β-LACTAMASE ACTIVITIES AND RESISTANCE TO ANTIBIOTICS OF *HAEMOPHILUS INFLUENZAE*, *H. PARAINFLUENZAE* AND *H. APHROPHILUS* STRAINS IDENTIFIED IN THROAT CULTURES FROM CHILDREN

Güven Uraz, Hülya Şimşek and Berrin Çelik

Microbiology Department, Faculty of Science and Arts
Gazi University, Ankara, Turkey

SUMMARY

Haemophilus bacteria are normally present in the upper respiratory tract of healthy individuals. However, these bacteria could be opportunistic pathogens especially in children. The present study was conducted to determine β-lactamase activity of *Haemophilus* from the throat cultures of children with upper respiratory tract infections. 154 *Haemophilus* strains were isolated from throat swabs of 208 children whom had upper respiratory tract infections. Among the 154 *Haemophilus* strains isolated, 117 *H. influenzae* (76%), 35 *H. parainfluenzae* (22.7%), and two *H. aphrophilus* (13%) were identified by API NH. β-Lactamase activity was positive in 42 isolates of 117 *H. influenzae* isolates, while it was negative in 75 isolates. β-Lactamase activity was positive in 20 *H. parainfluenzae* isolates, and negative in 15. All the *H. aphrophilus* isolates were β-lactamase negative.

It is known that β-lactamase positive *Haemophilus* bacteria are resistant to some antibiotics. Therefore, the antibiotic resistance of *Haemophilus* was further investigated in relation to β-lactamase activity. The *in vitro* antibacterial susceptibilities of *Haemophilus* strains for ampicillin, sulbactam-ampicillin, trimethoprim-sulfamethoxazole, gentamicin, chloramphenicol and ciprofloxacin were tested by disk diffusion method on chocolate agar. In 42 β-lactamase-positive *H. influenzae* isolates, 32 isolates were resistant against ampicillin. In 20 β-lactamase-positive *H. parainfluenzae* isolates, 16 were resistant against ampicillin. The two β-lactamase negative *H. aphrophilus* were sensitive to ampicillin.

Biotypes and serotypes were also investigated. Biotypes of *H. influenzae* strains were as follows: 40 strains biotype II, 25 strains biotype I, 14 strains biotype III, and 38 strains biotypes VII, VIII, V, and IV. Biotypes of 35 *H. parainfluenzae* strains were: 6 strains biotype III, 5 strains biotype I, 5 strains biotype IV. Biotypes of remaining 19 isolates were II, VIII, VI and VII. The serotypes of *H. influenzae* strains were determined by specific antiserums. Serotypes of 117 *H. influenzae* found were type a, b, c, d, and f.

KEY WORDS

Haemophilus, β-lactamase, antibacterial susceptibility

INTRODUCTION

It has recently been reported that increased numbers of β-lactamase-producing *Haemophilus* strains have been isolated from infections such as those of the respiratory tract in children younger than 4-5 years old. *Haemophilus* bacteria, isolated from the upper respiratory tract of children in this age group, were identified by the API NH method in this study.

The aims of our study were to identify *Haemophilus* bacteria isolated from clinical specimens and to determine β-lactamase enzyme activity and antibiotic susceptibility.

MATERIALS AND METHODS

Throat swabs (n = 208) were obtained children aged from 0 to 15 years and were examined at the Department of Child Health and Disease, SSK Ankara Hospital, and 154 *Haemophilus* strains were isolated.

Isolation of *Haemophilus* bacteria

The specimens were inoculated into horse blood and chocolate agar containing bacitracin, vancomycin and clindamycin, and were incubated at 37°C in 5-10% CO_2. After 48 h incubation, very small colonies appeared. Slides were prepared from these colonies and were

stained with Gram stain. When Gram-negative coccobacilli were seen by microscopic investigation, they were again inoculated into sheep or horse blood and chocolate agar with antibiotics /1/.

Use of API NH method for the identification of *Haemophilus* species

The API NH method was used to identify the isolated *Haemophilus* bacteria /2/. The API NH method (Bio Merieux-France) requires the following materials: API NH strip, 0.85% NaCl bulb, ZYMB solution, JAMES solution, swab, incubator, results chart and results table. In addition to the above materials, standard bulb, mineral oil, bulb stand, and identification software were also used. Strips and solutions used for the API NH method were stored at 2-8°C, while suspensions were stored at 2-30°C. For identification by API NH, first an incubator box was prepared and just one bulb containing 0.85% NaCl was opened for each isolate. Then *Haemophilus* colonies, whose McFarland turbidity was adjusted to 4, were inoculated into the bulb containing 0.85% NaCl. Fifty ml of this suspension were inoculated into 7 microtubes, and 150 ml of suspension were inoculated into 3 other microtubes. Mineral oil was added to the 7 microtubes containing penicillinase, glucose, fructose, maltose, saccarose, ornithindecarboxylase, and urease tests. Mineral oil was also added to the other 3 microtubes containing lipase, alkaline phosphatase, and β-galactosidase tests. The incubator was then closed and all the tubes were incubated under anaerobic conditions at 35-37°C for 2 hours. After the results of these 10 biochemical tests were obtained, ZYMB solution was added to the eighth and ninth microtubes, and JAMES solution was added to the tenth microtube. Thus, proline arylamidase, gamma glutamyl transferase and indol were also tested.

Evaluation of API NH method

Haemophilus species were classified according to negative and positive reactions (Table 1) by comparing the colors obtained to those in the standard table. The penicillinase test results were also determined with the API NH method. According to the results of the 12 biochemical tests used in the API NH method, *Haemophilus* species and their biotypes were determined. Biotypes of *Haemophilus* strains were determined according to the results of the indol, urease and ornithindecarboxylase tests (Table 2).

TABLE 1

Biochemical determination tests for *Haemophilus* species by API NH method

Haemophilus species	GLU	FRU	MAL	SAC	ODC	UREA	LIP	PAL	β-GAL	ProA	GGT	IND
H. influenzae	+	–/+	–	–	+	+	–	+	–	–	–	+
H. parainfluenzae	+	+	+	+	+	+	–	+	–	–	–	+
H. aphrophilus	+	+	+	–	–	–	–	+	+	–	+	–

Glucose (GLU), fructose (FRU), inulobiose (MAL), saccharose (SAC), ornithine carboxylase (ODC), urease (UREA), lipase (LIP), alkaline phosphatase (PAL), β-galactosidase (β-GAL), proline arylamidase (Pro A), γ-glutamyl transferase (GGT), indol (IND).

TABLE 2

Biochemical biotype determination tests
for *H. influenzae* and *H. parainfluenzae*

***Haemophilus* species and their biotypes**	**Biochemical biotype tests**		
H.influenzae	**Indol**	**Urease**	**Ornithinde-carboxylase**
Biotype I	+	+	+
Biotype II	+	+	–
Biotype III	–	+	–
Biotype IV	–	+	+
Biotype V	+	–	+
Biotype VI	–	–	+
Biotype VII	+	–	–
Biotype VIII	–	–	–
H. parainfluenzae			
Biotype I	–	–	+
Biotype II	–	+	+
Biotype III	–	+	–
Biotype IV	+	+	+
Biotype V	–	–	–
Biotype VI	+	–	+
Biotype VII	+	+	–
Biotype VIII	+	–	–

Serotypes of *H. influenzae* isolates

Serotypes of the isolates were determined by a slide agglutination method /3/. Bacto polyvalent a, b, c, d, e, f antiserums (DIFCO, Lot 60912LA) of *H. influenzae* were used. *H. influenzae* colonies were

mixed with 0.9% serum. If agglutination occurred, the result was recorded as positive. The serotype was defined according to agglutination in the corresponding antiserum. *H. influenzae* isolates which agglutinated with more than one antiserum were defined as non-serotype.

Determination of antibiotic susceptibility of *Haemophilus* species

The antibiotic susceptibility of the *Haemophilus* strains was examined by Kirby Bauer disk diffusion method /4,5/. McFarland turbidity of *Haemophilus* colonies was adjusted to 0.5 in broth. Antibiotic disks (DIFCO, Lot 60912LA) were placed in 2-2.5 cm wells inoculated with the suspension, and then incubated at 37°C, 8% CO_2 for 48 hours. Antibiotic sensitivities were determined by measuring the zone diameters formed around the antibiotic disks.

RESULTS

In this study, 154 *Haemophilus* strains (74%) were isolated from 208 throat swabs of children who had upper respiratory tract infections. Among these strains, 117 were *H. influenzae* (76%), 35 were *H. parainfluenzae* (22.7%) and two were *H. aphrophilus* (1.3%) (Table 3).

TABLE 3

Distribution of 154 *Haemophilus* species isolated from throat swabs

Isolated *Haemophilus* species	Number of isolated *Haemophilus*	%
H. influenzae	117	76.0
H. parainfluenzae	35	22.7
H. aphrophilus	2	1.3

Table 4 shows the biotype distribution of *H. influenzae* and *H. parainfluenzae* strains. Serotypes of *H. influenzae* strains were determined by slide agglutination method. Serotypes could be detected for 50 (42.8%) of 117 *H. influenzae* isolates. Serotype e was not

TABLE 4

Biotypes distribution of isolated *H. influenzae* and *H. parainfluenzae* strains

Biotypes	I	II	III	IV	V	VI	VII	VIII	Non-Biotype	Total
H. influenzae (n)	25	40	14	2	8	–	10	9	9	117
H. influenzae (%)	21.4	34.2	11.9	1.7	6.8	–	8.6	7.7	7.7	100
H. parainfluenzae (n)	5	4	6	5	–	2	2	3	8	35
H. parainfluenzae (%)	14.3	11.5	17.1	14.3	–	5.7	5.7	8.6	22.8	100

present among the *H. influenzae* strains. Sixty-seven of the *H. influenzae* isolates (57.2%) were non-serotype (Table 5).

Among 117 *H. influenzae* isolates, β-lactamase enzyme activity was positive in 42 (35.9%) isolates. Among the 35 *H. parainfluenzae* isolates, 20 (57.2%) were β-lactamase positive (see Table 6).

TABLE 5

Serotype distribution of *H. influenzae* strains

Serotypes	**Distribution of *H. influenzae* serotypes**	
	n	**%**
A	17	14.5
B	18	15.4
C	5	4.3
D	5	4.3
E	0	–
F	5	4.3
Non-serotype	67	57.2
Total	117	100

TABLE 6

β-Lactamase enzyme activity of *Haemophilus* isolates

Isolated *Haemophilus* species	**β-Lactamase positive**	**%**	**β-Lactamase negative**	**%**	**Total**
H. influenzae	42	35.9	75	64.1	117
H. parainfluenzae	20	57.2	15	42.8	35
H. aphrophilus	–	–	2	100	2

Tables 7 and 8 show antimicrobial susceptibility in β-lactamase positive and negative *Haemophilus* isolates.

TABLE 7

Susceptibility and resistance in β-lactamase positive *Haemophilus* isolates against various antibiotics

Antibiotics	*H.influenzae* n=42		*H. parainfluenzae* n=20	
	S	**R**	**S**	**R**
Ampicillin	10	32	4	16
Sulbactam-ampicillin	23	19	6	14
Trimeth.-sulfamet	13	29	5	15
Gentamicin	11	31	6	14
Chloramphenicol	28	14	10	10
Ciprofloxacin	36	6	18	2

S = susceptible, **R** = resistant

TABLE 8

Susceptibility and resistance in β-lactamase negative *Haemophilus* isolates against various antibiotics

Antibiotics	*H. influenzae* n=75		*H. parainfluenzae* n=15		*H. aphrophilus* n=2	
	S	**R**	**S**	**R**	**S**	**R**
Ampicillin	52	23	15	–	2	–
Sulbactam-ampicillin	58	17	14	1	2	–
Trimeth.-sulfamethox	17	58	3	12	1	1
Gentamicin	11	64	3	12	2	–
Chloramphenicol	42	33	10	5	1	1
Ciprofloxacin	71	4	15	–	2	–

S = susceptible, **R** = resistant

DISCUSSION

Although *Haemophilus* bacteria are normally present in the upper respiratory tract of healthy individuals, they may be opportunistic pathogens in children; they can cause meningitis, pneumonia, endocarditis, bronchitis, and otitis media.

In this study, 154 (74%) *Haemophilus* strains were isolated from 208 throat cultures. This is comparable to the results of Mamal *et al.* /6/, who isolated 60 (60%) strains of *Haemophilus* from throat cultures of 100 children. We found 117 (76%) strains of *H. influenzae* from 208 throat cultures. This is high compared to the results of Howard *et al.* /7/, who isolated 304 (30.5%) *H. influenzae* from 996 throat cultures, but similar to those of another Turkish study by Karaaslan *et al.* /8/, who isolated 117 (71%) *H. influenzae* from 165 throat cultures. The number of *H. influenzae* isolated by Chapin *et al.* /1/, 609 (71.3%) from 852 throat cultures, is also similar to our results.

Doern *et al.* /9/ found 26.9% of *H. influenzae* were serotype b; these results are similar to ours. In our study, 25 strains of 117 *H. influenzae* isolates were determined as biotype I, 40 strains were biotype II, 14 strains were biotype III, 2 strains were biotype IV, 10 strains were biotype VII, and 9 strains were biotype VIII. Brabender *et al.* /10/ found 5 biotype I, 10 biotype II, 11 biotype III, 3 biotype IV, and 2 biotype V of 93 *H. influenzae* isolates; these results are similar to ours, as are those of Sturn /11/, who found 6 of 52 *H. influenzae* isolates to be biotype I, 22 biotype II, 17 biotype III, 2 biotype IV, 2 biotype V, 1 biotype VII, and 1 biotype VIII.

Jorgensen *et al.* /12/ isolated 35 (38.9%) *H. parainfluenzae* strains from 90 *Haemophilus* isolated from throat cultures; our results were lower.

In the present study, 75 (64.1%) of 117 *H. influenzae* isolates were β-lactamase negative, and 42 (35.9%) were β-lactamase positive. Thirty of the 42 β-lactamase-positive *H. influenzae* isolates were resistant to ampicillin. Brabender *et al.* /10/ found 7 strains of 101 *H. influenzae* isolates β-lactamase positive. Farley *et al.* /13/ found 13 β-lactamase positive strains were resistant to ampicillin. Our findings are similar to theirs. Powell *et al.* /5/ found 9.4% β-lactamase positive isolates of 1272 *H. influenzae* strains were resistant to ampicillin. Jorgensen *et al.* /12/ stated that all β-lactamase positive *H. parainfluenzae* isolates were resistant against ampicillin; this finding is

higher than ours. Jorgensen /14/ found a greater number of β-lactamase positive *H. parainfluenzae* isolates than of *H. influenzae*. This finding is similar to ours.

Ampicillin is a frequently used antibiotic. However, our findings suggest that it will not be effective against a large proportion of β-lactamase positive *Haemophilus* strains commonly found in children. Although sulbactam-ampicillin inhibits β-lactamase activity, it is not much more effective. Trimethoprim-sulfamed, gentamicin, and chloramphenicol have been thought to produce resistance based on these antibiotics' own structures. Ciprofloxacin has been suggested to be the most effective drug for *Haemophilus* infections. Our findings have shown once again that *Haemophilus* bacteria in the upper respiratory tract are an important source of infection. Future studies are needed to determine the relationship between β-lactamase activity and the antibiotic susceptibility of *Haemophilus* strains.

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