Rev Esp Quimioterap, Junio 2003; Vol.16 (Nº 2): 195-203
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Original

Haemophilus influenzae and betalactam resistance: Description of bla_{TEM} gene deletion

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SUMMARY

The resistance to betalactam antibiotics in a total of 177 clinical isolates of Haemophilus influenzae, *mostly from respiratory tract samples and characterized by their betalactamase production, was studied using the cephalosporin chromogenic assay and by detecting* bla_{TEM} *and* bla_{ROB} *genes. A substantial number of clinical isolates carrying the* bla_{TEM} *gene, which presented a mutation consisting of the absence of a fragment of 136 base pairs, located upstream from the coding region including the –35 but not the –10 region of the promoter were found. This suggests that a new* bla_{TEM} *promoter exists in these strains. This finding was associated with increased resistance to the antibiotics cefaclor and loracarbef compared with normal isolates. It was also found that 3.9% of the isolates carried the* bla_{ROB-1} *gene.*

Key words: *Haemophilus influenzae* - Betalactamases - bla_{TEM} gene deletion

Haemophilus influenzae *y resistencia a los betalactámicos: Descripción de una supresión en el gen* bla_{TEM}

RESUMEN

Se ha estudiado la resistencia a antibióticos betalactámicos mediante la producción de betalactamasas, detección de cefalosporina cromogénica y de los genes bla_{TEM} *y* bla_{ROB} *en 177 aislamientos clínicos de* Haemophilus influenzae, *la mayoría de ellos procedentes de muestras respiratorias. Se detectó un número importante de aislamientos clínicos portadores del gen* bla_{TEM} *que presentaban una mutación consistente en la ausencia de un fragmento de 136 pares de bases (pb), localizada por encima de la región codificante incluyendo la región del promotor –35 pero no la región –10 del mismo. Esto sugiere la existencia de un nuevo promotor en estas cepas. Este hallazgo está asociado con un aumento de la resistencia a los antibióticos cefaclor y loracarbef en comparación con los aislamientos normales. Un 3,9% de los aislamientos eran portadores del gen* bla_{ROB-1}.

Palabras clave: *Haemophilus influenzae* - Betalactamasas - Supresión en gen bla_{TEM}

INTRODUCTION

Mutations in the genes that encode resistance to betalactam antibiotics have been described in several studies (1-7). These include mutations in extended-spectrum betalactamases TEM at positions 21, 164 and 265 (3). These mutations, however, are not always associated with an increase or decrease in bacterial resistance to betalactam antibiotics. It was therefore considered of interest to study the resistance to betalactam antibiotics conferred by the bla_{TEM} (bla_{TEM-1} and bla_{TEM-2}) and bla_{ROB} genes in clinical isolates of *Haemophilus influenzae* from our area of Spain, with the aim of establishing its distribution in the presence or absence of mutations of these isolates.

MATERIALS AND METHODS

A total of 177 clinical isolates of *H. influenzae* from children (77%) and adults (23%) (52% females and 48% males) were studied. All the isolates were obtained from different samples (sputum 25%, nasopharynx 47%, blood 9%, middle ear fluid 5%, eye 14%) from 5% of outpatients and 95% of inpatients (one isolate per patient) in our department, as previously identified using the automatic *Vitek*® (bioMerieux, Marcy-l'Etoile, France) system and factors X and V. The isolates were cultured in agar-chocolate (*Difco*®) (Difco Laboratories, Ltd., USA), and after 24 h of incubation at 36 ºC in an atmosphere of 10% CO_2, the cephalosporin chromogenic test (Cefinase, BBL Microbiology System, USA) was performed. The minimum inhibitory concentrations for the antibiotics ampicillin, coamoxiclav, cefaclor and loracarbef were then determined using the *E-test*® system (AB Biodisk, Solna, Sweden) in *Haemophilus* test medium. *H. influenzae* 49247, and *H. influenzae* 10211 were used as control organisms. The isolates were conserved in powdered 4% skim milk at a temperature of –80 ºC, and the colonies were separated for DNA extraction.

Molecular tests

DNA extraction

DNA extraction was performed by resuspending a colony of the isolates in 100 µl of sterile distilled water and heating at 100 ºC for 10 min, in order to lyse the bacterial wall and release the DNA. Subsequently, the lysate was centrifuged for 30 seconds at 15,000 rpm to eliminate cellular debris, and the supernatant was used for the bla_{TEM} and bla_{ROB} gene amplification reaction.

Amplification

Following extraction of the genetic material, the specific amplification of the bla_{TEM} and bla_{ROB} genes was conducted. Using the DNA extract, amplification of the bla_{TEM} gene was carried out using the primers described by Scriver *et al*. (8). The reaction was performed in a volume of 100 µl of the following composition: reaction buffer 1X (Tris-HCl 10 mM, KCl 50 mM, Triton X 100 0.1%, $MgCl_2$ 1.5 mM); dNTPs 200 µM, 50 pmol of the TEM[1] primer (5'-GTG TTA TCA CTC ATG GTT ATG-3') and 50 pmol of the TEM[2] primer (5'-GAA TTC TTG AAG ACG AAA GGG-3'); 2.5 U of Taq-polymerase (Promega); and 10 µl (1 ng) of the DNA extracted from the different isolates.

The reaction was performed in a Perkin Elmer 9600 thermal cycler with the following thermal profile: one cycle of 94 ºC for 5 min; 30 cycles of 94 ºC for 45 seconds, 55 ºC for 45 seconds and 72 ºC for 45 seconds; and one cycle of 72 ºC for 10 min.

Detection was performed using agarose gel electrophoresis with 2% ethidium bromide dye. Samples were considered positive if they gave an amplification product of 600 bp with homology to a molecular weight standard (100 base-pair ladder and ϕX 174/Hinf I; Pharmacia, Uppsala, Sweden) and the corresponding positive controls (Fig. 1). Subsequently, the samples were characterized using the restriction enzyme *Mbo* I (Promega Corp, Madison, WI, USA), which allows confirmation of the amplification results, as well as differentiating between *TEM-1* and *TEM-2* betalactamases. *TEM-2* differs from *TEM-1* in the substitution of C for A at position 4046 of the *TEM-1* gene (8). This substitution produces a loss of a restriction site for the enzyme. Following enzymatic digestion, this results in *TEM-1* betalactamase generating fragments of 317, 230, 36 and 17 bp, whereas *TEM-2* betalactamases yield fragments of 353, 230 and 17 bp (8).

Restriction was achieved using 20 µl of the amplification products of the bla_{TEM} gene and 10-12 U enzyme. Tubes were incubated for 1 h at 37 ºC and the restriction product was submitted to agarose gel electrophoresis with 4% ethidium bromide dye. In order to confirm the molecular weights of the bands obtained during digestion, molecular weight standards 100 base-pair ladder and ϕX 174/Hinf 1 (Pharmacia, Uppsala, Sweden; Pharmacia Biotech) were used (Fig. 2).

For the amplification of the bla_{ROB} gene, the same reaction mixture as above was used, with the same thermal profile, but with the specific primers for this gene[8] : ROB[1] (5'-CGC CCA ATT CTG TTC ATT-3') and ROB[2] (5'-GTT GAT ATT GTT CCA CGC-3'). The amplification product obtained had a size of 434 bp, which was determined as

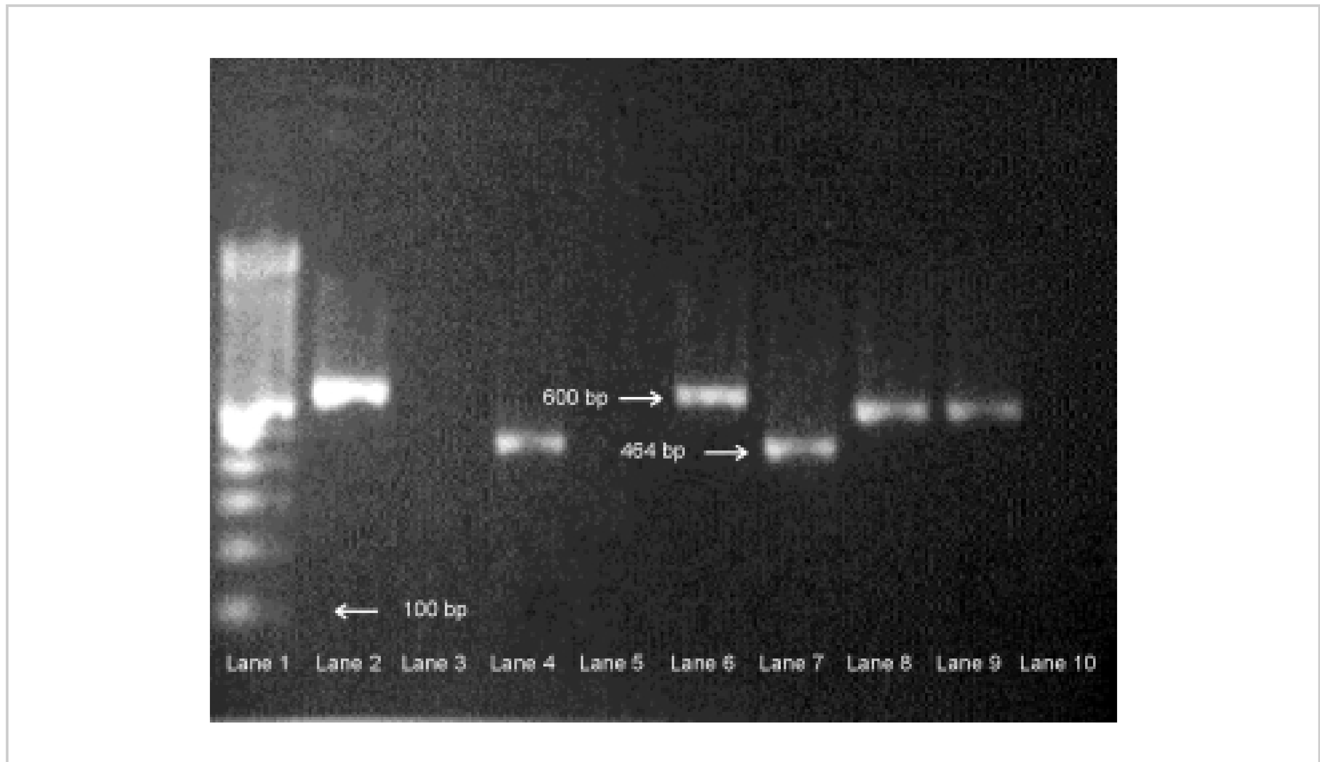

Figure 1. Agarose gel electrophoresis of amplifications products of gene *bla$_{TEM}$*. Lane 1: molecular size standards (100 base pair ladder; Gibco BRL). Lane 2 to lane 10: clinical isolates 141, 140, 139, 138, 137, 136, 135, 134, 133.

Figure 2. Agarose gel electrophoresis of restriction products with *Mbo* I of gene *bla$_{TEM}$*. Lane 1 to lane 5: clinical isolates 159, 154, 152, 148, 145. Lane 6: molecular size standards (100 base pair ladder; Gibco BRL). Lane 7 and lane 8: clinical isolates 134 and 132, respectively.

above using agarose gel electrophoresis with 2% ethidium bromide dye.

The specificity of the amplification products was confirmed using restriction fragment length polymorphism (RFLP) techniques with the restriction enzyme *Dra* I (Promega Corp., Madison, WI, USA.) *ROB-1* betalactamases generate fragments of 256 and 178 bp after digestion. Restriction of the amplification products of the bla_{ROB} gene was achieved using the same procedure as for the bla_{TEM} gene. *H. influenzae* ATCC 42947 was used as a negative control organism for both *TEM* and *ROB-1* polymerase chain reaction (PCR) assays. *H. influenzae* clinical isolates 2 and 48 were used as *TEM*-positive and *ROB-1*-positive control organisms respectively.

Sequence determination

Sequence determination was performed using the automatic ALF DNA sequencer (Pharmacia, Uppsala, Sweden). Cyclical sequencing was used based on a variation of the Sanger method of dideoxynucleotide-mediated chain termination. For the sequencing reactions, the Thermo Sequenase fluorescent-labelled primer cycling sequencing kit (Amersham Life Science) was used.

The primers used for sequencing reactions were the same as those used in the PCR reaction, marked with Cy5 fluorescent label in 5'. The primers used to amplify the bla_{TEM} genes generate a 600 bp fragment. The amplified fragment includes 208 bp upstream from the ATG codon and the first 392 bp out of the 858 bp of the coding gene region.

For sequencing, 0.2-4 µg of amplified DNA was used. The sequencing reactions were performed according to the instructions of each manufacturer.

RESULTS

Results obtained for the bla_{TEM} gene

For the bla_{TEM} gene, 65 (36.7%) positive amplification products were obtained from the 177 samples studied. Of these, 35 (53.8%) had the expected molecular weight of 600 bp and 30 (46.2%) had an unexpected molecular weight (< 600 bp). When we analyzed the results from the nitrocefin assay for the 65 samples, 16 (24.6%) were negative, 10 (62.5%) of which showed the expected molecular weight (600 bp) and six (37.5%) other values (< 600 bp). Of the 65 samples, 49 (75.4%) were positive in the nitrocefin assay, 25 (51%) of which corresponded to the expected molecular weight of 600 bp and 24 (49%) to another molecular weight (< 600 bp).

Of the total of 177 samples, 110 (62.15%) were negative for the bla_{TEM} gene, seven (6.25 %) of which were positive in the nitrocefin assay and 103 (93.75%) of which were negative.

With regard to the restriction analysis with the *Mbo* I enzyme, 27 samples were determined to be bla_{TEM-1}, 10 (37%) of which were negative in the nitrocefin test; eight samples were bla_{TEM-2}, all of which tested positive for nitrocefin.

Thirty samples had a characteristic restriction pattern different from the others, with bands common to the two expected patterns (17, 36, 230 bp) and another band of molecular weight of about 180 bp, not described for bla_{TEM-1} or bla_{TEM-2} (8) (Fig. 2). These 30 samples corresponded to those amplification products with a molecular weight below 600 bp for the bla_{TEM} gene, six (20%) of which were negative in the nitrocefin assay. None of the samples was positive for both *TEM-1* and *TEM-2*.

As for the sequence analysis of the amplification products of the *TEM* genes, the results confirmed the restriction analysis. The sequence described by Sutcliffe (10) for *TEM-1* and *TEM-2* was obtained, except for those samples with amplification products < 600 bp. The latter presented a sequence similar to that of *TEM-1* with the same restriction points as described for *Mbo* I, but displaying a loss of the fragment of 136 bp between positions 4208 and 4344. The 136 bp fragment which was deleted in the bla_{TEM} gene in 30 isolates corresponded to a deletion located upstream from the coding region including the –35 but not the –10 region of the promoter.

The sequence initially described for the amplified region is shown in Figure 3.

Results obtained for the bla_{ROB-1} gene

Of the 177 strains, seven were positive for the bla_{ROB-1} gene, which represents 3.9% of the 177 isolates and 9.7% of the 72 samples positive for the *TEM-1, TEM-2* and *ROB-1* resistance genes. All the positive samples were confirmed by restriction analysis with the *Dra* I enzyme. All the samples positive for the bla_{ROB-1} gene were also positive for nitrocefin, but none of the samples was positive for both bla_{TEM} and bla_{ROB-1} genes.

MIC results

Determination of the minimum inhibitory concentration (MIC) was performed on all isolates presenting resistance, independent of the type of resistance. Table 1 shows the MICs obtained in mg/l for the different samples.

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             4364
blaTEM-1       GAATTCTTGA AGACGAAAGG GCCTCGTGAT ACGCCTATTT TTATAGGTTA ATGTCATGAT
(Tr)blaTEM-1   GAATTCTTGA AGACGAAAGG G--------- ---------- ---------- ----------
               4304  -35Pa                -35Pb  -10Pa                -10Pb
blaTEM-1       AATAATGGTT TCTTAGACGT CAGGTGGCAC TTTTCGGGGA AATGTGCGCG GAACCCCTAT
(Tr)blaTEM-1   ---------- ---------- ---------- ---------- ---------- ----------
               4244
blaTEM-1       TTGTTTATTT TTCTAAATAC ATTCAAATAT GTATCCGCTC ATGAGACAAT AACCCTGATA
(Tr)blaTEM-1   ---------- ---------- ---------- --------TC ATGAGACAAT AACCCTGATA
               4184                 -35P3                     -10P3
blaTEM-1       AATGCTTCAA TAATATTGAA AAAGGAAGAG TATGAGTATT CAACATTTCC GTGTCGCCCT
(Tr)blaTEM-1   AATGCTTCAA TAATATTGAA AAAGGAAGAG TATGAGTATT CAACATTTCC GTGTCGCCCT
               4124
blaTEM-1       TATTCCCTTT TTTGCGGCAT TTTGCCTTCC TGTTTTTGCT CACCCAGAAA CGCTGGTGAA
(Tr)blaTEM-1   TATTCCCTTT TTTGCGGCAT TTTGCCTTCC TGTTTTTGCT CACCCAGAAA CGCTGGTGAA
               4064
blaTEM-1       AGTAAAAGAT GCTGAAGATC AGTTGGGTGC ACGAGTGGGT TACATCGAAC TGGATCTCAA
(Tr)blaTEM-1   AGTAAAAGAT GCTGAAGATC AGTTGGGTGC ACGAGTGGGT TACATCGAAC TGGATCTCAA
               4004
blaTEM-1       CAGCGGTAAG ATCCTTGAGA GTTTTCGCCC CGAAGAACGT TTTCCAATGA TGAGCACTTT
(Tr)blaTEM-1   CAGCGGTAAG ATCCTTGAGA GTTTTCGCCC CGAAGAACGT TTTCCAATGA TGAGCACTTT
               3944
blaTEM-1       TAAAGTTCTG CTATGTGGCG CGGTATTATC CCGTGTTGAC GCCGGGCAAG AGCAACTCGG
(Tr)blaTEM-1   TAAAGTTCTG CTATGTGGCG CGGTATTATC CCGTGTTGAC GCCGGGCAAG AGCAACTCGG
               3844
blaTEM-1       TCGCCGCATA CACTATTCTC AGAATGACTT GGTTGAGTAC TCACCAGTCA CAGAAAAGCA
(Tr)blaTEM-1   TCGCCGCATA CACTATTCTC AGAATGACTT GGTTGAGTAC TCACCAGTCA CAGAAAAGCA
               3824
blaTEM-1       TCTTACGGAT GGCATGACAG TAAGAGAATT ATGCAGTGCT GCCATAACCA TGAGTGATAA
(Tr)blaTEM-1   TCTTACGGAT GGCATGACAG TAAGAGAATT ATGCAGTGCT GCCATAACCA TGAGTGATAA
               3764
blaTEM-1       CAC
(Tr)blaTEM-1   CAC
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Figure 3. Sequence of the 600 bp amplified fragment of the *bla*$_{TEM-1}$ gene. Regions –35 and –10 of *Pa*, *Pb*, and *P3* are underlined. The start codon (ATG) is shown in bold and underlined. Delected nucleotides are indicated by dashes. Note: (Tr) *bla*$_{TEM-1}$: truncated *bla*$_{TEM-1}$ gene.

A statistical analysis using the Mann-Whitney U-test was performed to determine any significant differences between different isolates (Table 2). As can be seen in Table 2, significant differences were found between *TEM-1* isolates and the mutants in terms of resistance to the antibiotics cefaclor and loracarbef, with higher range and median values (mg/l) in the mutant isolates (3 [0.5-256], 3.5 [0.5-256]) than in the *TEM-1* isolates (2 [0.5-48], 1.5 [0.38-24]). In the case of cefaclor, the mode values for the mutant isolates (256 mg/l) were much greater than for the *TEM-1*

Table 1. Distribution of MICs (mg/l) for *H. influenzae*. Cefinase = PCR negative and cephalosporin chromogenic test positive. CF+ = cefinase positive. CF– = cefinase negative.

	CMI mg/l																						
	0.125	0.19	0.25	0.38	0.5	0.75	0.94	1	1.5	2	3	4	6	8	12	16	24	32	48	64	96	192	>256
Deletion/Cf+ (24 isolates)																							
Ampicillin									1					1	2	3	3	2	4	3			5
Coamoxiclav					8	6		4	4	2													
Cefaclor					1			3	4	2	3	1	1	3	1		1						4
Loracarbef						2		5	2	2	1	4	2	1	1	1	2						1
Deletion/Cf– (6 isolates)																							
Ampicillin					1												1	1		1			2
Coamoxiclav			1		1	1		1			1												1
Cefaclor						1			1		1				2								1
Loracarbef					1					1	1					2							1
TEM-1/Cf+ (17 isolates)																							
Ampicillin									1			1		3	1	1	2	3	1	2	1		1
Coamoxiclav			2	1	4	2	1	5	2														
Cefaclor						1		3	2	4	3	2		1					1				
Loracarbef				1	3			1	6	4						1	1						
TEM-1/Cf– (10 isolates)																							
Ampicillin		1		1										1	1	1			1		1		3
Coamoxiclav	1	1		2	1	1		1		1	1	1											
Cefaclor					1			3	3	1		1				1							
Loracarbef						2		4	1	1					2								
TEM-2 (7 isolates)																							
Ampicillin					1													1	1	2	1	1	
Coamoxiclav						3		3		1													
Cefaclor									1	1	2	1			1								1
Loracabef									3	2	1												1
ROB-1 (7 isolates)																							
Ampicillin																							7
Coamoxiclav				1				1	3	2													
Cefaclor																		1					6
Loracarbef																	1						6
Cefinase (7 isolates)																							
Ampicillin	1														2		2						2
Coamoxiclav			1	1	1	1		1		1													1
Cefaclor								3	1	1						1							1
Loracarbef					1	2		1	1	1					1								1

Table 2. Differences (*p*) between the various resistance groups.

Groups	Ampicillin (*p*)	Coamoxiclav (*p*)	Cefaclor (*p*)	Loracarbef (*p*)
TEM-1 vs. mutant	0.1764	0.0733	**0.0223**	**0.0189**
TEM-2 vs. mutant	0.5841	0.5834	0.7533	0.7080
TEM-1 vs. ROB-1	**0.0005**	0.0612	**0.0001**	**0.0001**
TEM-2 vs. ROB-1	**0.0008**	0.1161	**0.0050**	**0.0041**
Mutant *vs. ROB-1*	**0.0013**	0.1557	**0.0008**	**0.0002**
TEM-1 vs. others	0.9829	0.7615	0.8591	0.5660
TEM-2 vs. others	0.4042	0.7652	0.1715	0.0621
Mutant *vs.* others	0.3464	0.4931	0.2534	0.0713
ROB-1 vs. others	**0.0090**	0.3103	**0.0049**	**0.0010**

Note: p <0.05 denotes significant differences.

isolates (1 mg/l), although this difference was not seen for loracarbef (1 mg/l and 1.5 mg/l, respectively).

There were no significant differences between the *TEM-2* and mutant isolates.

When *TEM* (*TEM-1, TEM-2*, mutant) and other isolates (PCR–/nitrocefin+) were compared with *ROB-1* isolates, significant differences were seen for all the antibiotics except coamoxiclav, with the median being much higher in the *ROB-1* isolates than in the others. Lastly, no significant differences could be found between the *TEM* (*TEM-1, TEM-2*, mutant) and other isolates for any of the antibiotics studied.

DISCUSSION

In the *bla$_{TEM}$* gene amplification products, it is noteworthy that a considerable percentage (46.2%) of fragments had a molecular weight below 600 bp. It is reasonable to assume that this unexpected molecular weight is the result of a mutation (disappearance of 136 bp), which was confirmed by restriction analysis with the *Mbo* I enzyme, with the appearance of fragments (230, 36 and 17 bp) similar to those described by Scriver *et al*. (8) and another fragment with a molecular weight below 180 bp not previously described. This indicated that the initial amplification product prior to restriction would be of 463 bp (17 + 36 + 230 + 180).

When we determined the sequence of these amplification products, we found the loss of a fragment of 136 bp (from the base 4208 to the base 4344) from the sequence originally described by Sutcliffe *et al.* (10). This implies that the amplification products of a substantial percentage of isolates (46.2%) in this study do not present the expected molecular weight of 600 bp, but rather of 464 bp (600 -136 = 464 bp), as reflected approximately (463 bp) in the restriction with *Mbo* I.

The study of gene expression in the isolates with the wild-type or mutated (loss of 136 bp) *bla$_{TEM}$* gene also uncovered differences between the two types of isolates. Of the 16 PCR-positive/nitrocefin-negative isolates (*i.e*., carrying but not expressing the gene), 10 (62.5%) presented amplification products with the expected size and six (37.5%) the deletion. When we analyzed the 49 PCR-positive/nitrocefin-positive isolates, we found that 25 (51%) corresponded to amplification products with a molecular weight of 600 bp and 24 (49%) were amplification products with the deletion.

Overall, it can be concluded that a substantial number of isolates (24.6%) encode but do not express the gene for *TEM* betalactamase, and that this may be related to the deletion identified in this study.

This finding is confirmed by the results of the statistical analysis of the MIC values. In this analysis, a significant difference was found between *TEM-1* and mutant isolates for cefaclor and loracarbef, as the mutant strains were more resistant than wild-type *TEM-1*-bearing strains. A previous study showed that a high level of production of *TEM-20* could result from a 135 bp deletion in the promotor region (11). The sequence of region 10 of the *bla$_{TEM-20}$* gene differed from that of the truncated *bla$_{TEM-1}$* gene by one nucleotide but was the same *bla$_{TEM-22}$* sequence used for comparison in this study.

The sequencing results of the deleted fragment suggest that a new *bla$_{TEM}$* gene promoter (if the betalactam hydrolytic activity is related to the expression of this gene) or that another undetected betalactamase exists in these isolates. Furthermore, as six of the 30 isolates with the truncated *bla$_{TEM}$* gene were negative in the cephalosporin chromogenic test, further analysis is required to determine the phenotypical difference observed among the isolates with a truncated *bla$_{TEM}$* gene.

With regard to the normal *bla*$_{TEM}$ genes found in the 10 isolates with a negative cephalosporin chromogenic test and identified as *bla*$_{TEM-1}$, as only the first 392 bp of the *bla*$_{TEM}$ gene coding region were studied, it is not known whether any genetic activity occurred downstream that would explain the apparent nonexpression of the gene. This requires further sequencing analysis.

Another interesting finding is the presence of seven samples which were positive in the nitrocefin assay but gave negative results for *bla*$_{TEM-1}$, *bla*$_{TEM-2}$ and *bla*$_{ROB-1}$ gene amplification. This finding may be explained by attributing the coding of this betalactamase to a gene other than *TEM-1, TEM-2* or *ROB-1* (12). However, the MIC values for these isolates do not present significant differences from the *TEM* isolates as a whole, although there are differences with respect to *ROB-1* isolates. With the exception of two of these isolates the restoration of the amoxicillin activity in the presence of clavulanic acid strongly suggests the production of a class A betalactamase.

As for the distribution of the different types of *bla*$_{TEM}$ gene, 41.5% were *TEM-1*, 12.3% *TEM-2* and 46.2% presented the deletion. Thus, a significant proportion of the isolates carry the deletion, similar to isolates with the most frequent *TEM* resistance genes (*TEM-1* and *TEM-2*).

On the other hand, mixed *TEM-1/TEM-2*, *TEM-1*/mutant or *TEM-2*/mutant forms were not detected. However, as the nucleic acid sequence determination demonstrated that the sequence of the mutants is the same as that of the *TEM-1* isolates (the three restriction points coinciding) except for the lost sequence, these mutants are considered to be derivatives of the *TEM-1* betalactamases. Overall, it appears that this may be a new type of *TEM* betalactamase.

This study confirms the low frequency of the *bla*$_{ROB-1}$ gene, although its appearance in 9.7% of the PCR-positive isolates is an important finding from an epidemiological viewpoint. It should be pointed out that, in contrast to the *TEM* genes, all the isolates positive for the *bla*$_{ROB-1}$ gene were also positive for nitrocefin, which indicates a much more efficient expression of this gene in the isolates studied.

Examination of the MIC values for the isolates positive for the *bla*$_{ROB-1}$ gene confirmed the existence of substantial resistance to ampicillin (resistance to which is mediated by *ROB-1* betalactamase), with an MIC > 250 mg/l. Similar resistance was also seen to cefaclor and loracarbef, but very low levels of resistance to coamoxiclav (MIC = 0.38-1.5 mg/l) were detected. Of the seven isolates positive for the *bla*$_{ROB-1}$ gene, only one sample (number 98) had moderate MIC values for cefaclor and loracarbef.

Although some studies have been reported in other countries (13-15), few have been performed in Spain. Consequently, the substantial proportion (9.7%) of isolates positive for the *bla*$_{ROB-1}$ gene in our study and their high level of resistance to betalactam antibiotics indicate that epidemiological studies should be considered.

Overall, these findings suggest the need to use molecular determinations in epidemiological studies as well as in those cases failing betalactam antibiotic therapy but giving contradictory results in routine tests for betalactamase detection.

CONCLUSIONS

In the *H. influenzae* isolates studied, an large percentage of *bla*$_{TEM}$ and *bla*$_{ROB-1}$ genes were detected (37.2% and 3.9%, respectively). Phenotypic expression of the *bla*$_{TEM}$ gene was not detected in 24.6% of these cases. Resistance to betalactam antibiotics was not due to the *bla*$_{TEM}$ and *bla*$_{ROB-1}$ genes in 8.8% of the isolates studied (nitrocefin-positive, PCR-negative isolates). Molecular tests may be useful in epidemiological studies, as well as in cases where betalactam antibiotic therapy fails but routine phenotypic tests are unable to clarify the situation. A mutation in *TEM-1* isolates consisting of the loss of 136 base pairs in the amplified region was identified. The *TEM-1* mutants presented statistically significant differences with respect to the original *TEM-1* isolates, with greater levels of resistance to the antibiotics cefaclor and loracarbef in the mutants.

REFERENCES

1. Gallego, L., Basaras, M., Alonso, R., Sarria, L., Cisterna, R. *Aplicación de PCR para la detección de mutaciones en los genes codificantes de β-lactamasas.* Enferm Infecc Microbiol 1993; 11: 80-83.
2. Geha, D.J., Uhl, J.R., Gustaferro, C.A., Persig, D.H. *Multiplex PCR for identification of methicillin-resistant staphylococci in the clinical laboratory*. J Clin Microbiol 1994; 32: 1768-1772.
3. Stobberingh, E.E., Arends, J., Hoogkamp-Korstanje, J.A. et al. *Occurrence of extended-spectrum betalactamases (ESBL) in Dutch hospitals*. Infection 1999; 27: 348-354.
4. Goussard, S., Sougakoff, V., Mabilat, C., Bauerfeind, A., Courvalin, P. *An IS1-like element is responsible for high-level synthesis of extended-expectrum lactamase TEM-6 in Enterobacteriaceae.* J Gen Microbiol 1991; 137: 2681-2687.
5. Jacoby, G. A., Sutton, L. *β-lactamases and β-lactam resistance in Escherichia coli.* Antimicrob Agents Chemother 1985; 28: 703-705.
6. Jacoby, G. A., Medeiros, A.A. *More extended-spectrum β-lactamases*. Antimicrob Agents Chemother 1991; 35: 1697-1704.
7. Jouvenot, M., Deschaseaux, M.L., Royez, M. *Molecular hybridization versus isoelectric focusing to determinate TEM-type β-lactamases in gram-negative bacteria*. Antimicrob Agents Chemother 1987; 31: 300-305.

8. Scriver, S.R., Walmsley, S.L., Kau, C.L. et al. Canadian *Haemophilus* study group). *Determination of antimicrobial susceptibilities of Canadian isolates of Haemophilus influenzae and characterization of their β-lactamases*. Antimicrob Agents Chemother 1994; 38: 1678-1680.
9. Juteau, J.M., Sirois, M., Medeiros, A., Levesque, R. *Molecular distribution of ROB-1 β-lactamase in Actinobacillus pleuronumoniae.* Antimicrob Agents Chemother 1991; 35: 1397-1402.
10. Stucliffe, J.G. *Nucleotide sequence of the ampicillin resistance gene of Escherichia coli plasmid pBR322*. Proc Academy of Sci 1978; 75: 3737-3741.
11. Arlet, G., Goussard, S., Courvalin, P., Philippon, A. *Sequences of the genes for the TEM 20, TEM 21, TEM-22, and TEM-29 extended-spectrum β-lactamases*. Antimicrobl Agents Chemother 1999; 43: 969-971.
12. Livermore, D.M. *β-lactamases in laboratory and clinical resistance*. Clin Microbiol Rev 1995; 8: 557-584.
13. Livrelli, V., Darfeuille-Michaud, A., Rich, C., Joly, B., Dabernat, H. *Isolation and molecular characterization of an ROB-1 beta-lactamase plasmid in a Haemophilus influenzae strain in France*. Europ J Clin Microbiol Infect Dis 1988; 7: 583-585.
14. Medeiros, A.A., Levesque, R.C., Jacoby, G.A. *An animal source for the ROB-1 β-lactamase of Haemophilus influenzae type b.* Antimicrob Agents Chemother 1986; 29; 212-215.
15. Karlowsky, J.A, Verma, G., Zhanel, G.G., Hoban, D.J. *Presence of ROB-1 betalactamase correlates with cefaclor resistance among recent isolates of Haemophilus influenzae*. J Antimicrob Chemother 2000; 45: 871-875.