Specific PCR, bacterial culture, serology and pharyngeal sampling to enhance the aetiological diagnosis of cellulitis

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It is often difficult to obtain a bacteriological diagnosis in patients with cellulitis. We examined the utility of molecular techniques and skin and throat cultures, as well as serology, in providing evidence of either *Staphylococcus aureus* or group A *Streptococcus* (GAS) presence in patients with cellulitis. Samples were collected from patients with a clinical diagnosis of cellulitis who were recruited into a prospective placebo-controlled clinical trial (C4C study, EudraCT 2013-001218-14). Specific PCR, paired serology and culture for both organisms were carried out on a variety of samples where appropriate. Despite utilizing a range of diagnostic methods, a bacteriological diagnosis was only achieved in 43% of patients with a clinical diagnosis of cellulitis. Seventeen per cent of patients tested positive for GAS by any method but only 4% were positive by PCR, whilst *S. aureus* was detected in 34% of samples. Bacterial diagnosis in cases of cellulitis remains challenging. This is probably due to a very low bacterial burden with toxin production resulting in inflammation mediating skin damage. Further consideration for the need for long courses of antimicrobial therapy for cellulitis therefore appears merited.

**INTRODUCTION**

The bacterial aetiology of limb cellulitis is largely attributed to group A *Streptococcus* (GAS) and *Staphylococcus aureus*, but it is often difficult to get bacteriological confirmation (Jeng et al., 2010). As part of a National Institute for Health Research-funded clinical trial; Adjunctive Clindamycin for Cellulitis (C4C) trial (EudraCT Number: 2013-001218-14, ISRCTN: 86329346), which aims to evaluate the role of clindamycin in addition to flucloxacillin for the treatment of cellulitis, we wanted to secure a bacteriological aetiology in order to be able to increase our ability to generalize the findings to other GAS infections. Protein-inhibiting antibiotics are commonly recommended for serious toxin-mediated streptococcal and staphylococcal infections. If the trial demonstrates improvement with clindamycin, then this would be powerful supporting evidence for the use of such antibiotics in conditions such as necrotizing fasciitis and toxic shock syndrome.

We aimed to compare the utility of molecular techniques and skin and throat cultures, as well as serology, in providing evidence of either *S. aureus* or GAS presence in clinical cases of cellulitis.

**METHODS**

**Bacteriology.** Specimens from 77 patients were collected from October 2013 to July 2014. Patients were selected based on a clinical diagnosis of unilateral limb cellulitis using a standard pro forma. Twenty-six patients presented with recurrent cellulitis. The patients may have received up to 48 h of a β-lactam antibiotic depending on time to recruitment (Table 1). Up to two swabs of the erythematous area, including any local skin lesions or portals of entry, were carried out at recruitment. Only patients with purulent wounds or ulcers had swabs for bacterial culture as well as molecular testing. Swabs were placed into a universal transport medium (UTM; COPAN) for molecular testing and Amies charcoal transport medium (Transwab; MWE) for culture.

Fig. 1 summarizes the testing of the samples. Swabs of skin lesions were processed for culture of haemolytic streptococci and *S. aureus* using routine clinical laboratory methods. Some patients had swabs for culture taken during follow-up if lesions developed. A throat swab was taken from each patient, stored in Amies charcoal transport medium and cultured for streptococcal species. Patients were screened for meticillin-resistant *S. aureus* (MRSA) carriage with nasal swabs and selective media. We did not receive every specimen from every patient, which meant that some patients did not have every test performed.

**Streptococcal serological testing.** Blood sample pairs (baseline and day 10) from each patient were used to determine anti-streptolysin O
using an ASO Latex Test kit (Lab21 Healthcare Ltd) and anti-DNase B titres in serum using an ASD-kit (bioMérieux) according to the manufacturers’ instructions. They were regarded as positive if there was a twofold increase in titre to either antigen.

**Molecular diagnostics.** Bacterial DNA was extracted directly from the transport medium of swabs from clinical samples. Sample buffer (200 μl) was prepared for bacterial DNA extraction using QIA-symphony automated DNA extraction, producing an elution volume of 60 μl. Real-time PCR for GAS and *S. aureus* was performed on the extracted DNA using the ViiA7 Real-time PCR System (Life Technologies). PCR primers and probes (Table 2) were custom synthesized by Sigma-Aldrich. For *S. aureus*, a 96 bp fragment of the muc gene was amplified and detected (Kilic et al., 2010), whilst for GAS, a 154 bp fragment of the ntpC gene was amplified and detected (Hung et al., 2012). PCR of a T4 bacteriophage internal process control confirmed successful DNA and inhibitor-free nucleic acid extracts. The methods and determination of the detection limit of the PCR method were as described by Thors et al. (2015).

**RESULTS**

Overall, a bacteriological diagnosis was achieved in 33 out of 77 patients (43 %). Samples from 73 patients were tested by PCR.

**GAS**

Thirteen of the 77 (17 %) patients tested positive for GAS by any combination of culture, serology and PCR. Three patients were positive by PCR only (4 %). Of 66 samples tested serologically, only 48 patients had paired sera, and of these 11 patients were positive (23 %). Only one of these was also positive by PCR. None of these 11 patients cultured GAS. Twenty-four patients had swabs cultured: two of these cultured GAS, and both were PCR positive. One patient’s sample was culture negative for GAS but tested positive by PCR and by serology. None of the 71 throat swabs cultured GAS, although one group G Streptococcus was cultured.

**S. aureus**

Twenty-five of the 73 (34 %) samples tested positive for *S. aureus* by PCR. Of the 73 patients, 24 had swabs tested by culture, of which eight were positive (33.3 %). All of the samples in which *S. aureus* was identified by culture and determination of the detection limit of the PCR method were as described by Thors et al. (2015).

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**Table 1.** Number of samples positive for the presence of *S. aureus* or group GAS when samples were collected at <3 h, 3–6 h and >6 h after flucloxacillin administration

<table>
<thead>
<tr>
<th>Test</th>
<th>Time post-flucloxacillin treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;3 h</td>
</tr>
<tr>
<td>PCR positive</td>
<td>12</td>
</tr>
<tr>
<td>Culture positive</td>
<td>6</td>
</tr>
</tbody>
</table>

All culture-positive samples were also PCR positive.

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**Fig. 1.** Flow diagram summarizing the confirmatory tests undertaken on prospective clinical samples from patients with a clinical diagnosis of cellulitis. *Four patients tested positive for both GAS and *S. aureus* via a combination of method; **one group G Streptococcus identified. Pos, positive; Neg, negative.*
were also positive by PCR. No MRSA was isolated on nasal screens or from skin swabs.

**DISCUSSION**

This study used a combination of PCR from skin swabs, skin swab culture, throat swab culture and serology to detect evidence of GAS or *S. aureus* in clinical cases of cellulitis; other studies have used immunofluorescence of skin biopsies (Bernard *et al.*, 1989), PCR of tissue aspirates (Johnson *et al.*, 2012) and serology (Jeng *et al.*, 2010; Karppelin *et al.*, 2014; Leppard *et al.*, 1985). Despite this wide repertoire of testing, we were unable to achieve a confirmed bacteriological diagnosis in as many patients as those using serology alone. The most likely reason for our poorer diagnostic yield from serology is the short period between the acute and convalescent samples. The two most recent previous studies had a period of 4 weeks (Karppelin *et al.*, 2014) and 2–12 weeks (Jeng *et al.*, 2010) between sampling. Our trial follow-up was limited to 10 days, and this may be insufficient time to generate a detectable difference between samples, especially as we were unable to determine absolute antibody values. It is also possible that the strains of GAS in our population were less likely to produce sufficient streptolysin or DNase B to stimulate an immune response. A high proportion of our patients were given antibiotic therapy prior to sampling for PCR and culture, and half had subsequently received clindamycin, which may have suppressed toxin production. Antibiotic therapy has previously been associated with a poorer serological response (Karppelin *et al.*, 2014).

We found PCR to be more sensitive than culture for detecting both GAS and *S. aureus*; this is in contrast to the results of Johnson *et al.* (2012), who used 16S rRNA gene PCR. We used primers specific for GAS and *S. aureus* and achieved a positive result from 26/73 (36 %) patients, predominantly *S. aureus*. There was a substantial reduction in the number of positive culture results with increased time after the administration of flucloxacillin (Table 1). The effect of antibiotic administration on PCR positivity appeared to be less marked and was expected as, unlike culture, PCR can successfully detect non-viable organisms. The detection of GAS by both PCR and culture was poor and is probably a reflection of the paucibacillary nature of cellulitis in which the majority of clinical features are toxin mediated, and the non-invasive skin samples.

Throat carriage of GAS in the general UK population is low, at less than 2 % (Turner *et al.*, 2013). We anticipated a higher carriage rate in a population experiencing clinical disease often attributed to GAS. However, GAS could not be cultured from any of 71 throat swabs, although β-haemolytic group G *Streptococcus*, which can also cause cellulitis, was cultured from one sample. Thus, cellulitis is not associated with throat carriage of GAS, even in those patients who had not been treated with antibiotics prior to swabbing.

We support the view of others that, despite the more frequent isolation of *S. aureus*, GAS is the predominant pathogen in cellulitis. The failure to detect more evidence of GAS could be attributed to the sensitivity of the sampling and detection methods; however, other studies have reported mixed results, even when using more invasive sampling methods such as skin biopsies and needle aspiration. Direct immunofluorescence of skin biopsies has demonstrated that a very low number of *in situ* pathogens is present (typically within the dermis and/or hypodermis) (Bernard *et al.*, 1989). This probably contributes to the low incidence of positive culture results in this and other studies. However, it is increasingly thought that in uncomplicated cases of cellulitis, GAS is present in small numbers on the skin surface or structures and that the development of cellulitis is mediated through toxin production rather than bacterial invasion. *S. aureus* may be detected more commonly because it is present in larger numbers than GAS on the skin, and in particular within skin lesions such as ulcers and wounds.

The large repertoire of GAS toxins is able to produce extensive tissue damage, even when small numbers of organisms are present. It is possible that *S. aureus* is not the causative agent in most cases of cellulitis where it was isolated but rather adopts an opportunistic bystander role. This is supported by studies that demonstrate no extra benefit when adding an antibiotic active against MRSA in the presence of detectable MRSA (Pallin *et al.*, 2013). The extensive tissue damage caused by streptococcal toxins may produce an environment that promotes *S. aureus* multiplication – this is supported by the detection of both organisms in

<table>
<thead>
<tr>
<th>Organism (gene)</th>
<th>Primer/probe</th>
<th>T&lt;sub&gt;m&lt;/sub&gt;</th>
<th>Sequence (5′→3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Streptococcus pyogenes</em> (nuc)</td>
<td>F</td>
<td>62.8</td>
<td>TGCACACGCTACGCTATCACCC</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>62.5</td>
<td>AGGATAGGTCTTGGTTAGCTC</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>73.4</td>
<td>6FAM-TGACGATCTCAACGATTGATCGG-BHQ1</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> (nuc)</td>
<td>F</td>
<td>56.7</td>
<td>GTGACGTATTGTTAACCTTAGTTA</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>63.5</td>
<td>AATGTCGACGTTCTTATGTAATTT</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>64.1</td>
<td>6FAM-AAGTCTAAGTCGCTAGCAAAATGCA-BHQ1</td>
</tr>
</tbody>
</table>

T<sub>m</sub>, Calculated melting temperature; F, forward primer; R, reverse primer; P, probe; 6-FAM, 6-carboxyfluorescein; BHQ1, Black Hole Quencher-1.
five of the patients in our study. These data further support the previously described hypothesis of a low level of bacteria and the role of toxins, with the consequent inflammatory response, being important drivers in the pathogenesis of cellulitis (Bernard et al., 1989; Johnson et al., 2012). The role of the inflammatory cascade triggered by a small bacterial burden generates uncertainty regarding the appropriate use of prolonged courses of broad-spectrum intravenous antibiotics in the management of cellulitis.

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REFERENCES


