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Phosphorylcholine-containing antigens in bacteria from the mouth and respiratory tract

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Phosphorylcholine (PC)-containing antigens were sought in 269 bacterial isolates from the mouth and respiratory tract by an enzyme immunoassay method. Only 41 (15%) isolates were PC-positive and of these 29 (70%) were strains of *Haemophilus influenzae*. Other species that produced positive results included two of five isolates of *Gemella haemolysans*, two of five isolates of *Micrococcus* spp., and a single strain each of *Bacillus* sp., *Corynebacterium jeikeium*, *Lactococcus* sp. and *H. parainfluenzae*. The presence of PC-containing antigens in *H. influenzae* may be an important source of cross-reaction in antigen detection techniques that detect the C-polysaccharide antigen of *Streptococcus pneumoniae* in respiratory specimens and would result in false positive results.

Introduction

The diagnosis of *Streptococcus pneumoniae* infection has been attempted by methods that detect species-specific C-polysaccharide (PnC) antigen which contains phosphorylcholine (PC) [1–5]. Doubts about the specificity of this technique have been raised on the grounds that many other organisms share PC which is the immunodominant epitope. These organisms include protozoa (the excreted factor of *Leishmania donovani*) [6], helminths (*Toxocara canis* excretory secretory antigen and *Ascaris suum*) [7, 8] and fungi (*Aspergillus* spp. and *Mentagrophytes* spp.) [9]. Of more importance, potentially, is cross-reaction with other oral ('viridans') streptococci [10–13] and group C β -haemolytic streptococci, as contamination of sputum samples with these organisms could lead to false positive results. Studies of cross-reactions in α -haemolytic streptococci have shown that up to 36% bind anti-PC antibodies [12] and PC-containing antigen has been found in members of the *S. oralis* group — which also includes *S. mitis* and *S. pneumoniae* — but not in other representative strains of oral streptococci [13]. The aim of this study was to demonstrate the expression of PC-containing antigens by other bacteria from the mouth and respiratory tract and to estimate the potential for cross-reaction in PnC-based antigen detection methods.

Materials and methods

Bacterial isolates tested

The majority of bacteria studied were obtained from respiratory tract specimens or saliva samples investigated at the Royal Free Hospital, London. Mixed saliva (≥ 3 ml) was collected from healthy subjects and processed immediately. A portion (100 μ l) was inoculated on to four media: blood agar, chocolate agar incorporating bacitracin 80 mg/L, blood agar incorporating aztreonam 4 mg/L and Hoyle's Medium (all from Oxoid). Plates were incubated at 37°C for up to 48 h in an aerobic atmosphere of increased humidity and CO₂ 5–10%. A subculture was made of all the different colonial types represented on each plate and the number and dilution were recorded. β -Haemolytic streptococci from oral specimens were isolated on blood agar and identified by Gram's stain and the catalase test. Isolates were differentiated into Lancefield groups with the Slidex Strepto kit (bioMérieux, Marcy L'Etoile, France) and API STREP (bioMérieux) where appropriate. Other oral streptococci were identified by the Rapid Strep 32 (bioMérieux) following the manufacturer's instructions. Organisms were also obtained from routine sputum samples plated on to blood agar and chocolate agar.

Neisseriaceae were isolated on blood agar and chocolate agar and organisms were selected for study by Gram's stain and the oxidase test. Individual isolates were speciated by their ability to grow at 22°C on blood agar, on New York City Medium (Oxoid) at 37°C and on Nutrient Agar (Oxoid) at

37°C, and by their ability to reduce nitrate and produce DNAase [14, 15]. Oxidation of glucose, maltose, sucrose and lactose was assessed on serum-free agar slopes [16]. It was not possible to differentiate between *N. flavescens* and *N. cinerea* by this combination of tests and all DNAase-negative asaccharolytic species were identified as '*N. cinerea*'.

Haemophilus spp. were identified by Gram's stain, oxidase reaction and X and V factor dependence by a paper disk method (Mast Diagnostics Liverpool) on nutrient agar. A further group of 98 isolates of non-typable *H. influenzae* was obtained as a gift from the culture collection of Professor J.D. Williams (London Hospital Medical College). These had been collected previously as part of a survey of antimicrobial resistance in respiratory isolates of *H. influenzae*.

Isolates of the genus *Corynebacterium* and other oral gram-positive rods were selected for study on the basis of their colonial morphology, Gram's stain and catalase test. Isolates were speciated with the API Coryne kit (bioMérieux) following the manufacturer's instructions.

Representative isolates of Enterobacteriaceae isolated from faeces and urine from our culture collection previously identified by API 20E (bioMérieux) and agglutination tests were selected for study.

Preparation of bacterial isolates

Each isolate was subcultured into Brain Heart Infusion (BHI) Broth and incubated aerobically for 18 h at 37°C. To assist in quantification of antigen the viable count was determined by the method of Miles and Misra [17]. The bacterial cells were harvested by centrifugation at 3000 *g* and washed twice by centrifugation in PBS. The washed cells were digested by incubating with lysozyme 50 mg/ml (Sigma) for 1 h at 37°C as described previously [13].

Immunoassays

The quantitative PnC antigen capture ELISA has been described previously [13]. Briefly, 96-well microtitration plates (M29A, Dynatech, UK) were coated with a 1 in 2000 dilution of a mouse IgM monoclonal anti-PC antibody (mAb 5/88) (Universal Biologicals) in 0.06 M bicarbonate buffer (pH 9.6) by overnight incubation at 4°C. After washing with 10 mM Tris containing 0.15 M NaCl, Tween-20 0.05% and 1 mM Ca²⁺ (TBSTC) (Sigma), samples were added in 100-µl volumes in duplicate and incubated for 1 h at room temperature. After further washes, 100 µl of C-reactive protein (1.7 µg/ml) conjugated to horseradish peroxidase by the periodate method, diluted in TBSTC, was added and the plates were incubated for 3 h at room temperature. ABTS peroxidase substrate (Kirkegaard-Perry, Gaithersburg, MD, USA) was added after the plates had been washed four times and the optical density was read at

405 nm with an automated ELISA reader (Titretek Multiscan MC, Flow Laboratories).

The specificity of the result with all isolates that gave a positive result was confirmed by repeating the assay but including PC (Sigma) 10 mg/ml in the buffer at the antigen capture stage of the test as described previously [13]. The concentration of antigen expressed was determined by performing multiple 10-fold dilutions of the extracts. The concentration was calculated by comparing the optical density of the result with a standard curve with purified C-polysaccharide antigen (generously provided by Dr Lui, Rockefeller University, NY, USA) and the final concentration was calculated as described previously [13]. The amount of PC-containing antigen (pg/cfu) was calculated by dividing PC antigen concentration detected in the broth culture by the viable count.

Immunogold localisation of PC antigen

Two strains of *H. influenzae* — one PC- and one non-PC-containing strain — were taken and cultivated overnight in BHI broth. Bacterial cells were fixed in paraformaldehyde 1% and washed in PBS. The cells were then suspended in agar 3%, cut into 1-mm pieces and washed with distilled water. The specimen was dehydrated with graded dimethyl formamide (Sigma) then embedded in fresh LR white resin (Taab Laboratories, Reading) and polymerised with a UV lamp at 4°C. Ultra-thin sections were cut and placed on Poliform grids. The sections were floated on 0.5 M ammonium chloride (BDH, Poole) to quench free aldehyde groups, then washed twice in PBS. The grids were placed on normal goat serum (Dako, High Wycombe) 10% in BSA/PBS 0.1% for 10 min and then transferred to a drop of anti-PC MAb 5/88 (Universal Biologicals) (diluted 1 in 100) for 2 h. The grids were washed five times in PBS and then transferred on to drops of goat anti-mouse gold conjugate (British Biocell International, Cardiff) diluted 1 in 10 and incubated for 1 h. The grids were washed again in PBS and stained with uranyl acetate saturated in ethanol (BDH) 50% and Reynold's lead citrate.

Results

A total of 47 strains of β -haemolytic streptococci and seven enterococci was tested and of these one group C streptococcal isolate and two group G streptococcal isolates were PC-containing antigen positive. Among the gram-positive cocci tested, two of five strains of *Gemella haemolysans*, two of five strains of *Micrococcus*, and a single *Lactococcus* sp. isolate expressed a PC-containing antigen (Table 1). A single isolate of *C. jeikeium* out of four studied also gave a positive result. Negative results were obtained with other *Corynebacterium* spp. (8), *Staphylococcus aureus* (1), coagulase-negative staphylococci (11), *Propionibacterium* sp.,

Table 1. Species of oral and respiratory tract bacteria tested for the presence of PC-containing antigen

Bacteria	Number of strains tested	Number of strains positive	Range of PC concentration (pg/cfu)
<i>S. equisimilis</i>	8	1	0.022
<i>Streptococcus</i> sp. Group G	6	2	1.8
<i>Gemella haemolysans</i>	5	2	2.3–2.6
<i>Lactococcus</i> sp.	1	1	146
<i>Micrococcus</i> spp.	5	2	0.04–890
<i>Bacillus</i> spp.	4	1	–
<i>C. jeikeium</i>	4	1	*
<i>Corynebacterium</i> sp.	1	1	*
<i>H. influenzae</i>	100	29	0.001–0.045
<i>H. parainfluenzae</i>	14	1	*

Four *Proteus* spp., four *Shigella* spp., four *Salmonella* spp. and four *E. coli* isolates were tested and gave negative results.

*Antigen not quantified.

Fig. 1. a, Results of PC enzyme immunoassay for lysozyme-digested preparations of 98 *H. influenzae* and four *S. pneumoniae* isolates; b, Results of PC enzyme immunoassay with and without PC inhibition for 10 selected *H. influenzae* isolates.

S. pyogenes (19), *S. agalactiae* (10), group F streptococci (4), *Enterococcus* spp. (7), *Neisseria* spp. (9), *N. cinerea* (5), *N. meningitidis* (1), *N. mucosa* (9), *N. polysaccharae* (1), *N. sicca* (11), *N. subflava* (10) and *Moraxella catarrhalis* (5). Forty-three strains of bacteria from the Neisseriaceae family were tested and all gave negative results. In addition, 16 isolates of Enterobacteriaceae were tested and found to give negative results (four *Proteus* spp., four *Shigella* spp., four *Salmonella* spp. and four *Escherichia coli*).

Of 100 strains of *H. influenzae* and 14 strains of *H. parainfluenzae* examined for PC-containing antigen, 29 strains of *H. influenzae* and one of *H. parainfluenzae* gave positive results (Fig. 1a). The binding for all strains was specifically inhibited by PC at 10 mg/ml, although the results for only 10 strains of *H. influenzae* are illustrated in Fig. 1b.

The results in Fig. 2 indicate that of the 29 strains of *H. influenzae* that possessed a PC-containing antigen,

Fig. 2. Quantification of PC-containing antigen in 20 selected isolates of *H. influenzae*.

Fig. 3. Immunogold preparation of *H. influenzae* to locate PC-containing antigen. Immunogold particle size 10 nm.

the range of concentrations when compared to a *S. pneumoniae* PnC antigen standard were 0.9–45 µg/ml or *c.* (0.9×10^{-3}) – (4.5×10^{-2}) pg/cfu.

A representative PC-positive and a PC-negative strain were studied by immunogold staining in an attempt to localise the antigen. The negative control strain did not bind the gold-labelled immunoglobulin, indicating that the anti-PC MAb was not bound (data not shown). In the positive strain, gold particles can be seen scattered over the cell, but many are localised on the cytoplasmic membrane (Fig. 3). The gold particles also appear to be relatively concentrated around the cell wall septum.

Discussion

PC-containing antigens are found in diverse groups of micro-organisms including helminths and fungi [7, 9]. Among bacteria, PC-containing antigens have been found in gram-positive genera and those present in *S. pneumoniae* have been studied in most detail. The pneumococcal species-specific teichoic acid C-polysaccharide is ribitol teichoic acid [18] and each repeat unit possesses two PC residues linked through the two GalNAc residues [19]. The C-polysaccharide acts as an anchor for *S. pneumoniae* autolysin enzyme N-acetylmuramyl-L-alanine amidase binding specificity through the PC residue. This position is in the optimum position for peptidoglycan degradation, a function which is essential for growth and cell division. Organisms in which the PC of C-polysaccharide is

replaced by ethanolamine cannot bind autolysin and, consequently, daughter cells fail to separate. They are no longer bile soluble and are penicillin tolerant. Teichoic acid-containing portions of the cell wall activate the alternative complement pathway and have been shown to provide the stimulus for the acute inflammatory response [20–23]. The lipoteichoic acid 'F antigen' has a similar polysaccharide-PC component linked to a membrane anchor region [24] and as it is located in the plasma membrane it is thought to regulate autolysin function [25] by binding autolysin in a site where it is unable to act on peptidoglycan. Bacterial autolysis is preceded by a loss of F antigen from the plasma membrane [25]. The PC residue is the binding site for *S. pneumoniae* bacteriophages that have evolved an enzyme homologous to autolysin [26].

The presence of PC in cell wall teichoic acid has been found to be specific to the *S. oralis* group of organisms which includes *S. pneumoniae* [13]. In this study, cross-reacting antigens were found in a few gram-positive genera, including isolates of *G. haemolysans*, *Lactococcus*, *Bacillus*, *Corynebacterium* and *Micrococcus* spp. In none of these genera is the presence of a PC epitope of taxonomic significance as it is among oral streptococci [13].

The finding of PC-containing epitopes in approximately one-third of *H. influenzae* strains and a single strain of *H. parainfluenzae* was unexpected. Immunogold studies localised this antigen to the plasma membrane and mainly near the cell wall septum. This latter observation is of interest in view of the

importance of the C-polysaccharide-autolysin interaction in cell division in *S. pneumoniae* but further studies are required to establish the nature and function of this antigen. In view of the ability of PC-containing antigens to stimulate inflammatory mediators this is a potentially important finding and may indicate a mechanism whereby *H. influenzae* strains generate the inflammatory response associated with chronic bronchial sepsis.

The clinical use of antigen detection immunoassays has been limited by concerns about cross-reacting antigens. Quantitatively, the main organisms isolated from saliva are oral streptococci, *Haemophilus* spp. and *Neisseria* spp. All members of the *S. oralis* group possess a PC-containing teichoic acid, although the quantity expressed on *S. oralis* is between 10 and 1000-fold less than on *S. pneumoniae* [13]. This difference is enhanced as the number of organisms/ml is lower in saliva than in sputum, making false positive results less likely. This has been confirmed in clinical studies of quantitative enzyme immunoassays in which colonisation and infection can be distinguished [27]. The presence of PC-containing antigen in approximately one-third of *H. influenzae* isolates from patients with lower respiratory tract infection may raise the possibility of false positive results in this clinical situation. If the overnight culture tested here were estimated to contain *c.* 100⁹ cfu/ml and sputum from a patient with a 'significant' infection to contain 10⁸ cfu/ml, one can calculate that the amount of PC antigen found in patients infected with these strains would have been up to 4.5 µg/ml. With a cut-off of 1 µg/ml reported previously [27], three of the *H. influenzae* isolates in this study would have given a positive result.

In previous studies of serological diagnosis by a PnC method, false positive results were obtained from patients infected with *H. influenzae*. Although one group noted that cross-reacting antigen might be a problem they concluded that as patients from whom *Mycoplasma pneumoniae* had been isolated also gave positive results, the results were probably due to an unrecognised double infection [3]. A similar conclusion was reached by Holmberg *et al.* [4], who found that samples from 22.6% of a group of patients with LRTI gave positive results without any significant bacterial isolation and it is possible that this latter group gave positive results due to cross-reacting antigens from oral bacteria. Holmberg and Krook [1] also found false positive results in patients with *Legionella* and *Haemophilus* infection. They state that 'It is not known if there are C-polysaccharide-like structures in *Legionella* spp. or *Haemophilus influenzae* that are responsible for immunological cross-reactions or that if positive ELISA reactions are true positives, indicating double infection...'. Therefore, it can be concluded, from the information presented here in quantitative binding experiments that indicate PC-containing epi-

topes in *H. influenzae*, and also from previous trials of PC-based antigen detection methods, that cross-reactions between PnC and *H. influenzae* are clinically significant and will result in incorrect diagnoses in between 1 and 3% of cases.

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