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MIC Distributions and Epidemiological Cut-off Values for Azithromycin in Neisseria gonorrhoeae as Determined by Agar Dilution

Kathryn A. Lupoli

MIC Distributions and Epidemiological Cut-off Values for Azithromycin in *Neisseria gonorrhoeae* as Determined by Agar Dilution

By

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APPROVAL PAGE

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ABSTRACT

Background: Clinical breakpoints and epidemiological cut-off values for *N. gonorrhoeae* azithromycin antimicrobial susceptibility testing have not been established. This study utilized existing minimum inhibitory concentration (MIC) data from CDC's Gonococcal Isolate Surveillance Project (GISP) to establish epidemiological cut-off values for azithromycin and *N. gonorrhoeae* as determined by agar dilution.

Methods: MIC distributions for the pooled dataset and each data year (2005-2012) were constructed. Epidemiological cut-off values were calculated using two methods. Method 1 considers the wild-type MIC distribution, the modal MIC for the distribution, and the inherent variability of the test (±1 twofold-dilution). Method 2 defines the epidemiological cut-off value as two twofold-dilutions higher than the MIC_{50}.

Results: Taking into consideration the wild-type MIC distributions and the inherent variability of the test, the epidemiological cut-off value chosen for the pooled dataset and each data year using Method 1 was ≤1.0 µg/mL. The MIC_{50} for the pooled dataset and each data year was 0.25 µg/mL. Two twofold-dilutions higher than the MIC_{50} (0.25 µg/mL) for the pooled dataset and each data year was 1.0 µg/mL.

Discussion: The epidemiological cut-off values chosen using methods 1 and 2 (≤1.0 µg/mL) were identical for the pooled dataset and each data year, indicating the epidemiological cut-off value has not changed from 2005-2012. The epidemiological cut-off value for *N. gonorrhoeae* azithromycin agar dilution antimicrobial susceptibility testing established during this study can be used to help set clinical breakpoints and identify isolates with reduced susceptibility to azithromycin.

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Curriculum Vitae

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Education

Georgia State University, College of Health and Human Sciences, School of Public Health — Atlanta, GA
Master of Public Health, Prevention Sciences — Expected Dec. 2013

The University of Georgia, Franklin College of Arts and Sciences
Bachelor of Science, Genetics — Athens, GA, May 2008

Computer Skills
Microsoft Office including Microsoft Access and Visio, EndNote, SAS, and SPSS

Research

Independent Student Researcher — Jan. - Dec. 2007
Dr. Brian Condie
Developmental Biology, The University of Georgia

- Sequenced portions of viral genome via primer walking
- Analyzed and compared sequence data of several P1-like phages to identify regions of homology between structural genes using BLAST and Vector NTI
- Presented research findings at the UGA 2008 Undergraduate Research Symposium and twice during weekly departmental laboratory meetings

Work Experience

Microbiologist — Nov. 2009 - Present
Centers for Disease Control and Prevention, Division of STD Prevention, International Affairs Unit

- Assists in the development of protocols, budgets, and supply procurement for international behavioral and surveillance studies
- Performs diagnostic quality control testing on international study samples. Assays performed include DNA extraction via Qiagen kit, Real-time multiplex PCR, TPPA, RPR, and syphilis rapid point of care tests (Bioline and Determine).
- Develops training materials for laboratory staff in study countries
- Provides in-country technical assistance and training on the above techniques- Maputo, Mozambique (August 2011), Panama City, Panama (November 2011), Lusaka, Zambia (September 2013)

Microbiology Laboratory Technician — Sept. 2008 - Nov. 2009
Centers for Disease Control and Prevention, National Antimicrobial Resistance Monitoring System

- Receives and logs isolates
- Maintains inventory
- Assists with antimicrobial susceptibility testing of *Salmonella*, *Shigella*, *E. coli*, and *Campylobacter* species

- Performs antimicrobial susceptibility testing on *Salmonella typhi*, *Enterococcus* species, and outbreak isolates
- Performs *Salmonella* serotyping using commercial antisera and Bioplex assay techniques
- Responsible for preparing isolates for phage typing and the subsequent entry of phage typing results into STARLIMS

Additional Experience

2010 Haiti Cholera Response Detail Oct. - Dec. 2010
Centers for Disease Control and Prevention, Emergency Operations Center

- Coordinated ordering and shipment of emergency laboratory supplies
- Created and maintained "Laboratory Diagnosis" section of Haiti Cholera Outbreak webpage

2010 "Principles of STD/HIV Research" summer course participant July 2010
University of Washington Center for AIDS and STD

Immigrant, Refugee, and Migrant Health Intern May 2008
Centers for Disease Control and Prevention, Division of Global Migration and Quarantine

- Assisted in the development of standard operating procedures for the United States Quarantine Stations
- Assembled information to include in the Immigrant, Refugee, and Migrant Health homepage regarding refugee facts and statistics for World Refugee Day
- Performed data entry requiring the use of several departmental databases

Publications

Sjolund-Karlsson, M., Howie, R., Krueger, A., Rickert, R., Pecic, G., K. Lupoli, Folster, J.P., & Whichard, J.M. 2010. CTX-M-producing isolates of non-Typhi Salmonella from humans in the United States. *Emerg Infect Dis*, 17(1):97-99.

Presentations

Poster Presentation, National STD Prevention Conference, Minneapolis, MN March 2012
"Field Evaluation of Dried Tube Specimens for Syphilis Rapid Point of Care Test Proficiency in Suba District, Kenya"

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CHAPTER I

INTRODUCTION

Gonorrhea is the second most commonly reported notifiable disease in the United States. It is estimated that there are over 820,000 new gonorrhea infections annually. Untreated gonorrhea infections can cause serious and permanent health conditions in both men and women. Gonorrhea can be treated with appropriate antimicrobial therapy, but increasing resistance to commonly prescribed drugs may complicate our ability to treat infections in the near future.

Because *Neisseria gonorrhoeae* has developed resistance to many antibiotic therapies used to treat gonococcal infections (penicillin, fluoroquinolones, oral cephalosporins), antimicrobial susceptibility testing is becoming increasingly important to monitor resistance trends and guide treatment. National, state, and private laboratories performing antimicrobial susceptibility testing utilize the clinical breakpoints, or interpretive criteria, established by the Clinical and Laboratory Standards Institute (CLSI) to differentiate between susceptible, intermediate, and resistant bacterial isolates. Unfortunately, clinical breakpoints have not been established for any *N. gonorrheae* azithromycin antimicrobial susceptibility testing method. CDC currently recommends azithromycin, along with single-dose injectable cephalosporin regimens, to treat uncomplicated gonococcal infections of the cervix, urethra, and rectum. As such, interpretive criteria for *N. gonorrhoeae* azithromycin antimicrobial susceptibility testing are needed to monitor resistance and guide treatment regimens.

CLSI's methodology for establishing clinical breakpoints requires microbiological, pharmokinetic, and clinical data. In the absence of established clinical breakpoints, epidemiological cut-off values can be used to identify isolates without resistance mechanisms

(wild type) from non-wild type isolates. This study will utilize existing Minimum Inhibitory Concentration (MIC) data from CDC's Gonococcal Isolate Surveillance Project (GISP) to establish epidemiological cut-off values for azithromycin and *N. gonorrhoeae* as determined by agar dilution.

Research Questions

1. Do the MIC distributions for *N. gonorrhoeae* azithromycin agar dilution antimicrobial susceptibility testing differ from 2005-2012?
2. What is the epidemiological cut-off value for azithromycin for 2005-2012? Does the epidemiological cut-off value differ from 2005-2012?

CHAPTER II

REVIEW OF THE LITERATURE

2.1 *Neisseria gonorrhoeae* and Gonococcal Infection

Neisseria gonorrhoeae (gonococci) is the bacterium that causes gonorrhea and its associated clinical syndromes. Gonorrhea is transmitted through vaginal, oral, or anal sex and can also be transmitted from a mother to her unborn baby during childbirth. The World Health Organization (WHO) estimated there were 106.1 million new cases of gonorrhea infections worldwide in adults in 2008 (1). Gonorrhea is the second most commonly reported notifiable infection in the United States. There were 321,849 cases reported in 2011 yielding a 4% increase in incidence from 2010. Rates are highest in the Southern region of the United States, among women, and persons 20-24 years of age (2).

The broad spectrum of clinical manifestations includes symptomatic and asymptomatic local infection, complicated local infection, and systemic infection. While the majority of uncomplicated gonococcal infections are asymptomatic, the most common presentation of gonococcal infection in men is acute anterior urethritis accompanied by urethral discharge and/or dysuria (painful urination). Untreated gonococcal infections typically resolve over a period of several weeks and most patients become asymptomatic within six months. Complications, while rare in developed countries, include epididymitis, lymphangitis, penile edema, acute or chronic prostatitis, and periurethral abscesses (3).

The most common infection site in women is the endocervical canal, but urethral colonization followed by infection of the periurethral (Skene's) gland or Bartholin's gland ducts are also common in the absence of endocervical infection. Symptoms may include increased

vaginal discharge, dysuria, intermenstrual uterine bleeding, and heavy or prolonged menstrual periods (menorrhagia) (4). Pelvic inflammatory disease (PID), or the infection and inflammation of the upper genital tract, is the most common complication of gonorrhea in women. PID is accompanied by endometritis, tubo-ovarian abscess, or pelvic peritonitis and is also the most important complication in terms of public health impact due to its associated long term sequelae which include infertility, ectopic pregnancy, and chronic pelvic pain. It is estimated that between 10-20% of women with gonorrhea develop PID, and approximately eight percent of women in the United States develop PID in their lifetime. Prevalence is much higher in developing countries with rates as high as 32% (5-6).

Rectal infection is common in men who have sex with men (MSM), and up to 60% of women with gonococcal cervicitis also have infection of the rectal mucosa. While infection of the rectum in women is normally asymptomatic, rectal infection in men can be associated with overt proctitis (7). Isolated pharyngeal infection has been documented in 3-7% of heterosexual men, 10-20% of heterosexual women, and 10-25% of men who have sex with men. The majority of infections are asymptomatic, but association with acute pharyngitis, tonsillitis, fever, or cervical lymphadenopathy has been reported (4).

Gonococcal conjunctivitis and primary cutaneous gonorrhea, characterized by localized ulcers of the genitals and skin lesions, are rare (4). Disseminated (or systemic) gonococcal infection (DGI) is also rare and occurs in 0.2-1.9% of cases. DGI can occur in both males and females, but incidence is thought to be higher in females. Symptoms include fever, joint pain, skin rashes, and tenosynovitis. In very rare cases, disseminated gonococcal infection may progress to endocarditis (8).

N. gonorrhoeae and *Chlamydia trachomatis* co-infection is common. One cross sectional study of new clients presenting to a hospital-based STD clinic in the United Kingdom found that 39% of 1,239 women and 24% of 1,141 heterosexual men with gonorrhea also had chlamydia. In addition, more than half of the women and a third of the men 15-19 years of age were co-infected (9). Similarly, a study of adolescents entering selected United States detention centers found that 54% of females and 51% of males with gonorrhea were also infected with chlamydia (10). It is also widely understood that sexually transmitted infections (STIs) such as gonorrhea increase the risk of acquiring and transmitting human immunodeficiency virus (HIV) by two to five-fold and that aggressive STI prevention, screening, and treatment reduces the transmission of HIV (11).

There are several populations that have a higher risk of acquiring STIs or experiencing adverse health outcomes as a result of acquiring an STI. It is estimated that young people 15-24 years of age account for more than half of new STI cases in the United States. Risk factors such as engagement in high-risk sexual behaviors and barriers to accessing quality STI prevention services and care (concerns about confidentiality, lack of health insurance or ability to pay) increases adolescents' and young adults' risk of acquiring gonorrhea (2). MSM represent an estimated 2% of the United States male population, but they account for 59% of the people living with HIV in the country. Because the risk factors that contribute to the transmission of STIs (higher number of lifetime sex partners, higher partner acquisition rates, unprotected sex) also increase the acquisition and transmission of HIV, MSM also bear a disproportionately high burden of STIs (12, 13).

Gonorrhea affects approximately 13,200 pregnant women annually (14). Untreated infections can lead to adverse pregnancy outcomes such as early onset of labor, spontaneous preterm birth,

low birth weight, preterm rupture of membranes surrounding the uterus, miscarriage, and stillbirth (15-17). Gonorrhea can also be transmitted from an infected mother to her baby during delivery. Gonococcal ophthalmia neonatorum is the most common presentation of gonorrhea in neonates, but scalp abscesses, wound infections, systemic disease (meningitis and sepsis), and colonization of the oropharynx and gastric fluid can also occur (18). Genital infection in children is rare and typically acquired through sexual abuse (19).

2.2 History of Treatment and Antibiotic Resistance Trends

Silver proteinate or Protargol was used to treat gonorrhea from the late 1890's up to the introduction of antibiotics in the mid 1930's. The sulfonamides were the first effective antimicrobials against gonorrhea, but resistance was widespread by the mid-1940's. Penicillin became the first line drug for treatment in 1943. Within 10-15 years treatment failures had been reported, and higher doses were required for successful treatment. By 1989 penicillin was no longer recommended for the treatment of gonorrhea. Streptomycin and chloramphenicol (introduced in 1949), erythromycin (introduced in 1952), spectinomycin (introduced in 1961), and tetracycline (introduced in 1962) were used when treatment with penicillin was contraindicated, but strains resistant to these antibiotics emerged rapidly due to chromosomal mutations and other gene acquisition events. By the late 1980's most of these alternatives were no longer recommended for treatment (20).

Fortunately third generation cephalosporins such as ceftriaxone and cefixime and fluoroquinolones such as ciprofloxacin were highly effective against gonococci. By 1985 ceftriaxone became the recommended treatment for uncomplicated gonococcal infections, and in 1993, the oral fluoroquinolones (ciprofloxacin and ofloxacin) and cefixime were the

recommended first line treatment (21). Resistance to fluorquinolones was recorded as early as the mid-1990's in South East Asia and in the United States as early as 1991 (Hawaii). Increasing resistance to fluoroquinolones in the United States prompted the CDC to recommend the use of cephalosporins over fluorquinoloes in Hawaii and California in 2002. This recommendation was later expanded to include MSM in 2004, and fluoroquinolones were no longer recommended in the United States by 2007 (22).

In recent years decreased susceptibility to third generation cephalosporins has been reported from Asia and the Pacific region as well as Europe, Canada, and the United States. Cefixime treatment failures were first reported in Japan in 2003 and have subsequently been reported in the United Kingdom, Norway, Austria, and France. Widespread resistance to oral cephalosporins prompted Japan to discontinue the use of cefixime for the treatment of gonorrhea in 2006.

In 2009, a strain with high-level ceftriaxone resistance was isolated in Kyoto, Japan from a woman presenting with pharyngeal gonococcal infection. Subsequently, isolates with high-level resistance to ceftriaxone have been identified in men with urogenital infections in Spain and France (23). While ceftriaxone treatment failures have not yet been documented in the United States, data from CDC's GISP suggest the number of isolates with elevated ceftriaxone MIC's has markedly increased since 2006, particularly in the western region and among MSM (24). CDC's current treatment guidelines recommend combination therapy with 250 mg ceftriaxone intramuscularly and either 1 g azithromycin orally as a single treatment or 100 mg doxycycline twice daily for seven days (25).

2.3 Azithromycin and the Treatment of Gonorrhea

Azithromycin is an azalide drug derived from the macrolide class of antibiotics. The mechanism of action is inhibition of RNA-dependent peptide synthesis of bacteria by binding to the 50s ribosomal subunit. Azithromycin became available for the treatment of gonorrhea in 1983, and it has proven effective as a single dose alternative to oral cephalosporins in combination with ceftriaxone. It has also proven highly effective against pharyngeal infection, genital co-infection with *C. trachomatis*, and penicillin-resistant strains (26). Azithromycin, in combination with ceftriaxone, is one of the currently recommended first-line treatment regimens for uncomplicated gonococcal infections in the United States.

Resistance to azithromycin was first reported in the United States in New Mexico in 1993. The first isolate demonstrating high level resistance in the United States was identified in Hawaii in 2011 (27); however, data from GISP suggests the proportion of isolates with high level resistance to azithromycin remains low (28).

2.4 The Gonococcal Isolate Surveillance Project (GISP)

CDC established a national sentinel surveillance system known as the Gonococcal Isolate Surveillance Project (GISP) in 1986 to 1) to monitor trends in antimicrobial resistance in *N. gonorrhoeae* strains, 2) to characterize male patients with gonorrhea, especially those infected with strains that are resistant to currently recommended therapies, and 3) to describe the diversity of antimicrobial resistance in *N. gonorrhoeae* by phenotypically characterizing resistant isolates via the agar dilution method. This method provides an MIC (vs. zone diameter), is well-characterized, and well-standardized, and as such, is considered the gold standard for *N. gonorrhoeae* antimicrobial susceptibility testing. CDC prepares and distributes an annual report

of the project's findings, and data generated by GISP is used to inform selection of therapies for gonococcal infection and to revise CDC's STD Treatment Guidelines (29).

2.5 Clinical Breakpoints and Epidemiological Cut-off Values

Clinical breakpoints (CBPs) are values used by laboratories to classify MIC or zone inhibition data generated from antimicrobial susceptibility assays into clinically relevant categories i.e., susceptible, intermediate, or resistant to a particular antimicrobial agent (30). Clinical breakpoints influence local, regional, and national treatment guidelines by categorizing the susceptibility of previously tested isolates for a particular antimicrobial. In addition, clinical breakpoints guide empiric treatment (31).

The Clinical and Laboratory Standards Institute (formerly known as the National Committee for Clinical Laboratory Standards), established in 1968, is the international organization responsible for developing the clinical laboratory testing standards used in the United States. CLSI laboratory standards are established utilizing input from and consensus among government, industry, and healthcare professionals. CLSI's methodology for establishing clinical breakpoints requires four main data types: (i) MIC (minimum inhibitory concentration) distributions and epidemiological cut-off values (ECVs); (ii) phenotypic and genotypic in vitro resistance markers; (iii) pharmokinetic data from animal models and human studies; and (iv) clinical and bacteriological outcome data from clinical studies (32).

Currently there are no CLSI-established azithromycin clinical breakpoints for any *N. gonorrhoeae* antimicrobial susceptibility testing method. In the absence of established clinical breakpoints, epidemiological cut-off values can serve as a guide to differentiate wild-type strains from strains with acquired resistance mechanisms. The construction of MIC distributions and the

determination of epidemiological cut-off values is the first step in the development of clinical breakpoints for azithromycin agar dilution antimicrobial susceptibility testing for *N. gonorrhoeae*.

CHAPTER III

METHODS

3.1 Definitions

MIC, or minimum inhibitory concentration, is defined as the lowest concentration of antibiotic needed to inhibit visible growth of a microorganism in a laboratory. **Wild-type,** in the context of this study, is defined as lacking acquired or mutational antibiotic resistance mechanisms. **ECV**, or epidemiological cut-off value (synonymous with wild-type cut-off value), is defined as the MIC value which best describes the end of the wild-type distribution. It is expressed as ECV ≤ X μg/mL (33). **Pooled dataset** refers to data from 2005-2012. **Raw MIC data** refers to untransformed MIC values. **MIC_{50}** is defined as the value at which 50% of the isolates are inhibited. **MIC_{99}** is defined as the value at which 99% of the isolates are inhibited.

3.2 GISP Specimen Collection and Agar Dilution Susceptibility Testing

GISP sentinel STD clinics and regional laboratories are chosen for a 5-year term via an application process administered by a CDC Funding Opportunity Announcement (FOA). STD clinics and laboratories chosen as GISP sentinel sites and regional laboratories receive funding from CDC to assist with GISP program requirements. Each month around 24 sentinel STD clinics submit urethral isolates from the first 25-30 male patients presenting with urethral gonococcal infection as well as clinical and demographic data to one of five regional laboratories. The five current regional laboratories, located in Atlanta, Austin, Birmingham, Cleveland, and Seattle, test the isolates for β-lactamase production via the Nitrocefin test and antimicrobial susceptibility (minimum inhibitory concentrations, MIC's) to penicillin G,

tetracycline, spectinomycin, cefixime, ceftriaxone, ciprofloxacin, and azithromycin via the agar dilution method. Results are reported to CDC on a monthly basis, and any isolate meeting Alert Value MIC criteria (currently ≥ 2.0 μg/mL for azithromycin) undergoes confirmatory retesting by the regional laboratory (34).

3.3 GISP Azithromycin MIC Data

Azithromycin was added to the GISP panel in 1992, and azithromycin MIC data is available from 1992-2012. In 2005 there was a change in media used for agar dilution testing among all GISP regional laboratories. This media change resulted in an observational shift of the MIC distribution approximately equal to one twofold-dilution higher (35). Data from 1992-2004 were excluded from this analysis to ensure the consistency of laboratory methods for data collection.

3.4 MIC Distributions

MIC distributions for azithromycin were constructed for 2005-2012 using Microsoft Excel v.2010.

3.5 Statistical Analysis

All statistical analyses were performed using SAS/STAT® v.9.3 software (SAS Institute, Cary, North Carolina, USA). The univariate procedure was used to fit pooled azithromycin MIC values to normal and lognormal curves to test for normality. Data were then $\log_2$ transformed as described previously, and the univariate procedure was again used to fit $\log_2$-transformed MIC values to normal and lognormal curves to test for normality. Levene's test (glm procedure) and

one-way analysis of variance (ANOVA) were used with raw MIC data to test for homogeneity of variances and differences in mean azithromycin MIC from 2005-2012 (36). The Kruskal- Wallis test was conducted on raw MIC data using the npar1way procedure to test for underlying differences in the azithromycin MIC distributions from 2005-2012 ($\alpha = 0.05$). The Wilcoxon-Mann-Whitney test was then used to test for differences in the azithromycin MIC distribution from year to year (i.e., 2005-2006, 2006-2007, etc.) using raw MIC data ($\alpha = 0.05$). The univariate procedure was then used to calculate the median, mode, MIC_{50}, MIC_{90}, and MIC_{99} for each data year and the pooled dataset.

3.6 Calculation of Epidemiological Cut-off Values

Two methods were used to calculate epidemiological cut-off values. Method 1, often called the "eyeball method," considers the wild-type MIC distribution, the modal MIC for the distribution, and the inherent variability of the test (±1 twofold-dilution) to determine the epidemiological cut-off value. In addition, the epidemiological cut-off value should encompass at least 95% of the isolates in the wild-type distribution (37). The mode and modal MIC ±1 twofold-dilution were calculated for each data year. MIC values that 1) were larger than the modal MIC +1 twofold-dilution and 2) included at least 95% of isolates in the wild-type distribution were used to identify epidemiological cut-off values via visual inspection of each MIC distribution. Method 2 defines the epidemiological cut-off value as two twofold-dilutions higher than the MIC_{50} (38).

CHAPTER IV

RESULTS

4.1 MIC Distributions and Descriptive Statistics

Azithromycin MIC distributions for data years 2005-2006 can be found below in Figures 1-8.

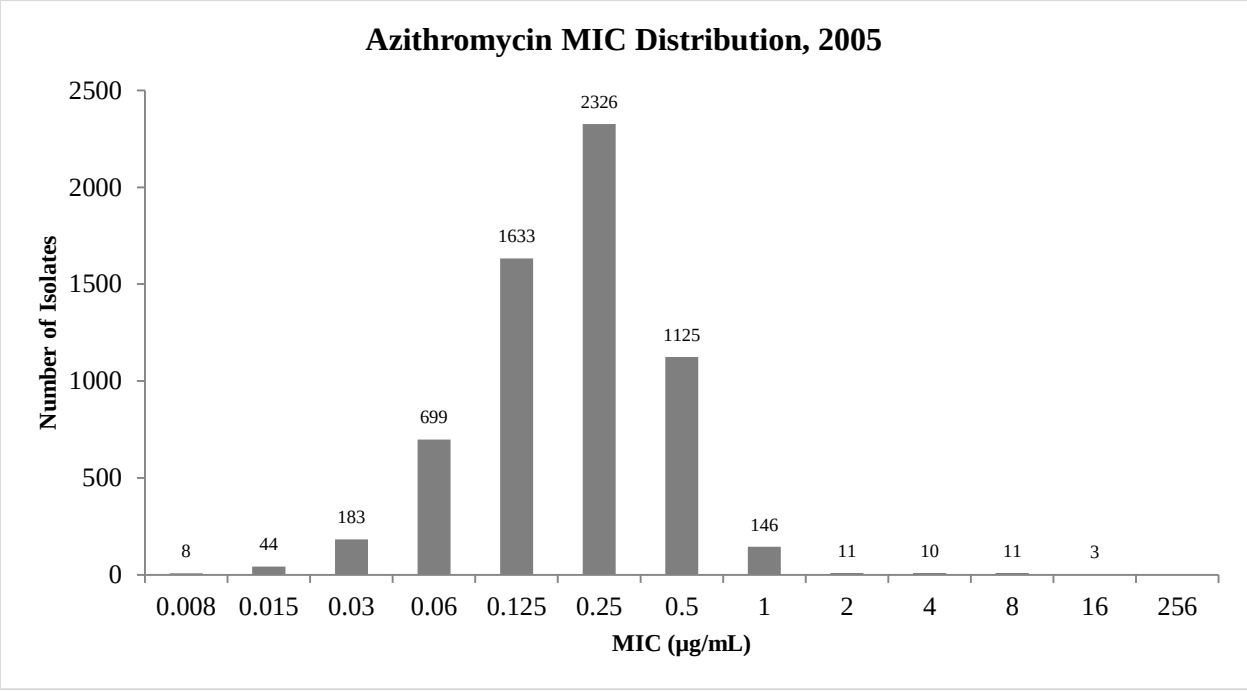

Figure 1. MIC distribution for azithromycin, 2005.

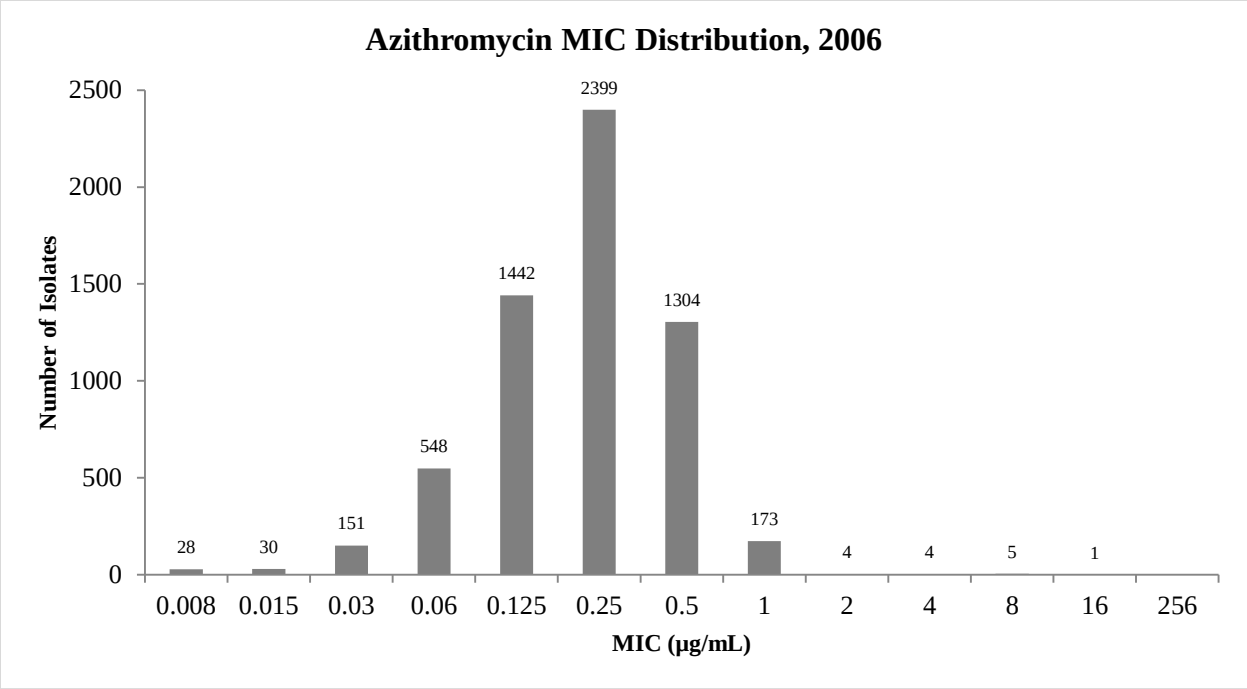

Figure 2. MIC distribution for azithromycin, 2006.

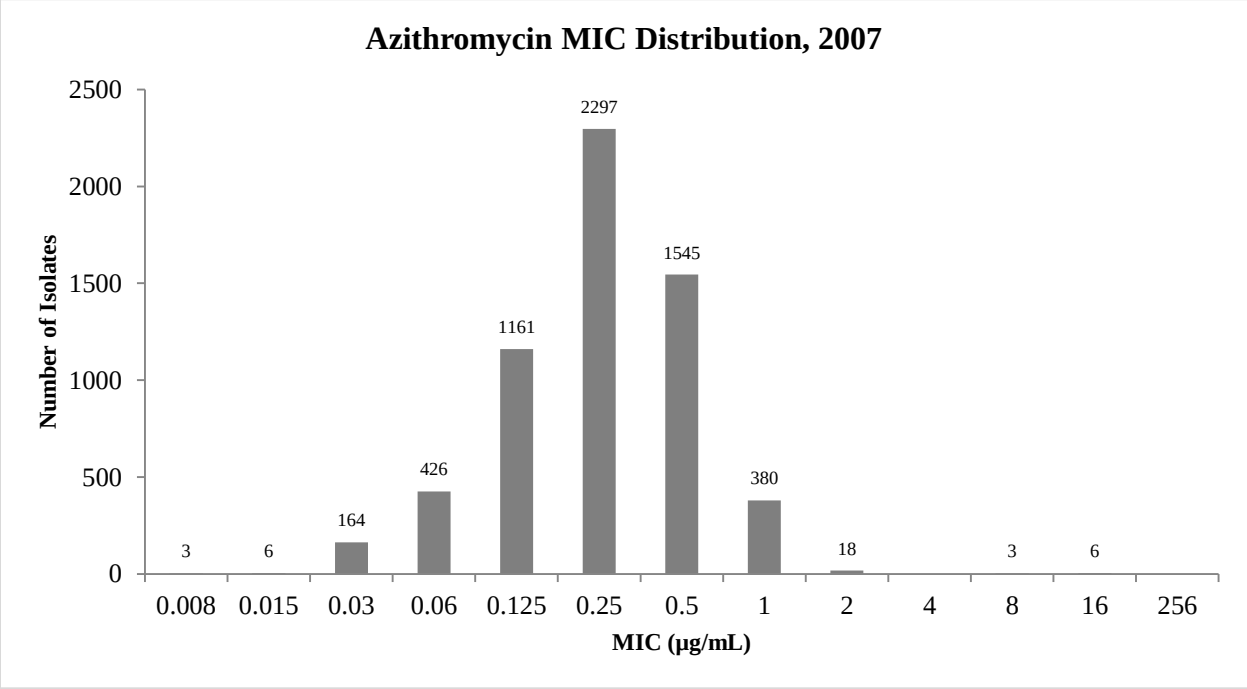

Figure 3. MIC distribution for azithromycin, 2007.

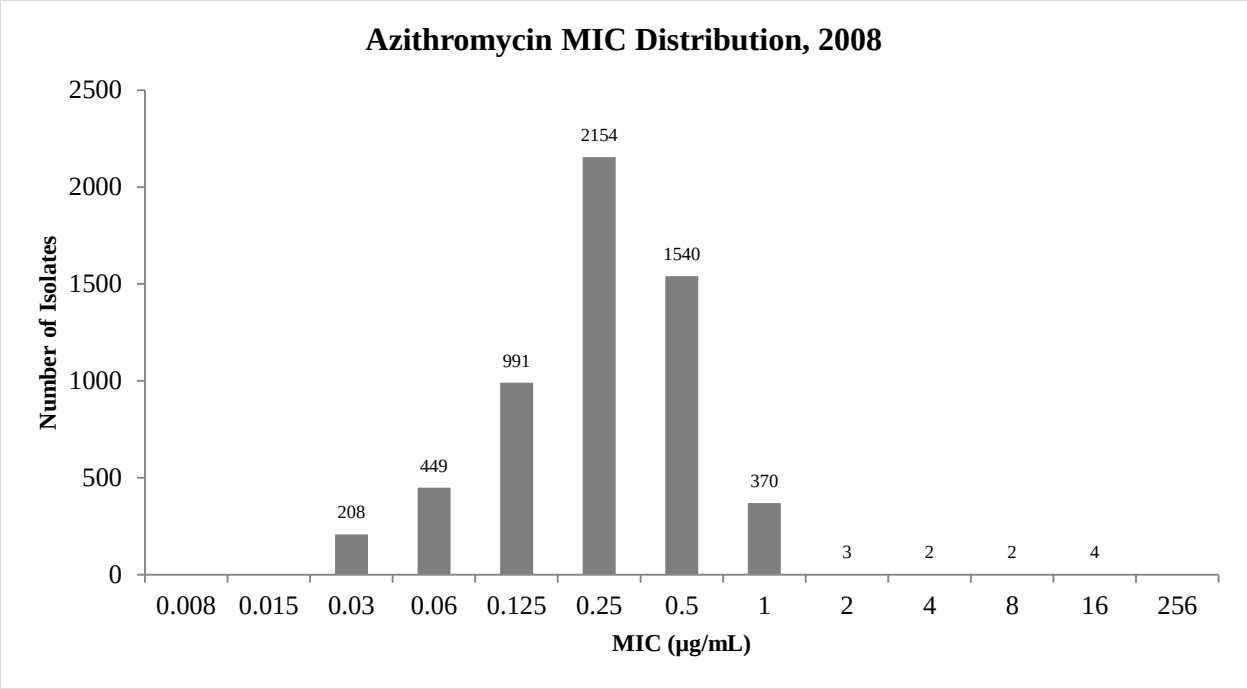

Figure 4. MIC distribution for azithromycin, 2008.

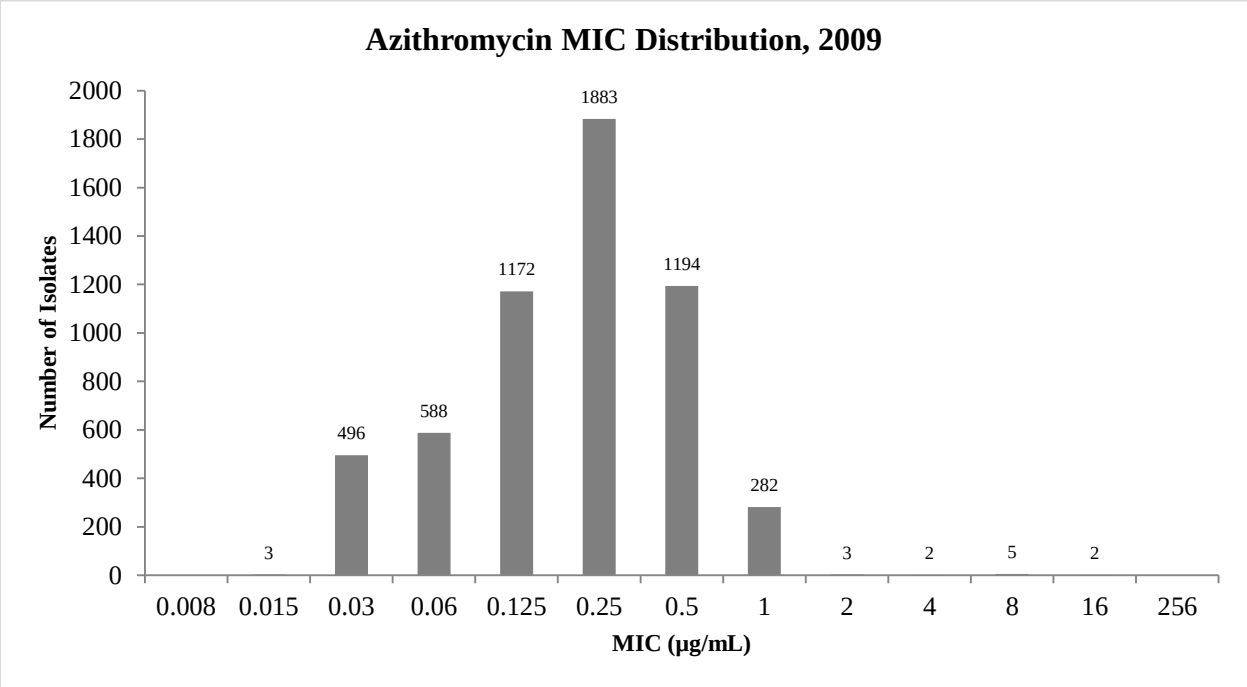

Figure 5. MIC distribution for azithromycin, 2009.

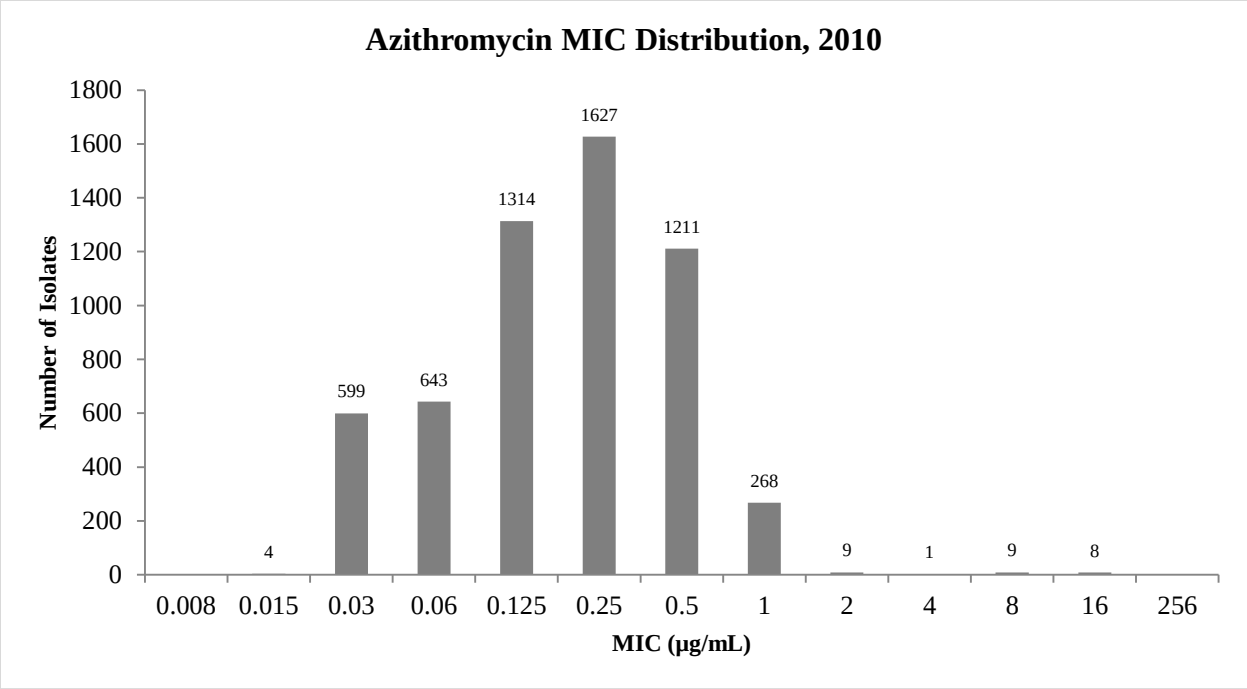

Figure 6. MIC distribution for azithromycin, 2010.

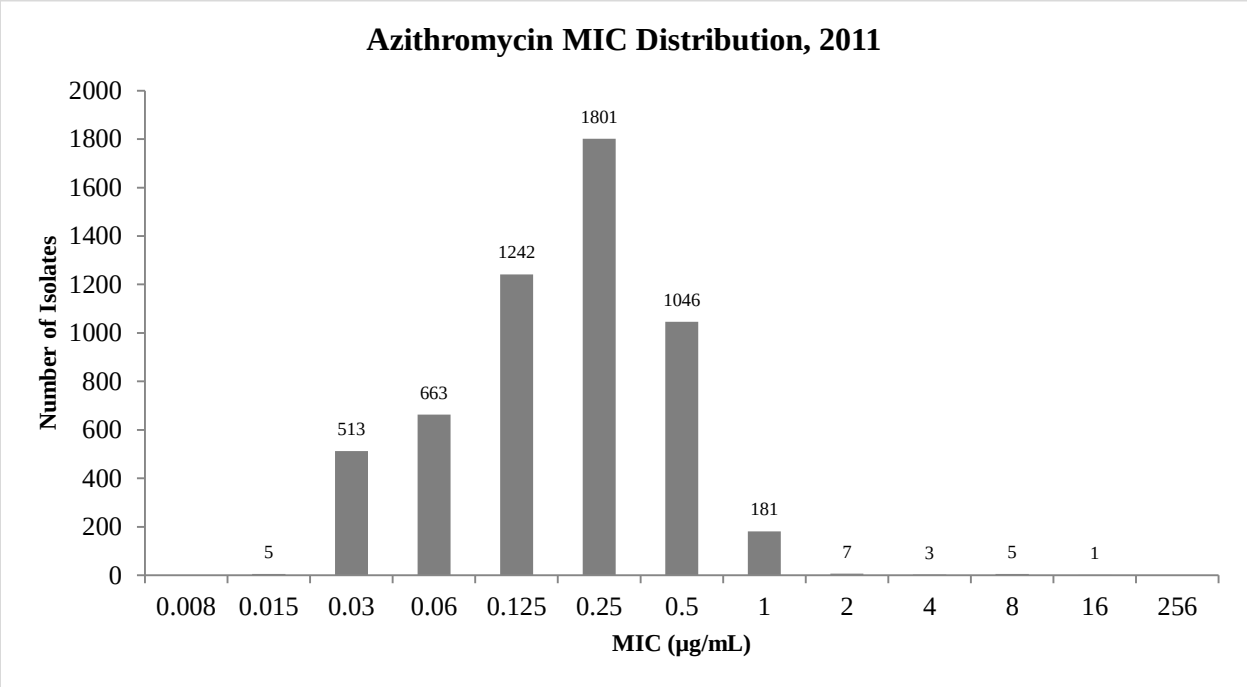

Figure 7. MIC distribution for azithromycin, 2011.

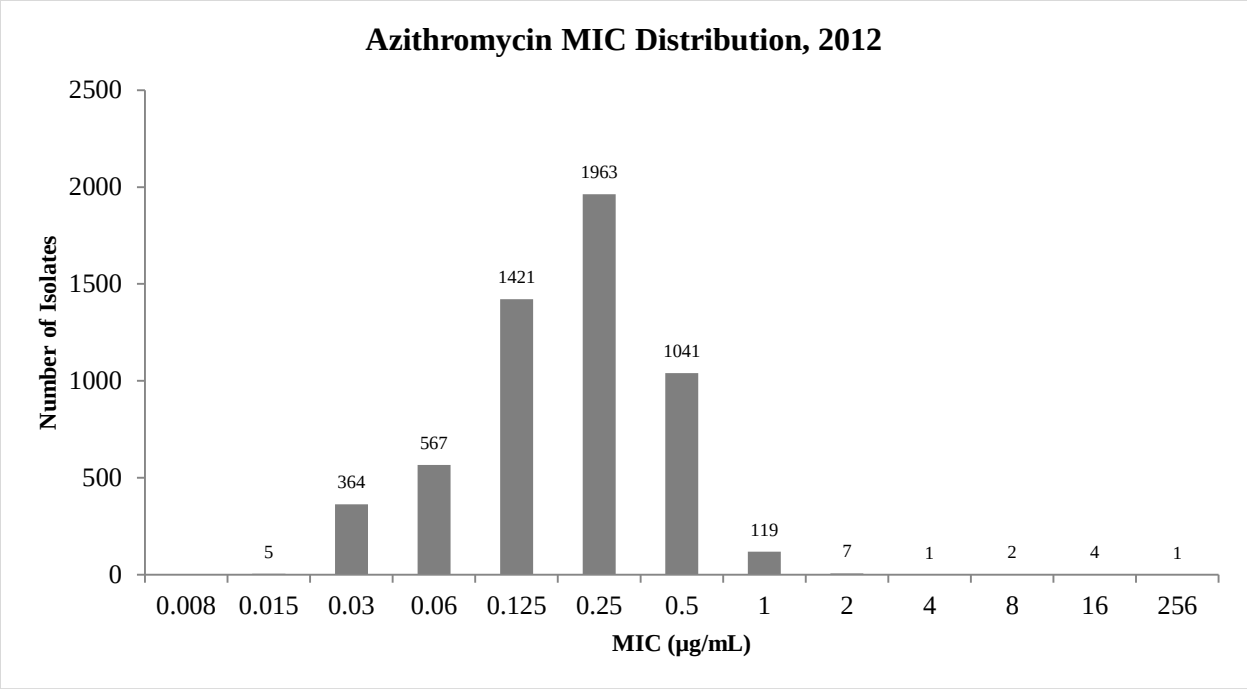

Figure 8. MIC distribution for azithromycin, 2012.

There were 6,199 observations in 2005, 6,089 observations in 2006, 6,009 observations in 2007, 5,723 observations in 2008, 5,630 observations in 2009, 5,693 observations in 2010, 5,467 observations in 2011, and 5,495 observations in 2012 for a total of 46,305 observations from 2005-2012. The mean MIC values for each data year, 2005-2012, were 0.281 µg/mL, 0.283 µg/mL, 0.343 µg/mL, 0.337 µg/mL, 0.290 µg/mL, 0.303 µg/mL, 0.264 µg/mL , and 0.311 µg/mL, respectively. The mean MIC value for the pooled dataset was 0.301 µg/mL. The median azithromycin MIC (or MIC_{50}) for each data year and the pooled dataset was 0.250 µg/mL. Descriptive statistics are summarized in Table 1.

Table 1. Descriptive statistics, GISP azithromycin MIC data, 2005-2012.

	N	Mean (µg/mL)	Median (µg/mL)	Mode (µg/mL)	Range (µg/mL)
2005	6,199	0.281	0.250	0.250	15.99
2006	6,089	0.283	0.250	0.250	15.99
2007	6,009	0.343	0.250	0.250	15.99
2008	5,723	0.337	0.250	0.250	15.97
2009	5,630	0.290	0.250	0.250	15.99
2010	5,693	0.303	0.250	0.250	15.99
2011	5,467	0.265	0.250	0.250	15.99
2012	5,495	0.311	0.250	0.250	255.99
2005-2012	46,305	0.301	0.250	0.250	255.99

4.2 Goodness-of-fit Tests for Normality and Equal Variances

There is statistically significant evidence that the pooled dataset is not normally distributed. The azithromycin MIC distribution for 2005-2012 (pooled dataset) fit poorly to normal and lognormal distributions as indicated by the Anderson-Darling goodness-of-fit tests (Table 2). The p values for both normal and lognormal distributions were <0.005 ($\alpha = 0.05$). Log_2 transformation of MIC values did not improve fit for either distribution. Levene's test for homogeneity of variance indicated equal variances among the eight data years (2005-2012) with an F statistic of 1.06 and a p value of 0.3883 ($\alpha = 0.05$).

Table 2. Anderson-Darling goodness-of-fit tests, GISP azithromycin MIC data, 2005-2012.

	A^2	p (α =0.05)
Normal Distribution	12361.38	<0.005
Lognormal Distribution	1515.70	<0.005

4.3 Tests for Differences in Azithromycin MIC Distributions

ANOVA indicated the mean azithromycin MIC differed significantly among the data years, 2005-2012 (F statistic = 2.62 and p = 0.0104; $\alpha = 0.05$). While the median MIC for the pooled dataset and each data year were equivalent at 0.25 µg/mL, the Kruskal-Wallis test also indicated a statistically significant difference in the underlying azithromycin MIC distributions for the pooled dataset ($\chi^2 = 849.87$; p = <0.0001). There were no statistically significant differences in the azithromycin MIC distributions from 2007-2008 (Z = 0.44; p =0.6585), 2010-2011 (Z = -1.50; p = 0.1330), and 2011-2012 (Z = -1.89; p = 0.0593). Table 3 shows the results of the Wilcoxon-Mann-Whitney two-sample comparisons.

Table 3. Comparison of azithromycin MIC distributions using the Wilcoxon-Mann-Whitney two-sample test, 2005-2012.

	Z	**p (α =0.05)**
2005-2006	6.35	<0.0001
2006-2007	10.91	<0.0001
2007-2008	0.44*	0.6585*
2008-2009	-13.26	<0.0001
2009-2010	3.55	0.0004
2010-2011	-1.50*	0.1330*
2011-2012	-1.89*	0.0593*

***No statistically significant difference in azithromycin MIC distributions.

4.4 Epidemiological Cut-off Values (ECVs)

As referenced in Table 1, the modal MIC for the pooled dataset and each azithromycin MIC distribution (2005-2012) was 0.25 µg/mL. The modal MIC ±1 twofold-dilution for the pooled dataset and each data year ranged from 0.125 to 0.5 µg/mL. Taking into consideration the wild-type MIC distributions (Figures 1-8) and the inherent variability of the test, the epidemiological cut-off value chosen for the pooled dataset and each data year using Method 1

was ≤1.0 µg/mL (Table 4). This cut-off encompassed 99% of the MICs in the pooled dataset and each data year.

The MIC_{50} for the pooled dataset and each data year was 0.25 µg/mL. Two twofold-dilutions higher than the MIC_{50} (0.25 µg/mL) for the pooled dataset and each data year was 1.0 µg/mL. The epidemiological cut-off value chosen for the pooled dataset and each data year using Method 2 was ≤1.0 µg/mL (Table 4). Again, the chosen epidemiological cut-off value encompassed 99% of MICs in the pooled dataset and each data year.

The epidemiological cut-off values were identical for Methods 1 and 2 for each data year and the pooled dataset (Figure 9).

Table 4. ECVs obtained from GISP Azithromycin MIC† data, 2005-2012

Year	N	Mode	MIC_{50}	MIC_{99}	Method 1 ECV*	Method 2 ECV**
2005	6,199	0.25	0.25	1.0	**1.0**	**1.0**
2006	6,089	0.25	0.25	1.0	**1.0**	**1.0**
2007	6,009	0.25	0.25	1.0	**1.0**	**1.0**
2008	5,723	0.25	0.25	1.0	**1.0**	**1.0**
2009	5,630	0.25	0.25	1.0	**1.0**	**1.0**
2010	5,693	0.25	0.25	1.0	**1.0**	**1.0**
2011	5,467	0.25	0.25	1.0	**1.0**	**1.0**
2012	5,495	0.25	0.25	1.0	**1.0**	**1.0**
2005-2012	46,305	0.25	0.25	1.0	**1.0**	**1.0**

†MIC (µg/mL)
*ECV considers the wild-type MIC distribution, the modal MIC, the inherent variability of the test (±1 twofold dilution), and should encompass at least 95% of isolates in the wild-type distribution.
**ECV = two twofold dilution steps higher than MIC_{50}

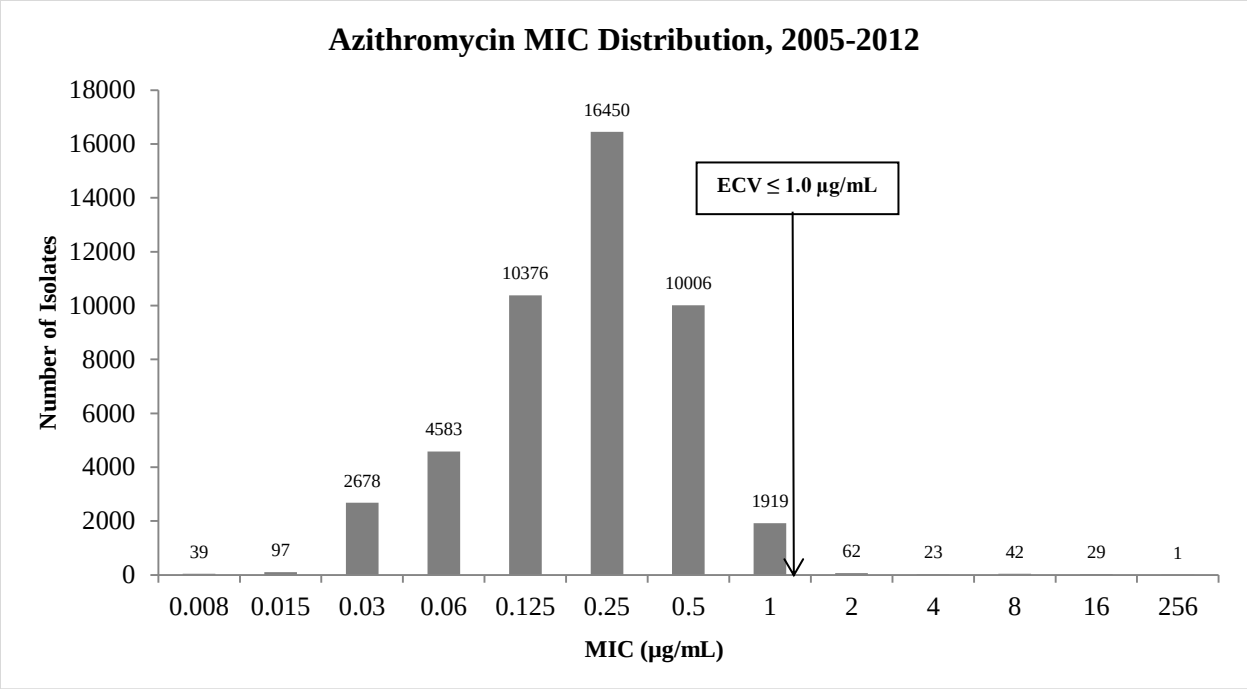

Figure 9. Epidemiological cut-off value for azithromycin, 2005-2012.

CHAPTER V

DISCUSSION

5.1 Implications

This study aimed to 1) determine if azithromycin MIC distributions for *N. gonorrhoeae* have changed from 2005-2012 and 2) to calculate epidemiological cut-off values for 2005-2012 GISP azithromycin agar dilution MIC data and determine whether or not the epidemiological cut-off values differ during this time period. A statistically significant difference in the underlying MIC distributions was observed between the eight data years; however, no statistically significant differences were found in MIC distributions from 2007-2008, 2010-2011, and 2011-2012. Interestingly, while the Wilcoxon-Mann-Whitney test demonstrated statistically significant differences in the MIC distributions from 2005-2006, 2006-2007, 2008-2009, and 2009-2010, the median azithromycin MIC for the pooled data set and each data year were identical at 0.25µg/mL. This is most likely due to the large sample sizes as the difference in ranks were large enough to be significant despite equal medians.

The epidemiological cut-off value selected for each data year and the pooled dataset was ≤1.0 µg/mL indicating the epidemiological cut-off value did not change from 2005-2012. While the Kruskal-Wallis test indicated a statistically significant difference in the underlying MIC distributions from 2005-2012, the lack of change in epidemiological cut-off value indicates the wild-type distribution has not shifted during this time period. In addition, the epidemiological cut-off values chosen using Method 1 and Method 2 were identical, suggesting that, for this dataset, both methods were comparable. Using this epidemiological cut-off value, isolates with

an MIC less than or equal to 1.0 µg/mL may be considered wild-type, whereas isolates with an MIC of 2.0 µg/mL or higher may have decreased susceptibility to azithromycin.

5.2 Future Considerations

More robust statistical procedures for the calculation of epidemiological cut-off values have been described previously for different bacteria-antimicrobial combinations, but comparison studies of these methods to Methods 1 and 2 used in this study show comparable results, usually within one twofold-dilution (39-42). As a result, only methods 1 and 2 were used for this analysis. Application of these statistical methods may be useful in the future for the establishment of clinical breakpoints for azithromycin agar dilution antimicrobial susceptibility testing for *N. gonorrhoeae*. In addition, genotypic markers of resistance were not included in this study. Examination of MIC distributions in tandem with molecular markers of resistance can provide a better understanding of the clinical importance of isolates with reduced susceptibility to azithromycin by confirming if such isolates harbor resistance mechanisms. Lastly, agar dilution antimicrobial susceptibility testing capacity for *N. gonorrhoeae* is limited in the United States. Similar studies should be conducted utilizing zone diameter and MIC data collected via E-tests to establish epidemiological cut-off values for azithromycin disk diffusion and E-test procedures.

One gram azithromycin given orally in combination with 250mg ceftriaxone given as a single intramuscular dose is one of two currently recommend treatment regimens for uncomplicated gonococcal infections of the cervix, urethra, and rectum. As such, clinicians require azithromycin antimicrobial susceptibility data to monitor resistance. Clinical laboratories may be reluctant to perform azithromycin antimicrobial susceptibility testing for *N. gonorrhoeae*

because results are difficult to interpret without established clinical breakpoints. The lack of established breakpoints for *N. gonorrhoeae* azithromycin antimicrobial susceptibility testing hinders surveillance and hampers the management of patients who fail treatment. While epidemiological cut-off values cannot replace clinical breakpoints, they are the first crucial step in the establishment of clinical breakpoints by CLSI and other standard-setting institutes. The epidemiological cut-off value for azithromycin and *N. gonorrhoeae* agar dilution antimicrobial susceptibility testing established here can be used to help set clinical breakpoints and identify isolates with reduced susceptibility to azithromycin.

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