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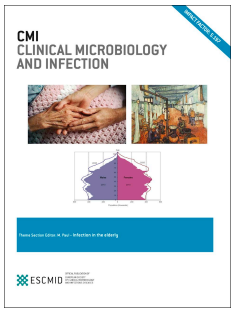

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Mycobacterium tuberculosis drug resistance testing: challenges, recent developments and perspectives

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Abstract (246 words)

Drug resistance testing, or antimicrobial susceptibility testing (AST), is mandatory for *Mycobacterium tuberculosis* (MTB) in cases of failure to standard therapy. We reviewed the different methods and techniques of phenotypic and genotypic approaches. Although Multi- and extensively- drug resistant (MDR-XDR) tuberculosis is present worldwide, AST-MTB is still mainly performed according to the resources available rather than the drug resistance rates. Phenotypic methods, i.e., culture based AST, are commonly used in high income countries to confirm susceptibility of new cases of tuberculosis. They are also performed to detect resistance in tuberculosis cases with risk factors, in combination with genotypic tests. In low income countries, genotypic methods screening hot spot mutations known to confer resistance, were found to be easier to perform because they avoid the culture and biosafety constraint. Given that genotypic tests can rapidly detect the prominent mechanisms of resistance, such as the *rpoB* mutation for rifampicin resistance, we are facing new challenges with the observation of false-resistance (mutations not conferring resistance) and false-susceptibility (mutations different from the common mechanism) results. Phenotypic and genotypic approaches are thus complementary for obtaining a high sensitivity and specificity for detecting drug resistances and susceptibilities to accurately predict MDR/XDR cure and to gather relevant data for resistance surveillance. Although AST-MTB was established in the 1960s, there is no consensus reference method for MIC determination against which the numerous AST-MTB techniques can be compared. This information is necessary for assessing the *in vitro* activity and setting breakpoints for future anti-tuberculosis agents.

Keywords: Antimicrobial susceptibility testing, genetic resistance, critical concentration, critical proportion, MIC, hot spot mutations

Objectives – definitions - history of drug resistance testing in tuberculosis

Drug resistance testing (DRT) for *Mycobacterium tuberculosis* (MTB) has two objectives: (i) individual management (treatment and prevention) of tuberculosis (TB) cases and (ii) anti-TB drug resistance surveillance. Since the first use of anti-TB drugs in the late 1940s [1], relapse cases of tuberculosis with acquired resistance have been repeatedly observed for all new effective drugs [2-4]. Because the resistant strains were then transmitted to treatment-naïve patients, DRT for MTB was recommended for all definite cases of tuberculosis [5]. Multi-drug resistant (MDR) TB, defined as resistance to the two key drugs isoniazid and rifampicin, has emerged as a global threat and occurs more frequently in previously treated cases (secondary resistance) than in untreated patients (primary resistance) [6]. However, DRT was estimated to be performed in only 58% of previously treated TB cases (12% of untreated cases), and DRT is mostly done in high income countries where the resistance rates are the lowest [6].

Because the criteria for drug resistance in MTB were defined in patients with treatment failure (clinical resistance) [7,8], the so-called "resistant" MTB strain is unlikely to show clinical responsiveness to the drug, and conversely, the "susceptible" MTB strain is likely to show response to treatment. Acquired resistance in MTB was suspected to be due to the selection of resistant mutants because similar mutants were observed in wild-type strains as pre-existing mutants. However, these mutants were present in lower proportions in previously untreated patients (e.g., $1/10^5$ for isoniazid, $1/10^8$ for rifampicin) than in the relapsed TB cases where proportions ranging from $1/10^2$ (1%) to 1/1 (100%) have been observed [7]. Genetic confirmation of this phenomenon was obtained when point mutations conferring resistance were observed in strains isolated from patients with treatment failure [9,10]. Major advances in laboratory methods for drug resistance testing were achieved in the 1960s when TB was endemic to western countries [11]. However, the testing methods have not changed much

since then and remain isolated to specialized laboratories, even at a time when detection of resistance for other bacteria, such as staphylococci and gram-negative bacilli, has been well organized by national and international committees such as European Committee on Antimicrobial Susceptibility Testing (EUCAST). Consequently, drug resistance testing in MTB appears today as using "old school" methods [5].

Although phenotypic culture based methods were first designed to detect drug resistance, there is an increasing need for the development of antimicrobial susceptibility testing (AST) as is done for other bacteria according to the principles created by the EUCAST. In 2016, there is no universal reference method for AST-MTB, which is challenging when setting the clinical reference points for old as well as new drugs, including bedaquiline and delamanid [12]. Additionally, any evaluation of potential drug resistance mutations using whole genome sequencing (WGS) data is heavily dependent on a uniform and well-defined reference method for AST-MTB.

Phenotypic methods and challenges for predicting resistance

In 1969, drug resistance in MTB was defined as "a decrease in the sensitivity of sufficient degree to be reasonably certain that the strain concerned is different from wild-type strains, which are isolates that have never come into contact with the drug". Isolates that cannot grow at "critical" concentrations were then defined as susceptible, whereas those that can grow were considered resistant. This was the rationale for the resistance ratio method described by Mitchison [13] and the proportion method described by Canetti [7]. However, Canetti showed that in wild-type strains, there was a certain proportion of resistant mutants pre-existing in the tubercle bacillus population and calculated that a proportion of 1% was the higher limit between the susceptible and resistant isolates [7]. The AST-MTB techniques used today are

based on these two methods with some techniques considering only the critical concentration or both the critical concentration and the critical proportion (Table 1) [7,8,14].

One of the most discussed points in relation to AST-MTB is the "critical concentration" (CC), i.e., the drug concentration (in mg/L or µg/ml) included in the culture medium. These concentrations were not based on pharmacokinetics/pharmacodynamics (PK/PD) but were determined experimentally by comparing the growth of wild-type versus "non" wild-type strains [7]. One should be aware that CC varies with the medium used (egg-based media, such as Löwenstein-Jensen, which are cooked at 80°C, and synthetic Middlebrook [7H9, 7H10, 7H11 or 7H12] media to which oleic acid albumin, dextrose and catalase [OADC] are added) because this value depends on the concentration of the drug that remains active in the media (Table 2).

The challenges for research and development of new DRT or AST techniques are reducing the time to results and producing easy-to-perform kits. There is also a necessity to reconsider breakpoints or discuss the need in reporting MICs to improve clinical relevance of DST results The new tests are limited by the complexity of the resistance development process in MTB. This process involves dynamic emergence of spontaneous mutants and their stability with regard to the selective pressure, which is based on PK variability dependent within the treatment regimen and the patient [15]. One recently developed technique uses the MGIT 960TB system EpiCenter software equipped with the TB eXiST module (Becton, Dickinson and Company, New Jersey, USA). This enables simultaneous testing of different drug concentrations on different bacterial inocula with "real time" visualization of the results (Fig. 1). This technical improvement is worthwhile for personalized therapeutic purposes and may have an impact on the cure rate of MDR/XDR-TB infected patients as well as on the prevention of acquired resistance [16,17]. TB eXiST AST is also used to quantify the resistance level and may be important for some drugs (see below) [16,18].

Minimum inhibitory concentrations in relation to resistant and susceptible populations of *M. tuberculosis*

In light of the increasing rates of drug resistance, a modernized approach for setting clinical AST breakpoints is needed to optimize treatment decisions [19]. According to EUCAST, clinical breakpoints should be defined by combining minimal inhibitory concentration (MIC) distributions, clinical outcomes and PK/PD data [19,20]. As described above, MTB breakpoints were mainly based on experimental data using different media, techniques and drugs. Most of all, there were no predefined dosing standards, and precise documentation of previous experiments is mainly inaccessible [19,21,22]. In general, phenotypic AST methods have the advantage of being able to quantitatively predict not only resistance but also susceptibility [15,16,20]. However, several obstacles exist for large-scale MIC determination for MTB as it demands resources, time and biosafety precautions. No MIC reference method is universally accepted for MTB, and several AST techniques are used (Table 2) [21].

The epidemiological cut-off (ECOFF) is outlined by the Gaussian distribution formed when MICs are compiled from several laboratories using the same method or technique [20]. By definition, the ECOFF is the highest MIC for organisms devoid of phenotypically detectable resistance (the wild-type distribution). For antimicrobial agents considered clinically active for the organism, the ECOFF is also the lowest possible susceptibility breakpoint [20]. The ECOFF is the cornerstone for validating rapidly emerging whole genome sequencing data and for defining clinical breakpoints but has not been systematically used to define breakpoints for MTB [19,20]. To illustrate MIC distributions (references outlined in the supplementary reference file), the MIC data for rifampicin were compiled from several studies including commonly used methods (Fig. 2). The MIC distribution shows a clearly visible separation between resistant and susceptible populations (Fig. 2A). For the

fluoroquinolones (FQ), most MIC data is currently available for ofloxacin as a class representative (Fig. 2B), but clearly there is a need to define ECOFFs for newer FQs, such as moxifloxacin. There are several examples where the determination of ECOFFs could help in providing rational breakpoints for MTB [19,23,24].

As genotyping detection of resistance is increasingly used, drug resistance mutations are reported as associated with a low increase in MICs, which tend to be close to the ECOFFs. There are several examples where ECOFFs could improve breakpoints for MTB. For ethambutol, the current breakpoint and the ECOFF are very close to the MICs of isolates with resistance mutations, such as in codon 306 of *embB* [23,25]. This might be the reason for the poor reproducibility of ethambutol testing even among reference laboratories. Recent data indicated that isolates harbouring some rare specific mutations in codons 516, 526 and 533 of *rpoB* may show only a low level rifampicin resistance or even be classified as susceptible in BACTEC 960 MGIT [26,27]. For rifabutin, the current breakpoint from the Clinical and Laboratory Standards Institute (CLSI) categorizes non-wild-type isolates with *rpoB* mutations in D516V and rifampicin resistance as rifabutin susceptible, without having systematically evaluated PK/PD and clinical outcome data [21,24]. Such isolates harbouring these resistance mutations may be identified and separated from wild-type isolates by introducing an intermediate category since increased dosing of rifampicin is promising according to recent clinical trials [28]. For fluoroquinolones, ECOFFs are also useful for identifying low level resistance, such as in codon 90 of *gyrA*, where MICs can be lower than current breakpoints [23, 29] but above the ECOFF [30] (Fig. 2B). In contrast to rifampicin, there is a close relationship between MICs for the susceptible and resistant populations where there may be a selection bias in favour of resistant isolates, which is important to consider when MIC distributions for MTB drugs are evaluated (Fig. 2B). For some of the drug resistance mutations, poorly defined breakpoints result in oscillating AST reports between susceptibility

and resistance, which may negatively affect clinical management in difficult-to-treat patients with MDR/XDR-TB and introduce bias in studies comparing genotypic and phenotypic resistance. It may be possible that such isolates, commonly referred to as having low-level resistance, are accessible for treatment using optimized dosing and therapeutic drug monitoring (TDM) [29,30]. However, PK/PD and clinical outcome data need to be investigated in clinical studies in order to confirm this hypothesis. An intermediate category, which indicates that such isolates may be treatable with increased dosing and TDM, should be considered, as is currently the case for other bacterial pathogens, to separate wild-type, fully susceptible isolates from those with resistance mutations [20]. Inadequate dosing given for patients with low level resistant isolates erroneously classified as susceptible may lead to poor treatment outcomes and further development of resistance [30].

Genotypic resistance

The vast majority of drug resistance in the *Mycobacterium tuberculosis* complex is caused by single-nucleotide-polymorphisms, although insertions or deletions are also possible [31]. Therefore, molecular assays represent a valuable option to accelerate the detection of resistance from weeks to hours or days. A variety of techniques have been proposed to detect resistance mutations, which has resulted in the development of more than 30 commercial drug-susceptibility assays [32]. Thus far, only line probe assays (LiPAs) and the Xpert MTB/RIF assay (Cepheid, Sunnyvale, CA) have been endorsed by the World Health Organization (WHO) for the rapid detection of MDR tuberculosis cases [33]. Several commercial tests are available to detect rifampicin resistance including InnoLipa RIF-TB, GenoType MTBDR*plus*, AID test and the LiPA Nipro NTM+MDRTB assay, and all of these tests, except the InnoLipa RIF-TB, also detect mutations that confer resistance to isoniazid [34-36].

LiPAs are based on the targeted amplification of specific regions of the *M. tuberculosis* genome followed by hybridization of the amplicons to oligo probes immobilized on nitrocellulose strips. They were the first genotypic tests used to detect MDR-MTB in the 1990s, by detecting *rpoB* mutations in the rifampicin resistance-determining region (RRDR) [37]. The pooled sensitivity of the GenoType MTBDR*plus* (version 1) for the detection of rifampicin resistance was found to be 98.1% (95% CI 95.9-99.1) with a specificity of 98.7% (95% CI 97.3-99.4). The results for isoniazid showed a lower sensitivity (84.3%, 95% CI 76.6-89.8) but a high specificity (99.5%, 95% CI 97.5-99.9) [35]. More recently, non-inferiority of version 2 of the GenoType MTBDR*plus* assay and the newly developed LiPA Nipro NTM+MDRTB assay was demonstrated [36]. Among real time PCR assays, the Cepheid Gene Xpert MTB/RIF, a fully automated real time PCR-based assay for the detection of *M. tuberculosis* DNA and mutations associated with rifampicin resistance, has a pooled sensitivity and specificity of 95% (95% CI 90-97) and 98% (95% CI 97-99), respectively [33,38,39]. Because this test requires less technical expertise, the Xpert MTB/RIF is today widely used, and some countries have even decided to use it as the initial diagnostic test for patients with presumptive TB.

Overall, the sensitivity of the genotypic tests for the detection of rifampicin resistance (a surrogate marker for detecting MDR-MTB) is generally over 95-98% [33,36,38,39], which shows that most of the resistant isolates harbour *rpoB* mutations located in the RRDR. The specificity is also generally close to 99% [33,36,38,39], which shows that most *rpoB* mutations found in the RRDR confer resistance. However, since the practice of genotypic testing has greatly increased during the last five years, *rpoB* mutations not associated with resistance are now being detected, which brings the genotype-phenotype correlation into question [40]. The Cochrane review performed in 2014 nicely showed that although the performances of these tests are excellent, one should calculate the predictive values for

resistance and susceptibility detection according to the resistance rate measured at the scale of the country, region or city [39].

For second line drug detection, especially for detecting XDR isolates, there is no fully automated point-of-care assay thus far. The WHO recently endorsed the GenoType MTBDR*sl* version 2 LiPA for the detection of resistance to fluoroquinolones (mutations in *gyrA* and *gyrB* quinolone resistance-determining regions) and to kanamycin, amikacin, and capreomycin (mutations at the *rrs* 1401-1402 positions and in the *eis* gene promoter) [41].

The results from the aforementioned assays have to be analysed with care. First, they can generally only be used to rule-in, as opposed to rule-out resistance given that they target a limited number of known resistance mutations. Second, systematic false-positive results are possible due to synonymous mutations, which can significantly affect the positive predictive values in settings where the true rate of resistance is low [42]. Third, the interpretation of their results is not always clear, such as the degree of cross-resistance to fluoroquinolones caused by particular *gyrA* mutations [26,29]. Additional research is therefore urgently required to address these shortcomings in our understanding of resistance on the genotypic as well as phenotypic level.

Gaps, challenges and prospective research

Over the past few years, steady progress has been made in elucidating novel resistance mechanisms, particularly for second-line drugs [4,43]. However, it is important to appreciate that the remaining discrepancies between genotypic and phenotypic results are not solely due to yet unknown resistance mechanisms [15,16]. Instead, factors such as the aforementioned shortcomings with the current critical concentrations, primary versus acquired resistance, the inadequate limits-of-detection of sequencing, random errors, and false associations between genotype and phenotype all play a role.

The relative importance of these factors differs among antibiotics and has to be dissected in carefully designed studies. For example, it is becoming increasingly clear that hetero-resistant mutations that occur at less than approximately 30% of the total population and are therefore not detected by Sanger sequencing play an important role in resistance to fluoroquinolones [16,44,45]. This inadequate limit-of-detection provided by Sanger sequencing to detect low-level hetero-resistance compared with phenotypic methods, which can be calibrated to detect resistance at 1% of the population for most drugs, can be overcome by either sequencing from the drug-containing medium (as opposed to the drug-free medium from which sequencing is usually performed) or by increasing the sequencing coverage. It should be noted, however, that even at high coverage current bioinformatics algorithms do not necessarily identify hetero-resistant insertions or deletions.

Random errors (i.e., usually false-resistant results) are an important factor for drugs for which the true prevalence of resistance is low. Therefore, repeating phenotypic and genotypic testing for discrepant results is warranted to identify candidate isolates with novel resistance mechanisms [46]. Finally, it is important to include large numbers of genotypically diverse susceptible control isolates to avoid false-associations between a resistant phenotype and mutations that are merely polymorphisms, as discussed elsewhere in more detail [42]. In this context, it is notable that the general assumption that resistance mutations confer resistance irrespective of their genetic background may not always apply, although the clinical relevance of these observations remains to be determined [47,48]. For example, *whiB7* mutations do not result in streptomycin resistance in isolates with an inactive Tap efflux pump, an underestimated physiological mechanism of resistance in MTB, which underlines the need for a detailed and broad-ranged mechanistic understanding of resistance mechanisms in MTB [43].

Conclusions and perspectives

Phenotypic AST is still the most reliable laboratory approach to determine antimicrobial resistance and susceptibility in MTB, its advantages being quality control networks and generally good clinical correlation [49]. Advantages of genotypic methods include the ability to easily and rapidly detect resistance, but there are still limitations regarding the accuracy (needs for robust quality control) and the correlation with treatment and clinical outcome because only some mutations have been demonstrated to consistently and reliably confer high-level resistance [25]. The discrepancies between genotype and phenotype AST approaches, which are limited to certain antibiotics and certain isolates [27,50], need to be solved by more research into resistance mechanisms. Because of the spread of drug resistance in MTB, patients with MDR-TB and XDR-TB infections should have the chance of being treated on the basis of antibiotic resistance profiles as determined by combined phenotypic and genotypic methods. Because there is good news about the development of new anti-TB drugs for the next decades, we must reach a consensus for a reference method according to the new standards of EUCAST in a timely fashion. This is one important goal of the newly formed EUCAST subcommittee on antimycobacterial susceptibility testing (http://www.eucast.org/mycobacteria/).

Transparency declarations

No conflicts of interest are declared for the submitted work, with the exception of Dr Köser who is a consultant for the Foundation for Innovative New Diagnostics. The Bill & Melinda Gates Foundation and Janssen Pharmaceutica covered Dr Köser's travel and accommodation to present at meetings.

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301 **TABLE 1.** Phenotypic methods and techniques used for drug susceptibility testing in

302 *Mycobacterium tuberculosis* [14]

Methods	Detection of MTB growth	
	Direct evidence	Indirect evidence
Critical concentration	Resistance ratio	Nitrate reductase assay
	Absolute concentration	Resazurin test
	Microscopy observed direct susceptibility testing (MODS)	Alamar blue test
	Microtitre plates (e.g., Versatrek)	
Critical proportion and critical concentrations	WHO and ECDC recommended protocol on Löwenstein Jensen	Automated BACTEC MGIT960
	CLSI recommended protocol on 7H10 or 7H11	

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304

305 **TABLE 2.** Critical concentrations (mg/L) used in various techniques of antimicrobial

306 susceptibility testing for *Mycobacterium tuberculosis* [14,16,21]

Antimicrobial agent	LJ	7H11	7H10	MGIT	MGIT TB eXiST qAST		
					Low	Intermediate	High
Isoniazid	0.2	0.2	0.2	0.1	0.1	1-3	10
Rifampicin	40	1.0	1.0	1.0	1	4	20
Ethambutol	2.0	7.5	5.0	5.0	5	12.5	50
Streptomycin	4.0	2.0	2.0	1.0	1	4	20
Kanamycin	30	6.0	5.0	2.5	-	-	-
Amikacin	30	-	4.0	1.0	1	4	20
Capreomycin	40	-	4.0	2.5	2.5	5	25
Ofloxacin	4.0	2.0	2.0	2.0	1	2	10
Moxifloxacin	0.5	-	0.5/2	0.5/2	0.25	0.5	2.5

307
308

Figure legends

FIG. 1. Representative results for quantitative resistance testing using the MGIT-TB eXiST technique performed for a *M. tuberculosis* isolate showing a low level resistance to moxifloxacin (R at 0.25 but S at 0.5 mg/L) and harbouring a *gyrA* A90V mutation.

The growth in control tubes appears as blue lines (dotted line for 1:1; discontinuous line for 1:10 and continuous line for 1:100). Resistance was detected as growth appearing in the tube containing 0.25 mg/L (pink line) at the same time (14 days) as the 100% inoculum, but growth at 0.5 mg/L (green line) was observed only after the 1% inoculum.

FIG. 2 MIC distributions for (A) rifampicin (395 isolates from 7 studies in 7H10, 7H11 and MGIT) and (B) ofloxacin (2538 isolates from 22 studies in 7H10, 7H11, MGIT, 7H9 and LJ). The references are listed in the supplementary files. The PubMed search strategies (until the 31st of May 2016) were "rifampicin" AND "*Mycobacterium tuberculosis"* AND "MIC" for rifampicin MIC data, and "ofloxacin" or "fluoroquinolones" AND "*Mycobacterium tuberculosis"* for ofloxacin MIC data. Abbreviations: 7H10, Middlebrook 7H10 media; MGIT, BACTEC 960 MGIT media; 7H11, Middlebrook 7H11 media; 7H9, Middlebrook 7H9 media; LJ, Löwenstein-Jensen media.

Supplementary FIG.1 PRISMA flow charts for searches on MIC data for (A) rifampicin and for (B) ofloxacin

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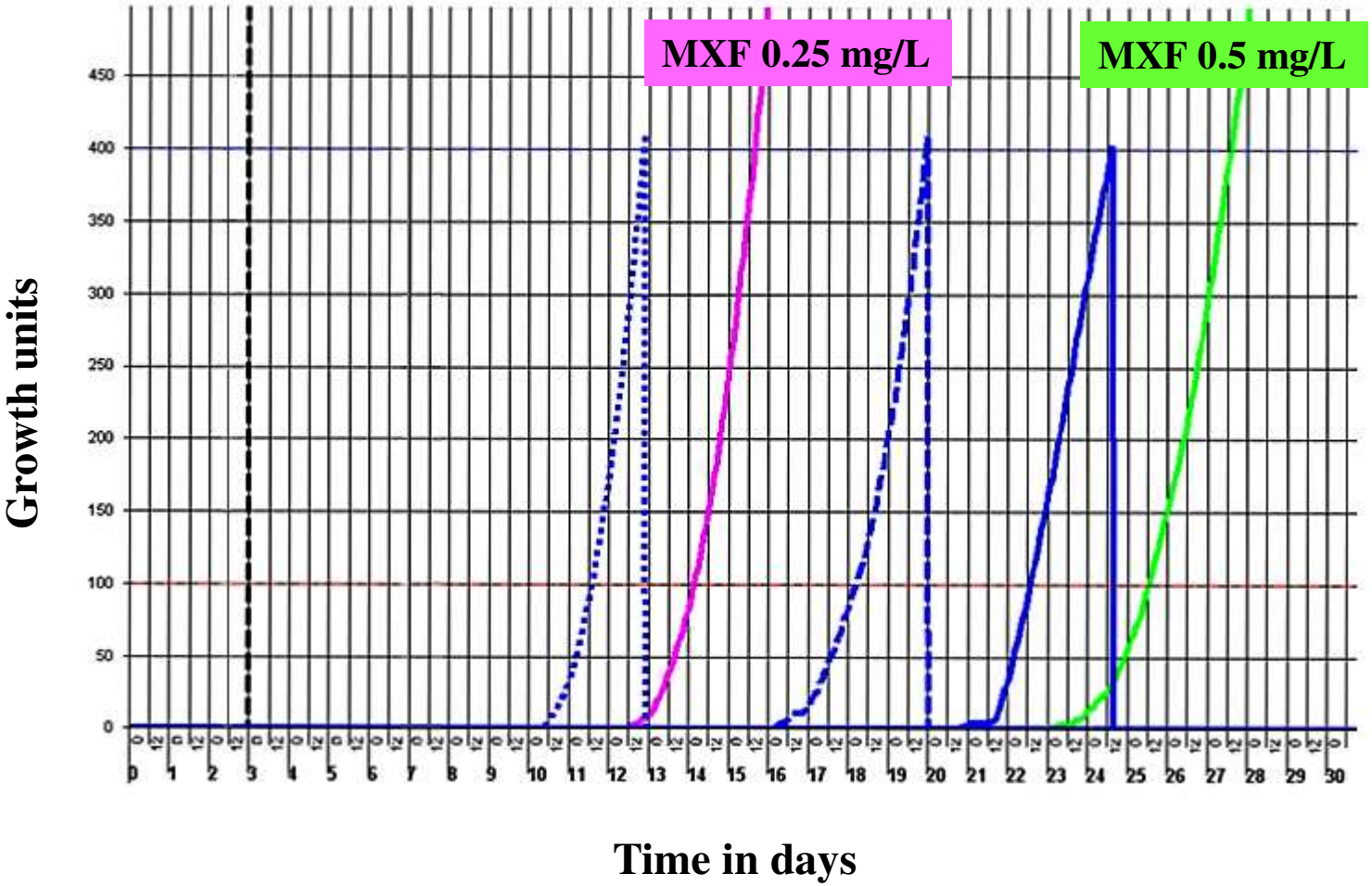
Figure 1



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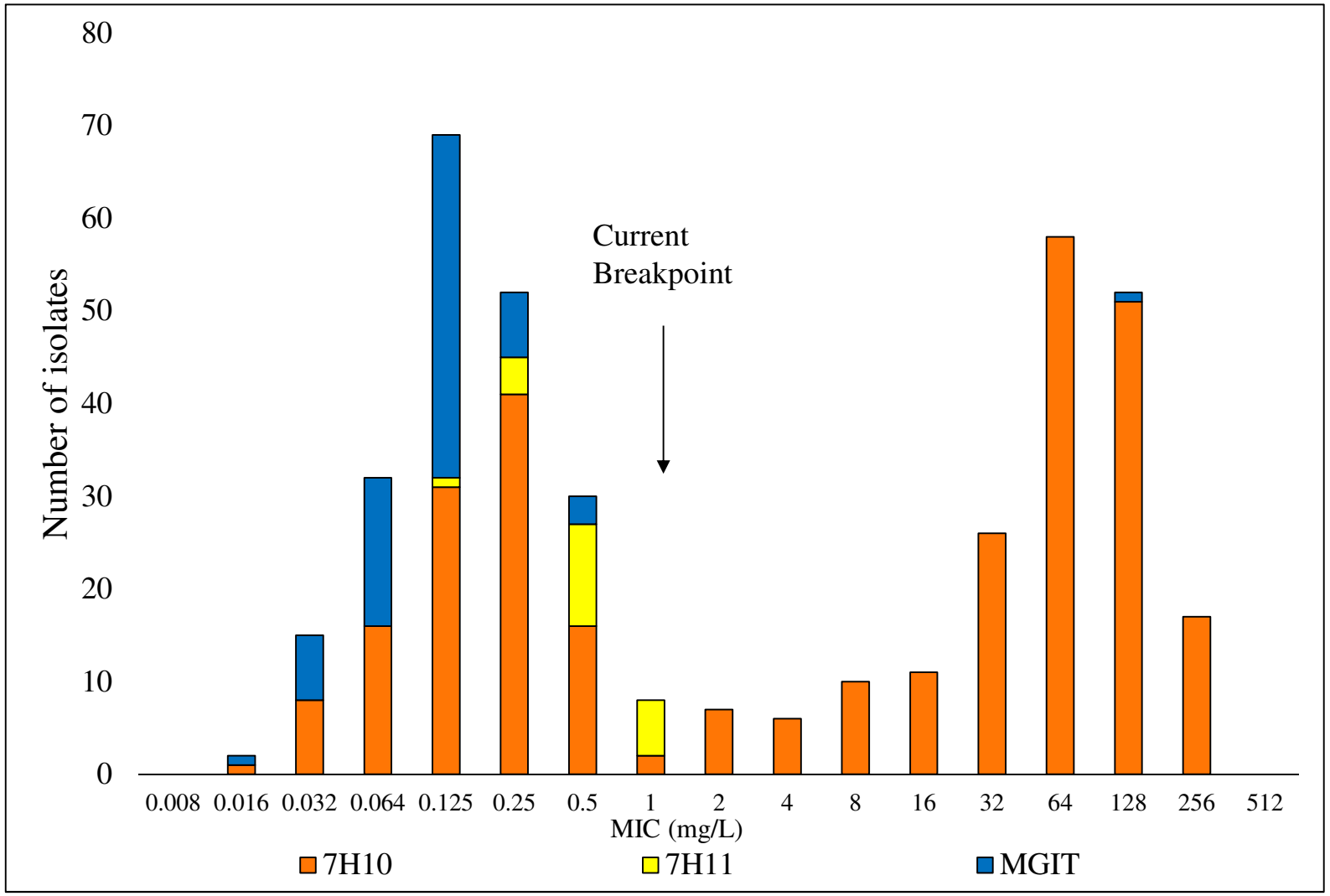

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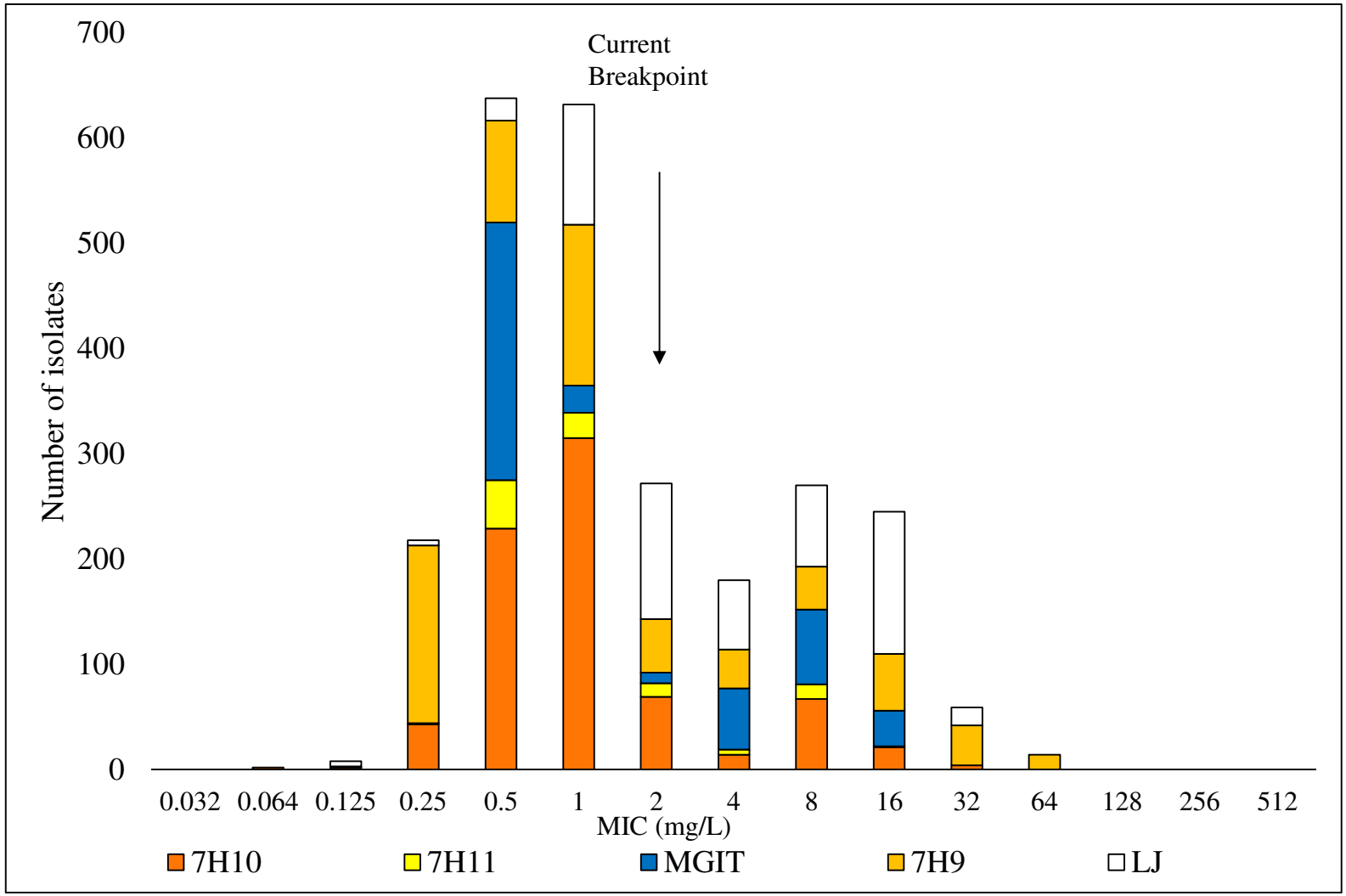

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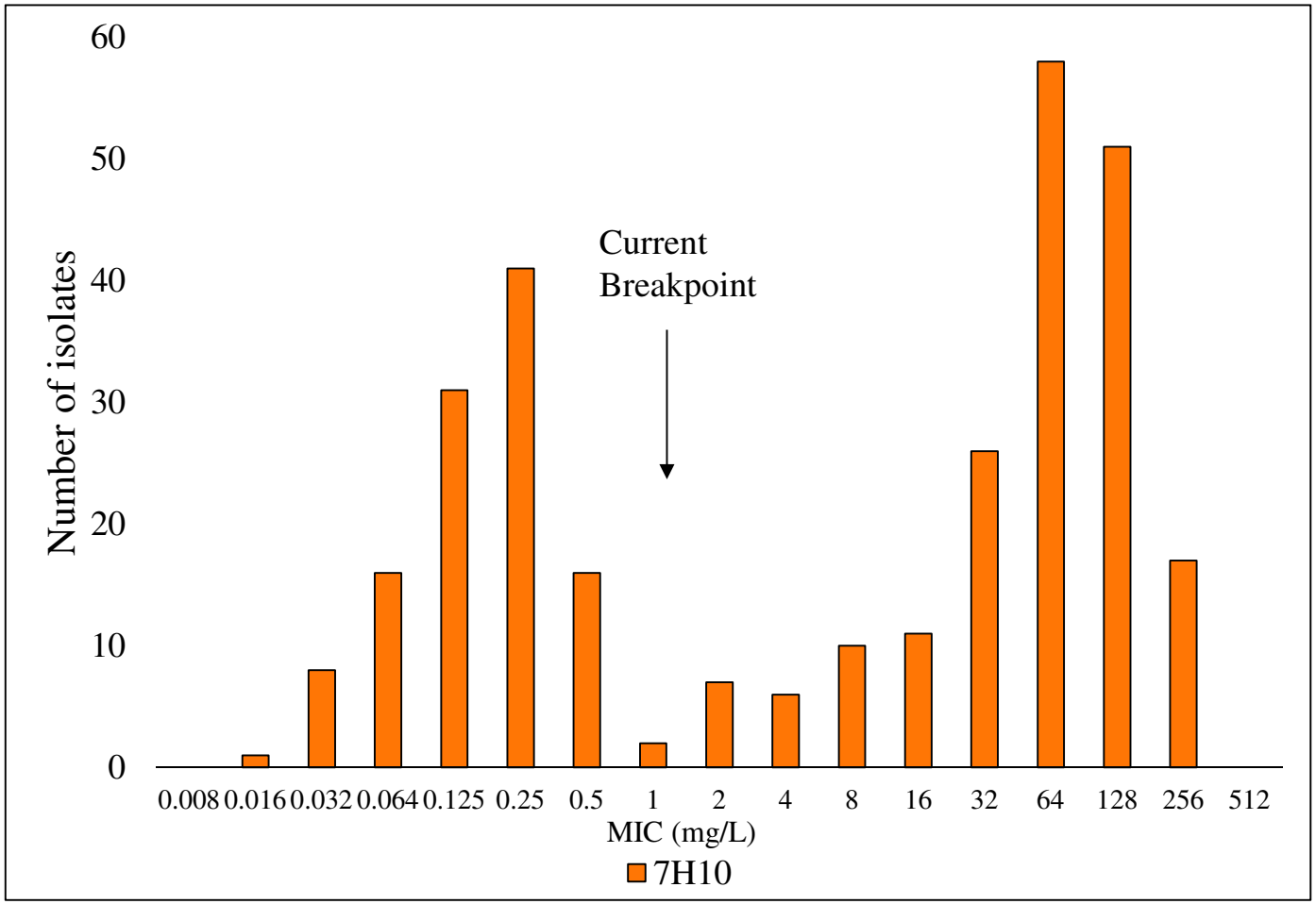

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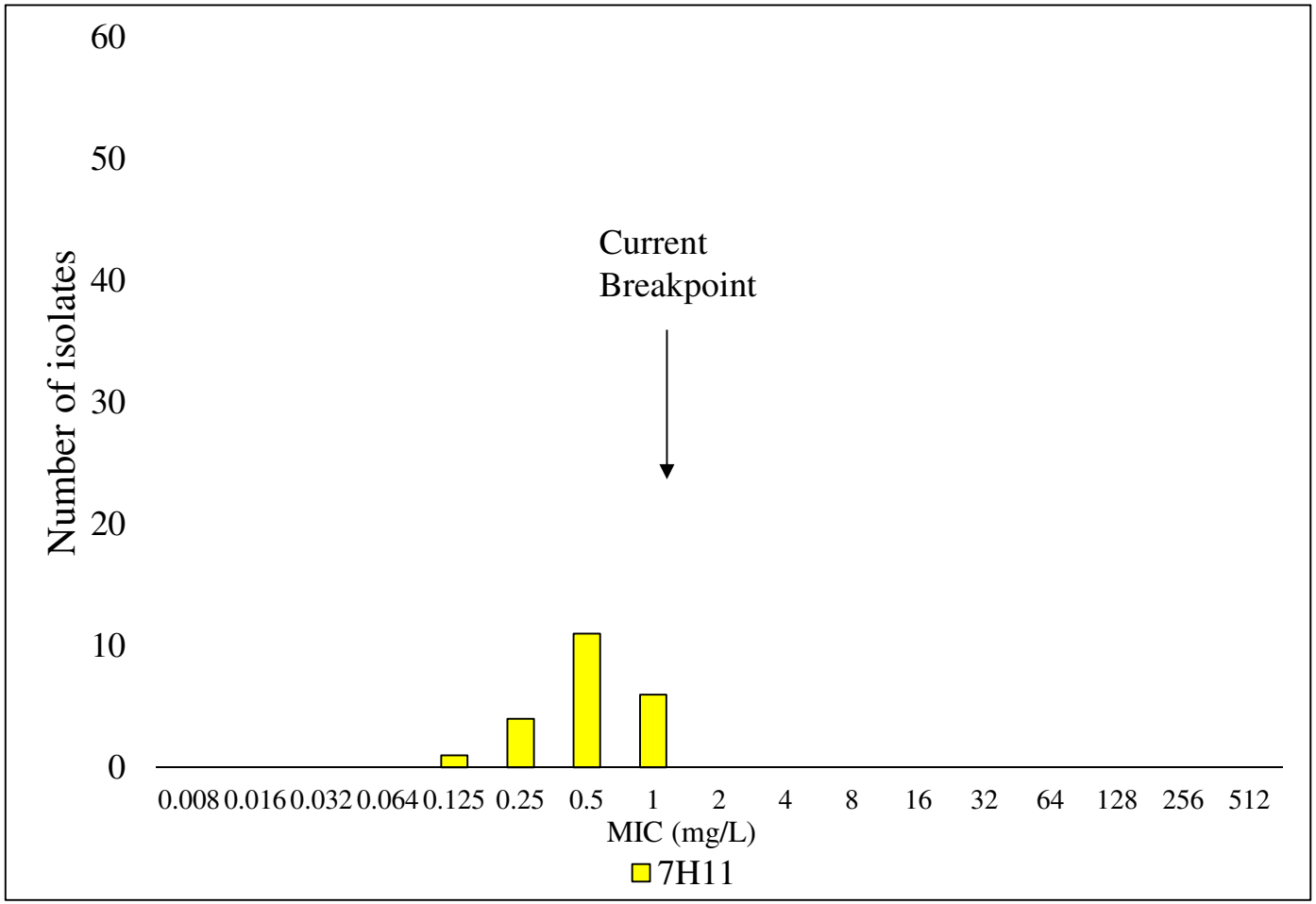

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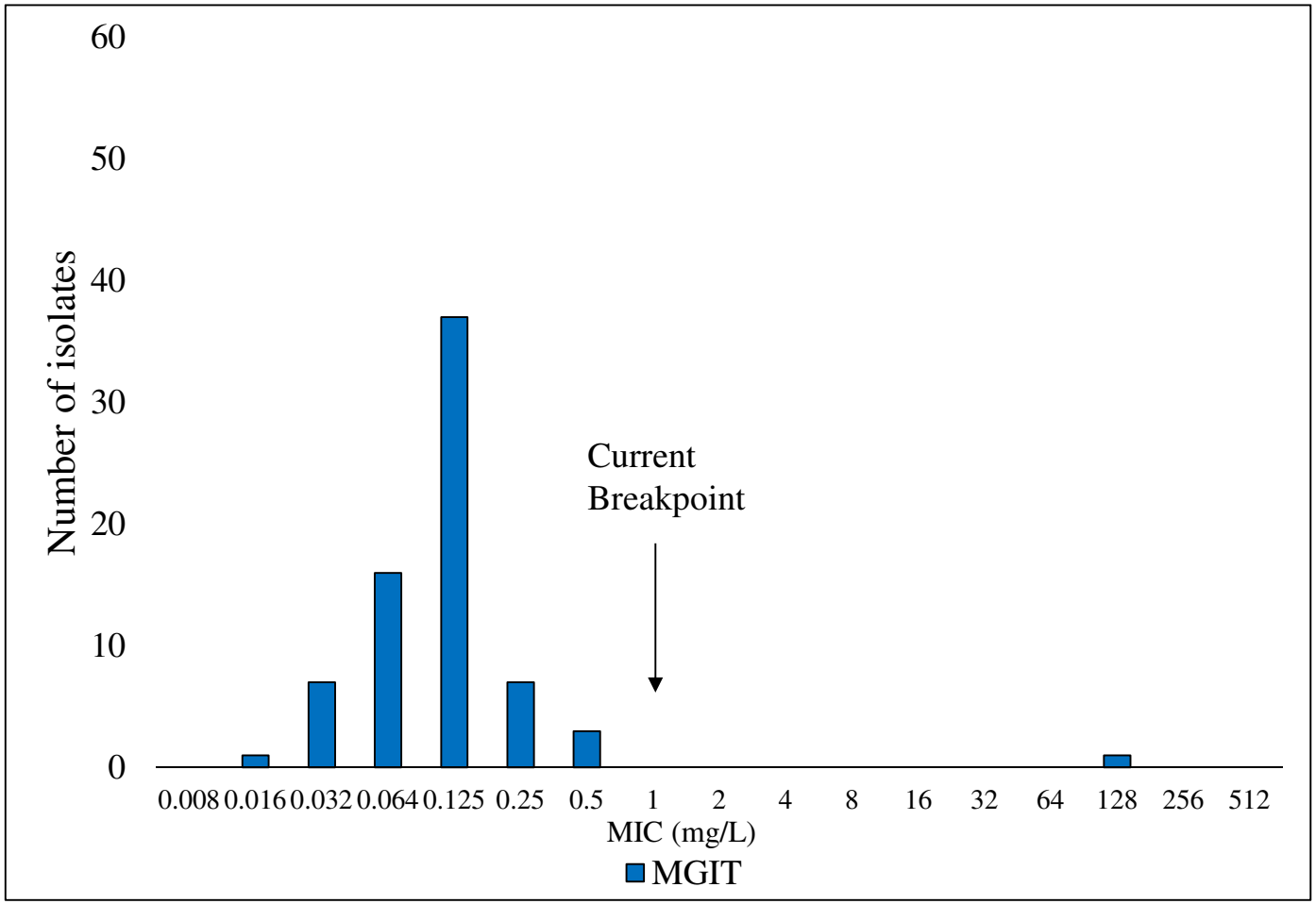

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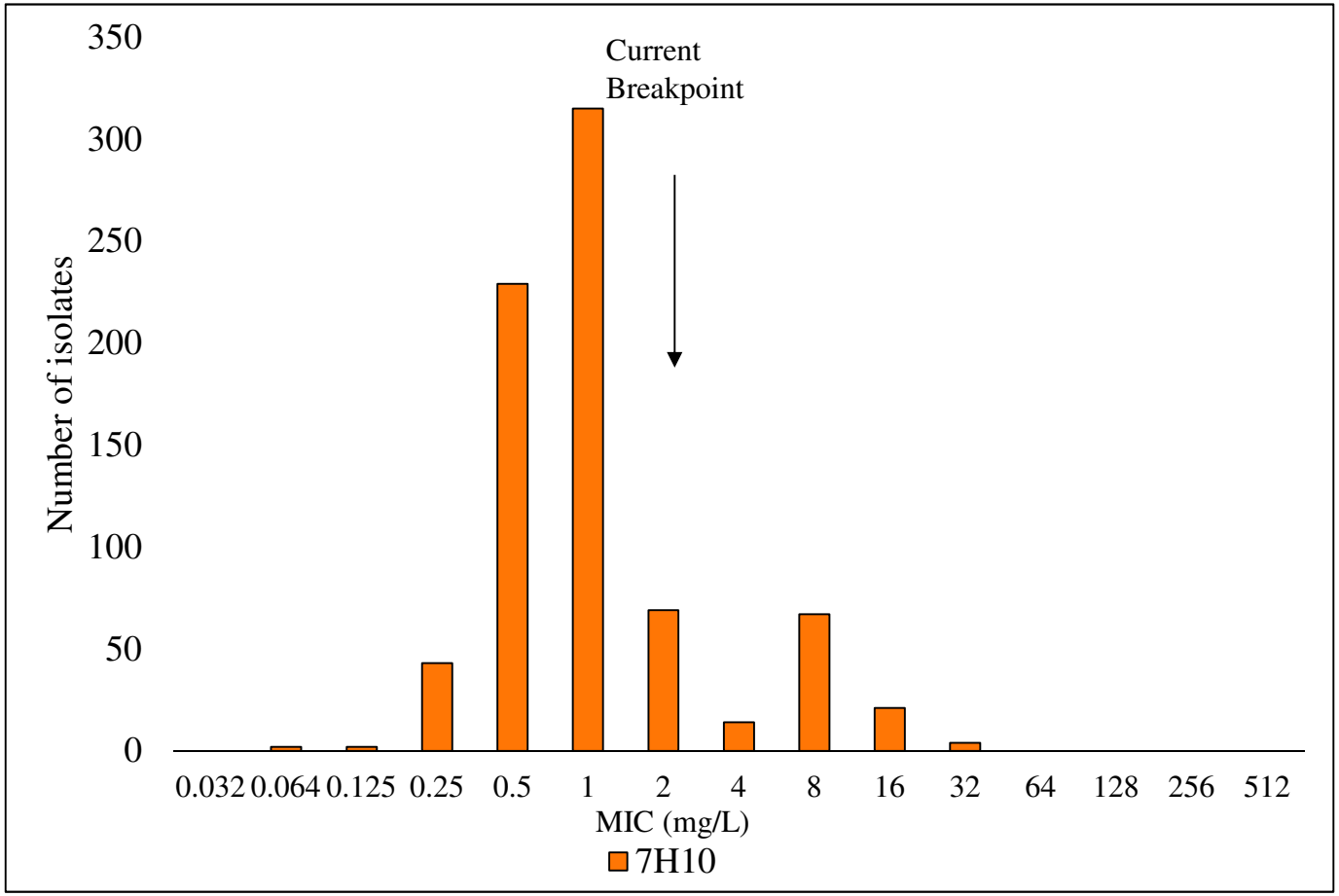

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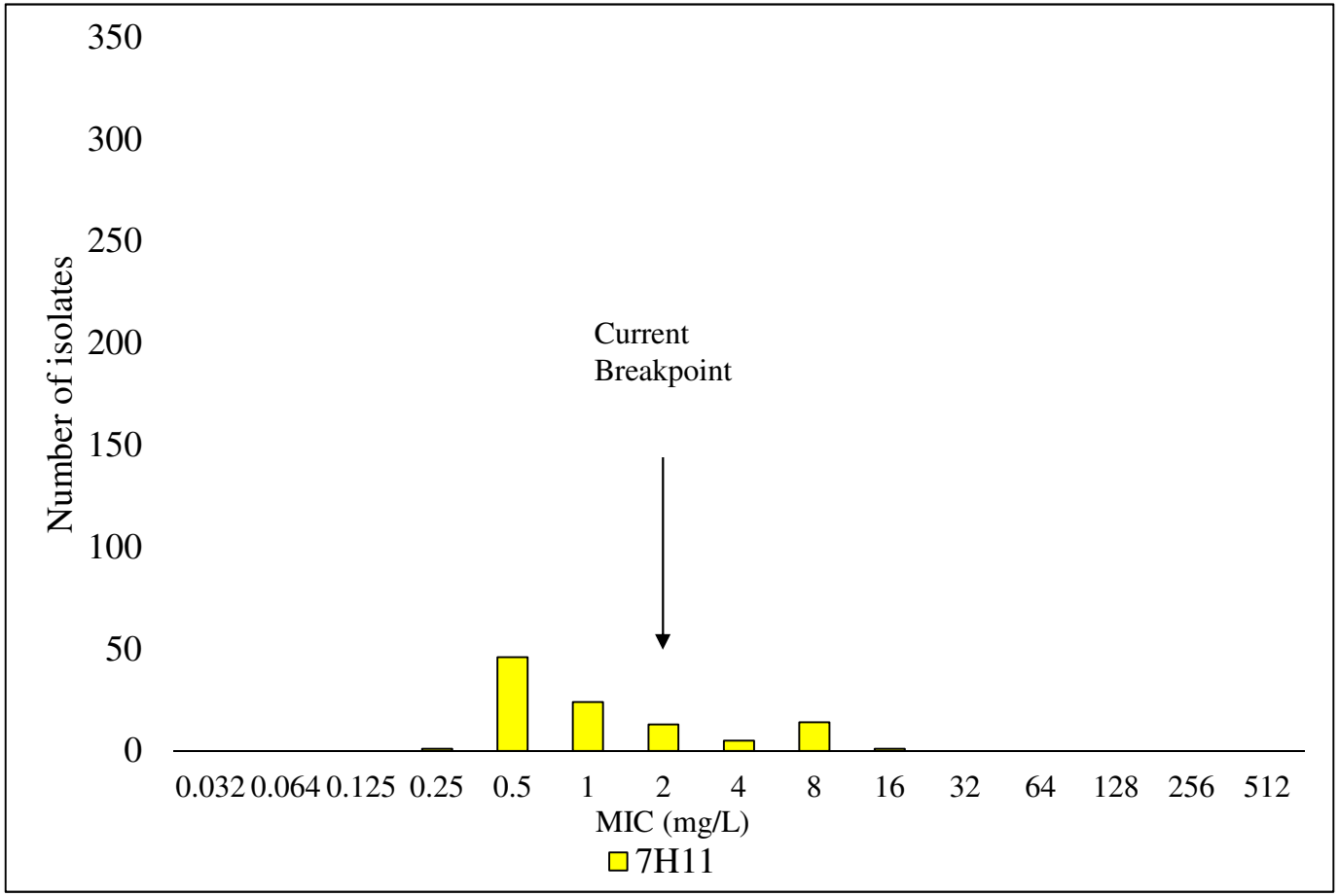

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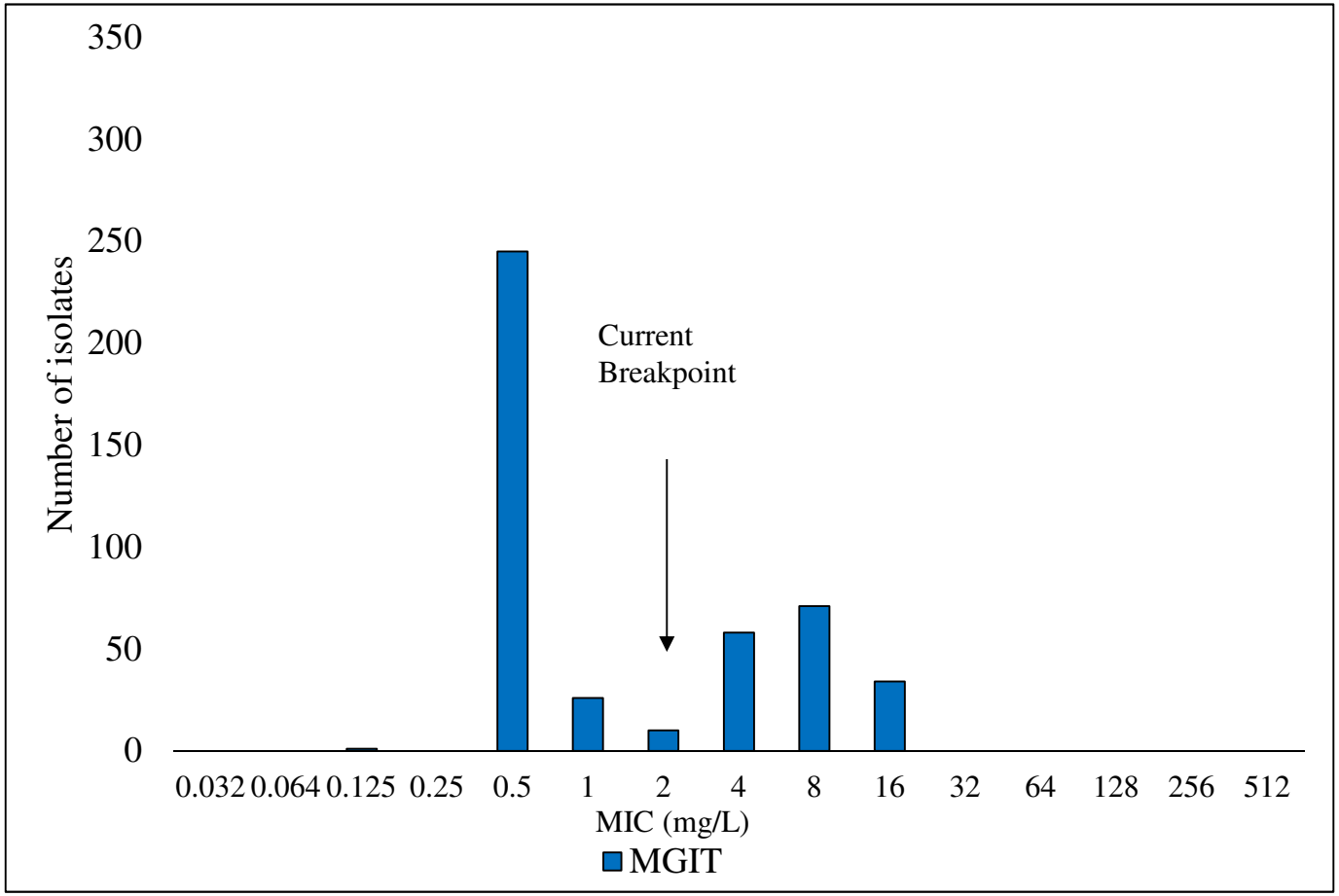

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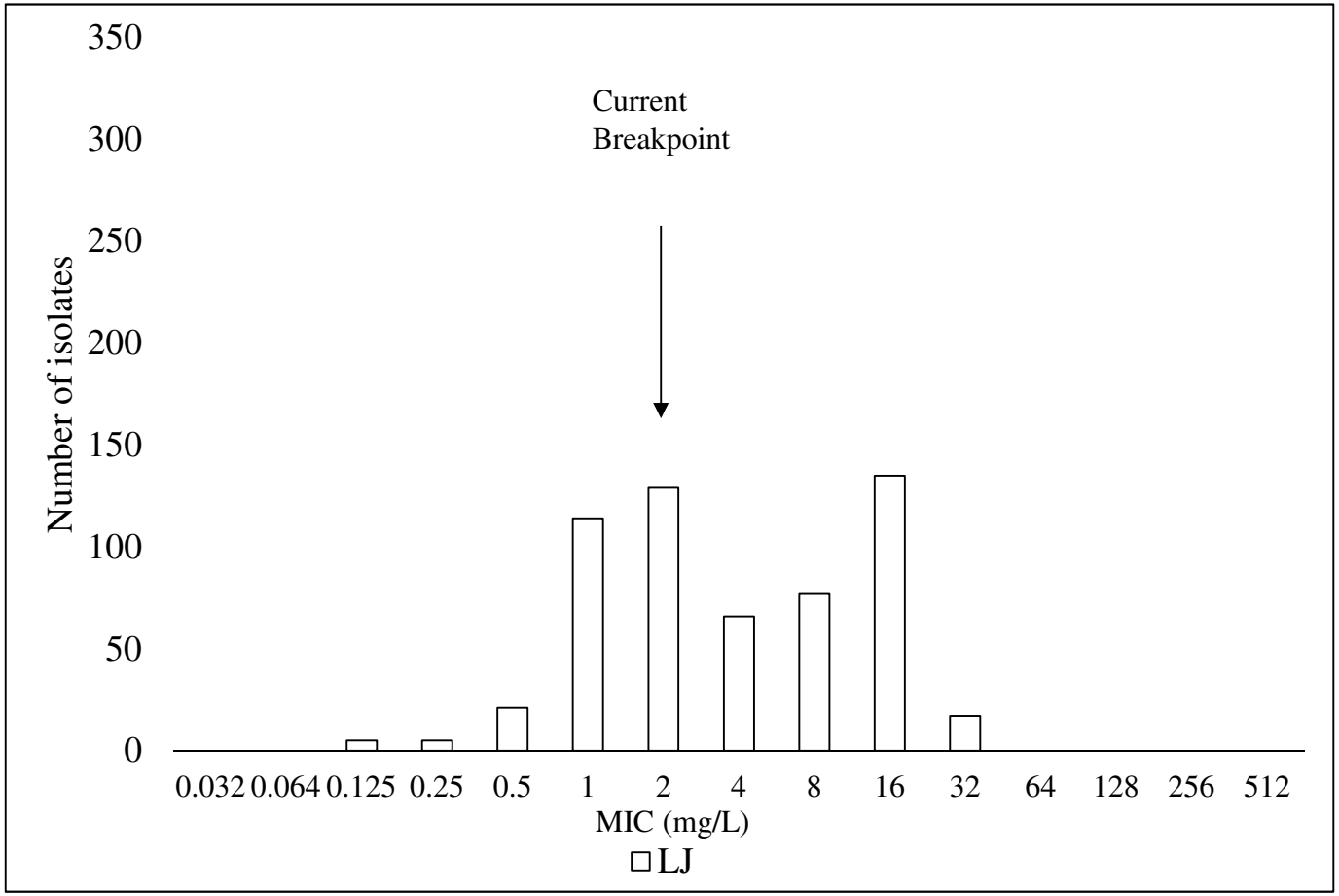

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