Rates of Detection of *Neisseria meningitidis* in Tonsils Differ in Relation to Local Incidence of Invasive Disease

Oliver Greiner, Christoph Berger, Philip J. R. Day, Gabriela Meier, Christoph M. Tang, and David Nadal*

**Divisions of Infectious Diseases and Oncology, University Children’s Hospital of Zurich, Switzerland, and Centre for Molecular Microbiology and Infection, Imperial College of Science Technology and Medicine, London SW7 2AZ, England**

Received 10 July 2002/Returned for modification 6 August 2002/Accepted 17 August 2002

Nasopharyngeal swabbing substantially underestimates carriage of *Neisseria meningitidis*. Real-time PCR assays were employed to examine the presence of a broad range of bacteria and of *N. meningitidis* groups B and C, respectively, in tonsils from 26 individuals from Oxford, England, and 72 individuals from Zurich, Switzerland. The detection limit of each PCR system was DNA from one bacterial cell per reaction mixture. Tonsillar DNA did not inhibit amplification of meningococcal gene sequences, and *N. meningitidis* was detected in tonsils exposed to the bacterium. Whereas in both sets of patients other bacteria were detected, *N. meningitidis* group B and group C were only found in tonsils from Oxford where the incidence of invasive meningococcal disease is much higher than in Zurich. These observations suggest that PCR-based methods could be used for the detection of meningococcal carriage and that differences in disease incidence could be explained by different transmission rates in the community rather than host genetics or coexisting infections.

*N. meningitidis* is the leading cause of bacterial meningitis in children and adolescents in more-developed countries (8, 13). However, the epidemiology and pathogenesis of meningococcal infection are not fully understood. For example, while most epidemiological data are based on nasopharyngeal swabbing, recent data suggest that this method considerably underestimates meningococcal carriage (12). Indeed, *N. meningitidis* was detected in tonsillar tissue by immunohistochemistry four times more frequently than in nasopharyngeal swabs (R. J. Sim, M. M. Harrison, E. R. Moxon, and C. M. Tang, Letter, Lancet 356:1653-1654, 2000). Thus, rapid and sensitive detection methods are needed to provide accurate epidemiological information.

Since changes in the rate of carriage of a specific group of *N. meningitidis* may contribute to changes in the incidence of invasive disease, we investigated operative samples of tonsils from two geographic areas, Oxford, England, and Zurich, Switzerland, for the presence of *N. meningitidis* group B and group C, respectively. Highly sensitive and specific PCR-based assays were developed, and the results corroborate immunohistochemical findings. However, PCR failed to detect meningococci in any operative tonsillar sample from individuals from Zurich. This may have important implications for the further study of the epidemiology, pathogenesis, and prevention of meningococcal infection.

**MATERIALS AND METHODS**

**Bacterial strains.** Strains of *N. meningitidis* serogroups B and C isolated from cerebrospinal fluid were used as reference strains (P. Rohner, Swiss National Reference Center for Meningococci, Geneva, Switzerland). To test the specificity of PCR, *Neisseria lactamica, Moraxella lacunata, Moraxella phenylpyruvica, Moraxella incolumis* (kindly provided by R. Zbinden, Institute of Medical Microbiology, University of Zurich), *Moraxella catarrhalis, Haemophilus influenzae* type b, and non-typeable *H. influenzae* (from the collection of the Division of Infectious Diseases, University Children’s Hospital of Zurich) were used. In addition, *Streptococcus pneumoniae* strain ATCC 49619 and *Streptococcus oralis* strain ATCC 10557 served as representatives of gram-positive bacteria from the upper respiratory tract.

**Clinical samples.** Tonsillar DNA from 26 patients 2 to 36 years old (mean, 11.9 years; median, 9.5 years) undergoing tonsillectomy in Oxford was available for investigation. In Zurich, tonsils were collected from 72 patients 3 to 15 years old (mean, 6.4 years; median, 6.0 years) who underwent necessary tonsillectomy in the same time period (August 1998 to July 2000). None of the patients had been treated with antibiotics in the 2 weeks prior to tonsillectomy. Immediately after removal, tonsils were frozen in liquid nitrogen and stored at −70°C. The study was approved by the institutional ethics committees. Informed consent was obtained from patients or their parents or guardians.

**DNA extraction.** For extraction of DNA from tonsils collected in Oxford, approximately 5 g of tonsillar tissue was frozen in liquid nitrogen, ground in a homogenizer, and then resuspended in 2 ml of phosphate-buffered saline with 5 mg of protease K. The mixture was then incubated overnight at 35°C, extracted three times with an equal volume of phenol-chloroform-isooamyl alcohol (25:24:1), and then extracted twice with chloroform-isooamyl alcohol (24:1). The DNA was precipitated by the addition of 100% ethanol; the pellet was washed twice in 70% ethanol, and finally the pellet was resuspended in Tris-EDTA-RNase A. The extraction of DNA from tonsillar tissue collected in Zurich was performed using the QIAmp tissue kit protocol (Qiagen, Basel, Switzerland) following the manufacturer’s instructions, and the extract was eluted in 50 μl of Tris-EDTA and stored at 4°C. Extraction of bacterial DNA from 1 ml of liquid culture of stock bacterial strains or patient sample from Zurich was performed as described previously (5). Extracts were stored at −20°C until required for analysis.

**Real-time PCR.** For the detection of a broad range of bacteria, we used a set of universal oligonucleotide primers specific for conserved eubacterial 16S rRNA gene sequences (GenBank accession no. X01859). The sequence of the forward primer was 5′-TCGTGTGTGGAAAAGTGGGCTT-3′ (positions 1070 to 1091), and the sequence of the reverse primer was 5′-CATCCCCACCTTCCGAGT-3′ (positions 1190 to 1171), and the sequence of the probe was 5′-AGTCCCGCA ACAGGCGCAACC-3′ (positions 1093 to 1113). The fluorescent reporter dye at the 5′ end of the probes was hexachloro-6-carboxy-fluorescein (6-HEX); the quencher at the 3′ end was 6-carboxy-N,N,N′-tetramethylrhodamine (TAMRA). For the detection of *N. meningitidis* groups B and C, we employed two different real-time PCRs each. One pair of real-time PCR systems targeting the sialyl-
transferase gene (siaD) of group B (GenBank accession no. M90503) and group C (GenBank accession no. M90505) of N. meningitidis was adopted from the literature (6). For the second pair, the primers and the fluorogenic probe for the siaD transferase gene for group B (siaB) and group C (siaC) of N. meningitidis were designed using Primer Express software. The nucleotide sequence of the forward primer for siaB was 5'-AGTTTTTTICAAGATTCAAATGGTGTT-3' (positions 41 to 66), the sequence of the reverse primer was 5'-TAAGCTGACCTAAATTAGATGACAAATAAT-3' (positions 136 to 104), and the sequence of the probe was 5'-AACATCTCTATTATTTCCAACCCCTTACG-3' (positions 69 to 98). The fluorescent reporter dye at the 5' end of the probe was 6-FAM; the quencher at the 3' end was a nonfluorescent dihydrothiophenyl-6-HEX; the quencher at the 3' end was 6-carboxy-fluorescin (FAM); the probe was 5'-TGATTCGATACACCATACAGCCCAAACAGGA-3' (positions 1053 to 1082). The fluorescent reporter dye of this probe was 6-carboxy-fluoresc cein (FAM); the quencher at the 3' end was again QSY-7. All primers and fluorogenic probes were obtained from Microsynth GmbH (Bal gach, Switzerland).

The principle of the real-time PCR has been described extensively (7) and includes the primer-specific extension from PCR amplifiers and the subsequent hybridization and hydrolysis of a fluorogenic probe. This permits the reporter dye to fluoresce in proportion to PCR product accumulation. The cycle threshold (C_T) value is defined as the cycle at which the reporting dye fluoresce first exceeds the calculated background level; a low C_T value corresponds to a high target concentration. Each run contains both negative (no template) and positive controls. The real-time PCR amplifications were performed in 25-μl reaction volumes containing 2 μl Platinum Quantitative PCR SuperMix-UDG (Life Technologies Inc., Gaithersburg, Md.), which includes dUTP and uracil-N-glycosylase and to which were added the following: Blue 636 in a final concentration of 0.1 pmol/μl, a 333 nM concentration of each primer, a 200 nM concentration of fluorescent labeled probe, and 1 μl of DNA extract. All reactions were performed in duplicate, and amplification and detection employed an ABI PRISM 7700 sequence detection system. Standard amplification parameters were as follows: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles comprising 95°C for 15 s and 60°C for 1 min. Real-time data were analyzed using the Sequence Detection Systems software version 1.7.

Sensitivity, detection range, and specificity of the real-time PCR assays. To determine the sensitivity and the detection range of the real-time PCR assays, standard curves for N. meningitidis serogroups B and C were generated as follows. Strains of N. meningitidis group B and group C were grown on chocolate blood agar plates at 37°C for 24 h. The bacteria were harvested in saline and diluted with saline to a concentration of 10^6 microorganisms/ml. A series of 10-fold dilutions was made in physiological saline. DNA was extracted from 1 ml of each dilution and used as the target in control PCR assays. The calculated C_T values were then plotted against the numbers of microorganisms. To determine the specificity of the PCR assays, dilutions of other bacterial species, including N. lactamica, M. lacunata, M. phencytrum, M. lincolnii, M. catarrhalis, H. influenzae type b, nontypeable H. influenzae, S. pneumoniae, and S. oralis, were tested.

RESULTS

Sensitivity, detection range, and specificity of the real-time PCR assays. For all bacterial strains tested, the sensitivity of the PCR detecting 16S rRNA sequences was 10^3 bacterial cells per reaction mixture; the detection range was 10^3 to 10^6 bacterial cells per reaction mixture if a cutoff C_T value of 30 was set to eliminate the results of negative controls. The assays targeting siaD detected N. meningitidis group B (Fig. 1A) and N. meningitidis group C (Fig. 1B) linearly from 1 to 10^6 bacterial cells per reaction. Moreover, none of the assays for N. meningitidis group B detected N. meningitidis group C, or vice versa. The assays for meningococcal detection were negative for other bacteria found in the upper respiratory tract (Fig. 1).

Exclusion of inhibiting factors and exposure of tonsils to meningococci. To exclude the presence of potential PCR-inhibiting factors in tonsillar tissue, a dilution series of N. meningitidis group B and group C, respectively, was spiked with tonsillar DNA from two patients; the addition of tonsillar DNA did not affect the sensitivity of the PCR assays (Fig. 2A). To explore whether meningococci only on the surface of the tonsil would be detected by real-time PCR, tonsils were exposed to a saline solution containing either 10^3 or 10^5 N. meningitidis cells/ml for 15 min and then were dried by dabbing on sterile paper towels. Figure 2B shows that N. meningitidis was detected in DNA extracted from the tonsils exposed to meningococci in vitro.

N. meningitidis in tonsils from patients. Next, we applied our real-time PCR-based systems to tonsillar DNA from a series of patients from Oxford (Sim et al., letter). In those patients, N. meningitidis was grown from swabs collected from three patients, while 13 samples were positive by immunohistochemistry (Sim et al., letter). Employing real-time PCR targeting conserved regions of eubacterial 16S rRNA with a cutoff C_T value of 30, bacterial DNA was detected in tonsils from 23 of 26 patients (88% of individuals; mean C_T value, 24.4; median C_T value, 24.6). Meningococcal DNA was detected in tonsils from 14 of 26 patients: of the three nasopharynx swab culture-positive and immunohistochemistry-positive samples, two tested positive for group B and one tested positive for group C meningococci; 8 of the 10 culture-negative but immunohistochemistry-positive samples were positive for group B, while only 3 of the 18 culture-negative and immunohistochemistry-negative samples tested positive for group C. Thus, for tonsils from individuals identified as meningococcal carriers by nasopharyngeal culture, a concordance between immunohistochemistry and real-time PCR of 100% was found, and for tonsils from individuals whose nasopharyngeal swabs were negative, a concordance of 72% was found.

Detection rates of N. meningitidis in tonsils from distinct geographical regions. Next, we examined the tonsils from 72 patients from Zurich. Employing real-time PCR targeting conserved regions of eubacterial 16S rRNA and setting a cutoff C_T value of 30, bacterial DNA was detected in tonsils from 58 (81%) patients (mean C_T value, 21.9; median C_T value, 22.0). Using the four different real-time PCR systems specific for meningococci, none of the tonsillar samples from the 72 patients was shown to contain either N. meningitidis group B or group C.

DISCUSSION

Using highly sensitive and specific real-time PCR-based assays for N. meningitidis group B and C, respectively, we detected these bacterial species in excised tonsils exposed in vitro to these bacteria and corroborated immunohistochemistry findings for tonsils from Oxford. However, we were unable to detect meningococci in tonsillar tissue samples from subjects from Zurich, where the incidence of invasive meningococcal disease is lower.

The four real-time PCR-based assays employed in this study to detect N. meningitidis B and C were able to detect the equivalent of one single bacterial cell per reaction mixture (Fig. 1). Furthermore, the four assays were highly specific for N. meningitidis since there was no amplification of DNA from a plethora of other bacteria (Fig. 1). Although other bacterial species were detected in the tonsils of more than 80% of the patients from both Oxford and Zurich, none of the samples...
from Zurich was positive for *N. meningitidis*. These negative results were not due to PCR inhibitory factors present in the tonsillar samples, as similar amplification of *N. meningitidis* was demonstrated in spiking experiments (Fig. 2A).

A potential limitation of this study is that nasopharyngeal swabs were not obtained from the patients from Zurich. However, given the inaccuracy of nasopharyngeal swabbing for assessing meningococcal carriage (Sim et al., letter), culture may not have been helpful in elucidating the discrepancies between donors from Oxford and from Zurich. Nevertheless, our real-time PCR-based assays, a method demonstrated to be at least as sensitive as immunohistochemistry (9), showed a concordance of 100% with immunohistochemistry for nasopharyngeal culture positive and of 72% for culture negative individuals in patients from Oxford.

The failure to detect meningococci in any tonsillar sample from 72 different donors from Zurich markedly contrasts with the findings in donors from Oxford (Sim et al., letter). One potential explanation for the discordant results could be differences in processing of tissues. However, the broad-range detection of bacterial species showed that organisms other than meningococci were well detected in both sets of samples. Another potential explanation for the discordant results could be epidemiological factors, including age-related or geograph-
N. meningitidis

vitro to meningitidis group B or C with DNA from tonsils shown to be negative for 3920 GREINER ET AL. J. CLIN. MICROBIOL.
carriage rates are lower in younger children (1.
dom employing nasopharyngeal swabbing revealed that the
letter). Cross-sectional studies in Norway and the United King-
dom employing nasopharyngeal swabbing revealed that the
carriage rates are lower in younger children (1–4). However,
age differences do not account for the lack of meningococci in
tonsils from individuals in Zurich. Although patients from Zu-
rich were younger than those from Oxford (means, 6.0 and 9.5
years, respectively), only 3 of the 14 subjects testing positive for
N. meningitidis by real-time PCR were older than 15 years; the
mean age of the meningococcal carriers from Oxford was in the same age range
as the patients from Zurich, the differences in ages between the
investigated cohorts did not contribute to the discordant results.
The incidence of invasive meningococcal diseases mirrors the
incidence (10). In Switzerland the yearly incidence of invasive meningococcal disease in 1999 to 2000 was 2.4 cases/100,000 inhabitants, and in England it was 5.4 cases/100,000 inhabitants (11). Thus, assuming that the prevalence of menin-
gococcal carriage in tonsillar tissues from at least around 15 subjects from Zu-
rich. Nevertheless, the relationship between carriage and dis-

case may not be linear. Finally, the discrepancies in tonsillar carriage of meningococci demonstrated in cohorts from two geographical areas with different epidemiology of N. meningitidis indicate that findings from one geographical region may not invariably be applicable to another region. Furthermore, the results may suggest that yet-to-be-identified pathogenic properties of circulating meningococcal strains may substantially determine both the rate and the anatomical localization of carriage.

Our findings demonstrate the application of PCR-based methods for detecting N. meningitidis in tonsillar material. Although such an approach would not be an effective epidemiological test, it could be adapted to other samples, including the direct detection of N. meningitidis from nasopharyngeal swabs without the need for culture and characterization of microorganisms. Thus, real-time PCR could be a valuable method for further understanding the epidemiology of meningococcal infection and for contact tracing and targeting prophylactic therapy during outbreak situations.

ACKNOWLEDGMENTS

Funding for the study was partially provided by a grant from the Müller-Grocholski-Stiftung (to D.N.) and by The Meningitis Research Foundation (to C.M.T.).

REFERENCES

probe system useful for detecting PCR product and nucleic acid hybridization, PCR Methods Appl. 4:357–362.


