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RESEARCH ARTICLE

ANTIMICROBIAL SUSCEPTIBILITY PATTERNS OF HAEMOPHILUS *INFLUENZAE ISOLATED* FROM LOWER RESPIRATORY TRACT INFECTIONS

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ABSTRACT

Objective: Determination of antimicrobial susceptibility patterns of *H. influenzae* isolated from lower respiratory tract infections to commonly used antimicrobials.

Materials and methods: 142 sputum samples were collected from patients with lower respiratory tract infection from June/2013 to April/2014. Specimens were cultured on chocolate agar supplemented with multivitex under 10% CO_2. Isolation and identification of *H. influenzae* were based on standard bacteriological and biochemical criteria. Serotyping with specific antisera (Bio-RAD laboratories-Japan) was carried out by slide agglutination method. Antibiotic sensitivity test to 12 antimicrobials was determined by disc diffusion method. Furthermore, the minimum inhibitory concentration (MIC) of these antibiotics was assessed by agar dilution method.

Results: Throughout the study period 40 isolates of *H. Influenzae* were obtained. 29(72.5%) were recovered from males (mean age 46 ±18 years), and 11 from females (mean age 41.1 ±13.3 years). All isolates were sensitive to cefotaxime, pipracillin and amikacin. The sensitivity rate to rifampicin was 77.5%, amoxacillin 67.5%, gentamycin 62.5%, vancomycin 60%, chloramphenicol 72.5%, tetracycline 57.5% and for ciprofloxacin 70%. The resistance rate to ampicillin was 72.5%. None of the isolates were sensitive to cloxacillin. The median MIC for cefotaxime, pipracillin and amikacin was 0.5 mg/ml for both type b and nontype b isolates. The median MIC for cloxacillin, gentamycin and rifampicin was >1024, 0.5 and 0.5 mg/ml respectively for both serotypes. Otherwise, the difference in the mean MIC between type b and nontype b was insignificant except for chloramphenicol (P= 0.03) and rifampicin (P=0.02). However, a part from cefotaxime, pipracillin, amikacin and cloxacillin, 30% of isolates that were resistant by disc method became sensitive when retested by agar dilution method. The disc diffusion method showed that 97.5% of isolates were resistant to more than one antimicrobial, while agar dilution method revealed that 87.5% of the isolates were resistant to more than three antimicrobials. The sensitivity of *H. influenzae* to different antimicrobials neither affected by patient's age and sex nor by serotype.

Conclusion: *H. influenzae* recovered from lower respiratory tract infections were variably resistant to antimicrobials. Continuous surveillance of antimicrobial susceptibility at the local and national level remains important, in order to guide appropriate empirical antimicrobial therapy.

INTRODUCTION

H. influenzae are recognized as important cause of community-acquired pneumonia, acute exacerbation of chronic bronchitis, acute sinusitis and acute otitis media **(**Dominguez *et al*., 2004**).** Encapsulated *H. influenzae* contain capsular polysaccharide antigen one of six serotypes (a-f), type b capsule is polyribitol phosphate polymer, these surface polysaccharides are strongly associated with virulence of *H. influenzae* type b (Hib) **(**Cary, 2010**)**. The increasing rates of antimicrobial resistance of Hib are a worldwide concern (Ginsburg *et al*., 2013; Torres *et al*., 2014). Several studies and worldwide national surveillance groups have reported increased rates of resistance of Hib to different antimicrobials **(**Maraki and Papadakis, 2014; Shuel *et al*., 2014**;** Shiro *et al*.,2015**)**. However, on the other hand there were some reports found that Hib still had high susceptibility for certain antimicrobials (Geelen *et al*., 2013; Tomic and Dowzicky, 2014; Baba *et al*., 2015).

Multiple bacterial mechanisms may contribute in the development of antimicrobial resistance of Hib; β-lactamase positive Hib strains were reported to be more resistant to certain antimicrobials compared with β-lactamase-negative strains (Goto *et al*., 2009; Kyd *et al*., 2011; Zhao *et al*., 2015), ability of Hib for biofilms formation (Cardines *et al*., 2012), exhibition of hypermutable phenotypes (Cardines *et al*., 2012). Additionally, high gene transfer and inter-species recombination of the *fts*I gene among isolates of Hib in a clinical setting, highlighting its importance in the emergence of altered penicillin-binding protein 3 and β-lactamase-negative ampicillin-resistance (Bae *et al*.,2010; Barbosa *et al*., 2011;Witherden *et al*.,2014). Moreover, most reports associate co-trimoxazole resistance to the presence of variants of

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dihydrofolate reductase *dfr*A genes which are responsible for trimethoprim resistance; while the sulfamethoxazole resistance are due to sulfonamide genes *sul*1 and *sul*2 and/or mutation in the chromosomal *folP* gene encoding dihydropteroate synthetase (Mohd-Zain *et al*.,2013).Whereas, the mechanism of quinolone-resistance was found to be an amino acid mutation in the type II topoisomerase (Shoji *et al*., 2014). Furthermore, the carriage of high molecular weight conjugate plasmid in Hib was reported to constitute the genetic basis to ampicillin, tetracycline and chloramphenicol resistance and strongly associated with single and multiple resistances of these antibiotics **(**Levy *et al*., 1993**)**.However, the resistance to trimethoprim and clarithromycin determinant is usually chromosomally mediated **(**Campos *et al*., 2004). Regarding the Patient's background factors; children aged < 3 years and prior treatment with antimicrobials were risk factors for development of antimicrobial resistance (Hoshino *et al*., 2013; Shiro *et al*., 2015).

MATERIALS AND METHODS

This study was conducted from Jun/2013 to April/2014. 142 early morning sputum samples were collected from patients with lower respiratory tract infections. These patients were attending TB and Chest Disease Center in Baquba- Diyala province. Sputum samples were collected in wide-mouth sterile containers with a tightly fitting screw-cupped. Before culturing, the specimens were mixed thoroughly using sterile glass beads, then a loopfull was taken and streaked on chocolate agar plate supplemented with multivitex ((Multi-vitamins, BDH-UK).The plates were placed in a candle jar (5-10% CO_2) and incubated overnight at 37^0C. Isolation and identification of *H. influenzae* were based on colonial morphology and standard bacteriological and biochemical criteria. These include indol production, hydrolysis of urea agar, positive oxidase and catalase tests and production of hemolysis on blood agar plates **(**Brooks *et al*., 2007). Serotyping with specific antisera (Bio-RAD laboratories-Japan) was carried out by slide agglutination method.

Antimicrobial sensitivity test to the commonly used twelve antimicrobials was determined by disc diffusion method on chocolate agar plates **(**Kirby *et al*., 1966). Furthermore, the minimum inhibitory concentration (MIC) of these antimicrobials was assessed by agar dilution method as described by **(**Slack, 2007).

RESULTS

Throughout the study period, 40 isolates of *H. influenzae* were recovered, 29(72.5%) were recovered from males (mean age 46 ±18 years), and 11 from females (mean age 41.1 ±13.3 years). Furthermore, serotyping revealed that 7(17.5%) were *H. influenzae type b* and the remaining 33(82.5%) were nontype b. The results of antimicrobial susceptibility of *H. influenzae* isolates by disc diffusion and agar dilution methods were shown in table (1) and (2) respectively.

Table 1 Sensitivity and resistant rates of *H. influenzae* isolates by disc diffusion method

Antibiotics	Sensitive		Resistant	
	No.	%	No.	%
Cefotaxime	40	100	0	0
Vancomycin	24	60	16	40
Pipracillin	40	100	0	0
Cloxacillin	0	0	40	100
Ampicillin	11	27.5	29	72.5
Tetracycline	23	57.5	17	42.5
Amoxacillin	27	67.5	13	32.5
Chloramphenicol	29	72.5	11	27.5
Rifampicin	31	77.5	9	22.5
Gentamycin	25	62.5	15	37.5
Ciprofloxacin	28	70	12	30
Amikacin	40	100	0	0

Table 2 Sensitivity and resistant rates of 16 *H. influenzae* isolates by agar dilution method.

Antibiotics	Sensitive		Resistant	
	No.	%	No.	%
Cefotaxime	16	100	0	0
Vancomycin	9	56.5	7	43.8
Pipracillin	16	100	0	0
Cloxacillin	1	6.3	15	93.7
Ampicillin	6	37.5	10	62.5
Tetracycline	11	68.8	5	31.3
Amoxacillin	11	68.8	5	31.3
Chloramphenicol	12	75	4	25
Rifampicin	13	81.3	3	8.8
Gentamycin	12	75	4	25
Ciprofloxacin	10	62.5	6	37.5
Amikacin	16	100	0	0

A part from cefotaxime, pipracillin and amikacin to which all *H. influenzae* isolates were sensitive by both methods, the difference in sensitivity to other antimicrobials by both methods was statistically insignificant as shown in table (3).

The median of MIC of cefotaxim, vancomycin, cloxacillin, ampicillin pipracillin and tetracycline for *H. influenzae* type b and non-type b isolates as determined by agar dilution method was illustrated in table (4 A). The median MIC of these antibiotics on type b isolates compared to non-type b isolates was statistically insignificant.

Additionally, the results were found that 97.5% of the *H. influenzae* isolates were resistant to more than one antimicrobial by disc diffusion method. Whereas 100% of the isolates were resistant to more than one antimicrobials, table (5).

DISCUSSION

Two major ways that modern medicine saves lives are through antibiotic treatment of severe infections and the performance of medical and surgical procedures under the protection of antibiotics. Recently, it has been stated that at least some clinical isolates of many pathogenic bacteria species; *M. tuberculosis, N. gonorrhoeae, E. faecium, S. aureus, K. pneumoniae, A. baumannii, P. aeruginosa,* and species of enterobacter, salmonella, and shigella are now resistant to most antibiotics; therefore, the problem seems out of control (Nathan and Cars, 2014). Unfortunately, in our country, the

antimicrobial resistance crisis has multiple contributes, since till date there is neither national guidelines controlling antimicrobial prescription in governmental health care settings and private clinics nor national rules restricting antimicrobial purchasing at private pharmacies, overlapped by patient's uncommitment.

Table 3 Comparison of antibiotic susceptibility of *H. influenzae* isolates by disc diffusion and agar dilution methods.

Sensitivity by disc method	Sensitivity by tube dilution method						P (McNemar)
	Sensitive		Resistant		Total		
	No.	%	No.	%	No.	%	
Vancomycin							
Sensitive	9	100	0	0	9	100	1 [NS]
Resistant	0	0	7	100	7	100	
Total	9	56.3	7	43.8	16	100	
Cloxacillin							
Sensitive	0	0	0	0	0	0	0.5 [NS]
Resistant	1	6.3	5	93.8	16	100	
Total	1	6.3	15	93.8	16	100	
Ampicillin							
Sensitive	4	100	0	0	4	100	0.25 [NS]
Resistant	2	16.7	10	83.3	12	100	
Total	6	37.5	10	62.5	16	100	
Tetracycline							
Sensitive	8	100	0	0	8	100	1 [NS]
Resistant	3	37.5	5	62.5	8	100	
Total	11	68.8	5	31.3	16	100	
Amoxacillin							
Sensitive	10	100	0	0	10	100	1 [NS]
Resistant	1	16.7	5	83.3	6	100	
Total	11	68.8	5	31.3	16	100	
Chlorampheicol							
Sensitive	12	100	0	0	12	100	1 [NS]
Resistant	0	0	4	100	4	100	
Total	12	75	4	25	16	100	
Rifampicin							
Sensitive	12	100	0	0	12	100	1 [NS]
Resistant	1	25	3	75	4	100	
Total	13	8.8	3	18.8	16	100	
Gentamycin							
Sensitive	10	100	0	0	100	100	0.5 [NS]
Resistant	2	33.3	4	66.7	100	100	
Total	12	75	4	25	100	100	
Ciprofloxacin							
Sensitive	8	100	0	0	8	100	0.5 [NS]
Resistant	2	25	6	75	8	100	
Total	10	62.5	6	37.5	6	100	

The results of the present study showed that in both disc diffusion and agar dilution methods, the cefotaxime, pipracillin and amikacin were 100% effective against *H. influenzae* isolates. This is probably because these antimicrobials are newly introduced in clinical practice in our country. However, similar results were documented in previous reports **(**Bae *et al*., 2010; Tomic and Dowzicky, 2014; Baba *et al*., 2015**)**. In this regard it has been found that the newer cephalosporins, the cefotaxime gives excellent therapeutic results in *H. influenzae* isolates (Marco *et al.,* 2001). On the other hand the results showed that 43.7% of the tested *H. influenzae* isolates were resistant to chloramphenicol, an antibiotic that sometimes was the drug of choice for treating *H. influenza* infections. Previous studies, however, reported different rates of chloramphenicol resistance among *H. influenzae* isolates **(**Ginsburg *et al*., 2013; Shuel *et al*., 2014). Other studies had reported a higher chloramphenicol resistance of β-lactamase producing compared to β-lactamase non-producing *H. influenza* strains (Bae *et al*., 2010; Zhao *et al*., 2015). Of note, in a previous study, we found that 20 % of *H. influenza* isolates was β-lactamase producers (Al-Azawi *et al*., 2008). Nevertheless, the emergence of chloramphenicol resistant isolates of *H. influenzae* may necessitate further studies as this may interfere with the empirical treatment of respiratory tract infections.

Table 4A Median MIC of cefotaxim, vancomycin, pipracillin cloxacillin, ampicillin and tetracycline on type b and non-type b H. influenzae isolates.

Antimicrobials	*H. influenzae* serotype		P value
	Non-type	Type b	
Cefotaxim			
Range of MIC	0.5-0.5	0.5-0.5	0.69 [NS]
Median MIC	0.5	0.5	
No. of isolates (%)	12 (100)	4 (100)	
Vancomycin			
Range of MIC	0.5-64	0.5-128	0.39[NS]
Median MIC	0.5	16	
No. of isolates (%)	7(58.3)	2 (50)	
Pipracillin			
Range of MIC	0.5-0.5	0.5-0.5	0.38 [NS]
Median MIC	0.5	0.5	
No. of isolates (%)	12 (100)	4(100)	
Cloxacillin			
Range of MIC	8- >1024	512->1024	0.71 [NS]
Median MIC	>1024	>1024	
No. of isolates (%)	1 (8.3)	0	
Ampicillin			
Range of MIC	0.5->1024	4->1034	0.07 [S]
Median MIC	32	>1024	
No. of isolates (%)	7(58.3)	1(25)	
Tetracycline			
Range of MIC	0.5-512	0.5-512	0.2 [NS]
Median MIC	4	64	
No. of isolates (%)	9 (75)	2 (50)	

Table 4B MIC of amoxacillin, chloramphenicol, rifampicin, gentamycin, ciprofloxacin and amikacin for both type b and non-type b isolates

Antimicrobial	*H. influenzae* serotype		P value
	Non-type b	Type b	
Chloramphenicol			
Range of MIC	0.5-32	0.5-64	0.03 [S]
Median MIC	0.5	16	
No. of isolates	10(83.3)	3(75)	
Rifampicin			
Range of MIC	0.5-128	0.5-0.	0.02 [S]
Median MIC	0.5	0.5	
No. of isolates (%)	9 (75)	4(100)	
Gentamycin			
Range of MIC	0.5-128	0.5-164	0.8 [NS]
Median MIC	0.5	0.5	
No. of isolates (%)	9 (75)	3(75)	
Ciprofloxacin			
Range of MIC	0.5- 1024	0.5-64	0.39[NS]
Median MIC	0.5	16	
No. of isolates (%)	8 (66.6)	2(50)	
Amikacin			
Range of MIC	0.5-0.5	0.5-0.5	0.4[NS]
Median MIC	0.5	0.5	
No. of isolates (%)	12 (100)	4(100)	
Amoxacillin			
Range of MIC	0.5-64	0.5-512	0.14 [NS]
Median MIC	0.5	16	
No. of isolates (%)	9(75)	2(50)	

The present results also found that 72.5% and 62.5% of *H. influenza* isolates were resistant to ampicillin by agar diffusion

and agar dilution methods respectively. A high resistance rate of *H. influenza* to ampicillin was reported by other studies, most of which were linked to β-lactamase production (Goto *et al*., 2009; Bae *et al*., 2010; Hoshino *et al*., 2013; Shuel *et al*., 2014). Whereas, other studies documented a higher sensitivity rate (Cardines *et al*., 2012; Mohd-Zain *et al*., 2012; Tomic and Dowzicky, 2014; Zhao *et al*., 2015). It was well documented that ampicillin resistance was associated with mutations in the transpeptidase domain of the *fts*I gene, including most β-lactamase negative ampicillin resistant and high percentage of β-lactamase positive ampicillin resistant strains (Barbosa *et al*., 2011; Park *et al*., 2013). Regarding the tetracycline, 68.8% of our *H. influenza* isolates were sensitive. However, different previous studies had yielded different sensitivity rates of *H. influenzae* strains from respiratory tract infections to tetracycline (Mohd-Zain *et al*., 2012; Shuel *et al*., 2014; Maraki *et al*., 2014; Zhao *et al*., 2015).

Table 5 Rate of multi-antibiotic resistance by disc diffusion and agar dilution methods.

No. of antimicrobial	Resistant isolates by disc diffusion method		Resistant isolates by agar dilution method	
	No.	%	No.	%
1	1	2.5	0	0
2	6	15	2	12.5
3	9	22.5	8	50
4	11	27.5	2	12.5
5	6	15	2	12.5
6	2	5	1	6.3
7	4	10	1	6.3
8	1	2.5	0	0
Total	**40**	**100**	**16**	**100**

A comparison of sensitivity patterns of *H. influenzae* to different antibiotics between the disc diffusion and agar dilution method showed insignificant difference. Although similar results were reported by other studies **(**Monoharan *et al*., 2003**)**. However, it can be suggested that the agar dilution method can be used more reliably in routine antibiotic susceptibility testing of *H. influenzae* isolates.

A part from chloramphenicol and rifampicin, the MIC of all other antibiotics for both type b and non-type b isolates were statistically insignificant. Probably the presence of polysaccharide capsule in type b isolates may have a role in antibiotic resistance. In this regard it as been reported that capsulated strains of *H. influenzae* were significantly more resistant to ampicillin, tetracycline and chloramphenicol than were non-capsulated strains **(**Park *et al*., 2013; Intakorn *et al*., 2014; Garmendia *et al*., 2014**)**.

The rate of multi drug resistance of *H. influenzae* was fascinating results, about one half of the isolates were resistant to 3 or more antimicrobials. Although similar results were found in previous studies **(**Dabernat and Delmas, 2012; Pfeifer *et al*., 2013; Skaare *et al*., 2014). However, the high rates of multi- drug resistant isolates of *H. influenzae* in the present study necessitate further investigations to verifying these results.

The worldwide antimicrobial resistance crisis has a potentially devastating effect on human beings, livestock, and the global economy. It has been estimates that 300 million people will die as a result of drug resistance during the next 35 years (Editorial, 2014). Therefore, continuous surveillance of antimicrobial susceptibility at the local and national level remains important, in controlling the prevalence of resistance, since actual resistance data should be taken into account to guide appropriate empirical antimicrobial therapy, beside improvement in the diagnostic methods and continued education are recommended to guide discontinuation of antimicrobials.

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