Transcription Levels and Sequence Analysis of *ton*B Gene in *Haemophilus parainfluenzae* versus *Haemophilus influenzae* Isolates from Patients Undergoing Tonsillectomy and/or Adenoidectomy

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ABSTRACT

Introduction: *Ton*B, an energy transduction protein, is a known virulence factor in *Haemophilus influenzae*. The *ton* B gene encoding this protein was previously PCR detected and transcribed by us in both *H influenzae* and *Haemophilus parainfluenzae* obtained from multiple tonsillar sites in patients undergoing tonsillectomy and/or adenoidectomy. In the present study we attempted to assess possible differences in the transcription levels sequence of the *tonB* gene between *H influenzae* and *H parain - fluenzae* isolates and among the members of each species.

Methods: Transcription levels and DNA sequence analysis of the *ton*B gene were assessed on 7 *H influenzae* isolates, 13 *H parainfluenzae* isolates, 1 *H influenzae* strain ATCC 49766, and 1 *H parainfluenzae* strain ATCC 1450. Isolates were obtained from patients undergoing tonsillectomy and/or adenoidectomy due to recurrent tonsillitis or idiopathic tonsillar hypertrophy. Isolates were identified and their RNA and DNA extracted. RNA extracts of all test isolates and ATCC strains were subjected to semi-quantitative RT-PCR of the *ton*B gene. DNA extracts of the same isolates were subjected to PCR and sequence analysis of the *ton*B gene. Sequencing of the *H parain - fluenzae ton*B gene is novel.

Results: The 7 *H influenzae* isolates, 13 *H parainfluenzae* isolates, and 2 ATCC strains had similar end-point titers of their RT-PCR amplicons of the order of 811 bp, denoting no differences in the transcription levels of the *ton*B gene between the 2 species and among members of the same species. Comparison of DNA sequences of the 811 bp *ton*B gene between *H influenzae* and *H parainfluenzae* isolates revealed an average homology of 32%.

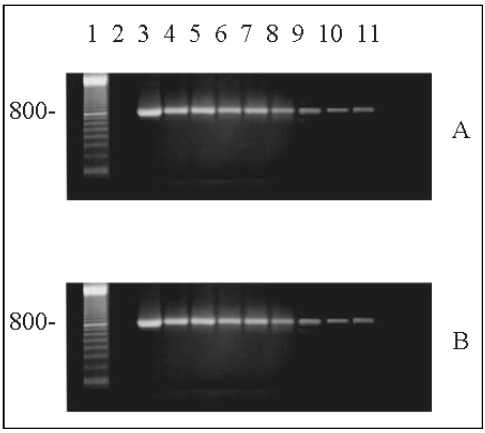

Figure 1: Semi-quantitative RT-PCR of the tonB gene of (A) a representative *H influenzae* isolate and (B) a representative *H parainfluenzae* isolate. (A) Lane 1: 100bp ladder, Lane 2: negative control, Lane 3: 1:1 dilution, Lane 4: 1:2 dilution, Lane 5: 1:4 dilution, Lane 6 1:8 dilution, Lane 7: 1:10 dilution, Lane 8: 1:20 dilution, Lane 9: 1:40 dilution, Lane 10: 1:60 dilution, Lane 11: 1:160 dilution. (B) Lane 1: 100bp ladder, Lane 2: negative control, Lane 3: 1:1 dilution, Lane 4: 1:2 dilution, Lane 5: 1:4 dilution, Lane 6 1:8 dilution, Lane 7: 1:10 dilution, Lane 8: 1:20 dilution, Lane 9: 1:40 dilution, Lane 10: 1:60 dilution, Lane 11: 1:160 dilution.

Conclusions: The degree of sequence heterogeneity of the *ton*B gene may reflect structural differences in the TonB protein. Moreover, *ton*B gene sequence heterogeneity was observed in *H influenzae* and *H parainfluenzae* isolates with the same genotype. Further investigation is needed to determine whether these differences relate to different functions of the encoded protein.

INTRODUCTION

Both *Haemophilus influenzae* and *Haemophilus parainfluenzae* are known to form a substantial part of the oral normal flora in humans and in animals.[1,2] Despite this fact, many types of *Haemophilus* infections occur in humans.[3] The role of *H influenzae* in causing both invasive and non-invasive infections has been well established and the role of *H parainfluenzae* in causing disease such as tonsillitis should not be overlooked.[2,4,5]

TonB, an energy transduction protein, is a known virulence factor in *H influenzae*.[6,7] The role of TonB in the active uptake of both heme and iron in *H influenzae* has been well established by several investigators.[8,9] TonB is also implicated in uptake of nutrients, efflux of antibiotics and toxic substances, in addition to signal transduction[7,10-13]

In our previous study, 88 *Haemophilus* isolates were identified in 32 of 60 patients undergoing tonsillectomy and/or adenoidectomy. Fifty-five of the 88 isolates grew *H influenzae* and 32 grew *H parainfluenzae*. PCR-based detection and transcription of the *ton*B gene, encoding the TonB protein, was observed in all 88 *Haemophilus* isolates.[14]

Since *H parainfluenzae* isolates were isolated from both surface and core tonsillar specimens of the patients and since the *ton*B gene encoding the TonB protein, a known virulence factor in *H influenzae*, was detected and transcribed in this species, we attempted to assess possible differences in the transcription levels of the *ton*B gene between *H influenzae* and *H parainfluenzae* isolates and among the members of each species using semiquantitative RT-PCR and detect structural differences in the *tonB* gene of the 2 species by sequence analysis.

MATERIALS AND METHODS

Sample Size and Source

Seven *Haemophilus influenzae* and 13 *Haemophilus parainfluenzae* isolates in addition to *H influenzae* ATCC 49766 and *H parainfluenzae* ATCC 1450 were used in the study. The 20 *Haemophilus* isolates were collected in a previous study from multiple tonsillar sites from patients undergoing tonsillectomy and/or adenoidectomy at the American University of Beirut-Medical Center.[14]

Sample Handling and Culture

In the previous study, detected *Haemophilus* organisms were isolated, identified at the

Table 1. Average percentage homology of the DNA sequence of the *ton*B gene in the *Haemophilus* isolates.

Category	Average homology
H influenzae ATCC vs. *H parainfluenzae* isolates	31%
H influenzae ATCC vs. *H influenzae* isolates	35%
H parainfluenzae ATCC vs. *H influenzae* isolates	33%
H parainfluenzae ATCC vs. *H parainfluenzae* isolates	32%

species level, subcultured, and stored at -70°C for later use. The isolates were identified using the X and V factors and the API NH Kit (Biomerieux, Vitek Inc., Hazelwood, Mo).[14] In this study the isolates were subcultured from -70°C on chocolate agar plates, incubated at 37°C under 5% CO_2 tension overnight, and subjected to further testing.

RNA Extraction

RNA was extracted from 20 *Haemophilus* isolates and 2 ATCC strains using the Rneasy mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's specifications. The concentration of RNA was determined using the Ultrospec 1000E UV visible spectrophotometer (Amersham Pharmacia Biotech Inc., Upsalla, Sweden).

Semi-quantitative RT-PCR

First strand cDNA synthesis was done using the Ready. To. Go. You Prime first-strand beads (Amersham Pharmacia Biotech, Inc, Upsalla, Sweden) according to the manufacturer's specifications. Five micrograms of extracted RNA were used in each reaction. Several dilutions of the cDNA were prepared and subjected to PCR amplifications as previously described.[14] Amplicons were then run on 1.5% agarose gel, stained using ethidium bromide, visualized on a UV transilluminator, and photographed with type 667 Polaroid film.

DNA Extraction

DNA was extracted from 20 *Haemophilus* isolates in addition to two ATCC strains using the GFX Genomic blood DNA kit (Amersham Pharmacia Biotech Inc, Upsalla, Sweden) according to the manufacturer's specifications.

Sequencing

The *ton*B gene was PCR-amplified in all the *Haemophilus* isolates and the two ATCC strains as previously described.[14] PCR product purification was done using the GFX PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech Inc, Upsalla, Sweden) according to the manufacturer's specifications. Samples were cycle sequenced using 0.5 dilution of the BigDye V.3 ready reaction mix by Applied Biosystems and data were collected on an ABI-PRISM 377 automated DNA Sequencer with XL upgrades (Biocompare, Inc., South San Francisco, CA, USA) The primers used were G1 and G2. Analysis of the data was done by the Chromas 145.exe program through the use of a base-calling algorithm and a chromatogram of dye intensity created over time.

RESULTS

Semi-quantitative RT-PCR of the tonB Gene

Semi-quantitative RT-PCR was performed on all 20 *Haemophilus* isolates used in the study in addition to 2 ATCC strains. The RNA yield of all the isolates ranged between 6 and 10 [µg]. cDNA was synthesized and the titer was determined by the maximum dilution of cDNA that gives a positive RT-PCR amplicon of 811bp. All *H influenzae* and *H parainfluenzae* isolates used in the study in addition to the 2 ATCC strains did not have amplification of the cDNA at dilutions above 1:160 (Figure 1).

Figure 2. Comparison of the DNA sequence of the *ton*B gene of *Haemophilus influenzae* (first line) and *Haemophilus parainfluenzae* (second line) ATCC strains. The bolded font indicates homology of the *ton*B gene sequence between the two strains.

PCR and DNA Sequencing of the tonB Gene

All 20 *Haemophilus* isolates used in the study in addition to the reference strains amplified the 811bp *ton*B gene by PCR. Comparison of the *ton*B gene DNA sequence between *H influenzae* ATCC 49766 and *H parainfluenzae* ATCC 1450 revealed a 28% homology (Figure 2). The homology ranged between 27% and 43% when comparison of the DNA sequence of the *ton*B gene between *H influenzae* ATCC 49766 and 5 randomly selected *H parainfluenzae* isolates was done. Comparison of the *ton*B gene DNA sequence of *H parainfluenzae* ATCC 1450 and 5 selected *H influenzae* isolates revealed a homology that ranged between 28% and 48% (Table 1).

DISCUSSION

TonB, an energy transduction protein, was found to be required for the acquisition of heme and iron from a variety of sources in *H influenzae*.[6,15] It was not, however, detected in *H parainfluenzae*. The gene encoding the TonB protein was detected and transcribed in *H parainfluenzae* in a previous study done in our laboratory.[14]

Since the *ton*B gene was also harbored by *H parainfluenzae*, its characteristics at the structural and transcriptional levels were compared to those of *H influenzae*. Levels of transcription determined in all *H influenzae* and *H parainfluenzae* isolates in addition to 2 ATCC strains revealed comparative titers between the 2 species. This denotes no differences at the transcription levels of the *tonB* gene between *H influenzae* and *H parainfluenzae*, as well as among members of the same species. Although *H parainfluenzae* does not have an absolute requirement for heme and does not acquire iron-bound transferrin or heme:hemopoxin or heme:haptoglobin, it might need the

TonB protein for the acquisition of other growth factors and nutrients necessary for its growth.[3,16-18] It is important also to note that *H parainfluenzae* has the ability to use an exogenous siderophore enterobactin and it is well established that the TonB-ExbB-ExbD complex supports the active transport of iron siderophores.[18,19] The TonB protein might also be needed by *H parainfluenzae* for the efflux of certain antibiotics and toxic substances. Moreover, the role of the TonB in *H parainfluenzae* can be in mediating signal transduction, as in the case of *Escherichia coli* K12 where TonB was found to play a role in signal transduction together with the ExbB and ExbD proteins.[10] These assumptions need further investigation. Initially, the complete expression of the gene needs to be detected and later determination of the regulation of the gene expression has to be assessed. Iron, for example, which plays a role in regulating the expression of the *ton*B gene in other gram-negative bacteria, may also play a role in *H parainfluenzae*.[20,21] This does not exclude other factors that may play a regulatory role.

As to comparative structural characteristics, the DNA sequence analysis of the *ton*B gene revealed nucleotide sequence heterogeneity between the 2 species. Heterogeneity was also found among members of the same species. The observed differences of the DNA sequences may reflect variations of the amino acid sequences that would determine different functions of the protein.

Moreover, comparison of the *ton*B gene sequence between 2 *H parainfluenzae* isolates with the same genotype indicated a 33% sequence homology. This observation denotes a high degree of heterogeneity of *ton*B gene even within the same strains of the same species.

In conclusion, the *ton*B genes in *H influenzae* and *H parainfluenzae* are different in structure as determined by sequence analysis, though its transcriptional level is identical in both species. Studies are underway to assess the role of mutated *H parainfluenzae ton*B gene *in vivo* and *in vitro*. Research into the ability of the wild type strain to induce pathology in mice compared to a strain with a mutation in its *ton*B gene will shed light on the role of the encoded TonB protein as a potential virulence factor in *H parainfluenzae*. The gene sequence also appears to be heterogeneous within *H parainfluenzae* isolates having identical genomic patterns.

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