Re-evaluations of *H. influenzae* Susceptibility Tests for Penicillins ± ß-Lactamase Inhibitors Against ß-Lactamase-Negative Ampicillin-Resistant (BLNAR) Strains: A SENTRY Program Report

Y HIRAKATA, PG AMBROSE, SM BHAVNANI, RN JONES

Tohoku Univ. Sch. Med, Miyagi, Japan; Ordway Res. Inst., Albany, NY, USA; JMI Laboratories, North Liberty, IA, USA

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JMI Laboratories
North Liberty, IA, USA
www.jmilabs.com
319.665.3370
fax 319.665.3371
ronald-jones@jmilabs.com

ABSTRACT

Background:
CLSI established *H. influenzae* (HI) susceptible (S) breakpoints (BKP) between 1990-1995, especially for penicillins to delineated ß-lactamase (+) strains. Few BLNAR strains (16-17) were available for analysis, but with their greater prevalence (e.g. Japan [J]) requires modification of BKPs and surrogate testing guidelines.

Methods:
We used 96 recent HI (J 85; other continents 11) having ampicillin ± sulbactam (AMP and A/S) MICs at ≥2 µg/ml by CLSI broth microdilution method. Comparison agents tested by MIC and disk diffusion (DD) methods were: amoxicillin/clavulanate (AMC), ceftriaxone (CRO), imipenem (IPM), meropenem (MEM) and piperacillin ± tazobactam (P/T). Results were used to validate CLSI and US-FDA (P/T) criteria or statements of BLNAR resistance regardless of test results. PCR-based identification and *ftsI* were evaluated.

Results:
Potency ranking for the BLNAR HI was (MIC_{90} in µg/ml): P/T (0.12) > CRO (0.25) > MEM 0.5) > AMP = IPM (4) > AMC (8). J and non-J isolates did not differ and ß-lactamase inhibitors did not enhance co-drug activity. BLNAR strain (with *ftsI* gr. II [66] and III [20] mutations) MIC values routinely span current CLSI BKPs causing serious interpretive discords most marked for DD tests. PK/PD analyses favor updated BKP criteria.

Antimicrobial	MIC category[a]	DD results by category: R	I	S
AMP	R	27	8	2
	I	24	26	9
A/S	R	24	-	8
	S	21	-	43
AMC	R	31	-	12
	S	28	-	25
CRO	Non-S	0	-	0
	S	26	-	70
IPM	Non-S	2	-	1
	S	20	-	73
MEM	Non-S	0	-	7
	S	4	-	85

a. I = intermediate.

Conclusions:
CLSI tests of contemporary BLNAR HI strains are associated with high intermethod error rates. Adjustment of S BKPs appears prudent for AMP and other potent anti-HI agents (CRO, MEM, P/T). P/T was 2- to 4-fold more active than other ß-lactams against BLNAR, but has been declared inactive by CLSI despite having S MIC values per US-FDA criteria.

INTRODUCTION

Haemophilus influenzae has long been a common cause of community-acquired respiratory tract infections (CA-RTI) and serious invasive disease (bacteremia and meningitis), although the later types of infection were markedly diminished by vaccine introduction. Resistances to commonly used treatment regimens remains rare except for ß-lactam hydrolysis mediated by a TEM-type enzyme. Standardized susceptibility methods for *H. influenzae* were developed by the Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) in the early 1990s based on the use of the modified Mueller-Hinton medium (Haemophilus Test medium or HTM).

Except for ß-lactamase-producing isolates, few resistant *H. influenzae* strains were available for test development and validation, that included the ß-lactamase-negative ampicillin-resistant (BLNAR) strains that occurred as rates of <0.1%. Subsequently, these BLNAR strains have become more frequent worldwide and particularly in the Western Pacific and some European nations. Also, fluoroquinolone-resistant *H. influenzae* having QRDR mutations have become more prevalent leading to confusion regarding the interpretation of non-susceptibility as tested/interpreted by CLSI methods.

Initial CLSI interpretive criteria for ampicillin were published in 1990 by Doern et al. and modifications by Jones and colleagues in 1992. As newer ß-lactams (oral cephems, carbapenems, penicillin/ß-lactamase inhibitor combinations) and other antimicrobial classes were developed, the CLSI added interpretive criteria to the yearly supplemental tables. However, no systemic re-evaluation of the *H. influenzae* breakpoints have been forthcoming as demanded by the greater emergence of BLNAR isolates and resistance to other drugs used to treat CA-RTI (mainly fluoroquinolones with MIC values of >0.06 µg/ml). Furthermore, discords between CLSI testing of *H. influenzae* by MIC and disk diffusion methods have been observed, additional problems with HTM lot-to-lot quality, suspected flaws in quality control ranges and unexpected favorable clinical outcomes using "supposed inactive" agents casts doubt on the contemporary validity of CLSI standards/breakpoints for *H. influenzae*. Also, the appearance and acceptance of pharmacodynamic principals as a significant contributor to breakpoint selection, places the current CLSI breakpoints in doubt, especially for those agents having interpretive criteria established prior to 2001.

In this study, we examined a highly selected collection of BLNAR *H. influenzae* strains from the global SENTRY Antimicrobial Surveillance Program. These 96 strains (with examined PBP3 gene data) were tested to determine intermethod accuracy of CLSI methods and interpretations for ampicillin, six other ß-lactams and ciprofloxacin. Also, piperacillin/tazobactam was reassessed for possible addition to the CLSI interpretive tables or modification of US-FDA product package insert.

MATERIALS AND METHODS

The SENTRY Program *H. influenzae* isolates were screened for strains having elevated ampicillin MIC values (≥2 µg/ml). These organisms (96; 85 from Japan) were confirmed by repeat testing with reference broth microdilution methods of the CLSI (M7-A7, 2006) and additional processing against ampicillin/sulbactam, amoxicillin/clavulanate, ceftriaxone, imipenem, meropenem, piperacillin/tazobactam and a non-ß-lactam control (ciprofloxacin). Disk diffusion testing was simultaneously performed using the CLSI M2-A9 (2006) method for the same antimicrobial agents. Interpretations of the test results used criteria published in M100-S17 (2007).

Results of MIC and disk diffusion testing were compared by scattergram analysis (Figure 1 A-H) with calculations of intermethod error. Limits of acceptable error were derived from CLSI M23-A2 (2001) where the occurrence of false susceptibility (very major error) was ≤1.5%, false resistance (major error) was ≤3.0% and minor error (intermediate by one test and resistant or susceptible by the other) was not to exceed 10.0%. An intermediate category criterion has not been established for the studied agents other than ampicillin. Furthermore, only susceptible breakpoints are currently published for ceftriaxone, the carbapenems and ciprofloxacin.

Please note the warning that BLNAR *H. influenzae* strains should be considered resistant to some ß-lactam compounds is found in M2-A2/M100-S17 tables (amoxicillin/clavulanate, ampicillin/sulbactam, cefaclor, cefetamet, cefonicid, cefprozil, cefuroxime and loracarbef) and in M7-A7/M100-S17 tables piperacillin/tazobactam and cefamandole were additionally listed, many of those statements present since the early 1990s. The *H. influenzae* quality control strain (ATCC 49747), a BLNAR organism, was utilized concurrently with all results recorded within CLSI ranges. Of note, piperacillin/tazobactam control ranges were 0.06-0.5 µg/ml and 33-38 mm; values considered to be highly susceptible.

H. influenzae isolates with BLNAR phenotype were tested using primers specific for *H. influenzae* and *H. haemolyticus* 16S rDNA, all were confirmed as *H. influenzae*. Categorization of BLNAR phenotype based on penicillin binding protein 3 (PBP3) into types I, II and III was achieved by PCR using primers that amplify specific regions of the *ftsI* gene.

RESULTS

- In global surveillance programs (SENTRY Program), the emergence of BLNAR *H. influenzae* was documented, greatest in Japan (85 of 96 isolates, Table 1).
- Susceptibility rates among eight tested agents was greatest for fluoroquinolones, ceftriaxone and piperacillin/tazobactam (100%), followed by the carbapenems (93-97%; Table 1).
- *ftsI* typing by PCR revealed group II (67) and III (20) mutations and resultant ß-lactam MIC values that span the currently applied CLSI breakpoints.
- When testing the BLNAR isolates, high rates of disk diffusion categorical errors were observed (Table 2; Figure 1A-H), with very major errors ranging from 0.0 (ceftriaxone and ciprofloxacin) to 12.5% (amoxicillin/clavulanate). Acceptable intermethod error rates and high categorical agreement were only observed for ciprofloxacin.
- Piperacillin/tazobactam only has a MIC susceptible breakpoint (CLSI and US-FDA) and a disclaimer about activity against BLNAR strains. However, this penicillin/ß-lactamase inhibitor combination was the most potent ß-lactam tested with activity two-fold superior to ceftriaxone with all MICs at ≤0.25 µg/ml. This was four-fold below the breakpoint (≤1 µg/ml) used for other *H. influenzae* (ß-lactamase-positive or -negative).
- Susceptible disk diffusion breakpoints for piperacillin/tazobactam are proposed at ≥21 mm that produces no serious interpretive errors and complete categorical agreement (100%), similar to that noted for very potent fluoroquinolones (Tables 1 and 2; Figure 1G).
- *H. influenzae* testing breakpoints were initially assessed over 15 years ago and clearly need to be re-evaluated due to the escalating occurrences of resistant isolates and the evolving role of pharmacokinetic/pharmacodynamic considerations in breakpoint selection. Adjustments appear necessary for aminopenicillin ± enzyme inhibitors, parenteral cephalosporins and carbapenems, each considering US-FDA approved clinical indications.

Table 2. Intermethod discord rates for the CLSI disk diffusion method when testing 96 BLNAR *H. influenzae* isolates against seven ß-lactam agents and ciprofloxacin.

Antimicrobial agent	Error rate (%) by category: Minor[a]	Major[a]	Very major[a]	Absolute categorical agreement (%)
Ampicillin	42.7	0	2.1	55.2
Ampicillin/sulbactam	NA[b]	21.9	8.3	69.8
Amoxicillin/clavulanate	NA	29.2	12.5	58.3
Ceftriaxone	NA	27.1	0.0	72.9
Imipenem	NA	20.8	1.0	78.1
Meropenem	NA	4.2	7.3	88.5
Piperacillin/tazobactam[c]	NA	0.0	0.0	100.0
Ciprofloxacin	NA	0.0	0.0	100.0

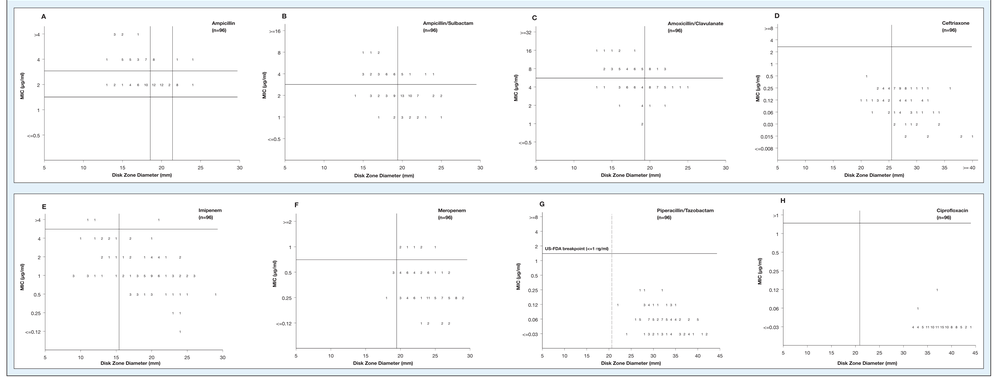
a. Very major = false-susceptible by disk diffusion method, major = false-resistant and minor = intermediate by one method and susceptible or resistant by the other (see ampicillin results only).
b. NA = not applicable due to unpublished intermediate criteria by the CLSI.
c. Results using proposed disk diffusion susceptible breakpoint at ≥21 mm to correlate with published MIC breakpoint (≤1 µg/ml) by CLSI and US-FDA.

Table 1. MIC distributions for seven ß-lactam antimicrobial agents tested by CLSI methods against ß-lactamase-negative ampicillin-non-susceptible *H. influenzae* (BLNAR; 96 strains from Japan [85] and worldwide [11]).

Antimicrobial agent	Cum % inhibited at MIC (µg/ml): ≤0.12	0.25	0.5	1	2	4	8	16	MIC (µg/ml) 50%	90%	% susceptible[a]
Ampicillin	0	0	0	0	61	94	-[b]	-	2	4	0
Ampicillin/sulbactam	0	0	0	13	67	96	100	100	2	4	67
Amoxicillin/clavulanate	0	0	0	2	9	55	94	100	4	8	55
Ceftriaxone	58	99	100	100	100	100	100	-	0.12	0.25	100
Imipenem	1	3	19	66	86	97	-	-	1	4	97
Meropenem	7	63	93	100	100	100	-	-	0.25	0.5	93
Piperacillin/tazobactam	97	100	100	100	100	100	100	100	0.06	0.12	100
Ciprofloxacin	100	100	100	100	-	-	-	-	≤0.03	≤0.03	100

a. CLSI MIC breakpoints only.
b. - = untested concentration.

Figure 1. Scattergrams illustrating the CLSI MIC breakpoints (solid horizontal lines) and disk diffusion (solid vertical lines) for ampicillin (A), ampicillin/sulbactam (B), amoxicillin/clavulanate (C), ceftriaxone (D), imipenem (E), meropenem (F), piperacillin/tazobactam (G) and ciprofloxacin (H) results tested against 96 ß-lactamase-negative, ampicillin non-susceptible *H. influenzae*. Broken vertical line shows the proposed disk diffusion breakpoint for piperacillin/tazobactam.



CONCLUSIONS

- BLNAR *H. influenzae* are occurring in clinical practice with greater regularity, especially in the Western Pacific (Japan).
- Current CLSI testing methods for *H. influenzae* do not accurately categorize the susceptibility of these BLNAR isolates, and a systematic re-evaluation appears urgently needed to prevent misguided chemotherapy (high rates of false-resistant errors by disk diffusion methods)
- Some agents previously considered as inactive against BLNAR *H. influenzae* (due to limited data) have now emerged as having great potency (piperacillin/tazobactam) and should be given accurate susceptible breakpoints as proposed here (MIC at ≤1 µg/ml [US-FDA] with correlate disk diffusion zones at ≥21 mm).

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