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PCR-Based Detection, Restriction Endonuclease Analysis, and Transcription of *tonB* in *Haemophilus influenzae* and *Haemophilus parainfluenzae* Isolates Obtained from Children Undergoing Tonsillectomy and Adenoidectomy

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We developed and evaluated a PCR-based-restriction endonuclease analysis method to detect and analyze the *tonB* gene of *Haemophilus influenzae* and *Haemophilus parainfluenzae* from pediatric patients undergoing tonsillectomy and adenoidectomy. Multiple sites from the same patient, including the surface of adenoids and tonsils, as well as the core of tonsils, were cultured on chocolate agar and identified using standard procedures and the API NH Kit. A total of 55 *H. influenzae* isolates were recovered from different sites of 20 patients, and 32 *H. parainfluenzae* isolates were recovered from various sites of 12 patients. DNA was extracted from American Type Culture Collection strains and test isolates by the PureGene kit. Two primers, G1 (21-mer) and G2 (23-mer), were designed by us to amplify by PCR the *tonB* gene that consists of an 813-bp fragment. A nested PCR using primers T1 (23-mer) and T2 (24-mer) that flank an internal sequence to the gene of the order of 257 bp and restriction endonuclease digestion using *Xho*I and *Bgl*II were done to detect whether heterogeneity within the gene exists between the two species. Reverse transcription-PCR (RT-PCR) was finally done to detect transcription of the gene in both species. Our data have shown that the *tonB* gene was detected in both species. It is known to encode a virulent protein, TonB, in *H. influenzae*; however, demonstration of its presence in *H. parainfluenzae* is novel. Nested-PCR and restriction endonuclease analysis have shown that the *tonB* gene is apparently structurally the same in both species, with possible differences that may exist in certain *H. parainfluenzae* isolates. RT-PCR done on selected numbers of *H. influenzae* and *H. parainfluenzae* have shown that the *tonB* gene was transcribed in both species. This shows that the TonB protein, if expressed, may play a different role in the virulence in *H. parainfluenzae* since it is not needed for heme or heme complexes uptake as with *H. influenzae.*

Chronic tonsillitis represents persistent inflammatory changes despite antibiotic therapy. Sore throat and cervical lymphadenopathy may persist, and peritonsillar abscesses, although rare, can complicate acute or recurrent tonsillitis, thus increasing the morbidity of these patients and the need for surgical management. Even though the symptoms are the same, tonsillitis may be caused by either viruses or bacteria. Generally, younger preschool children tend to have viral tonsillitis, caused mainly by adenoviruses, influenza viruses, parainfluenza viruses, enteroviruses, and herpes simplex viruses. Other children and adults acquire bacterial infections. The most common bacterial agents are beta-hemolytic group A streptococcus (20% of the cases), *Streptococcus pyogenes*, and *Haemophilus* spp. (6).

In a preliminary pilot study conducted by us on the determination of the most prevalent etiology of tonsillitis in a group of Lebanese patients presenting to the otolaryngology and Head and Neck Surgery department of the American University of Beirut Medical Center (AUBMC) over a period of 1 year, *Haemophilus influenzae* (40%) and *Haemophilus parainfluenzae* (24%) were found to be the most prevalent etiologies. Association of these two species was clinically established with the illness, since they were isolated from culture plates either singly or along with normal flora such as alpha-hemolytic *Streptococcus* or *Neisseria* species.

H. influenzae and *H. parainfluenzae* are facultatively anaerobic gram-negative coccobacilli (7). *H. influenzae* is a human-specific pathogen that must colonize the human mucosal surface to avoid extinction. Although it is in a commensal relationship with its host, it is also found in upper and lower respiratory infections in adults and children, in whom *H. influenzae* remains a leading cause of disease (11, 12, 13). *H. parainfluenzae*, on the other hand, is not human specific, and it is better known as a commensal bacterium that is part of the normal flora (2, 7). Both organisms have several virulence factors, but an unconventional factor that is recently drawing the attention of researchers is the TonB protein, an energy-transducing transmembrane protein responsible for the transport of different essential metabolites into various bacteria. TonB has proven to be a potent virulence factor in *H. influenzae* (5). It is responsible for the active intake into the periplasm of iron-bound transferrin (4), heme, heme:hemopexin (1), heme-albumin, hemoglobin, hemoglobin:naptoglobin (8), after each of these chemicals binds to its proper outer cell surface receptor.

Unlike *H. influenzae*, *H. parainfluenzae*'s requirement for

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porphyrin is not essential due to the ability of the latter to synthesize it from delta amino levulinic acid (ALA) (7). In addition, the fact that *H. parainfluenzae* is unable to bind or acquire iron from transferrin or heme from hemoglobin-haptoglobin or heme-hemopexin contributes to its avirulence and commensalism (3, 9, 10, 14, 15). However, since *H. parainfluenzae* was found to be the etiology of tonsillitis in our patients and since the TonB protein is considered a potent virulence factor in *H. influenzae* through the active uptake of iron, heme, and other essential metabolites (1, 4, 5, 8), we sought to detect and carry out a comparative analysis of the *tonB* gene encoding the TonB protein in both *H. influenzae* and *H. parainfluenzae* species by PCR amplification and endonuclease restriction analysis. In addition, we sought to check by reverse transcription-PCR (RT-PCR) the transcription of mRNA as a preliminary indication for expression of the protein. To do this, we designed primers that flank a 813-bp fragment which constitutes the whole *tonB* gene, and we developed and evaluated a PCR restriction endonuclease–RT-PCR-based method for the detection, analysis, and transcription of the gene in *H. influenzae* and *H. parainfluenzae*.

MATERIALS AND METHODS

Source and identification of the isolates. Isolates were collected over a period of 1 year from six different tonsillar sites of 32 patients suffering from tonsillitis and undergoing adenotonsillectomy at the AUBMC. Specimens were taken aseptically from the surface and core of the adenoids and similarly from the right and left tonsils. The specimens were cultured on blood and on chocolate agar plates and incubated overnight at 37°C. Chocolate agar plates were incubated in the presence of 5% CO_2. Isolates recovered were identified by using standard procedures and the API NH kit. A variety of bacteria were identified (unpublished data), including 55 *H. influenzae* and 32 *H. parainfluenzae* isolates that were used in this study.

DNA extraction, PCR, and Nested PCR. Total DNA was extracted from *Haemophilus* isolates and American Type Culture Collection (ATCC) strains (*H. influenzae* ATCC 49427 and *H. parainfluenzae* ATCC 9796 strains) using the Pure-Gene Kit (Gentra Systems, Inc.). PCR was done on all DNA extracts to amplify the whole 813-bp gene using the G1 (5′-ATTATGCAAACAAAACGTTCG-3′) and G2 (5′-GAAGAGTAAAACTAATTGCACAC-3′) (Amersham Pharmacia Biotech, Freiburg, Germany) primers designed by us using the GenBank database. Nested PCR was done using T1 (5′-GCAAGCACAACAAGTGCAGCTAA-3′) and T2 (5′-GCCGCCTTATCTAAACTTTCATCG-3′) on 813-bp amplicons to amplify a 257-bp amplicon.

PCR amplifications were carried out in 100-μl reaction mixtures consisting of 10 μl of DNA (2 ng/μl) and 90 μl of the amplification mix, which contained the following components: 20 pmol each of the G1 and G2 primers (for the amplification of the 813-bp amplicon) or the T1 and T2 primers (for the nested PCR that amplifies a 250-bp amplicon), 0.5 mM $MgCl_2$, 200 μm concentrations of each deoxynucleoside triphosphate, 10 μl of PCR buffer (Amersham Pharmacia Biotech), and 2.5 U of *Taq* DNA polymerase (Amersham Pharmacia Biotech). PCR amplification was performed in a minicycler (M.J. Research, Watertown, Mass.) for 34 cycles. Each cycle consisted of 1 min at 95°C for denaturation, 1 min at 55°C for annealing, and 1 min at 72°C for extension. A final extension for 10 min at 72°C was also done. Amplicons were detected by electrophoresis on a 1% agarose (Sigma, St. Louis, Mo.) gel in 1× Tris-borate-EDTA buffer at 100 V for 1 h. Gels were stained with ethidium bromide (1 mg/ml), observed under UV light, and photographed with type 667 Polaroid film. PCR controls included DNA from ATCC strains and a reagent blank.

Restriction endonuclease analysis. For digestion of the gene (813-bp amplicon), 10 μl of PCR-amplified DNA was restricted with 10 U of the restriction endonucleases *Xho*I and *Bgl*II in a total volume of 15 μl, according to the manufacturer's specification (Amersham Pharmacia Biotech). The restricted fragments were separated by electrophoresis on agarose gels (2.5% Nusieve agarose, 3:1; FMC Bioproducts, Rockland, Maine) at 60 V for 2 h. Gels were stained with ethidium bromide, and fragments were visualized under UV light and photographed as described above.

RT-PCR. RT-PCR was done on RNA from two *H. influenzae* and six *H. parainfluenzae* isolates of different patients to demonstrate possible transcription of the *tonB* gene detected in *H. parainfluenzae* in comparison to *H. influenzae*. To that purpose, RNA was extracted using the RNeasy Mini Kit (Qiagen) according to manufacturer's specifications. cDNA strand was synthesized from RNA using the Ready-To-Go Kit (Amersham Pharmacia Biotech) according to the manufacturer's specification. RT-PCR was done on cDNA-generated strand using the G1 and G2 primers and the PCR conditions described above. Control tubes of extracted RNA were subjected to PCR to rule out the presence of DNA in the starting RNA samples. Amplicons were visualized on an ethidium bromide-stained gel and photographed.

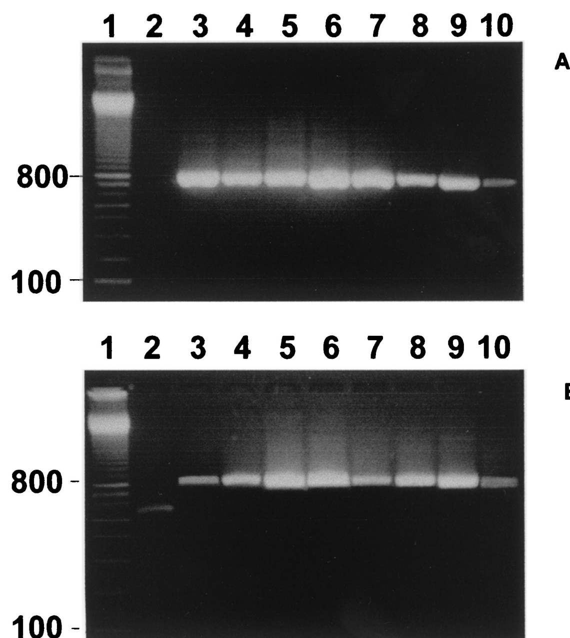

FIG. 1. (A) PCR 813-bp amplicons of a representative sample of *H. influenzae*, and *H. parainfluenzae* test isolates. Lane 1, 100-bp ladder; lane 2, negative control; lanes 3 and 4, *H. influenzae* test isolates; lanes 5 to 10, *H. parainfluenzae* test isolates. (B) The corresponding RT-PCR amplicons for the PCR amplicons in panel A. Lane 1, 100-bp ladder; lane 2, positive control; lanes 3 and 4, *H. influenzae* test isolates; lanes 5 to 10, *H. parainfluenzae* test isolates.

RESULTS

Our data show that all 55 *H. influenzae* and 32 *H. parainfluenzae* isolates, in addition to both *H. influenzae* and *H. parainfluenzae* ATCC strains, amplified the 813-bp sequence (Fig. 1A). Nested PCR amplified the 257-bp amplicon (Fig. 2) in all isolates with the exception of four *H. parainfluenzae* isolates, indicating that these have nucleotide sequence differences where the primers bind. Restriction analysis of the *tonB* gene with *Xho*I showed a similar DNA pattern between ATCC strains and the tested isolates (Fig. 3). The original 813-bp sequence from all isolates of both species was digested by *Xho*I into 200- and 600-bp fragments. However, the *Bgl*II restriction enzyme cut 28 of 55 *H. influenzae* and 15 of 32 *H. parainfluenzae* amplicons into two fragments (ca. 388 and 425 bp), and these are similar to the expected values derived from the DNA sequence. A total of 27 of 55 *H. influenzae* and 17 of 32 *H. parainfluenzae* strains, as well as both ATCC strains, were not digested by this enzyme. The six-base recognition sequence of *Xho*I cuts nucleotides of the *tonB* gene corresponding to the 130(K), 131(D), and 132(L) amino acids of the TonB protein, while the *Bgl*II recognition sequence cuts nucleotides of the

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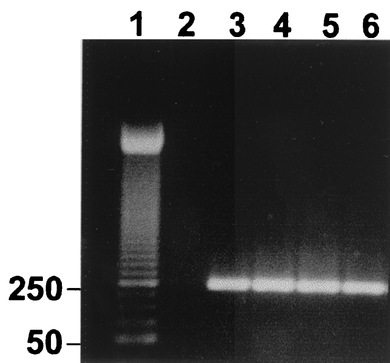

FIG. 2. Representative nested-PCR amplicons (257 bp) of the total *tonB* gene from *H. influenzae* and *H. parainfluenzae* ATCC strains and test isolates. Lane 1, 50-bp ladder; lane 2, negative control; lane 3, *H. influenzae* ATCC; lane 4, *H. influenzae* test isolate; lane 5, *H. parainfluenzae* ATCC strain; lane 6, *H. parainfluenzae* test isolate.

gene corresponding to the 190(T), 191(R), and 192(A) amino acids of the protein (5). Based on digestion with the two enzymes, two composite patterns, I and II, were generated (Table 1). RT-PCR has shown that the *tonB* gene in selected *H. influenzae* and *H. parainfluenzae* isolates was transcribed (Fig. 1B).

DISCUSSION

Generated data has shown that the *tonB* gene is present in all tested *H. influenzae* isolates, as well as in all tested *H. parainfluenzae* isolates, a fact not reported earlier for *H. parainfluenzae*. This observation indicates that *H. parainfluenzae* has the potential to exhibit virulence similar to that seen in *H. influenzae*. Nested-PCR analyses have shown minor heterogeneity at the primer annealing site of four *H. parainfluenzae* isolates, a fact that hindered the amplification of the internal sequence of the *tonB* gene by nested PCR of these four isolates. All other isolates of *H. influenzae* and *H. parainfluenzae* amplified the internal sequence of the gene by nested PCR.

TABLE 1. Restriction endonuclease analysis of *tonB* gene in *H. influenzae* and *H. parainfluenzae* isolates

Species	*Xho*I			*Bgl*II			Composite pattern (no.)
	Fragment length (bp)	No. of isolates		Fragment length (bp)	No. of isolates		
		Cut	Uncut		Cut	Uncut	
H. influenzae	204, 609	55		388,425	28		I (28)
	204, 609	55				27	II (27)
H. parainfluenzae	204, 609	32		388,425	15		I (15)
	204, 609	32				17	II (17)

Restriction endonuclease analysis, on the other hand, has shown two composite patterns, I and II, using *Xho*I and *Bgl*II in both *H. influenzae* and *H. parainfluenzae* (Table 1), showing that the gene is structurally the same in both species with minor nucleotide sequence variations also observed in both species. This nucleotide variation is reflected at the level of amino acids 190(T), 191(R), and 192(A) downstream of the TonB protein (4, 5). This observed alteration does not seem to affect either survival or the function of both species. The 130(K), 131(D), and 132(L) amino acids of the TonB protein, on the other hand, are well conserved in both *H. influenzae* and *H. parainfluenzae* (4, 5), reflecting the functional importance of this conserved region. Based on this molecular analysis, the *tonB* gene seems to have a common nucleotide sequence in both species, with minor variations. Being structurally the same, the *tonB* gene in *H. parainfluenzae* may encode a protein that could have the same functionality as that of *H. influenzae*; however, since iron is apparently not essential for the survival of *H. parainfluenzae*, it is believed that it may play an important

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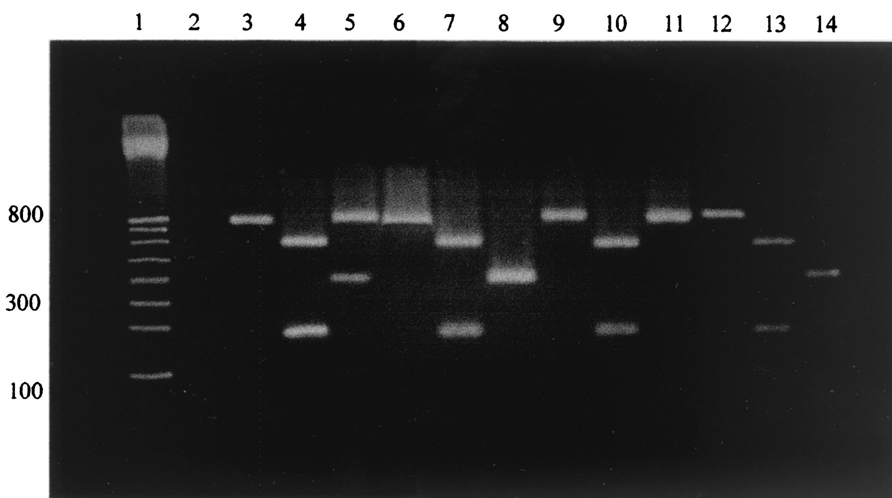

FIG. 3. Representative uncut and digested PCR amplicons of the *tonB* gene from *H. influenzae* and *H. parainfluenzae* ATCC strains and test isolates. Lane 1, 100-bp ladder; lane 2, negative control; lanes 3 to 8, *H. influenzae* amplicons; lane 3, ATCC strain uncut amplicon; lane 4, ATCC strain digested amplicon with *Xho*I; lane 5, ATCC strain digested amplicon with *Bgl*II; lane 6, test isolate uncut amplicon; lane 7, test isolate digested amplicon with *Xho*I; lane 8, test isolate digested amplicon with *Bgl*II; lanes 9 to 14, *H. parainfluenzae* amplicons; lane 9, ATCC strain uncut amplicon; lane 10, ATCC strain digested with *Xho*I; lane 11, ATCC strain digested with *Bgl*II; lane 12, test isolate uncut amplicon; lane 13, test isolate digested with *Xho*I; lane 14, test isolate digested with *Bgl*II.

role in enhancing *H. parainfluenzae*'s virulence if the species has an iron acquisition mechanism (10). For that reason the role of the *tonB* gene and TonB protein in *H. parainfluenzae* may be to allow either the acquisition of iron bound to certain carriers not yet tested or the acquisition of other essential metabolites. Both cases reflect the virulence potential of the *tonB* gene.

The *tonB* gene is shown to be transcribed into mRNA in both *H. parainfluenzae* and *H. influenzae*. This indicates that expression of the gene is initiated in both species. Since the *tonB* gene is transcribed in *H. parainfluenzae* and since the species is reported not to acquire iron-bound transferrin nor heme:hemopexin or hemoglobin:haptoglobin (15), however, it may be assumed that the TonB protein, if translated, may very well be involved in the acquisition of other essential metabolites, as is the case in other bacteria (5). It may constitute a virulence factor since its gene is carried by the species genome and is detected in patients with tonsillitis. On the other hand, it may be also assumed that the translation of the TonB protein is blocked and thus the protein is not expressed. Detection of the protein in *H. parainfluenzae* would clarify its role as a virulence factor. In addition, generation of a *tonB* mutant strain of *H. parainfluenzae* would enhance our understanding of its role in the pathogenesis of the organism.

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