NZ NAC New Zealand National Antimicrobial Susceptibility Testing Committee

Haemophilus influenzae antimicrobial susceptibility testing

Replies to the recent NZ NAC Antimicrobial Susceptibility Testing questionnaire indicated that several laboratories are having some difficulty with *Haemophilus influenzae* susceptibility testing.

If you have recently switched from the CLSI method (using ampicillin 10 µg disc on HTM) to the EUCAST method (using penicillin 1 unit and/or ampicillin 2 µg disc on MH-F media), you have probably found an alarming increase of β-lactamase-negative ampicillin-resistant (BLNAR) *H. influenzae*.

Background:

Ampicillin resistance in *H. influenzae* is either due to the production of β-lactamase (usually TEM-1, or less commonly ROB-1) or amino acid substitutions in PBP3 due to *fts*I gene mutations (i.e. chromosomally-mediated resistance). Changes to PBP3 can result in reduced susceptibility to aminopenicillins and cephalosporins, including extended-spectrum cephalosporins. Resistance due to changes in PBP3 can be defined as low-level (R517H or N526K substitutions), high-level (additional S385T substitution) or third stage (L389F substitution). A variety of other mutations can also be present (1,2,3). The two types of resistant mechanisms can be found together.

Some strains of *H. influenzae* with PBP3 mutations have ampicillin MICs as low as 0.5 mg/L and many others group around the susceptibility breakpoint (i.e. ≤1 mg/L). Gradient strips can underestimate the MIC (1). Other issues with susceptibility testing include under or over confluent growth, despite using a 0.5 McFarland inoculum; and difficult-to-read fuzzy zones when MH-F plates have not been adequately warm and dry before use. Broth microdilution is the recommended gold standard method, but this is not usually practical for routine clinical laboratories.

During 2017 Canterbury Health Laboratories, in conjunction with ESR, conducted a study on 100 *H. influenzae* isolates, comparing penicillin, ampicillin and cefuroxime disc zone sizes, and ampicillin MICS, using EUCAST guidelines, with *fts*I gene mutations and consequent PBP3 substitutions. The study included 77 β-lactamase negative, penicillin-resistant isolates (i.e. zone diameter with pen 1 unit disc <12 mm) and 23 penicillin-susceptible isolates. One of the penicillin-resistant isolates could not be sequenced, so results will be discussed for the remaining 99 isolates.

Results:

There was excellent correlation between isolates which were penicillin resistant by disc diffusion (DD) and those with PBP-3 substitutions. 77 isolates had PBP3 substitutions; 76 of which were penicillin resistant by disc (Table 1). One isolate was penicillin susceptible by DD but possessed mutation A502V. 22 isolates were penicillin susceptible and had no PBP-3 mutations. More than 90% of the isolates with PBP3 substitutions had low-level type N526K (which is also the predominant genotype in Australia, Europe and North America) (1).

Table 1: PBP3 substitutions compared to disc diffusion

BLNAR categorisation according to PBP3 substitutions		Penicillin 1 unit disc		Ampicillin 2 µg disc		Cefuroxime 30 µg disc	
	Number	S	R	S	R	S	R
High	6	0	6	0	5	0	6
Low I or II	71	1	70	0	30	0	65
Not BLNAR	22	22	0	64	0	23	5
Correlation[1]		98.7% (76/77)		45.5% (35/77)		92.2% (71/77)	

1 Correlation between disc results and BLNAR categorisation

6/77 isolates were classified as having high-level resistance, possessing S385T + R517H or N526K substitutions; three of which also had the L389F substitution. Table 2 shows DD and ampicillin MIC results along with the *fts*I gene mutation category. Of note is that one of these isolates would not have been classified as BLNAR by ampicillin MIC strip (MIC 1.0 mg/L).

Table 2: High-level resistance by *fts*I gene mutations

Zone diameter (mm)				**Amp MIC (mg/L)**	**Skaare[4]**
Pen 1 unit	**Amp 2 µg**	**Amp 10 µg**	**Cfur 30 µg**		
6	6	15	14	6	High III-like + L389F
6	8	10	6	4	High III-like + L389F
6	13	19	6	2	High III + L389F
6	14	23	16	1	High III-like
6	15	21	12	2	High III-like
7	17	20	14	2	High III-like

There were 35/77 isolates classified as BLNAR (zone diameter <16 mm) using the ampicillin 2 µg DD test. All ampicillin-resistant isolates had one or more PBP3 substitution (Table 1). However only 20/35 isolates would have been classified as BLNAR by ampicillin MIC strip.

71/77 isolates with PBP3 substitutions were categorised as cefuroxime resistant (zone diameter <26 mm, IV administration) in the cefuroxime 30 µg DD test. However, there were 5 cefuroxime-resistant isolates that did not have any PBP3 substitutions (Table 2). Of note, cefuroxime zone sizes <15 mm correlated well with high-level resistance.

Only 15 of the 77 isolates with PBP3 substitutions were categorised as ampicillin resistant (zone diameter ≤18 mm) in the CLSI ampicillin 10 µg DD test. However, this proportion improved to 55 of 77 if isolates categorised as having 'intermediate' ampicillin

resistance (zone diameter 19-21 mm) were included (data not shown). It is well recognised that CLSI breakpoints fail to classify BLNAR correctly.

Our study has shown that using the EUCAST recommended screening method of penicillin 1 unit has high correlation with *fts*I gene mutations, predominantly affecting cephalosporins rather than ampicillin. MIC gradient strips cannot be relied on to confirm ampicillin disc diffusion, and since performing broth microdilution is not practical for routine laboratories, we recommend following EUCAST guidelines. Laboratories switching from CLSI to EUCAST will usually see an increase in the number of isolates classified as BLNAR.

References:

1. Skaare D, Lia A, Hannisdal A, *et al*. *Haemophilus influenzae* with non-Beta-Lactamase-mediated Beta-Lactam resistance: Easy to find but hard to categorize. *J Clin Microbio* 2015; 53: 3589-3595.
2. Skaare D, Anhonisen I, Kahlmeter G, *et al*. Emergence of clonally related multidrug resistant *Haemophilus influenzae* with penicillin-binding protein 3-mediated resistance to extended-spectrum cephalosporins, Norway, 2006 to 2013. *Euro Surveill* 2014; 19: pii=20986.
3. Søndergaard A, *et al*. Detection of N526K-substituted penicillin-binding protein 3 conferring low-level mutational resistance to β-lactam antibiotics in *Haemophilus influenzae* by disc diffusion testing on Mueller-Hinton agar according to EUCAST guidelines, *J Antimicrob Chemother* 2012; 67: 1401–1404.
4. Skaare D, Anthonisen I, Caugand D, etal. Multilocus sequence typing and fstI sequencing: a powerful tool for surveillance of penicillin-binding protein 3-mediated beta-lactam resistance in nontypeable Haemophilus influenzae. BMC Microbiol 2014; 14:131.

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