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"Dodgy 6As": Differentiating Pneumococcal Serotype 6C from 6A by Use of the Quellung Reaction$^{\nabla}$

The Quellung reaction has long been the standard method for pneumococcal serotyping (7); however, the new serotype 6C is reportedly indistinguishable from serotype 6A using this method (8). We report an anomalous Quellung reaction associated with serotype 6C, which may aid in diagnostic interpretation.

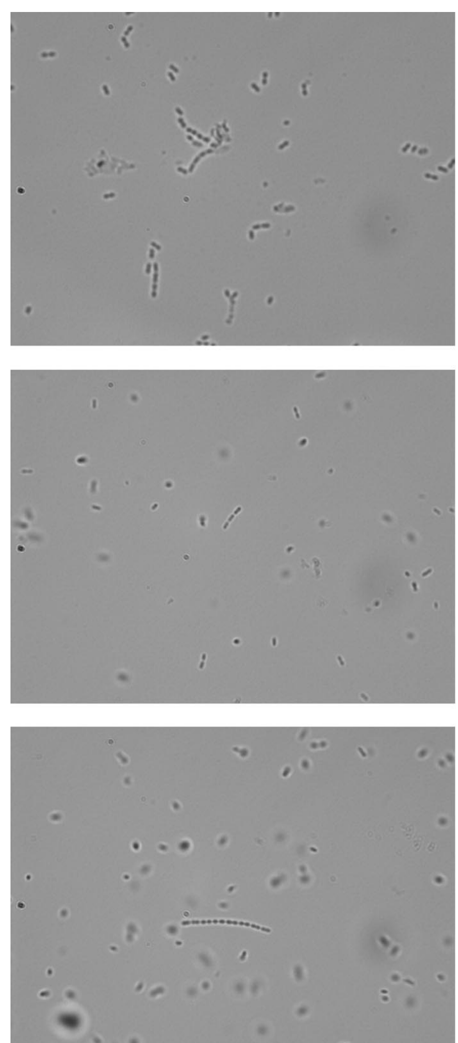
The 7-valent pneumococcal conjugate vaccine was introduced in Australia for indigenous and other high-risk infants in 2001. In 2002, a serotyping anomaly was noted in a subset of carriage isolates. These isolates appeared negative for all nine pool antisera A to I (covering 90 pneumococcal serotypes) but reacted positively with Omni serum. Following advice from the Pneumococcal Reference Laboratory in Queensland, Australia, we found that these isolates reacted strongly with group 6 antiserum but that factor reactions were weak. Group 6 is included in the B pool. A selection of isolates was sent to Queensland and serotyped as 6A. In 2003, the Statens Serum Institute (SSI) confirmed 10 such isolates as 6A; pool B reactions were mostly weak (+), while pool Q reactions were strongly positive (+++). (Pool Q, which includes group 6, is part of an alternative set of antisera used to identify types present in the 23-valent pneumococcal polysaccharide vaccine.) To distinguish "normal" 6As with strong pool B reactions, we referred to isolates with weak or apparent negative pool B reactions as "dodgy" 6As.

In 2007, when the new serotype 6C was announced (8), we queried whether our dodgy 6As were actually 6Cs. Using a *wciN* PCR (9), we revealed that 1 of 20 prevaccine isolates recorded as 6A was 6C, while 17 of 18 postvaccine isolates recorded as 6A were 6C, suggesting serotype replacement. All 17 postvaccine serotype 6C isolates had been recorded as dodgy 6As, while the one postvaccine serotype 6A isolate was recorded as a normal 6A. Atypical reactions had not been recorded prevaccine.

To confirm our results, fresh serum broths were prepared and serotyped blinded. All 20 isolates typed as 6A by PCR were normal 6As by the Quellung reaction, while all 18 isolates typed as 6C by PCR were dodgy 6As by the Quellung reaction. Subsequently, another 144 6A isolates from a 2003 to 2005 surveillance study were tested using *wciN* PCR. All 61 normal 6As were confirmed as 6A, while all 83 dodgy 6As were 6Cs. We have therefore matched PCR results to pool B reactions for 182 isolates previously recorded as 6A (81 [100%] normal 6A isolates were confirmed as serotype 6A by PCR and 101 [100%] dodgy 6A isolates were determined to be 6C by PCR).

We have used five different batches of pool B antiserum since 2002; thus, batch variation is unlikely to have influenced our findings. Furthermore, in a competitive inhibition enzyme-linked immunosorbent assay, 6C polysaccharide antigen did not significantly attenuate the binding of pool B to 6A and 6B polysaccharide antigens. This suggests that pool B may react weakly with serotype 6C. Interestingly, 6C antigen considerably inhibited the binding of pool Q to these antigens, suggesting a strong pool Q reaction with 6C (as reported by SSI).

The Quellung reaction is produced using the Neufeld test, described by Lund (6) and Austrian (1), and the SSI pneumococcal antisera insert (August 2001 revision). Briefly, a drop of specimen is mixed with a drop of typing serum, a coverslip is placed over the mixture, and the preparation is examined using a ×100 magnification oil immersion lens. A positive reaction occurs when type-specific antibody binds to the pneumococcal capsule, causing a change in its refractive index so that it appears "swollen" and more visible. A World Health Organization working group acknowledged the Quellung reaction as the standard method for serotyping pneumococcal isolates but did not detail a recommended method (7).



(a) 6A pool B reaction (strong/positive)

(b) 6C pool B reaction (weak/negative)

(c) 6C negative control

FIG. 1. Quellung reaction photographed using a ×100 magnification oil-immersion lens, giving an appearance similar to that seen at ×40 magnification. (a) 6A pool B reaction (normal); (b) 6C pool B reaction (dodgy); (c) 6C negative control (no antiserum added).

Recently, 11 reference laboratories in Europe participated in the validation of pneumococcal serotyping (5). A high degree of consensus was found between the Neufeld test and other serotyping methods (agglutination or gel diffusion). Six laboratories used the Neufeld test; however, the magnification used was not specified, nor was the use of oil immersion (5). Australian laboratories routinely use ×40 magnification (4), which is quicker and simpler than ×100. This protocol is accurate and reliable and has been used by experts for more than 30 years (2). According to Henrichsen (3), the Quellung reaction continues to be carried out essentially as described by Austrian, and we find no reference to any modified Neufeld method used outside Australia.

To our eyes, the differing strengths of the pool B reaction with 6A and 6C were clearly visible at both magnifications (Fig. 1). Furthermore, this phenomenon is not confined to pool B antiserum. We have observed that a strong positive pool G reaction indicates serotype 29 or 35B, while a weak positive pool G reaction invariably indicates serotype 34.

We have demonstrated that pneumococcal serotype 6C is associated with a weak pool B reaction which differentiates it from 6A. This information should prove useful for laboratories that use the Quellung reaction, particularly those without the capability for molecular typing.

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