Lack of horizontal gene transfer of methicillin-resistance genetic determinants from PBP2a-positive, coagulase-negative staphylococci to methicillin-sensitive *Staphylococcus aureus* using transcutaneous electrical nerve stimulation (TENS)

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ABSTRACT

Previous research shows that approximately half of the coagulase-negative staphylococci (CNS) isolated from patients in the intensive care unit (ICU) at Belfast City Hospital were resistant to methicillin. The presence of this relatively high proportion of methicillin-resistance genetic material gives rise to speculation that these organisms may act as potential reservoirs of methicillin-resistance genetic material to methicillin-sensitive *Staphylococcus aureus* (MSSA). Mechanisms of horizontal gene transfer from PBP2a-positive CNS to MSSA, potentially transforming MSSA to MRSA, aided by electroporation-type activities such as transcutaneous electrical nerve stimulation (TENS), should be considered. Methicillin-resistant CNS (MR-CNS) isolates are collected over a two-month period from a variety of clinical specimen types, particularly wound swabs. The species of all isolates are confirmed, as well as their resistance to oxacillin by standard disc diffusion assays. In addition, MSSA isolates are collected over the same period and confirmed as PBP2a-negative. Electroporation experiments are designed to mimic the time/voltage combinations used commonly in the clinical application of TENS. No transformed MRSA were isolated and all viable *S. aureus* cells remained susceptible to oxacillin and PBP2a-negative. Experiments using MSSA pre-exposed to sublethal concentrations of oxacillin (0.25 µg/mL) showed no evidence of methicillin gene transfer and the generation of an MRSA. The study showed no evidence of horizontal transfer of methicillin resistance genetic material from MR-CNS to MSSA. These data support the belief that TENS and the associated time/voltage combinations used do not increase conjugational transposons or facilitate horizontal gene transfer from MR-CNS to MSSA.

KEY WORDS: Electroporation.
Staphylococcus, coagulase-negative.
Staphylococcus aureus, methicillin-resistant.
Transcutaneous electric nerve stimulation.

Introduction

Hospital outbreaks due to methicillin-resistant *Staphylococcus aureus* (MRSA) are a major problem in nososcomial infection and warrant programmes to control dissemination, given the potential of MRSA to produce invasive infections, particularly in vulnerable patients, and its multiple resistance to antibiotics.[1,2] More recently, unlike its nosocomial-acquired (NA) relative, community-acquired MRSA (CA-MRSA) has been documented among healthy individuals without predisposing risk factors.[3] The appearance and spread of CA-MRSA represent a new challenge in medicine and has important clinical implications for treatment of *S. aureus* infection.

Previously, this research group reported that approximately half of the coagulase-negative staphylococci isolated from patients in the intensive care unit (ICU) at Belfast City Hospital showed resistance to methicillin (MR-CNS).[4] This relatively high proportion gives rise to speculation that these organisms may act as potential reservoirs of methicillin-resistance genetic material that can be passed to methicillin-sensitive *S. aureus* (MSSA).

Therefore, the mechanisms of potential horizontal gene transfer of such genetic material from penicillin-binding protein 2a (PBP2a)-positive CNS to MSSA are studied, as transformation of MSSA to MRSA is possible and MR-CNS may thus act as a reservoir of methicillin-resistance genetic determinants that prime the development of antibiotic resistance in sensitive *S. aureus*.

Electroporation is a molecular technique employed commonly to enhance genetic recombination between

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Gram-positive organisms, including staphylococci. Currently, electroporation-based transformation is used, as it is technically easier to perform, competent cells can be stored for a longer period of time, and transformant recovery is faster.[5] Electroporation is a means of inducing horizontal gene transfer which involves exposing bacterial cells to an electrical field (approximately 5–10 kV) that is sufficient to create pores in the cell membrane, allowing insertion of donor DNA into the recipient host cell.

The aim of the present study is to examine the effect of electroporation-type activities employed in clinical medicine, in particular transcutaneous electrical nerve stimulation (TENS). Similar time/voltage combinations to those used in TENS are applied *in vitro* to a population of viable MSSA organisms in the presence of genomic DNA containing methicillin genetic determinants obtained from non-viable MR-CNS, in order to determine whether or not TENS promotes genetic recombination events involving the horizontal transfer of methicillin-resistance genetic material.

Materials and methods

Description of staphylococci

Methicillin-resistant coagulase-negative staphylococci (n=21) were isolated from a variety of clinical specimens (wound and vaginal swabs, pus, urine and sputum). All isolates were confirmed phenotypically by Gram stain and catalase and coagulase reactions with rabbit plasma, as described previously.[2] Isolates were identified to species level by the BD BBL Crystal GP identification scheme (Becton Dickinson, Maryland, USA), following the manufacturer's instructions.

All isolates were screened for oxacillin resistance by standard NCCLS disc-diffusion assays. Presence of PBP2a was confirmed by the Mastalex latex agglutination assay kit (Mast Diagnostics, Merseyside, UK), which has been demonstrated as suitable for the detection of PBP2a in CNS.[6]

Methicillin-sensitive *S. aureus* (n=20) were isolated from clinical specimens, as above, and were characterised, as above, where they were shown to be coagulase-positive and not to express the PBP2a protein on latex agglutination testing. All isolates were screened for oxacillin susceptibility by standard NCCLS disc-diffusion assays, as well as by E-test against oxacillin (AB Biodisk, Solna, Sweden).

Electroporation

All MR-CNS and MSSA isolates were cultured on Colombia agar base (CM331, Oxoid, Basingstoke, England), supplemented with defibrinated horse blood (5% [v/v], Oxoid), and incubated at 37°C for 24 h. Genomic DNA from each MR-CNS isolate was prepared by harvesting a 20 µL loop of cells (approximately 10^{11} colony-forming units [cfus]) in 11 mL of a combined 1xTAE buffer solution (40 mmol/L Tris, 20 mmol/L acetic acid, 1 mmol/L EDTA), followed by boiling for 15 min.

After cooling, 1mL of the boiled suspension containing genomic DNA from all 21 isolates of MR-CNS was placed in nutrient broth (10 mL, Oxoid CM1) and incubated at 37°C for 24 h before subculturing on Colombia agar base (CM331, Oxoid), supplemented with defibrinated horse blood (5% [v/v], Oxoid). This was incubated at 37°C for a further 24 h to check for lethal destruction of viable MR-CNS cells in the suspension.

An MSSA suspension of viable cells from 21 confirmed isolates was prepared as above but omitting the boiling stage. The electroporation cell used a 17 cm horizontal electrophoresis tank that had been sterilised in absolute alcohol. To this, sterile, chilled (0°C) 1xTAE buffer was added (80 mL), together with genomic DNA from heat-killed MR-CNS (10 mL) and viable MSSA (10 mL), and mixed thoroughly. A time/voltage combination of 200 V (DC) for 25 min was applied across the cell, and the temperature of the cell was not allowed to exceed 37°C.

Previous experimental work was carried to demonstrate that the TAE/time/voltage combination was not lethal to the viable MSSA organisms present in the cell. A control experiment was set up excluding application of voltage to observe any spontaneous genetic recombination event(s) from the MSSA to the MRSA phenotype.

Post-electroporation recovery of viable staphylococci

Following electroporation, the entire buffer suspension (100 mL) was centrifuged at 3000 x***g*** for 5 min, and the supernatant discarded. The pellet was resuspended in Nutrient Broth No.2 (10 mL, Oxoid CM0067) and incubated for 24 h at 37°C.

Determination of the presence of MRSA

Following non-selective recovery, cells were harvested by centrifugation, as detailed above, and then selective MRSA broth (3 mL; MastaScreen MRSA kit, Mast Diagnostics, Merseyside, UK) was added and incubated at 37°C for 24 h. Following incubation, a colour change from red to yellow indicated the presence of MRSA, following which all presumptive positives were confirmed phenotypically by inoculation on nutrient agar (Oxoid) containing 5% (w/v) sodium chloride, with an oxacillin disc (1 µg) to observe susceptibility. A control solution was subjected to the same enrichment procedure and the complete experiment was replicated twice.

Pretreatment of MSSA with sublethal concentration of oxacillin

The above experiment was repeated using MSSA that had been pre-exposed to a sublethal concentration of oxacillin, prior to electroporation with genomic DNA from MR-CNS. Briefly, minimum inhibitory concentrations (MICs) of all 20 MSSA isolates were determined by E-test with oxacillin (AB Bio, Sweden). Isolates (n=20) were inoculated in Nutrient Broth No. 2 (10 mL, Oxoid) containing 0.25 µg/mL oxacillin (Sigma, St. Louis, USA) and incubated overnight at 37°C. Cells were harvested by centrifugation at 3000 x***g*** and the supernatant discarded. The pellet was resuspended in 1xTAE buffer (10 mL), and electroporation was performed, as described above.

Results

This study employed genomic DNA from 21 isolates of MR-CNS, including *S. epidermidis* (n=11), *S. haemolyticus* (n=7), *S. hominus* (n=1), *S. lugdunensis* (n=1) and *S. simulans* (n=1). Viable MSSA (n=20) isolates were employed with MICs to oxacillin in the range 0.5–1.0 µg/mL (MIC_{50}=0.58). Following

preparation of genomic DNA from the MR-CNS isolates, no viable MR-CNS organisms were detected prior to introduction to the electrophoresis tank.

Calibration experiments demonstrated that the temperature of the liquid phase in the tank at a constant 200 V did not exceed 37°C in 25 min, which mimicked a typical TENS time/voltage application. In addition, it was demonstrated that electroporation at 200 V for 25 min in 1xTAE was not lethal to viable MSSA cells.

Following electroporation with the viable MSSA cells and the genomic DNA from the MR-CNS isolates, the presence of an MRSA organism was not demonstrated, as all *S. aureus* remained susceptible to oxacillin and they did not change their susceptibility phenotype to express PBP2a. Likewise, no MRSA were detected after MSSA had been pre-exposed to oxacillin, prior to electroporation with genomic DNA from MR-CNS. No MRSA were demonstrated in the experimental controls.

Discussion

Currently, many therapeutic devices are used in electrical stimulation to aid pain relief or in other therapeutic modalities (e.g., electrical stimulation of heart muscle during cardiac arrest). Transcutaneous electrical nerve stimulation is a battery-operated electronic device used for pain relief. Adhesive electrodes are placed on the skin around the area of pain and an electrical impulse is transmitted to block the pain signals sent to the brain. There are many TENS units on the market and these are used widely to help reduce pain or to keep it under control.

Transcutaneous electrical nerve stimulation is used in conditions such as arthritis, lumbago, post-operative pain, sports injuries and other muscular pain, including that associated with labour. In addition, cosmetic devices now use some form of electrical impulse (e.g., muscle-stimulating devices designed to tone and tighten muscles).

With such wide application, it is important to establish that the electrical impulses from this equipment does not encourage transformation of antibiotic-resistance genetic determinants, from MR-CNS co-residing with MSSA, on the skin surface. Therefore, this study attempted to mimic the time/voltage conditions employed in the clinical delivery of TENS applications.

In staphylococci, methicillin resistance is under genetic regulation and is encoded by the *mec*A gene[7] and through expression of PBP2a.[8] This gene locus is located on a mobile genetic element called the staphylococcal cassette chromosome (SCC) in *S. aureus*, which may range in size from 21 to 67 kb, and is integrated in the MRSA chromosome at a unique site (*attBscc*).[8] The SCCmec elements are classified according to the type of recombinase carried and, at present, five subtypes have been defined, designated as SCCmec types I–V.

Hanssen *et al*.[8] suggest that *mec*A-positive CNS may be a potential reservoir for these elements and that SCC may be transferred between staphylococci. Other evidence exists to support this concept of horizontal gene transfer between staphylococci (Table 1). Therefore, it is important to have an understanding of conditions and events that promote such horizontal gene transfer between staphylococci, in order to estimate the frequency of occurrence of such recombination events and the potential *de novo* generation of MRSA strains.

The findings presented here suggest that, under the conditions described, there is no evidence of horizontal transfer of methicillin-resistance genetic material from MR-CNS to MSSA. These data support the view that use of TENS at typical voltage combinations does not act as a mechanism to promote conjugational transposons and horizontal gene transfer of methicillin-resistance genetic material from MR-CNS to MSSA. □

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Table 1. Reports of horizontal gene transfer between staphylococci.

Description	Reference
*mec*A-positive coagulase-negative staphylococci may be a potential reservoir for genetic elements	8
Mupirocin (*mup*A) gene transfer from *S. epidermidis* to MRSA	9
Horizontal gene transfer of SaPIbov2, a mobile staphylococcal pathogenicity island agent among several species of staphylococci	10
Horizontal gene transfer of antibiotic-induced virulence factors	11
Genetic transfer between the staphylococci appears to have shaped their virulence and resistance profiles	12
Horizontal gene transfer between *mec*A-resistant, coagulase-negative staphylococci and MRSA	7
Horizontal gene transfer responsible for dissemination of antibiotic resistance determinants	13
Horizontal gene transfer of methicillin resistance between a methicillin-resistant, coagulase-negative *Staphylococcus* sp. and a methicillin-susceptible *S. aureus*, thereby generating an MRSA in a male neonate	14

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