Random Amplified Polymorphic DNA Subtyping of *Haemophilus influenzae* from Middle Ear Effusion Fluid of Lebanese Patients with Otitis Media with Effusion

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Objective: **In this study, we evaluated a Random Amplified Polymorphic DNA (RAPD) method, in the subtyping of *H. influenzae* in middle ear effusions (MEE), obtained from 33 patients with otitis media with effusion (OME) admitted to 3 medical centers in Beirut, Lebanon.**

Method: **RAPD was initially evaluated on 15 H. influenzae isolates using three 10 mer primers along with a 21 mer primer.**

Results: **Primer 1 (10 mer) and Primer 2 (20 mer) were the most discriminatory when used in conjunction and were selected to be used on DNA lysates obtained from 33 MEE samples positive for *H. influenzae* by PCR.**

Conclusion: **Our data have shown that 4 RAPD patterns were obtained on DNA of *H. influenzae* isolates and a single RAPD pattern was seen on *H. influenzae* DNA from MEE samples. This indicates that a single strain may have been implicated in these infections. Studies are underway to determine the prevalence of *H. influenzae* as the etiology of otitis media with effusion in Lebanon and subtype the organism to determine a possible multi-strain involvement.**

Key words: *Otitis, PCR, subtyping*

Otitis media is a very common acquired pediatric infection, especially during the first years of life. Few children escape this disease and recurrent attacks are frequent (1). Chronic effusions have in the past been assumed to be sterile. However recent investigations by Healy and Teele, 1977 (2), Giebink et al, 1979 (3), have reported that 30-50% of children have bacteria in the middle ear fluid. In children with both acute otitis media with effusion (AOM) and secretory otitis media (SOM), *Streptococcus pneumoniae* and *Haemophilus influenzae* are the predominant pathogens (4). These pathogens can contribute to as much as 50% of cases with ear infections (4). Tympanocentesis from children with AOM has revealed the presence of bacteria in up to 74% of cases, the most common isolates being *S. pneumoniae* (30-50% of cases), and H. influenzae (12-25% of cases) (5,6). A third pathogen that is becoming increasingly important is *Moraxella catarrahlis*.

The higher percentage of Beta-lactamase producing strains of *H. influenzae* (15-30%) and *M. catarrahlis* (90%) strengthen the need for diagnostic means to identify these pathogens in a rapid fashion. Culture from middle ear efusion (MEE) proved not to be adequate as a diagnostic tool for bacterial recovery from MEE samples (7,6). PCR has recently been demonstrated to be efficient for detecting various types of microorganisms responsible for otitis media with effusion (OME) (7,6,8). Studies have also shown that PCR-based assays are more sensitive than conventional culture, as there was about 46-55% discordance between PCR and culture results (7,6). In addition to PCR-detection, subtyping of detected isolates is important in order to determine the genomic diversity among these isolates and identify possible strain variation implicated in such infections. This will be primordial in tracing the source of infection and in establishing treatment policies. Conventional subtyping methods proved to either lack of sensitivity, specificity, reproducibilty or discriminatory potential. Molecular typing methods on the other hand, proved to be more universal, discriminatory and reproducible.

Random amplification of polymorphic DNA (RAPD) subtyping, a simple, reproducible, fingerprints of genomic DNA, can be generated in a PCR by using a single primer that has no known homology to the target sequence. The amplification is performed at low stringency, allowing the primer to anneal to several locations on the 2 strands of target DNA. Primers of 10 base long and with at least 40 mol% G+C have been useful for RAPD subtyping. The method has been applied successfully to *Clostridium difficile*, *Listeria monocytogenes, Campylobacter jejuni*, *Bacillus cereus* (9,10,11) and others. In this study, we assessed the utility of RAPD in the subtyping of *H. influenzae* detected previously in MEE samples (6).

Material and Method

Fifteen *H. influenzae* isolates previously obtained from a variety of clinical specimens and identified by conventional methods (12), were used to assess the utility of RAPD. *H. influenzae* ATCC strain 49766 was used as a positive control. MEE samples aspirated from 33 Lebanese children (age: 2-10 years), undergoing tympanostomy-tube

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placement for OME in 3 Medical Centers in Beirut, Lebanon, were used. These samples were positive by PCR for *H. influenzae*. Nine of 33 were also positive by culture. All samples were part of a previous study where MEE samples were collected between September 1996 and May 1997 from children with AOM (6). Eleven MEE samples negative for *H. influenzae* by PCR were also collected during the same period and used as negative controls.

H. influenzae strains and MEE samples were cultured on chocolate agar plates. The latter were incubated in 5% CO_2 at 37^0 C overnight. Positive cultures were further identified by Gram staining and standard identification assays (12). DNA was extracted from bacterial isolates by the PureGene kit (Gentra Systems Inc., North Carolina, USA) according to the manufacturer's specifications. DNA was extracted from MEE samples by the method of Loutit and Tompkins (5), with some modifications. The modified method uses 800 µg of proteinase K per ml and 5% Triton X-100.

PCR was done initially on DNA from 15 *H. influenzae* isolates using universal and species/specific primers as described previously (6). Three 10 mer primers were used to assess their utility in RAPD along with a 21 mer primer. Primer 1 (5' TCA CGC TGC A 3') along with Primer 2 (CTT CTT CAG CTC GAC GCG ACG) were selected for subsequent RAPD testing on DNA from MEE samples, PCR-positive for *H. influenzae* and on DNA from MEE samples negative for the organism. RAPD method was carried out in 100-µl reaction mixtures containing each: 10 µl of DNA, 16 µl of dNTPs (0.2 Mm), 10 µl of 10 X buffer (100 mM Tris-HCl[pH 8.3], 500 mM KCl, 4 mM $MgCl_2$), 1 µl of primer 1 (0.3 µg/µl), 1µl of primer 2, 0.5 µl of Taq DNA Polymerase (5U/µl) and 56.5 µl of sterile distilled water. A PTC-100 thermal controller (MJ Research, Watertown, Mass.) was used for all amplifications. PCR conditions were as follows: Pre-DNA denaturation at 940C for 5 min., followed by 44 cycles including each: denaturation at 94 ^{0}C for 30 sec., annealing at 37 ^{0}C for 1 min., extension at 72 ^{0}C for 2 min. A final extension at 72 ^{0}C for 10 min. was also done. PCR products were electrophoresed on a 1.5% agarose gel (FMC Bioproducts, Rockland, Maine). DNA fragments were visualized on a UV transilluminator (Foto/Prep I; Fotodyne, New Berlin, Wis.) and photographed with type 667 Polaroid film (Polaroid, Cambridge, Mass.). DNA patterns were compared visually with a 50 base-pair ladder. Patterns that had the same number of bands and similar fragment sizes were considered identical.

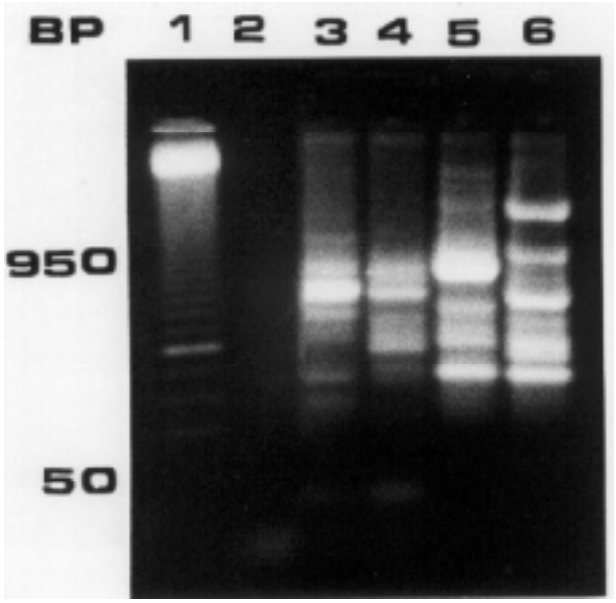

Figure 1. Representative RAPD patterns of *H. influenzae* isolates on agarose gel.
Lane 1: 50-bp ladder, lane 2: negative control, lanes 3-6: RAPD patterns of *H. influenzae.*

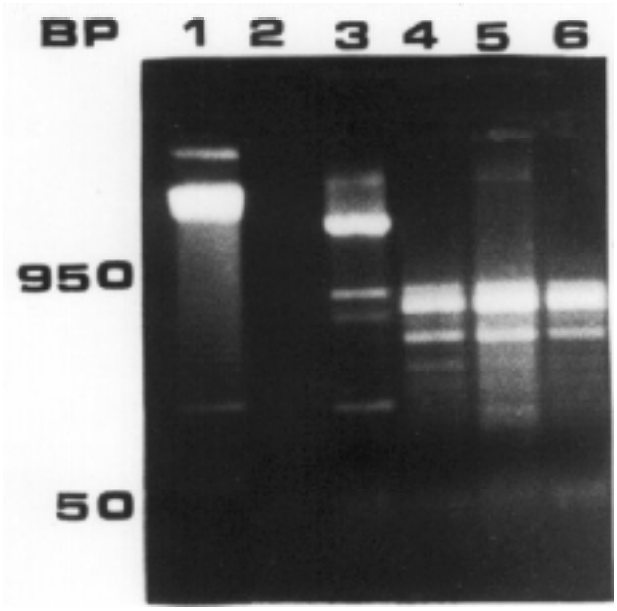

Figure 2. Agarose gel of RAPD pattern of 3 representative *H. influenzae* in MEE samples from the 3 medical centers in Beirut, Lebanon. Lane 1: 50-bp ladder; lane 2: negative control; lane 3: *H. influenzae* ATCC 49766; lanes 4-6: RAPD pattern in MEE samples.

Results and Discussion

Our data have shown that DNA from 15 *H. influenzae* isolates revealed 4 RAPD patterns with primer 1 and primer 2 (Figure 1). The patterns were reproducible. This indicates that RAPD using these primers is discriminatory amongst the species, since 4 patterns were observed in a relatively small number of isolates. This also implies that there may be genomic diversity amongst these isolates. DNA from the 33 MEE samples obtained from the 3 medical centers, gave the same RAPD pattern (Figure 2), denoting that no genomic variation is seen among the *H. influenzae* DNA in MEE samples and that all detected isolates may belong to one strain. The pattern observed is different from those of the 15 *H. influenzae* isolates and the ATCC strain (Figure 2).

The prevalence of one strain among tested isolates is possible given that all 33 MEE samples came from patients living in Beirut area. More genomic diversity may be encountered in *H. influenzae* isolates in MEE samples obtained from patients residing in another region in the country. Hence, application of this method on a larger scale of samples may reveal genomic variations among *H. influenzae*. If laboratory information is coupled with adequate epidemiologic data, this would allow RAPD subtyping on this organism to be evaluated as a marker to trace the source of identified strain(s) implicated in otitis media with effusion. With increasing prevalence of *H. influenzae* as the cause of chronic effusions, it becomes mandatory to determine the source of strain(s) implicated in this type of infections as well as other clinical and epidemiologic studies of the spectrum of diseases caused by this organism.

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