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, CH 8032 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 difference in disease incidence could be explained by different transmission rates in the community rather than host genetics or coexisting infections.

*MATERIALS AND METHODS*

**Bacterial strains.** Strains of *N. meningitidis* serogroups B and C isolated from cerebrospinal fluid were used as reference strains (F. Rohner, Swiss National Reference Center for Meningococci, Geneva, Switzerland). To test the specificity of PCR, *Neisseria lactamica*, *Moraxella lacunata*, *Moraxella phenylpyrolytica*, *Moraxella incognita* (kindly provided by R. Zbinden, Institute of Medical Microbiology, University of Zurich), *Moraxella catarhalis*, *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-isomyl alcohol (25:24:1), and then extracted twice with chloroform-isomyl alcohol (2:1). 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. 301859). The sequence of the forward primer was 5'-TCGTGTGGTAATAGTTG-3' (positions 1070 to 1091), the sequence of the reverse primer was 5’-CATCCACCTTCCAGT-3' (positions 1190 to 1171), and the sequence of the probe was 5’-AGTCGCGAAGCGACACCC-3' (positions 1093 to 1113). The fluorescent reporter dye at the 3' 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-
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 $10^6$ bacterial cells per reaction mixture; the detection range was $10^5$ to $10^9$ 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).

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 subbacterial 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.

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-
tical differences in the rates of meningococcal carriage in the Zurich cohort compared with the Oxford cohort (Sim et al., letter). Cross-sectional studies in Norway and the United Kingdom 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 Zurich 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 older than 15 years; the mean age of the meningococcal carriers from Zurich younger than 15 years was 6.1 years. Thus, since the vast majority of meningococcal carriers from Oxford were 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 carriage rates (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 meningococcal carriage in tonsillar tissues from at least around 15 subjects from Zurich was two times lower, we would have expected to detect \( N. \) meningitidis in tonsillar tissues from at least around 15 subjects from Zurich. Nevertheless, the relationship between carriage and disease 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**


**FIG. 2.** Detection of \( N. \) meningitidis group B and C by real-time PCR. (A) Absence of inhibition when spiking genomic DNA from \( N. \) meningitidis group B or C with DNA from tonsils shown to be negative for \( N. \) meningitidis. (B) DNA extracted from tonsils previously exposed in vitro to \( N. \) meningitidis group B or C for 15 min. The values are the means of duplicate measurements.
probe system useful for detecting PCR product and nucleic acid hybridization. PCR Methods Appl. 4:357–362.