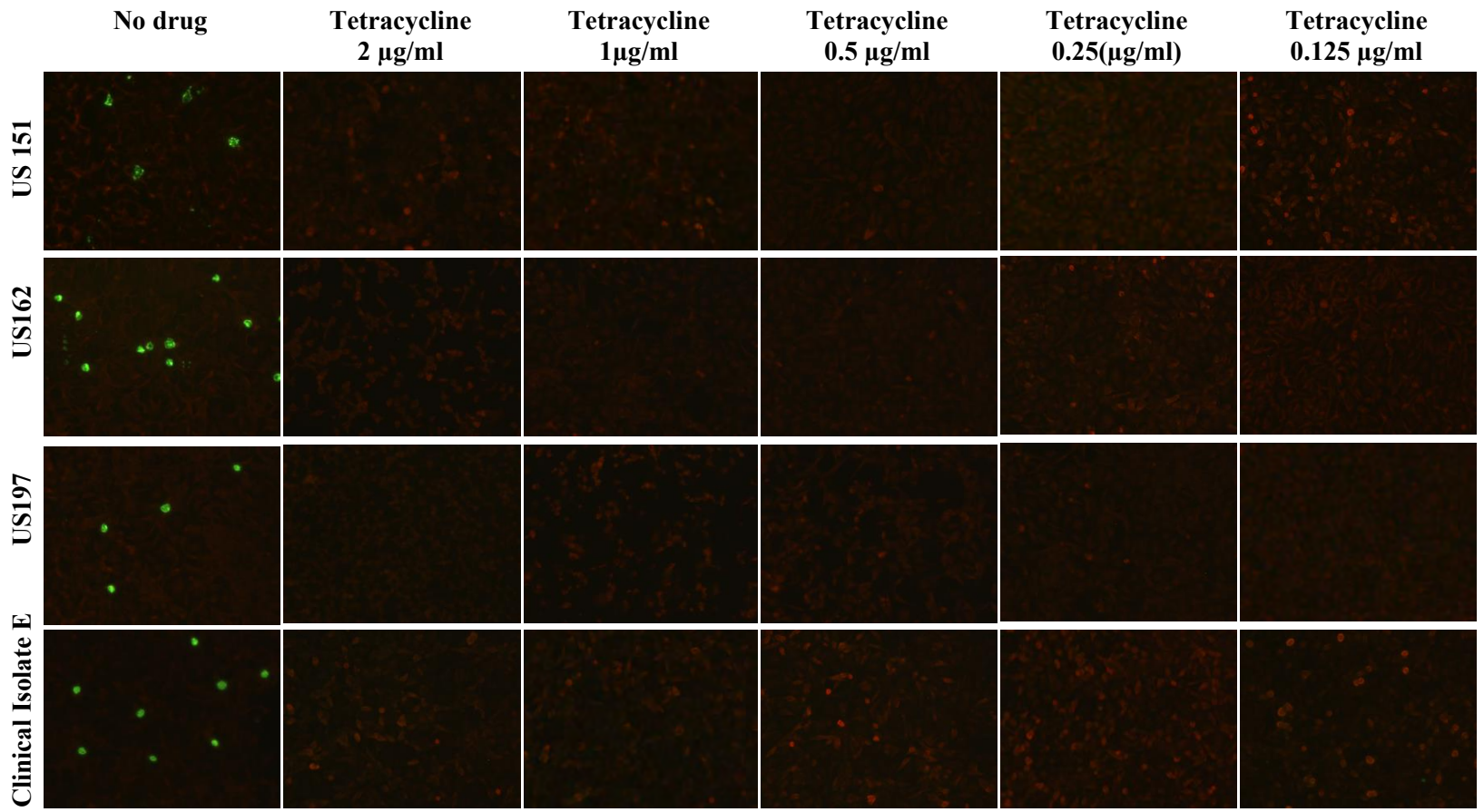

No drug
Tetracycline 2 µg/ml
Tetracycline 1µg/ml
Tetracycline 0.5 µg/ml
Tetracycline 0.25(µg/ml)
Tetracycline 0.125 µg/ml
US 151
US162
US197
Clinical Isolate E

Figure C2

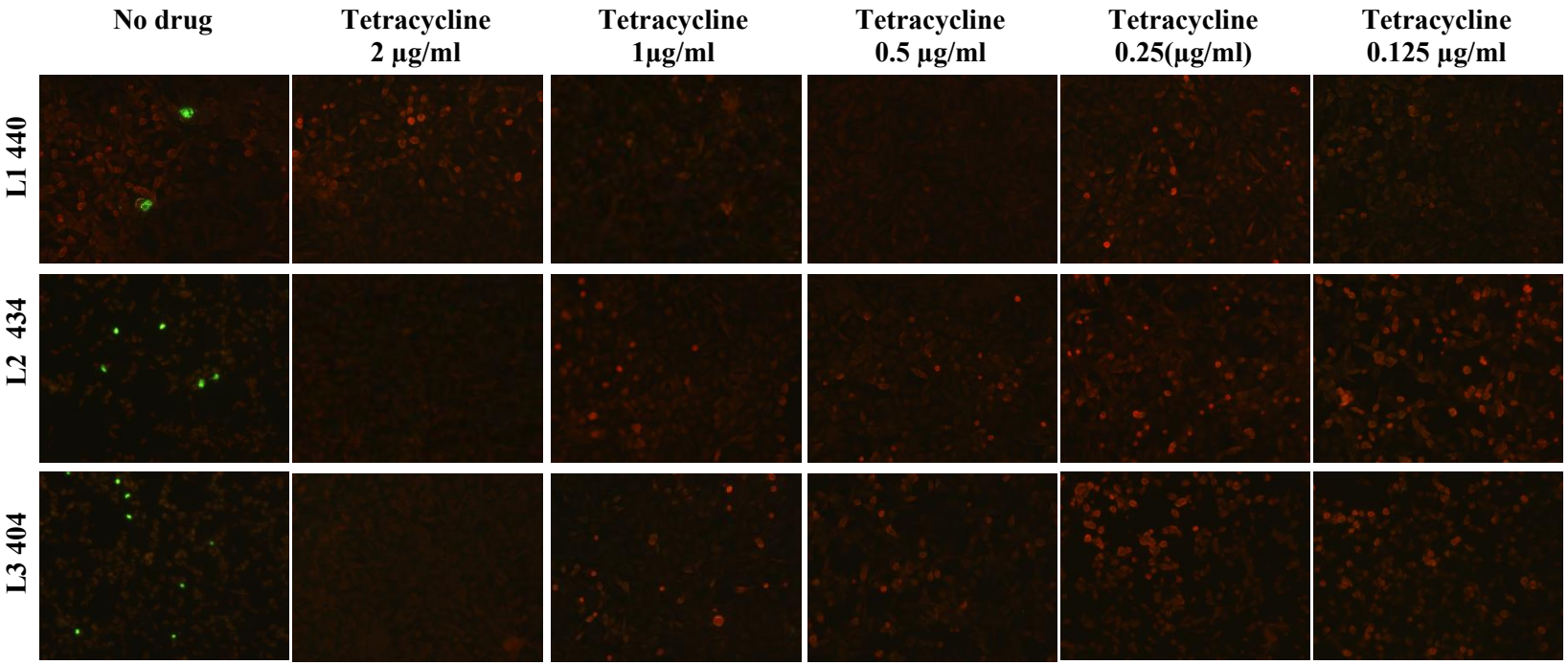

No drug
Tetracycline 2 µg/ml
Tetracycline 1µg/ml
Tetracycline 0.5 µg/ml
Tetracycline 0.25(µg/ml)
Tetracycline 0.125 µg/ml
L1 440
L2 434
L3 404

Figure C3

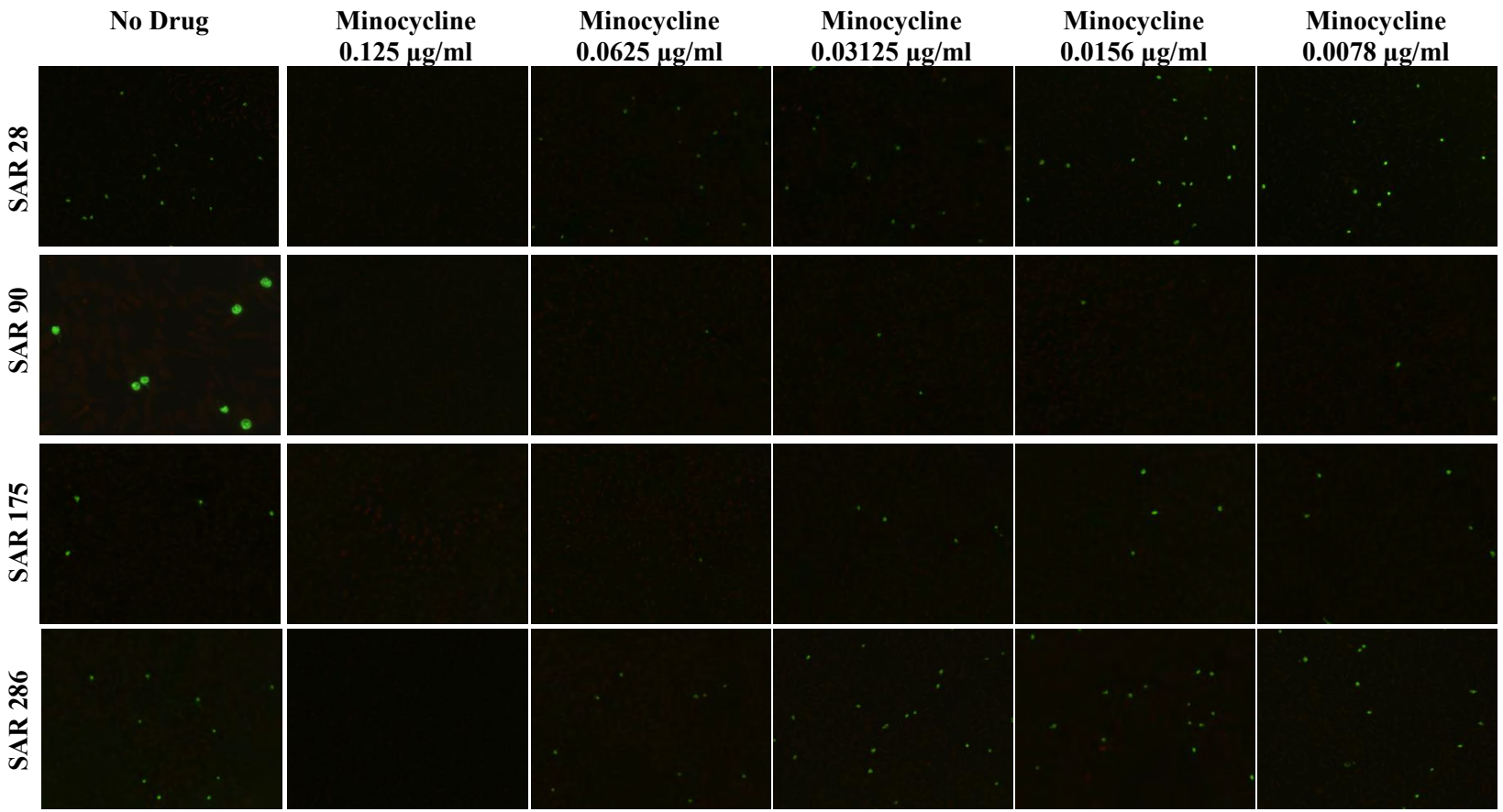

No Drug
Minocycline 0.125 µg/ml
Minocycline 0.0625 µg/ml
Minocycline 0.03125 µg/ml
Minocycline 0.0156 µg/ml
Minocycline 0.0078 µg/ml
SAR 28
SAR 90
SAR 175
SAR 286

Figure C4

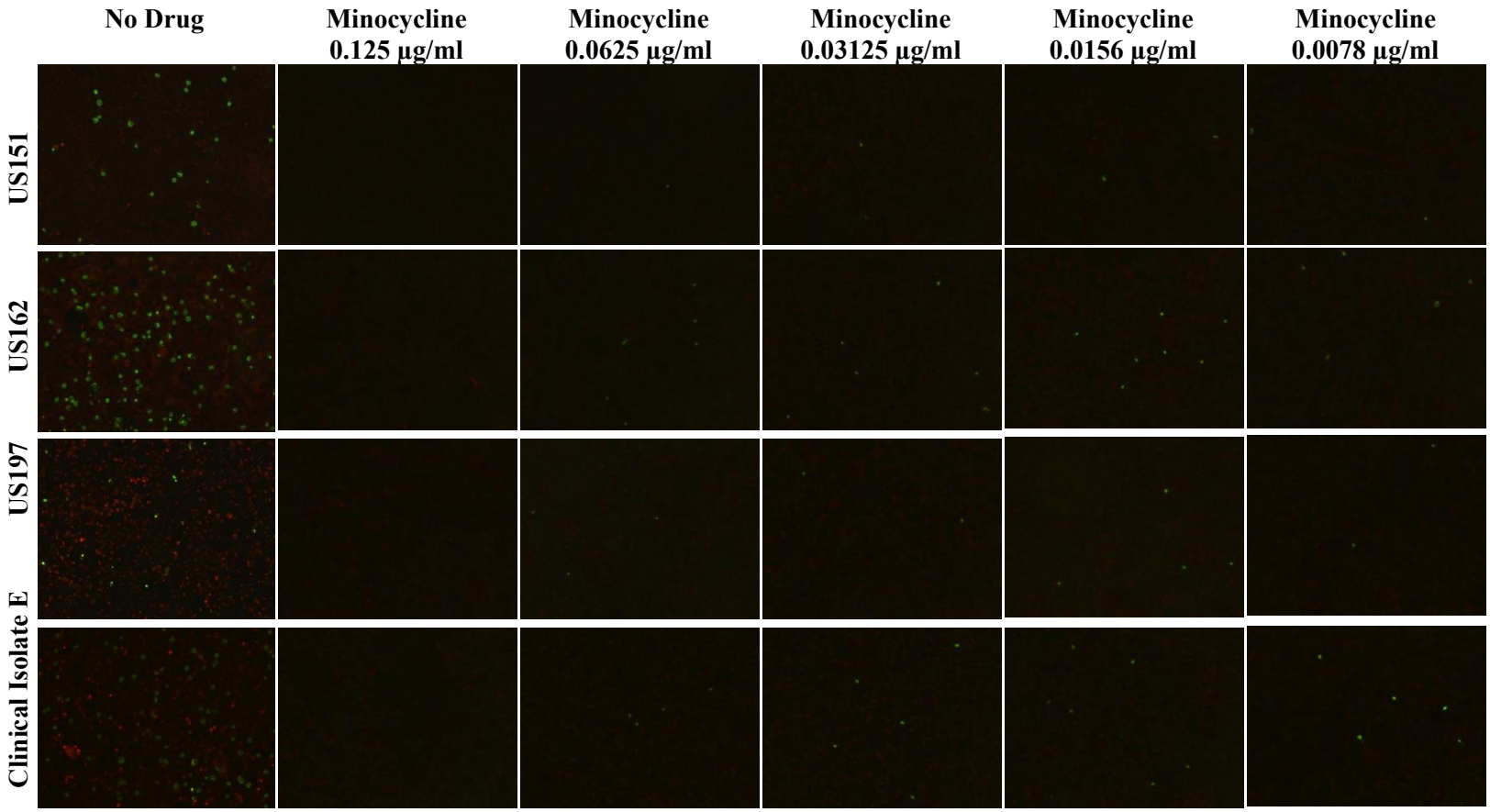

No Drug
Minocycline 0.125 µg/ml
Minocycline 0.0625 µg/ml
Minocycline 0.03125 µg/ml
Minocycline 0.0156 µg/ml
Minocycline 0.0078 µg/ml
US151
US162
US197
Clinical Isolate E

Figure C5

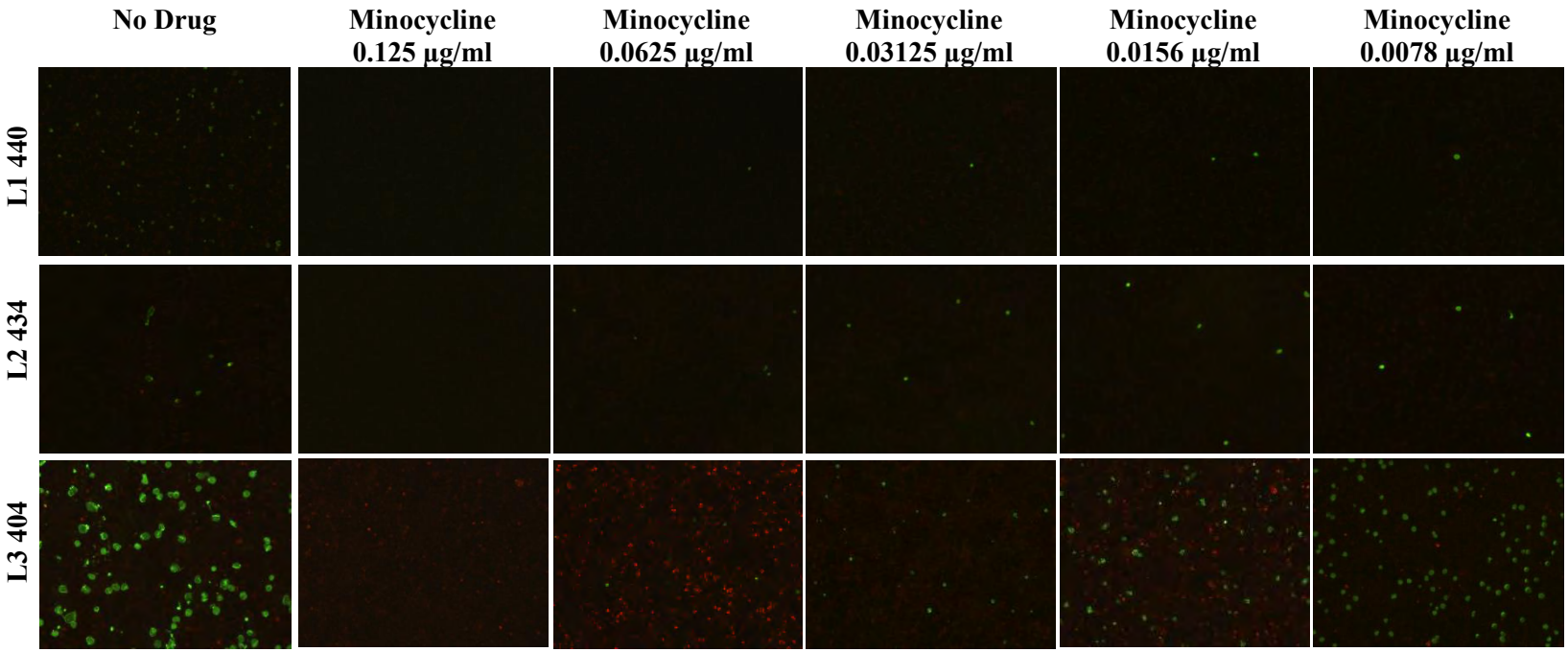

No Drug
Minocycline 0.125 µg/ml
Minocycline 0.0625 µg/ml
Minocycline 0.03125 µg/ml
Minocycline 0.0156 µg/ml
Minocycline 0.0078 µg/ml
L1 440
L2 434
L3 404

Figure C6

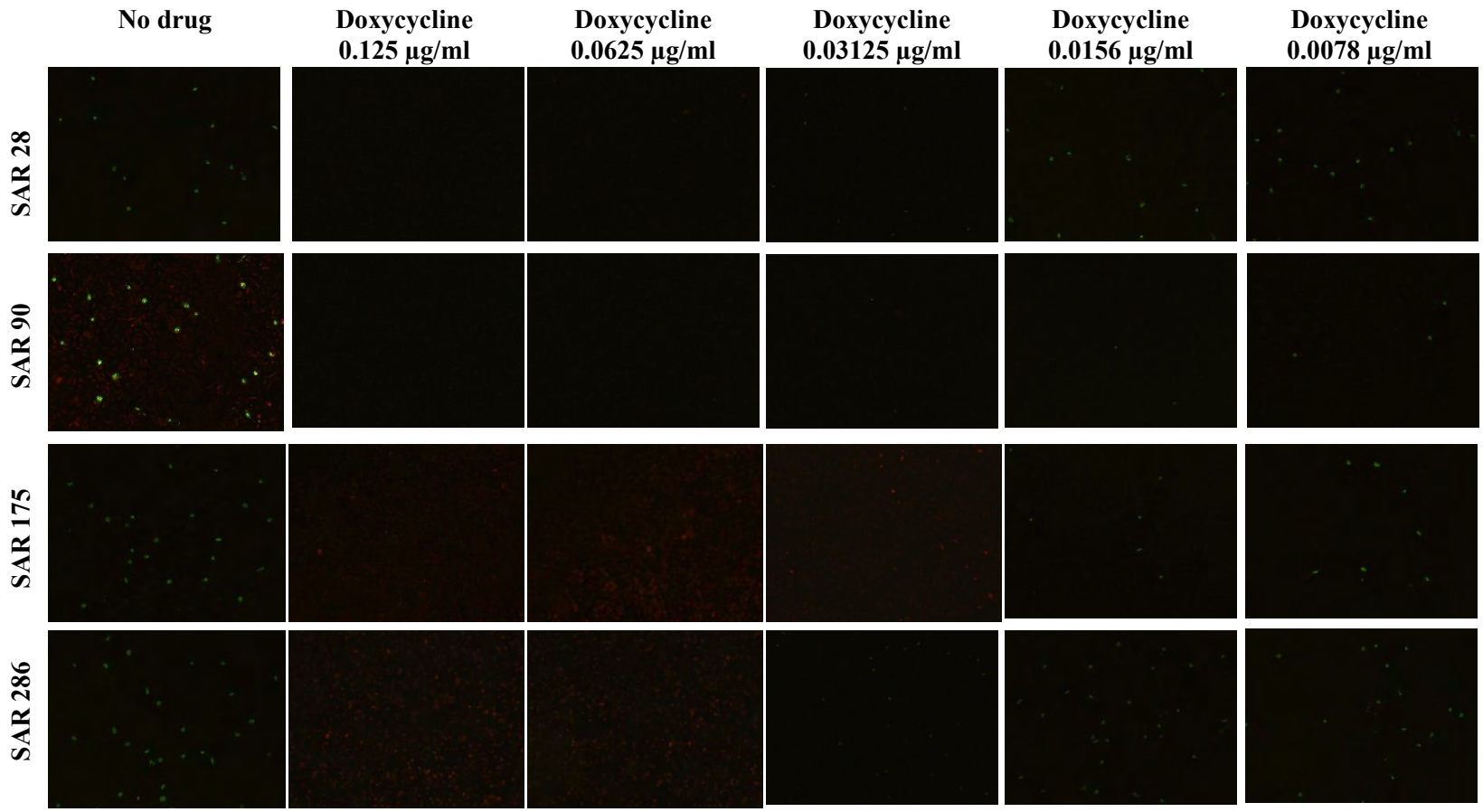

No drug
Doxycycline 0.125 µg/ml
Doxycycline 0.0625 µg/ml
Doxycycline 0.03125 µg/ml
Doxycycline 0.0156 µg/ml
Doxycycline 0.0078 µg/ml
SAR 28
SAR 90
SAR 175
SAR 286

Figure C7

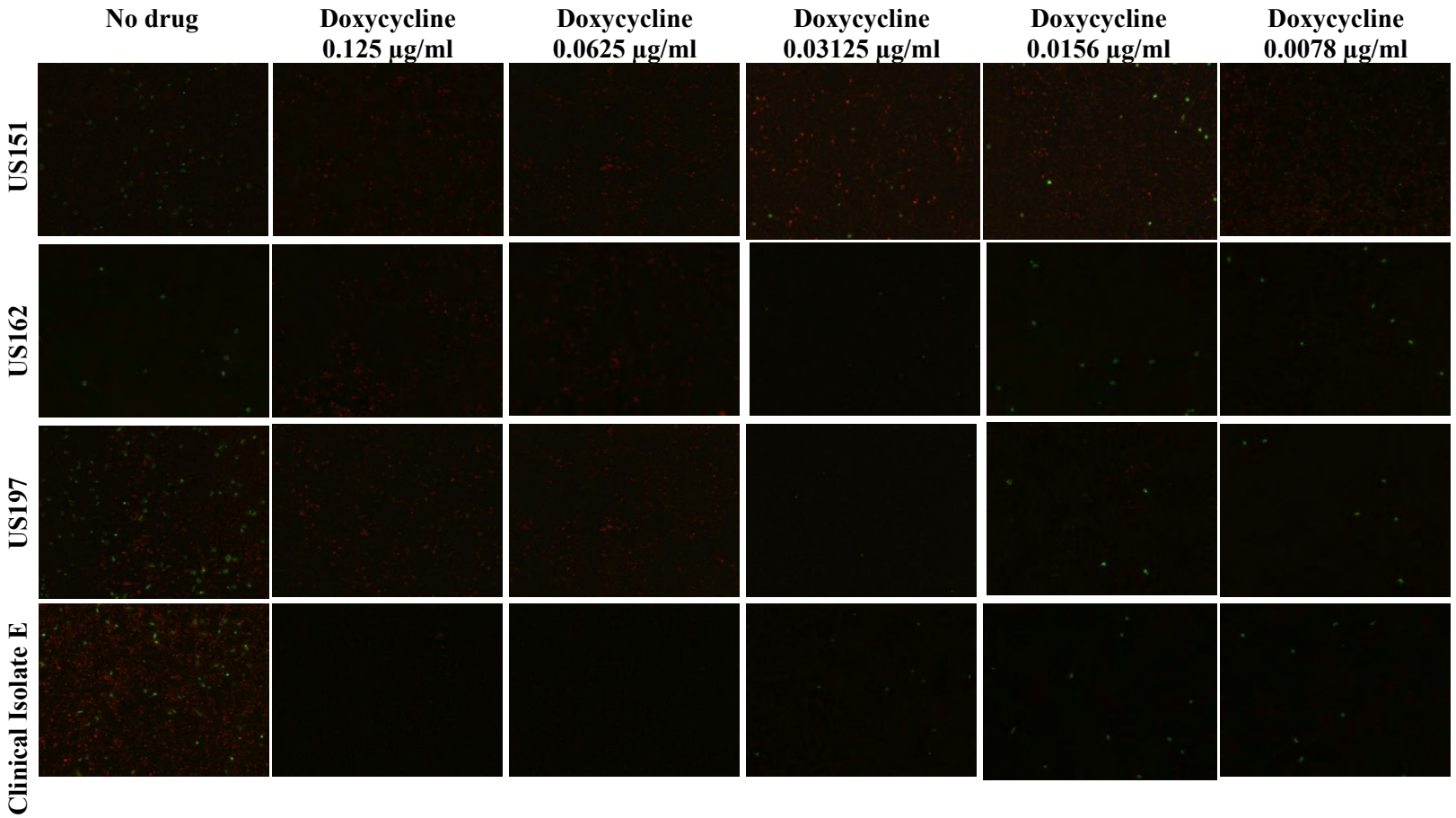

No drug
Doxycycline 0.125 µg/ml
Doxycycline 0.0625 µg/ml
Doxycycline 0.03125 µg/ml
Doxycycline 0.0156 µg/ml
Doxycycline 0.0078 µg/ml
US151
US162
US197
Clinical Isolate E

Figure C8

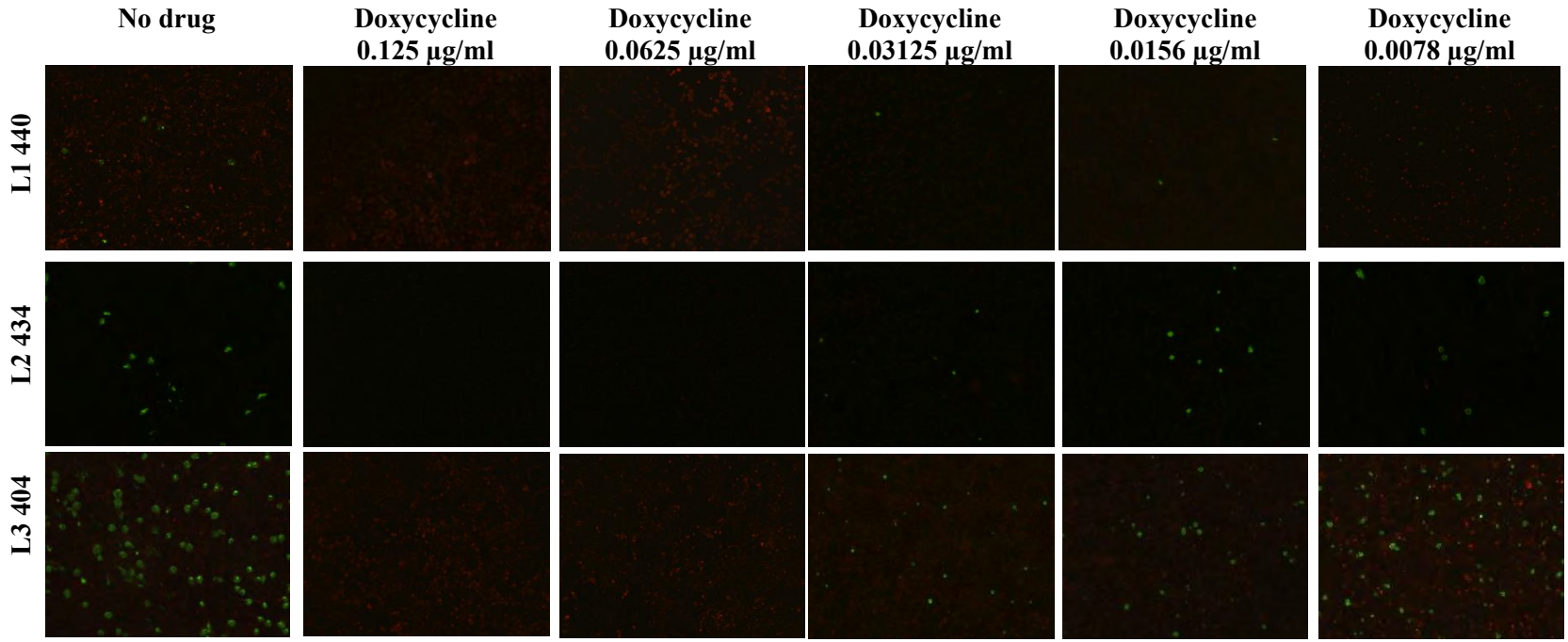

No drug
Doxycycline 0.125 µg/ml
Doxycycline 0.0625 µg/ml
Doxycycline 0.03125 µg/ml
Doxycycline 0.0156 µg/ml
Doxycycline 0.0078 µg/ml
L1 440
L2 434
L3 404

Figure C9

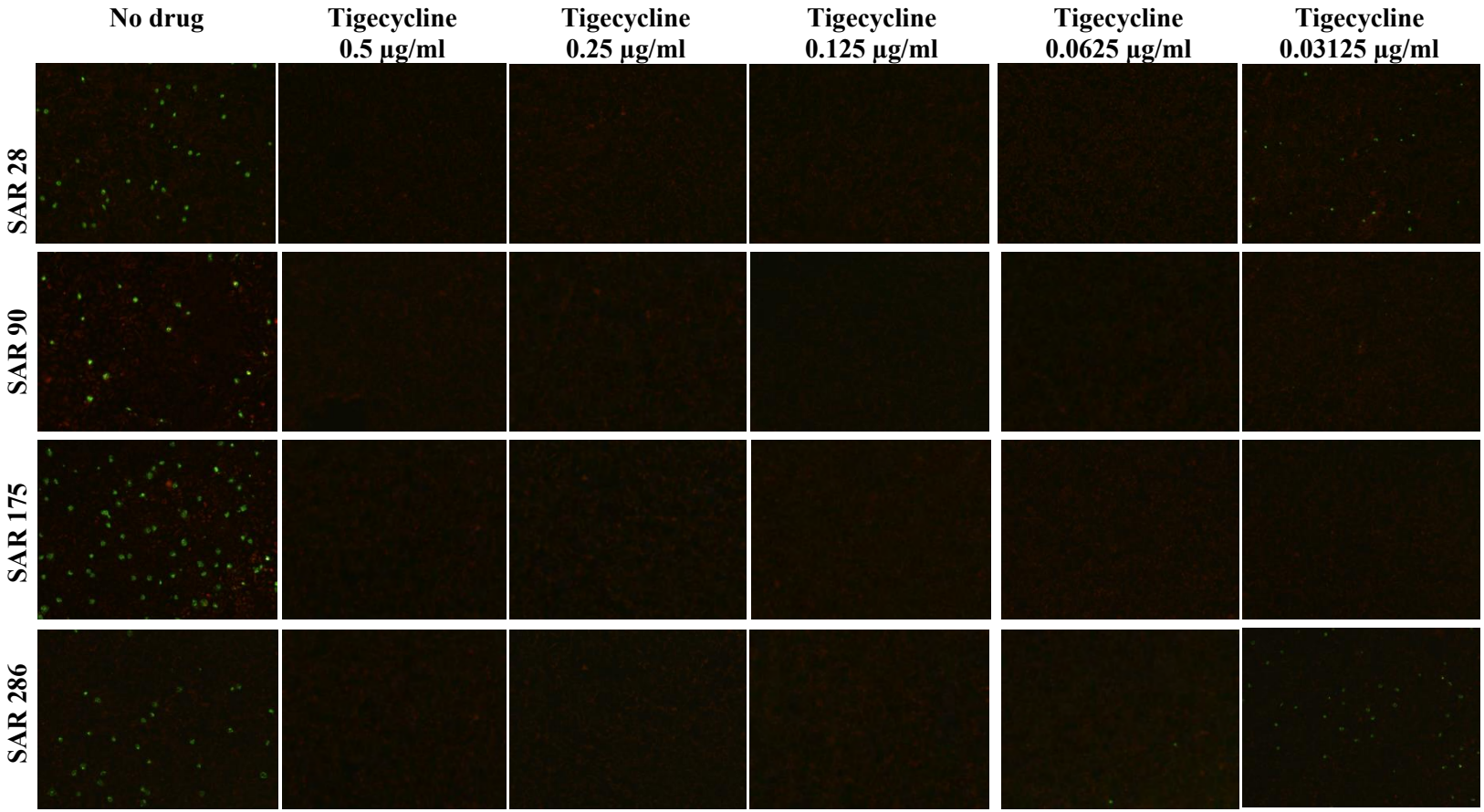

No drug
Tigecycline 0.5 µg/ml
Tigecycline 0.25 µg/ml
Tigecycline 0.125 µg/ml
Tigecycline 0.0625 µg/ml
Tigecycline 0.03125 µg/ml
SAR 28
SAR 90
SAR 175
SAR 286

Figure C10

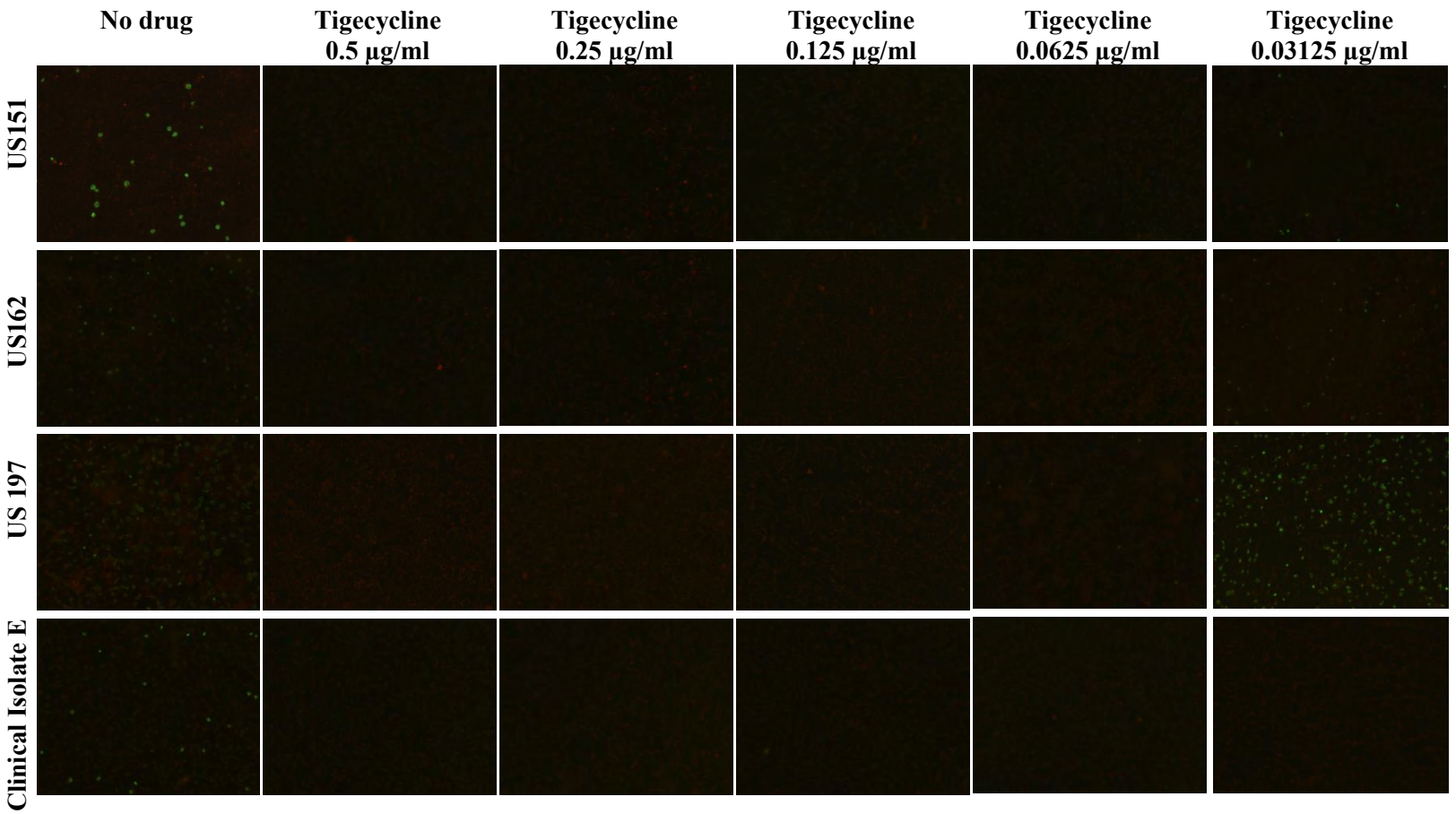
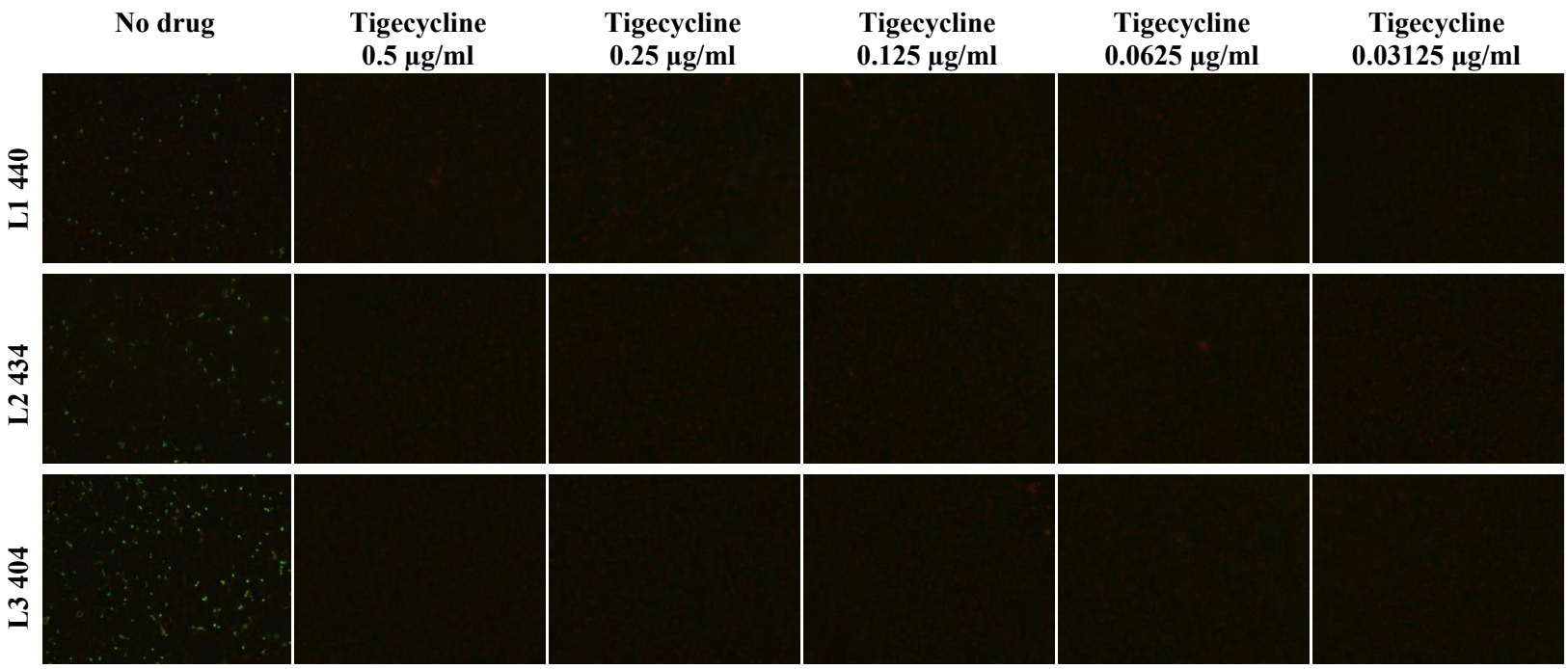

No drug
Tigecycline 0.5 µg/ml
Tigecycline 0.25 µg/ml
Tigecycline 0.125 µg/ml
Tigecycline 0.0625 µg/ml
Tigecycline 0.03125 µg/ml
US151
US162
US 197
Clinical Isolate E

Figure C11

Figure C12


No drug
Tigecycline 0.5 µg/ml
Tigecycline 0.25 µg/ml
Tigecycline 0.125 µg/ml
Tigecycline 0.0625 µg/ml
Tigecycline 0.03125 µg/ml
L1 440
L2 434
L3 404

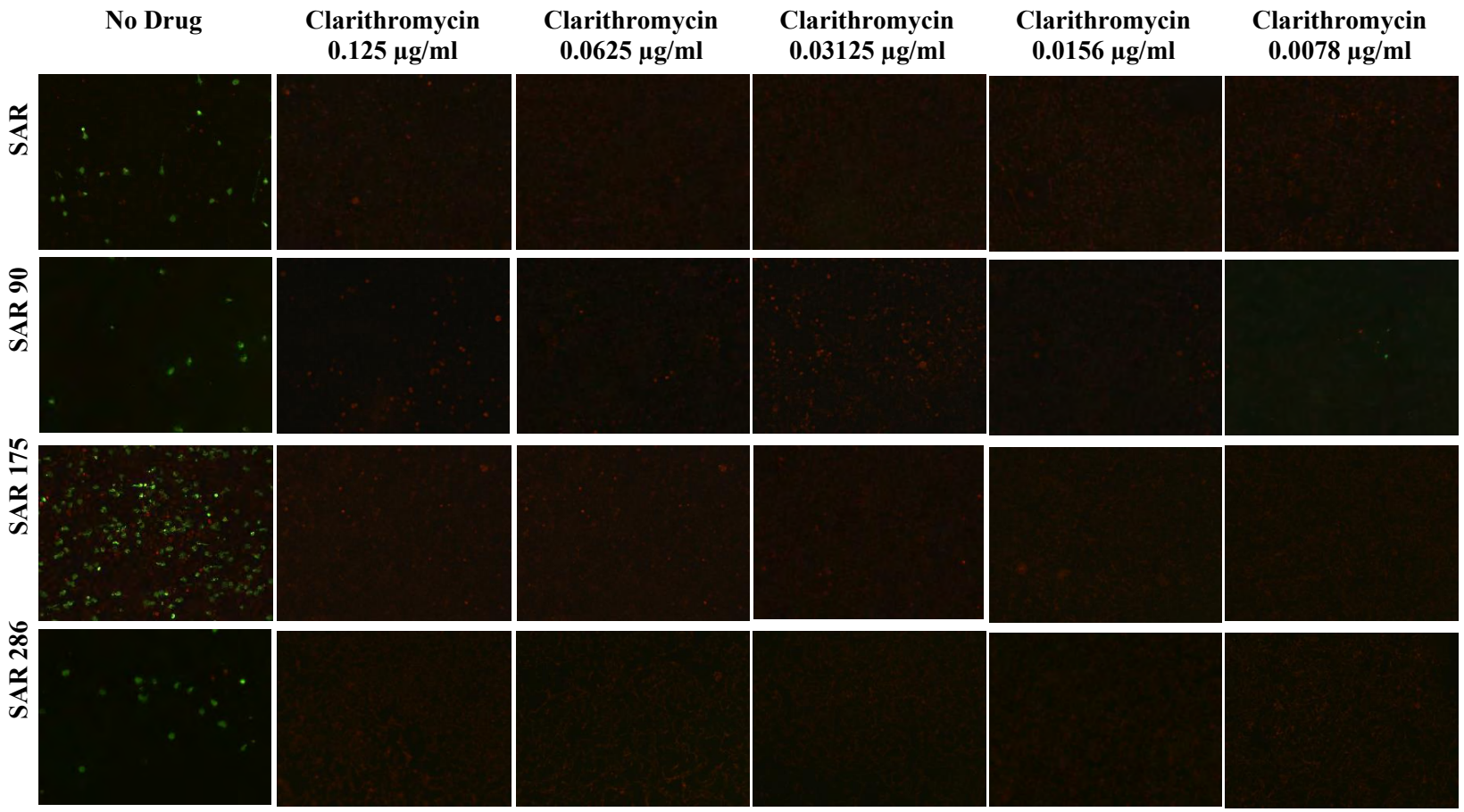

No Drug
Clarithromycin 0.125 µg/ml
Clarithromycin 0.0625 µg/ml
Clarithromycin 0.03125 µg/ml
Clarithromycin 0.0156 µg/ml
Clarithromycin 0.0078 µg/ml
SAR
SAR 90
SAR 175
SAR 286

Figure C13

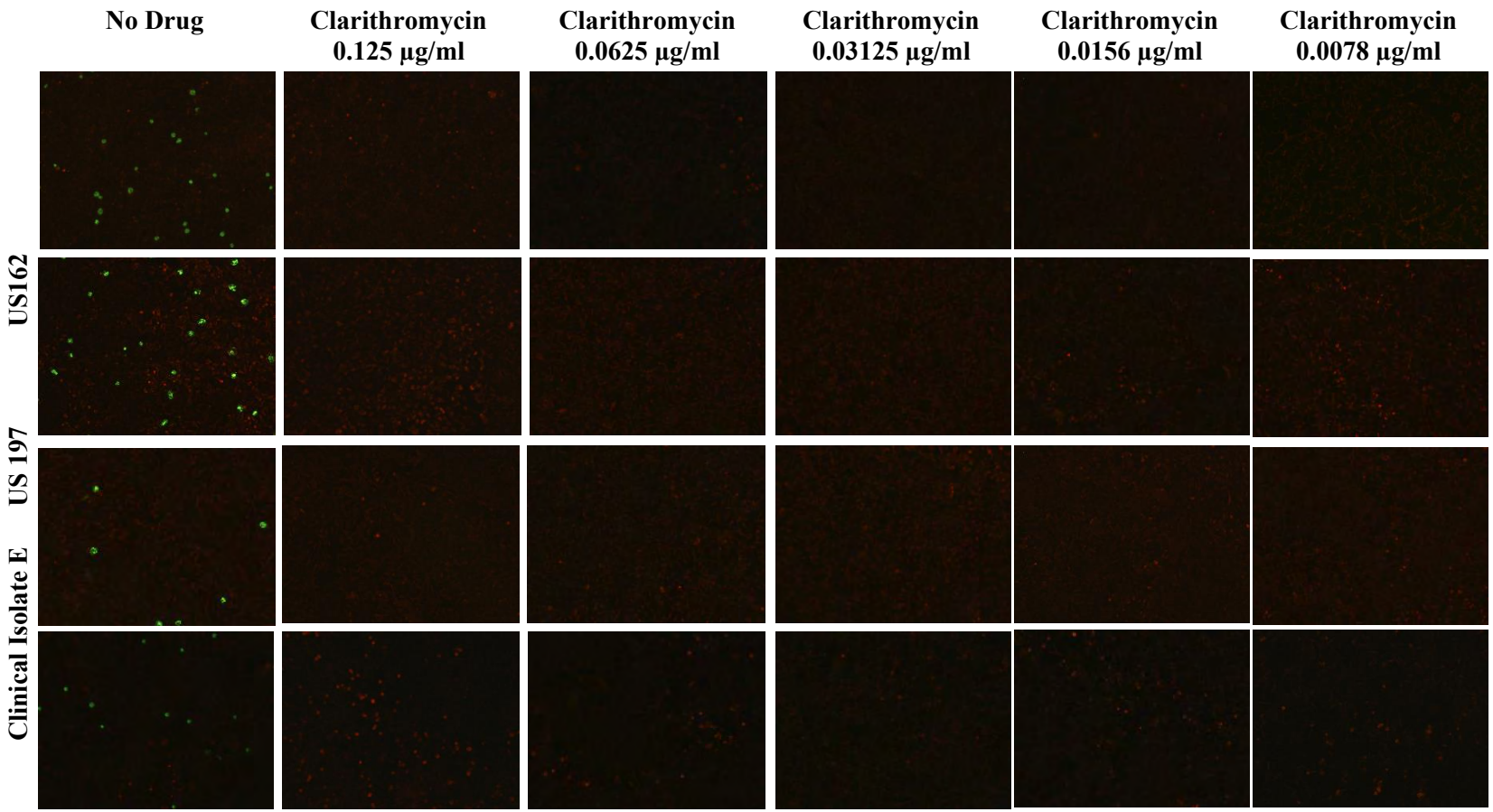

No Drug
Clarithromycin 0.125 µg/ml
Clarithromycin 0.0625 µg/ml
Clarithromycin 0.03125 µg/ml
Clarithromycin 0.0156 µg/ml
Clarithromycin 0.0078 µg/ml
US162
US 197
Clinical Isolate E

Figure C14

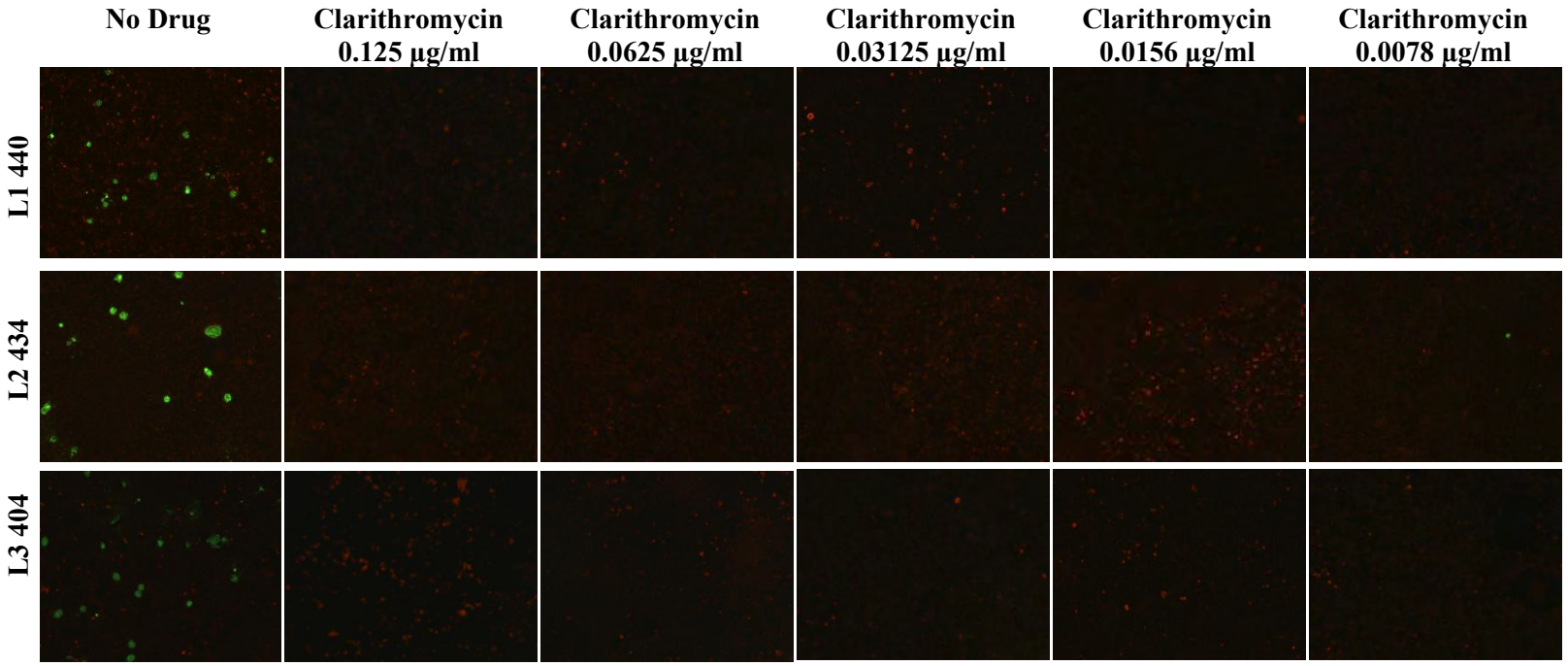

No Drug
Clarithromycin 0.125 µg/ml
Clarithromycin 0.0625 µg/ml
Clarithromycin 0.03125 µg/ml
Clarithromycin 0.0156 µg/ml
Clarithromycin 0.0078 µg/ml
L1 440
L2 434
L3 404

Figure C15

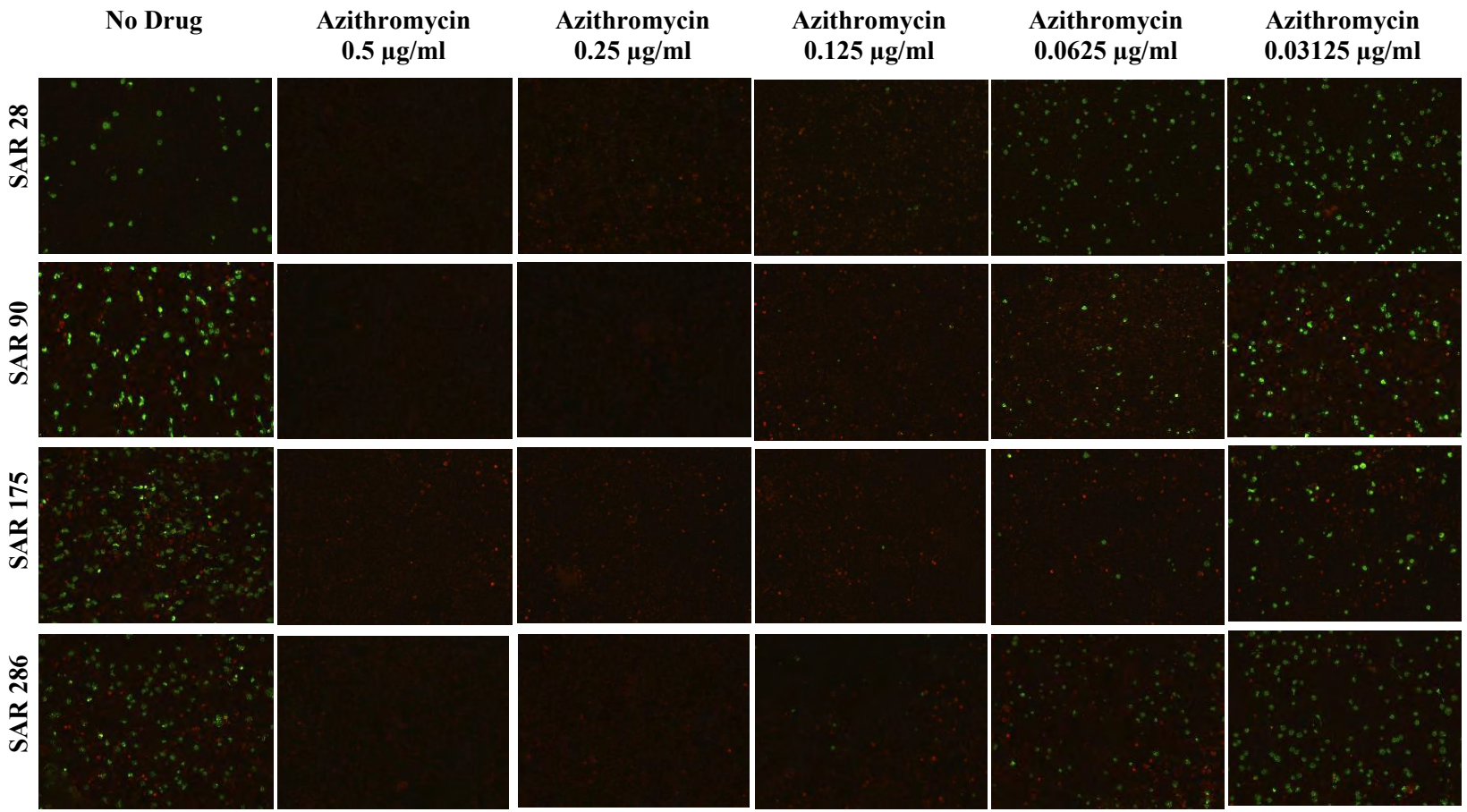

No Drug
Azithromycin 0.5 µg/ml
Azithromycin 0.25 µg/ml
Azithromycin 0.125 µg/ml
Azithromycin 0.0625 µg/ml
Azithromycin 0.03125 µg/ml
SAR 28
SAR 90
SAR 175
SAR 286

Figure C16

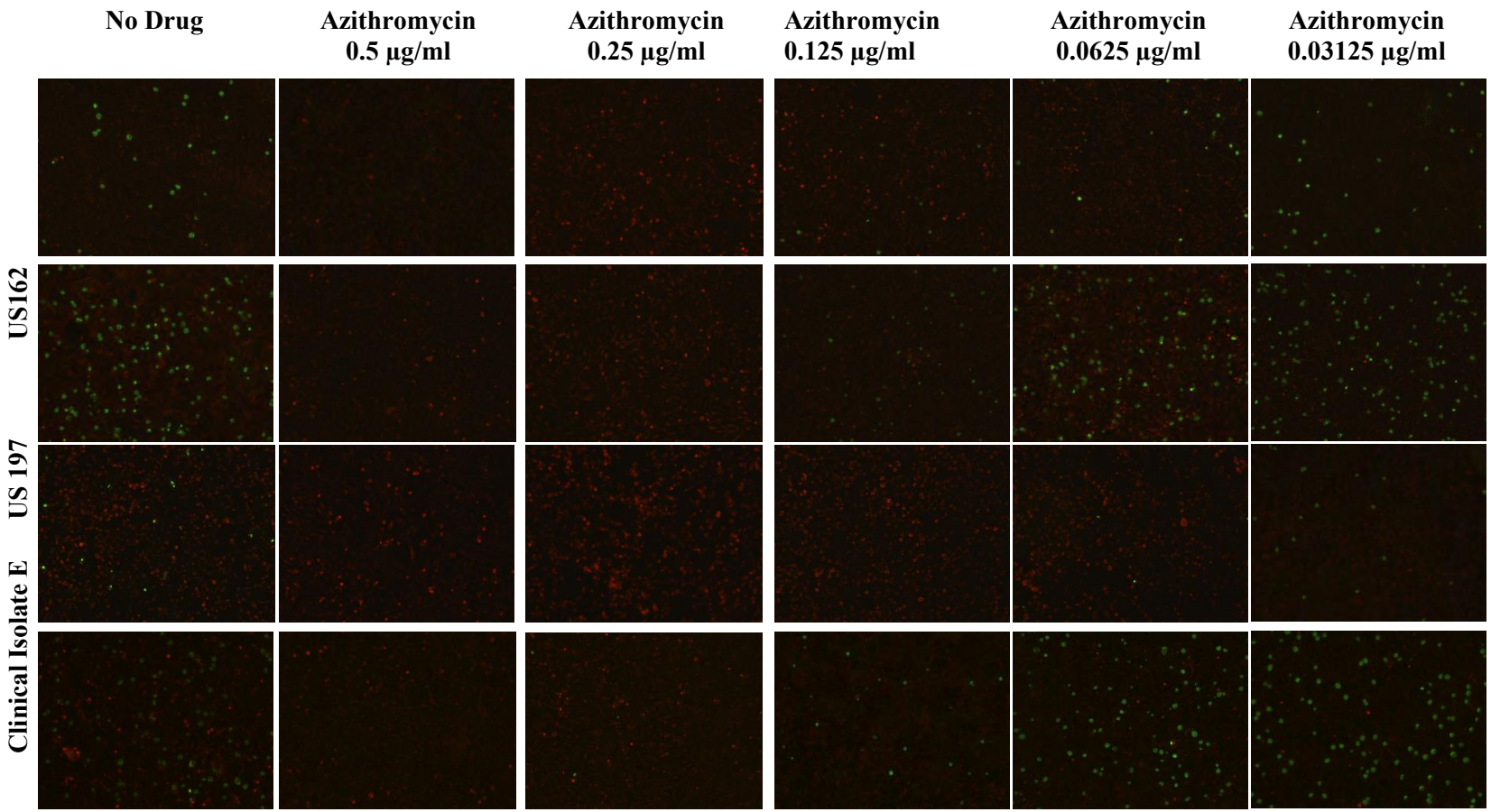

No Drug
Azithromycin 0.5 µg/ml
Azithromycin 0.25 µg/ml
Azithromycin 0.125 µg/ml
Azithromycin 0.0625 µg/ml
Azithromycin 0.03125 µg/ml
US162
US 197
Clinical Isolate E

Figure C17

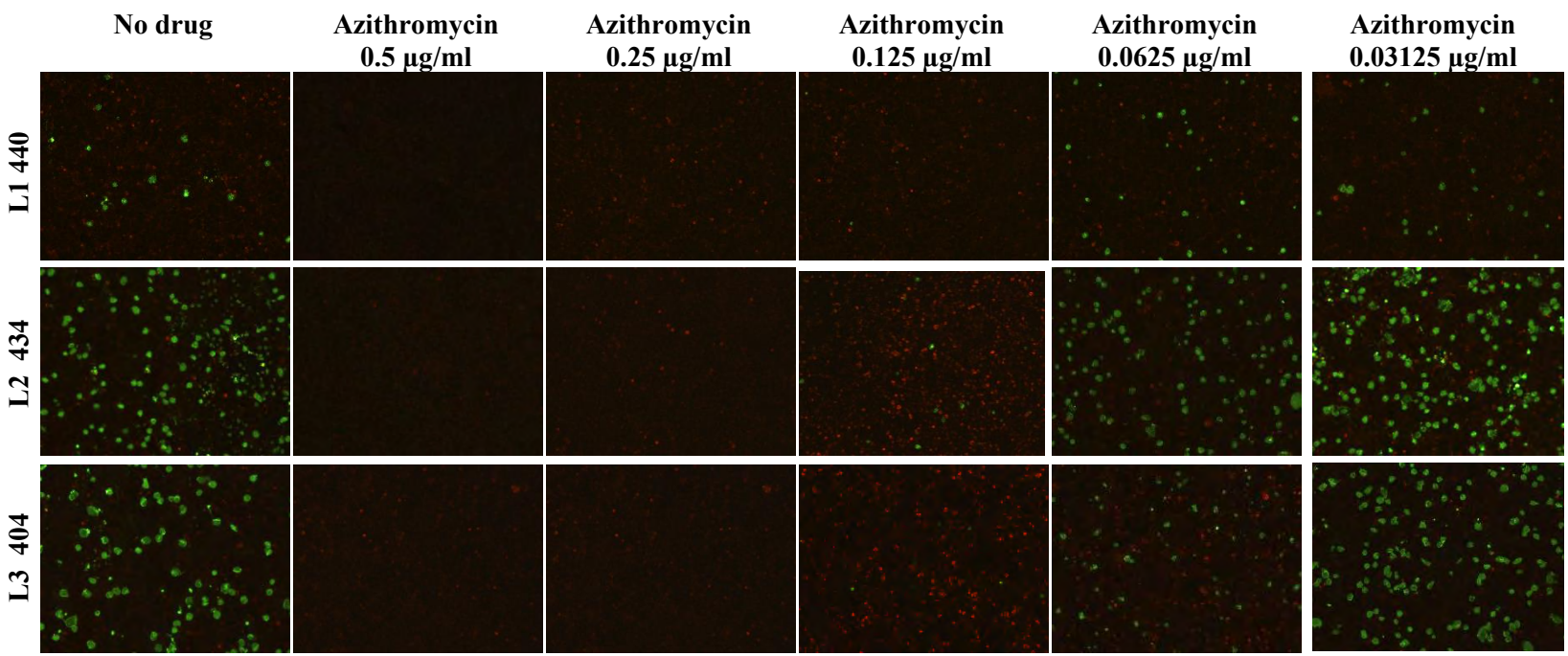

No drug
Azithromycin 0.5 µg/ml
Azithromycin 0.25 µg/ml
Azithromycin 0.125 µg/ml
Azithromycin 0.0625 µg/ml
Azithromycin 0.03125 µg/ml
L1 440
L2 434
L3 404

Figure C18

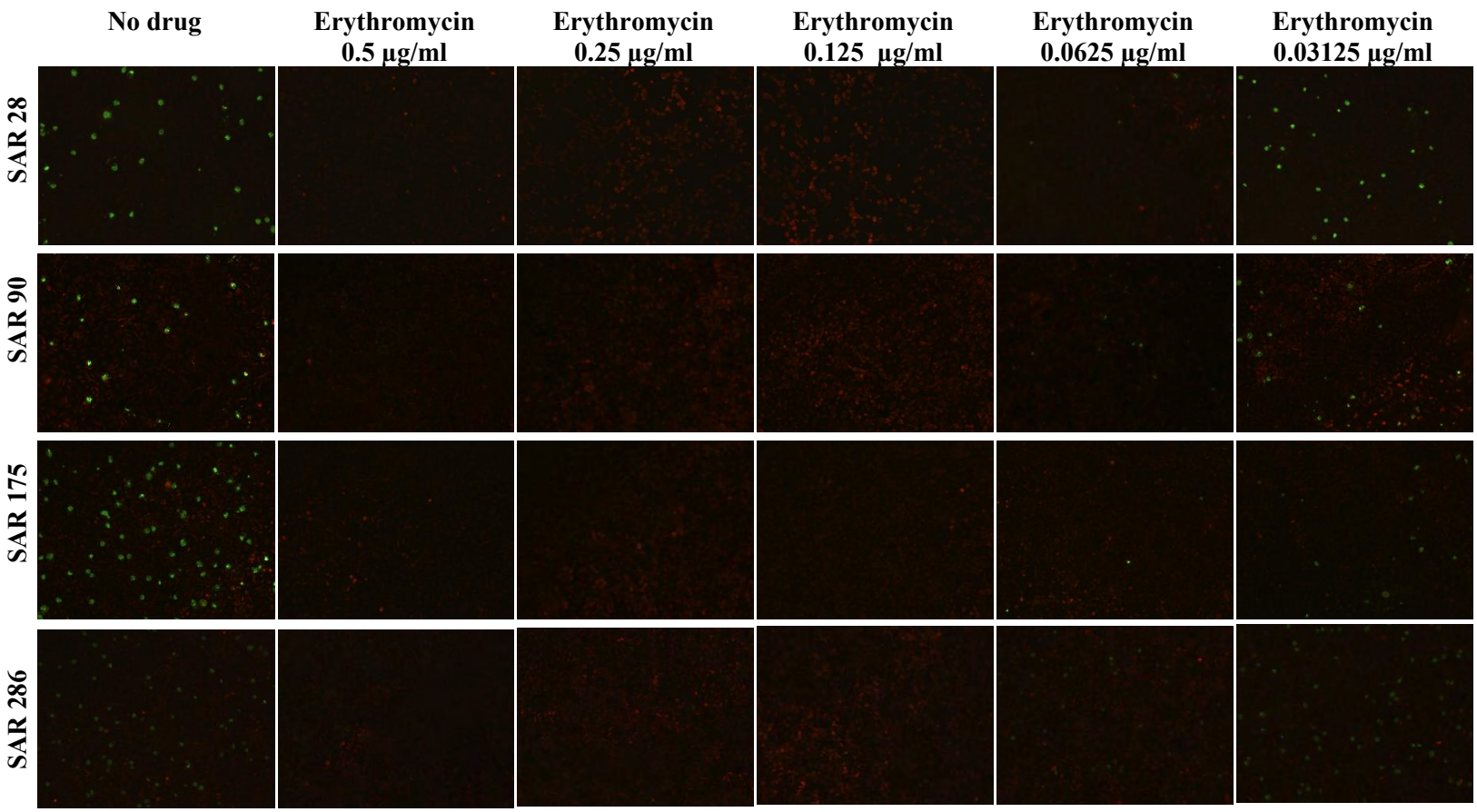

No drug
Erythromycin 0.5 µg/ml
Erythromycin 0.25 µg/ml
Erythromycin 0.125 µg/ml
Erythromycin 0.0625 µg/ml
Erythromycin 0.03125 µg/ml
SAR 28
SAR 90
SAR 175
SAR 286

Figure C19

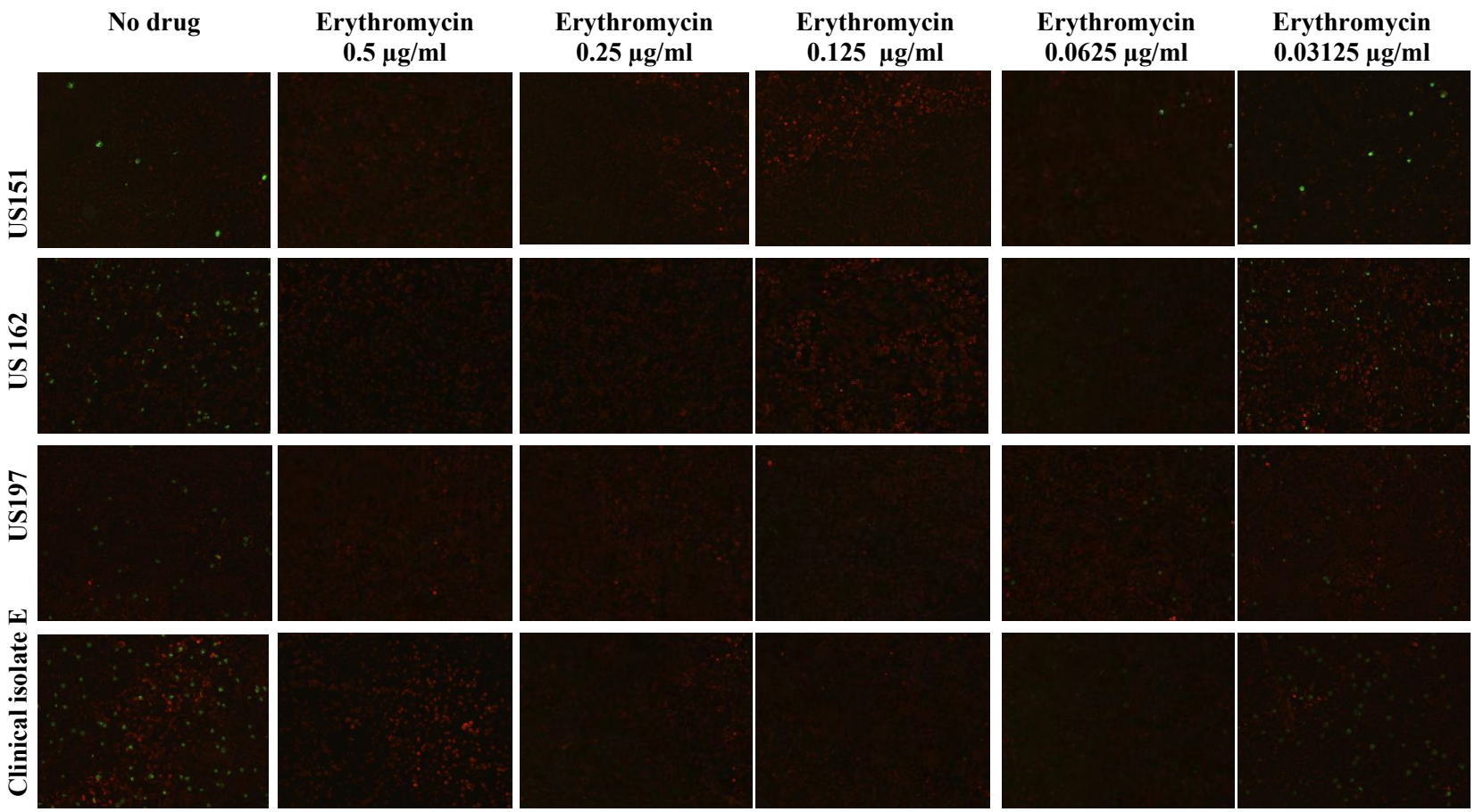

No drug
Erythromycin 0.5 µg/ml
Erythromycin 0.25 µg/ml
Erythromycin 0.125 µg/ml
Erythromycin 0.0625 µg/ml
Erythromycin 0.03125 µg/ml
US151
US 162
US197
Clinical isolate E

Figure C20

Figure C21

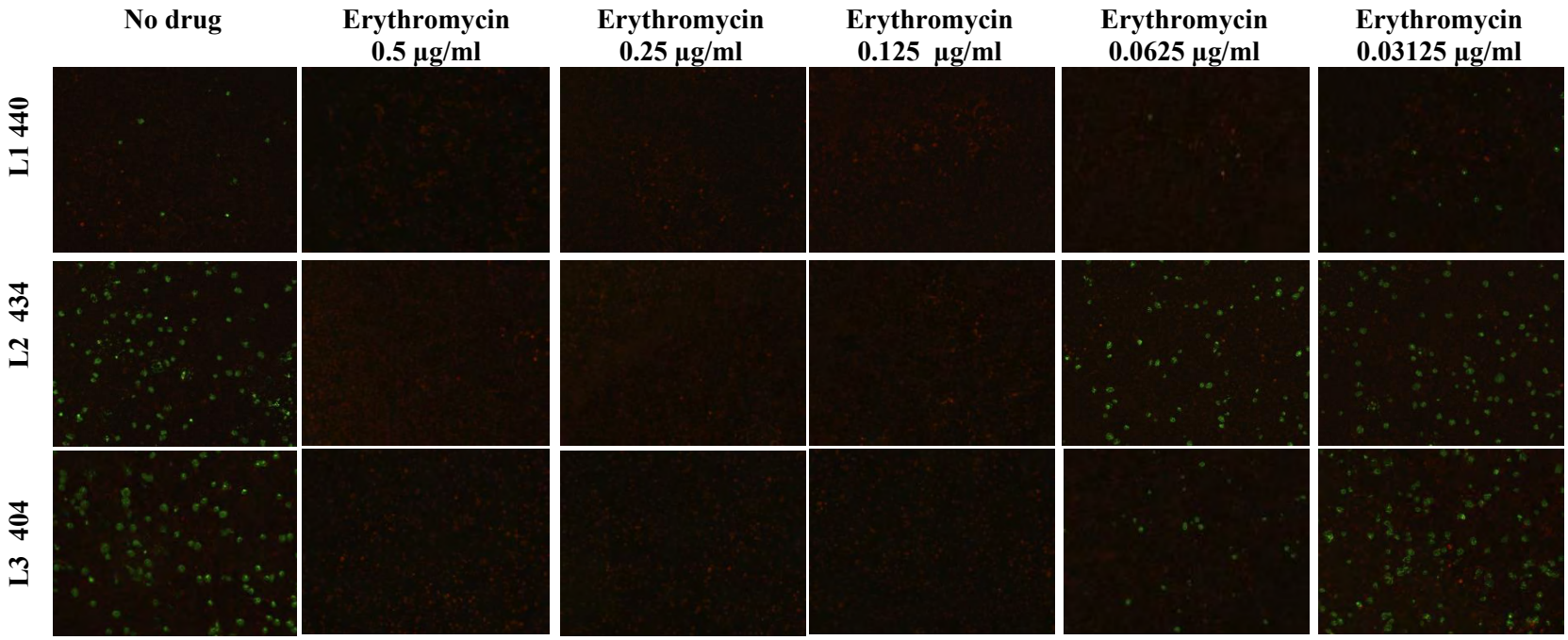
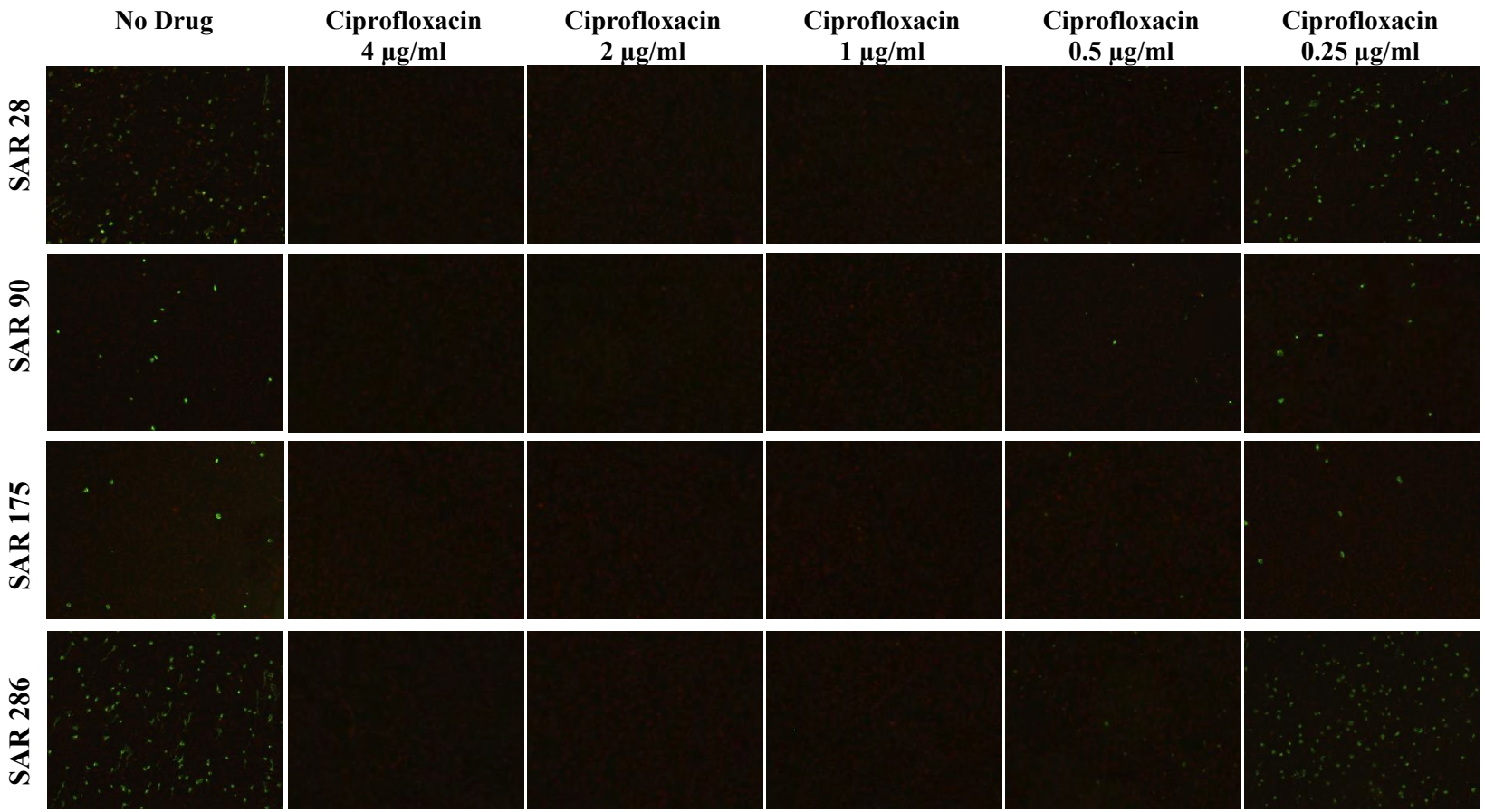

No drug
Erythromycin 0.5 µg/ml
Erythromycin 0.25 µg/ml
Erythromycin 0.125 µg/ml
Erythromycin 0.0625 µg/ml
Erythromycin 0.03125 µg/ml
L1 440
L2 434
L3 404

Figure C22


No Drug
Ciprofloxacin 4 µg/ml
Ciprofloxacin 2 µg/ml
Ciprofloxacin 1 µg/ml
Ciprofloxacin 0.5 µg/ml
Ciprofloxacin 0.25 µg/ml
SAR 28
SAR 90
SAR 175
SAR 286

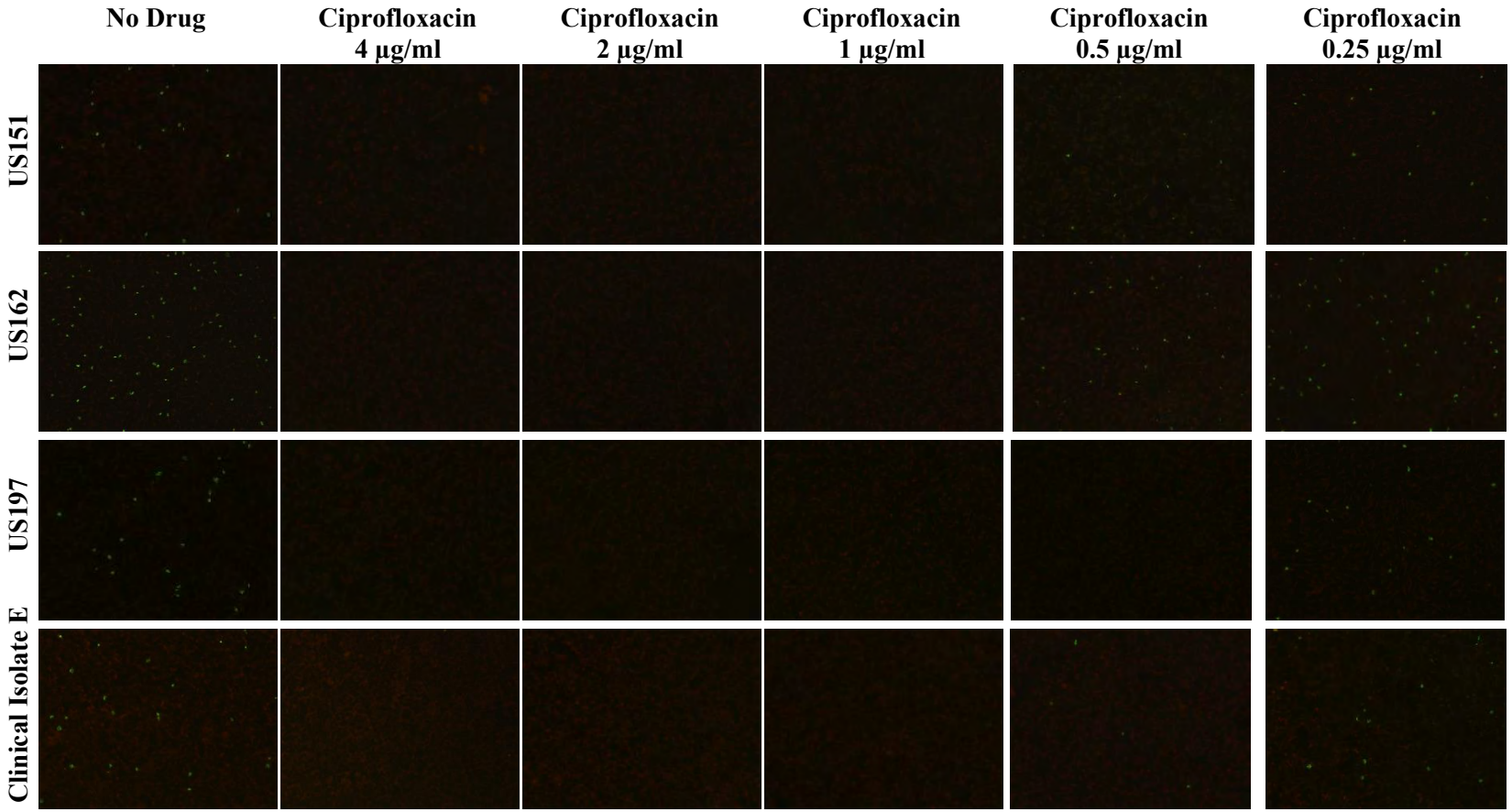

No Drug
Ciprofloxacin 4 µg/ml
Ciprofloxacin 2 µg/ml
Ciprofloxacin 1 µg/ml
Ciprofloxacin 0.5 µg/ml
Ciprofloxacin 0.25 µg/ml
US151
US162
US197
Clinical Isolate E

Figure C23

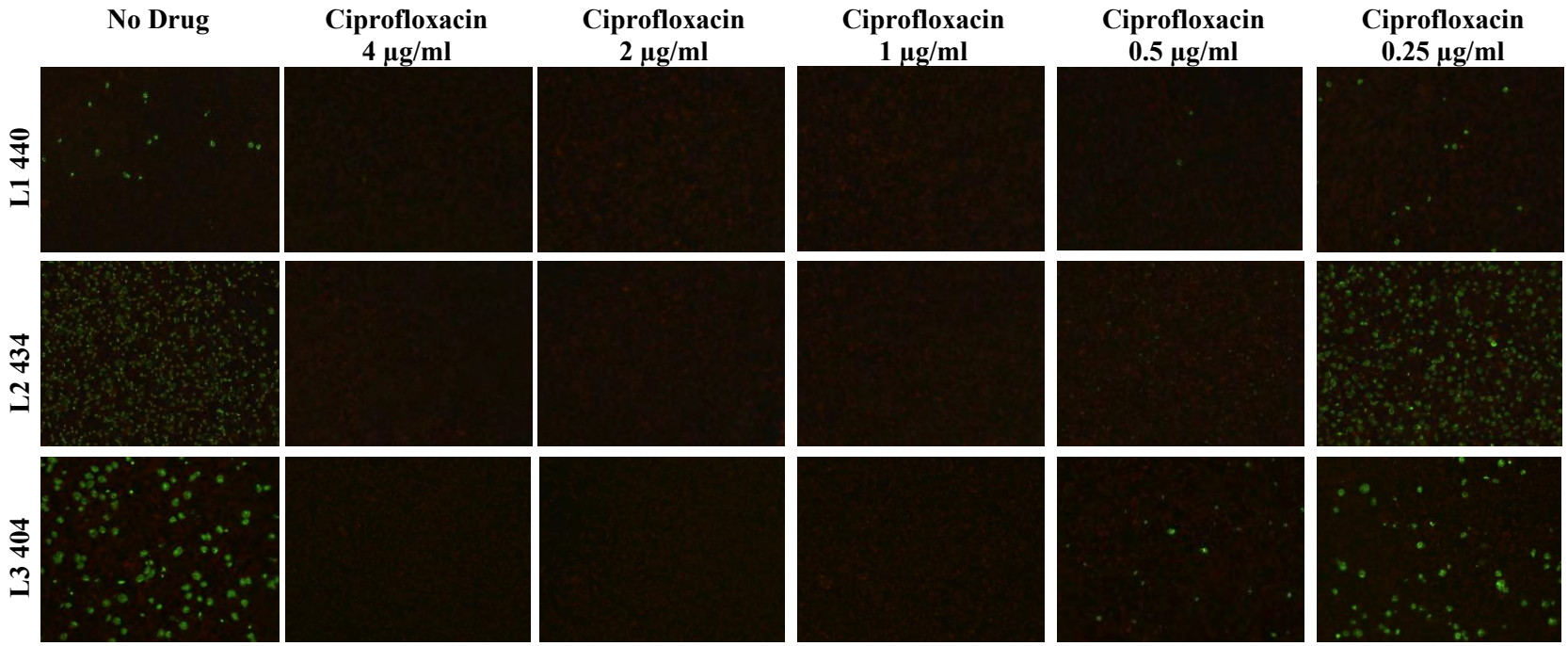

No Drug
Ciprofloxacin 4 µg/ml
Ciprofloxacin 2 µg/ml
Ciprofloxacin 1 µg/ml
Ciprofloxacin 0.5 µg/ml
Ciprofloxacin 0.25 µg/ml
L1 440
L2 434
L3 404

Figure C24