PCR-based assays for detection of *Streptococcus pneumoniae* serotypes 3, 14, 19F and 23F in respiratory specimens

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Current culture-based assays are insensitive for detection of simultaneous respiratory tract colonization by more than one pneumococcal serotype. Separate single-tube, nested PCR-based assays have been developed to detect *Streptococcus pneumoniae* serotypes 3, 14, 19F and 23F by amplifying unique DNA sequences in the capsular polysaccharide gene cluster of each serotype. Pairs of 27–32-base outer primers and 20–21-base inner primers and a 20–22-base probe were designed to amplify and detect a 200–221-base sequence by dot blotting using the labelled probe. Sensitivity of the assays was 0.01–10 fg using chromosomal DNA and ≤1 viable cell using DNA extracted from exponential-phase bacteria. Each serotype-specific assay detected chromosomal DNA from all of five to ten clinical isolates of the homologous type and did not detect DNA sequences from any of 190–204 strains from 51–52 different serotypes or 28 non-pneumococcal bacterial strains. Sixteen throat swabs from children that had been cultured for *S. pneumoniae* were tested in PCR assays following DNA extraction. All of six that grew *S. pneumoniae* serotype 3, 14, 19F or 23F were positive in the PCR assay for the homologous serotype (and in a PCR assay for sequences in *lytA*, present in all pneumococci) and were negative in assays for other serotypes. Of eight culture-negative specimens in children not receiving antimicrobials, three were positive for both the *lytA* assay and an assay for one of the four serotypes, suggesting true positive results; in three others all five PCR assays were negative and, in the remaining two, the *lytA* assay was positive but each of the four assays for individual serotypes was negative, suggesting either false-positive results or presence of DNA sequences from an *S. pneumoniae* serotype other than 3, 14, 19F or 23F. These preliminary clinical data suggest that these PCR-based assays are sensitive and specific for detection of individual serotypes of pneumococci and may be used with respiratory tract specimens.

INTRODUCTION

Infections due to *Streptococcus pneumoniae* (pneumococcus) are a major cause of morbidity and mortality among children and adults worldwide. Pneumococci are the leading cause of bacterial pneumonia and meningitis, accounting for over 40,000 deaths annually in the United States. In addition, pneumococci are the leading cause of sinusitis and of otitis media, the most common paediatric infection for which antibiotics are routinely prescribed (Centers for Disease Control and Prevention, 1997, 2000; Rubin, 2000; Siber, 1994). Virulent pneumococci express one of 90 chemically and antigenically distinct polysaccharide capsules that are the basis for classification into 46 serogroups and 90 serotypes (Henrichsen, 1995). Pneumococci asymptomatically colonize the pharynx, a prerequisite for pneumococcal infection. Furthermore, colonizing bacteria are the source of bacteria for person-to-person transmission.

Pneumococcal colonization is detected by culturing naso- or oropharyngeal swabs on blood-agar medium and serotype is determined by testing one or several colonies for reactivity with specific antisera (Lafong & Crothers, 1988; Lloyd-Evans *et al.*, 1996; Lund, 1960). However, culture of pneumococci from respiratory tract specimens is difficult (Larsen, 2000; Willett, 1988) and insensitive, as has been demonstrated by recovery of pneumococci from culture-negative specimens by mouse injection (Mackenzie *et al.*, 1940) or detection using a PCR-based assay on respiratory specimens that were culture-negative for pneumococci (Saukkoriipi *et al.*, 2002). Furthermore, detection of simultaneous carriage with more than one serotype using standard culture methods in which one to five pneumococcal colonies from each culture are serotyped is severely limited (Huebner *et al.*, 2000; Gundel & Okura, 1933; Hodges *et al.*, 1946; Mackenzie *et al.*, 1940), yet...
Serotype-specific PCR-based assays have the potential to detect specifically small numbers of one or more serotypes of pneumococci in respiratory specimens, but must be highly sensitive and suitable for use with clinical specimens. We describe new sensitive and specific single-tube nested PCR assays for detection of \textit{S. pneumoniae} serotypes 3, 14, 19F and 23F DNA, and their use with clinical respiratory samples.

**METHODS**

**Bacterial strains, growth and storage.** Pneumococcal isolates were obtained from the two largest clinical microbiology laboratories of the North Shore-Long Island Jewish Health System. In addition, isolates representing particular serotypes were obtained from the CAPSIR strain repository, Case Western Reserve University, Johns Hopkins Bloomberg School of Public Health and Boston Medical Center. Pneumococci were serotyped using latex particles sensitized with mono-specific typing sera (Statens Seruminstitut, Copenhagen, Denmark) and observing for agglutination (LaFong & Crothers, 1988; Lloyd-Evans et al., 1996; Lund, 1960). The Quellung reaction was used for isolates that gave equivocal results using latex agglutination (Avery, 1918).

Other bacterial species were obtained from our collection of clinical isolates, or were laboratory strains including \textit{Escherichia coli} strains ATCC 35218 and ATCC 35150, \textit{Pseudomonas aeruginosa} strains ATCC 27853 and ATCC 10145, \textit{Haemophilus influenzae} non-typable strains ATCC 9913, \textit{Neisseria meningitidis} strain ATCC 13419, \textit{Streptococcus salivarius} strain ATCC 13419, \textit{Streptococcus sanguis}, \textit{Streptococcus pneumoniae} strains ATCC 19615 and ATCC 12344\textsuperscript{T}, \textit{Streptococcus agalac-tiae} serotype IC strain ATCC 13813\textsuperscript{T} and \textit{Streptococcus intermedius}.

Bacteria were grown by subculture overnight on solid media (sheep blood agar) or in Todd–Hewitt broth (Becton Dickinson) to mid-exponential phase (OD\textsubscript{590} = 0.5, Spectronic 20 spectrophotometer; Bausch & Lomb) for studies of sensitivity of PCR. Bacteria were stored at –70°C in Todd–Hewitt broth containing 20% glycerol.

**DNA extraction from bacteria.** DNA was extracted from bacteria by one of two methods. The phenol/chloroform extraction method was used in early experiments but abandoned in favour of the Aquapure genomic DNA isolation kit method (Bio-Rad) because of a lower DNA contamination rate, presumably related to fewer steps and less handling using the latter procedure. For phenol/chloroform extraction, pneumococci were grown overnight on sheep blood agar, colonies were resuspended in 0.1 ml lysis buffer (0.1% deoxycholate, 0.01% SDS, 0.15 M sodium citrate) and incubated for 5 min at 37°C. Bacteria were stored at –70°C in Todd–Hewitt broth containing 20% glycerol.

DNA was isolated using the Aquapure genomic DNA isolation kit (Bio-Rad). DNA was extracted from 0.2 ml suspension buffer containing the swab using a High Pure PCR template preparation kit (Roche Diagnostics) according to the manufacturer’s instructions. Final volume was 0.2 ml and 10 μl was added to the reaction mixture for PCR.

**Selection of gene as target for PCR assay and nested PCR assay design.** DNA sequences of \textit{lytA} (Whatmore & Dowson, 1999) and the capsular polysaccharide (CPS) gene cluster for serotype 3 (Dillard et al., 1995), 14 (Kolkman et al., 1996), 19F (Morona et al., 1997) or 23F (Ramirez & Tomasz, 1998) were imported into GenBank and searched against the database. Sequences that had limited similarity with other pneumococcal serotypes and other sequences in GenBank were selected (typically an entire gene within the capsular gene cluster) and imported into the Primer3 Test program (Center for Genome Research at the Whitehead Institute for Biochemical Research, MIT, Cambridge, MA). Using this program, we developed a pair of outer primers, specifying a high melting point (68–72°C), a relatively high GC content (optimum 40–50%), a product size of 300–600 bases and a primer size of 27–32 bases. After outer primers were designed, inner primers were designed by specifying a melting temperature 10–15°C lower than that of the outer primers, a GC content of 40–50%, a product size of 300–600 bases and a primer size of 18–27 bases (Table 1). Probes of 20–22 bases were then designed using this program. Primers and probes were synthesized by the Molecular Genetics Core Laboratory of the North Shore-Long Island Jewish Research Institute using ABI 394 DNA/RNA Synthesizers (Applied Biosystems) and then purified by desalting using a 10DG desalting column (Bio-Gel P-6; Bio-Rad).

**Nested, single-tube PCR.** Preliminary studies were performed to optimize reaction conditions by varying the concentration of outer primers and inner primers to determine the minimum concentration that generated product, the optimal ratio of outer to inner primers (usually a 50–100-fold lower concentration of the outer primers than the inner primers), \textit{T\textsubscript{m}} of outer primers and MgCl\textsubscript{2} concentration (1.5–5 mM). The sample reaction mixture contained Eppendorf MasterMix (Eppendorf Scientific) in a final volume of 50 μl including 10 μl specimen and added MgCl\textsubscript{2} if required. Amplification was performed in a thermal cycler (PCR Master Cycler Gradient instrument; Eppendorf Scientific).
PCR conditions were: initial denaturation at 94 °C for 4 min, and 30 cycles of outer product amplification with denaturation at 94 °C for 1 min, annealing at 70 °C for 2 min and extension at 72 °C for 1.5 min. This was followed by five further cycles of 94 °C for 1 min, 55 °C for 2 min and 72 °C for 1.5 min. To generate inner product, the denaturation temperature was lowered to 87 °C for 1 min, annealing at 55 °C for 2 min and extension at 72 °C for 1.5 min for 35 cycles. Finally, one cycle of 87 °C for 1 min, annealing at 55 °C for 2 min and extension at 72 °C for 7 min was completed.

**Dot-blot detection of PCR products.** PCR products (1 μl) were applied to Gene Screen Plus hybridization transfer membranes (BioTechnology Systems, NEN Research Products) and product was detected using a labelled internal oligonucleotide probe. Probe labelling, hybridization and development of positives were performed using the Gene Images 39-oligolabelling module (Amersham Life Sciences) according to the manufacturer’s instructions.

**Agarose gel detection of PCR products.** A 2 % NuSieve-1 % SeaKem agarose gel (FMC BioProducts) containing 1 μg ethidium bromide ml⁻¹ was used for electrophoresis of 10 μl amplified product. Amplification products and size markers (phage 174 digest; Sigma) were visualized by placing the gel on a UV light box. Gels were inspected for the presence of a band of expected molecular mass and other bands, and to estimate DNA concentration.

### RESULTS

**Development of nested PCR assay for detection of the autolysin gene, lytA**

We developed a nested PCR assay to detect sequences in *lytA* because this gene is common to all pneumococci (Cherian et al., 1998) and should prove useful for screening of respiratory specimens for their presence. A nested assay was developed to maximize sensitivity (Picken et al., 1996).

After selected primers were synthesized, reaction conditions were optimized and used in the single-tube nested PCR with chromosomal DNA from pneumococci (Tables 1 and 2). Sensitivity of the assay was 2.5 fg purified chromosomal DNA and < 1 viable cell using DNA extracted from serial dilutions of mid-exponential phase bacteria added to a throat swab suspension from a healthy adult whose culture did not grow pneumococci. Chromosomal DNA from 158 pneumococcal strains representing 51 serotypes was subjected to PCR. A product was detected by dot-blot hybridization using labelled probe and a product of expected molecular mass was detected by agarose-gel electrophoresis using DNA from all strains (Table 3). After subjecting chromosomal DNA from 28 bacterial strains representing 14 species that may be found in the upper respiratory tract to PCR, no product was detected by dot-blot hybridization or agarose gel electrophoresis, demonstrating specificity of this assay for *S. pneumoniae* (Table 3).

**Development of nested PCR assay for type 14**

Our strategy for development of serotype-specific assays was to target genes in the CPS gene cluster of the serotype under study. Based on the comparative organization of CPS gene clusters presented by Garcia & Lopez (1997), we selected gene *cps14H* as it was without apparent significant similarity to the
CPS gene cluster of other serotypes (Kolkman et al., 1996). The sequence of the entire gene (1141 bases) was imported and searched (BLAST 2.2.1) against GenBank. A 720 base region without similarity to other sequences in GenBank was imported into the Primer3 Test program. Primers and probe were designed (Table 1) and individually tested in a GenBank BLAST search for similarity with prokaryotic and eukaryotic sequences. PCR was performed as described in Methods, with an MgCl₂ concentration of 4.5 mM. The assay was positive when chromosomal DNA from type 14 pneumococcal strains was tested and negative with chromosomal DNA from other pneumococcal serotypes or other bacterial species (Table 3). Sensitivity of the test was 10 fg for detection of purified chromosomal DNA from a serotype 14 strain and <1 viable cell using DNA extracted from serial dilutions of mid-exponential phase bacteria added to a throat swab suspension from a healthy adult whose culture did not grow pneumococci. Using a similar strategy, single-tube nested PCR assays were developed for serotypes 3, 19F and 23F with sensitivities of 2, 2-5 and 5 fg purified chromosomal DNA from the homologous serotype, respectively, each amplifying and detecting DNA from <1 viable cell.

Specificity of serotype-specific assays

Each of the four assays detected all strains of the homologous serotype tested as follows: five isolates of type 3, five isolates of type 14, ten isolates of type 19F, and six isolates of type 23F (Table 3). In each assay, 190–204 clinical pneumococcal isolates representing 51–52 different serotypes were negative, including other serotypes within the same serogroup for serotypes 19F and 23F (Table 3). Chromosomal DNA from 28 non-pneumococcal bacterial strains from 14 species including Haemophilus and Neisseria species, and non-pneumococcal α-haemolytic streptococci were negative in each of the four assays (Table 3).

Detection of pneumococcal DNA with human respiratory specimens

DNA from throat swabs from six children with acute otitis media that grew S. pneumoniae serotypes 3, 14, 19F or 23F, from one child whose culture grew serotype 6B and from eight children whose throat cultures were negative for S. pneumoniae was extracted and assayed by PCR (Table 4). Included in each PCR run for an individual serotype or lytA

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**Table 2. Target genes for PCR assays to detect S. pneumoniae lytA or serotype 3, 14, 19F or 23F DNA**

<table>
<thead>
<tr>
<th>Serotype specificity</th>
<th>Target gene</th>
<th>Putative function of predicted protein product</th>
<th>Sequence accession number and reference</th>
<th>PCR product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>lytA</td>
<td>Autolysin</td>
<td>AJ243413; Whatmore &amp; Dowson (1999)</td>
<td>200</td>
</tr>
<tr>
<td>3</td>
<td>cps3D</td>
<td>UDP-glucose dehydrogenase</td>
<td>U15171; Dillard et al. (1995)</td>
<td>221</td>
</tr>
<tr>
<td>14</td>
<td>cps14H</td>
<td>Putative polysaccharide polymerase</td>
<td>X85787; Kolkman et al. (1996)</td>
<td>219</td>
</tr>
<tr>
<td>19F</td>
<td>cps19fI</td>
<td>Putative polysaccharide polymerase</td>
<td>U09239; Morona et al. (1997)</td>
<td>208</td>
</tr>
<tr>
<td>23F</td>
<td>cps23fG</td>
<td>Putative polysaccharide polymerase</td>
<td>AF057294; Ramirez &amp; Tomasz (1998)</td>
<td>207</td>
</tr>
</tbody>
</table>

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**Table 3. Nested single tube PCR for detection of S. pneumoniae by dot-blot hybridization**

<table>
<thead>
<tr>
<th>Serotype assay</th>
<th>No. of bacterial strains tested in serotype-specific PCR assays</th>
<th>Non-pneumococcal bacterial strains†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pneumococcal strains, homologous serotype</td>
<td>Pneumococcal strains, serotype-related</td>
</tr>
<tr>
<td>All serotypes</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>–</td>
</tr>
<tr>
<td>14</td>
<td>5</td>
<td>–</td>
</tr>
<tr>
<td>19F</td>
<td>10</td>
<td>8‡</td>
</tr>
<tr>
<td>23F</td>
<td>6</td>
<td>8§</td>
</tr>
</tbody>
</table>

†Represents 14 non-pneumococcal species (see Methods section).
‡Serotype 19A, 5; serotype 19C, 3.
§Serotype 23A, 4; serotype 23B, 4.
was an additional positive-control specimen, consisting of a pneumococcal culture-negative suspension of oropharyngeal secretions to which was added 10^4 c.f.u. of exponential phase pneumococci of the homologous serotype, and a negative control consisting of oropharyngeal secretions from the same healthy adult whose culture was negative for *S. pneumoniae* and had assayed negative for lytA sequences. Assays for lytA, performed on five specimens that grew pneumococci, were positive in all specimens. Sequences corresponding to the homologous serotype but not heterologous serotypes were detected from the six specimens that were culture-positive for serotypes 3, 14, 19F or 23F, and no positive results were detected from the swab that grew serotype 6B. Follow-up throat swabs from two of these patients, obtained following a 10-day course of amoxycillin, were culture- and PCR-negative in all five assays. PCR positive results correspond to one to four bacterial cells (pneumococcal genome equivalents). Detection of *< 1* viable bacterial cell probably reflects detection of DNA from both non-viable cells present in the broth culture and viable bacteria detected by sub-culture. The 20–50 c.f.u. sensitivity of the assays developed by Brito et al. (2003) is lower than ours. Furthermore, the true sensitivity may have been overstated because their sensitivity measurement was based on detection of viable counts from broth cultures of bacteria that were likely to contain both viable and non-viable cells. The nested design contributed to the sensitivity of our assays; generally, we found the nested design to be 2- to 10-fold more sensitive than a similar assay amplifying the inner primers alone (data not shown). In addition, detection of product by dot-blot hybridization was two- to tenfold more sensitive than detection of product on agarose gel (data not shown).

**DISCUSSION**

Each assay for an individual serotype was designed to be specific by targeting sequences in the CPS gene cluster that encode a protein that appears to be specific for synthesis, transport or assembly of the particular polysaccharide that defines the serotype. Recently, a similar strategy was used by Lawrence et al. (2003) to develop PCR assays specific for five pneumococcal serotypes and three serogroups, and by Brito et al. (2003) to develop assays for nine serotypes. In contrast to the assays of Lawrence et al. (2003) and Brito et al. (2003), which were developed for typing pneumococcal isolates, our assays were developed to be highly sensitive and thus able to detect individual pneumococcal serotypes in clinical upper respiratory tract specimens including the smaller number of cells of a second or third serotype present in the same specimen (Huebner et al., 2000). Our assays were extremely sensitive; detection of 2–10 fg chromosomal DNA corresponds to one to four bacterial cells (pneumococcal genome equivalents). Detection of *< 1* viable bacterial cell probably reflects detection of DNA from both non-viable cells present in the broth culture and viable bacteria detected by sub-culture. The 20–50 c.f.u. sensitivity of the assays developed by Brito et al. (2003) is lower than ours. Furthermore, the true sensitivity may have been overstated because their sensitivity measurement was based on detection of viable counts from broth cultures of bacteria that were likely to contain both viable and non-viable cells. The nested design contributed to the sensitivity of our assays; generally, we found the nested design to be 2- to 10-fold more sensitive than a similar assay amplifying the inner primers alone (data not shown). In addition, detection of product by dot-blot hybridization was two- to tenfold more sensitive than detection of product on agarose gel (data not shown).

Selection of sequences within the CPS gene cluster is critical for reliable detection of a particular serotype because...
pneumococci undergo serotype switching in vivo by transformation and homologous recombination of the CPS gene cluster (Nesin et al., 1998). Although the serotype-specificity of the selected gene and gene sequence for the homologous serotype was supported by comparisons with sequences in GenBank, these analyses were not definitive because the database contained CPS gene cluster sequences for only a minority of the 90 serotypes. Specificity of each assay was demonstrated by absence of amplification using chromosomal DNA from other pneumococcal serotypes (and other bacterial species) as the DNA source for PCR. Although DNA from only 51 of the 90 serotypes was tested, these serotypes comprise more than 99% of the serotypes cultured from the nasopharynx of children in the United States and internationally (Finkelstein et al., 2003; Kellner et al., 1999; Lopez et al., 1999; Mbelle et al., 1999; Meats et al., 2003; Ridgway et al., 1995; Soewignjo et al., 2001; Syrijanen et al., 2001; Syroyiannopoulos et al., 2002). Furthermore, the serogroups/serotypes tested included those in the FDA-approved heptavalent pneumococcal conjugate vaccine, an investigational 11-valent ‘second-generation’ pneumococcal conjugate vaccine (Kayhty & Ahman, 2001) and 22 of the 23 serotypes in the polyvalent pneumococcal polysaccharide vaccine (Rubin, 2000). Moreover, the assays did not amplify DNA sequences from other bacterial species present frequently in respiratory secretions. Thus, each assay is serotype-specific, at least for pneumococcal serotypes likely to be encountered in respiratory secretions. Similarly, each of the PCR assays developed by Lawrence et al. (2003) for detection of serotypes 1, 3, 14, 19F and 23F DNA was found to be specific, in that none amplified DNA extracted from 22 heterologous pneumococcal serotypes or 18 other species of streptococci.

Using the High Pure PCR template preparation kit to extract DNA from throat swabs, the assays amplified serotype-specific DNA sequences from all of a small number of culture-positive clinical specimens tested, suggesting these assays are sensitive for detection of specific serotypes in clinical respiratory specimens. In Table 4, throat swabs 3, 4 and 10 were culture-negative for pneumococci but PCR-positive for both lytA and serotypes 14, 14 and 23F, respectively. Although these results could be interpreted as false-positive results, amplification and detection of both serotype-specific DNA sequences and lytA DNA sequences (and negative results in serotype-specific assays for three other serotypes) make it likely that these were true-positive results and that the assays were more sensitive than the cultures. Similarly, the three culture-negative specimens detected (Table 4; specimens 11A, 11B and 12) that were negative for DNA from serotypes 3, 14, 19F and 23F, but had lytA sequences, may contain pneumococci of another serotype(s). This interpretation is supported by the findings of Saukkoriipi et al. (2002), who, using real-time PCR to amplify and detect sequences in the pneumolysin gene present in all pneumococci, showed positive results in 96% of culture-positive nasopharyngeal specimens and in 52% of culture-negative specimens. By quantifying the genome equivalents, they found PCR-positive, culture-negative specimens had 10 genome equivalents compared with 10^6 or 10^7 genome equivalents in culture-positive specimens, corresponding to 10–100 or >100 colonies, respectively. These results support the idea that respiratory specimens from children frequently contain small numbers of pneumococci that are not readily recovered in culture as a more plausible explanation for the discrepancy between PCR and culture than false-positive results on the PCR-based assays. Similarly, using a real-time PCR assay to detect sequences in the pneumolysin gene present in all pneumococci, Greiner et al. (2001) found that 10% of their culture-negative respiratory specimens were PCR-positive. Thus, although only a small number of clinical specimens were tested, the results demonstrate the feasibility of this approach and provide a rationale for larger studies to determine sensitivity, specificity and predictive values of these assays to detect pneumococcal colonization with one or more serotypes.

During development of the assays using DNA extracted from bacterial colonies, we occasionally encountered false-positive results due to contamination. In all cases, this was traced to contamination occurring during extraction of DNA from bacterial cells with DNA of a second serotype that was being prepared simultaneously. Contamination was prevented by changing methods of DNA extraction from phenol/chloroform extraction to a commercial method (to minimize the number of steps in the procedure, thereby minimizing chances of contamination) and by performing the extraction in a laminar-flow biological safety cabinet rather than on a laboratory bench. Although nested PCR assays have been reported to have a higher rate of contamination than standard PCR due to amplicon contamination, we used a single tube assay that did not require the tube to be re-opened after the PCR had commenced, a method with a contamination rate that is equivalent to standard PCR (Picken et al., 1996).

It is likely that a similar strategy can be used to develop PCR assays specific for other pneumococcal serotypes. Complete CPS gene cluster sequence data for 80 serotypes are available from the Sanger Centre, which is under contract from the World Health Organization to sequence the CPS gene cluster of all 90 serotypes, and sequences of the CPS gene cluster for some serotypes are available in GenBank. Thus, it is likely that similar methods can be used to develop PCR assays to detect all clinically relevant pneumococcal serotypes that may be found in respiratory specimens. Use of these assays with upper respiratory tract specimens should further our understanding of the dynamics of pneumococcal carriage including carriage of multiple serotypes and the effect of vaccination on carriage. In addition, PCR-based assays should also be useful for serotyping pneumococci as was recently shown by Lawrence et al. (2003) and Brito et al. (2003).

**ACKNOWLEDGEMENTS**

Long Island Jewish Medical Center is the clinical campus for the Albert
REFERENCES


