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Major article

Use of a predictive protocol to measure the antimicrobial resistance risks associated with biocidal product usage

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Background: In this study we assessed the propensity of biocide exposure in the development of antimicrobial resistance in bacteria.

Methods: Our protocol is based on reporting changes in established antimicrobial susceptibility profiles in biocides and antibiotics after during use exposure to a product. The during use exposure reflects worse conditions of product use during application. It differs from the term low concentration, which usually reflects a concentration below the minimal inhibitory concentration, but not necessarily a concentration that occurs in practice.

Results: Our results showed that exposure to triclosan (0.0004%) was associated with a high risk of developing resistance and cross-resistance in *Staphylococcus aureus* and *Escherichia coli*. This was not observed with exposure to chlorhexidine (0.00005%) or a hydrogen peroxide–based biocidal product (in during use conditions). Interestingly, exposure to a low concentration of hydrogen peroxide (0.001%) carried a risk of emerging resistance to antibiotics if the presence of the oxidizing agent was maintained. We observed a number of unstable clinical resistances to antibiotics after exposure to the cationic biocide and oxidizing agent, notably to tobramycin and ticarcillin–clavulanic acid.

Conclusions: Using a decision tree based on the change in antimicrobial susceptibility test results, we were able to provide information on the effect of biocide exposure on the development of bacterial resistance to antimicrobials. Such information should address the call from the U.S. Food and Drug Administration and European Union Biocidal Products Regulation for manufacturers to provide information on antimicrobial resistance and cross-resistance in bacteria after the use of their product.

In January 2013, the U.S. Food and Drug Administration proposed a rule to determine the safety and effectiveness of antibacterial soap (http://www.fda.gov/newsevents/newsroom/pressannouncements/ucm378542.htm), whereby manufacturers of antibacterial hand soaps and body washes need to demonstrate that their products are safe for long-term daily use. This rule is based on the concern that long-term exposure to certain active ingredients, such as triclosan (TRI), may be associated with bacterial resistance and therefore pose a health risk.[1,2] This proposed rule echoes the European Biocidal Product regulation (effective from September 1, 2013; articles 19-b/ii, 37, and 47-1/b), which asks manufacturers to provide information on the antimicrobial resistance associated with their biocidal products.[3] This follows a number of European reports on the association of biocides with antimicrobial resistance.[4,5] Because of the increased use of biocidal products worldwide for a mounting number of applications, particularly domiciliary ones (eg, washing up liquid, surfaces, stationary, textiles), it is not surprising that biocidal products used at a low concentration, for example after dilution, or released in the environment at low concentrations, produce a selective pressure for bacteria to express resistance mechanisms.[1,2,4,6-9] In 2010, the European Scientific Committee on Emerging and Newly Identified Health Risks reported on the dearth of information concerning biocide exposure on the development of antimicrobial resistance in bacteria[5] and in particular the need for a standard protocol that could measure the ability of a biocide to induce or select for antimicrobial resistance in bacteria. Recently, a protocol reflective of the in use conditions of biocides was proposed.[7] Knapp et al reported on the use of this protocol to determine the effect of exposure to

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chlorhexidine, benzalkonium chloride, and 3 biocidal products to *Pseudomonas aeruginosa*, *Burkholderia cepacia*, *B lata*, *Klebsiella pneumoniae*, and 2 *Salmonella enterica* serovar Typhimurium strains.[10,11]

Bacterial resistance to cationic agents (eg, biguanides, quaternary ammonium compounds) and phenolics (eg, TRI) has been widely reported[2,4,6,7,9,11-14] and is often perceived to present a higher risk for the development of bacterial resistance to antimicrobials. A number of resistance mechanisms to these biocides have been described, including overexpression of efflux and changes in bacterial surface.[7,15] Bacterial resistance to highly reactive biocides such as alkylating and oxidizing agents has also been reported.[6,16,17] An outbreak of *Mycobacterium massiliense* in particular showed for the first time a clinical isolate, with resistance to glutaraldehyde and all the frontline antimycobacterial antimicrobials, causing significant public concern.[17]

In this study we explored the use of a predictive protocol[7] to determine changes in the antimicrobial susceptibility profile of *Staphylococcus aureus* and *Escherichia coli* when exposed to TRI, chlorhexidine gluconate solution (CHG), hydrogen peroxide, and a hydrogen peroxide–based product.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and storage of cultures

One representative gram-positive and 1 gram-negative bacteria were selected for testing against 1 formulated biocidal product and 3 biocides. The bacterial strains chosen were *S aureus* (NCIMB 9518) and *E coli* (NCIMB 8545).[18,19] Both bacteria are commonly used in standard efficacy test protocols. Liquid cultures of all strains were grown in tryptone soya broth (TSB) (Oxoid, Basingstoke, UK) at 37°C ± 1°C for 16-24 hours. Strains were stored on protect beads (Fisher Scientific, Loughborough, UK) at –80°C ± 1°C and restricted to a maximum of 2 subcultures from the original freezer stock prior to exposure to a given biocide. Test inocula were prepared from harvesting an overnight TSB culture centrifuged at 5,000 g for 10 minutes and resuspended in deionized water (diH20).

Formulations, actives, and neutralizer

A hydrogen peroxide–based foaming lotion for hand disinfection (Oxy BAC F31 RO 1331; DEB Group, Denby, UK) was tested at 1% and 0.001% H_2O_2 (final concentration). Three unformulated biocides, TRI (0.0004% in 5% dimethyl sulfoxide [DMSO]), CHG (0.00005%), and hydrogen peroxide (0.001%), were also used. All biocides were neutralized with 5 g/L sodium thiosulfate. Neutralizer toxicity and efficacy to quench the biocides were tested as described by Knapp et al[10] and confirmed (data not shown).

Antimicrobial susceptibility testing

The protocol to evaluate the effect of biocide exposure on the susceptibility profile and stability of bacterial isolates has been described.[7,11] Briefly, it consists of 3 parts: (1) an initial background antimicrobial susceptibility profile of test bacteria before biocide exposure, (2) exposure of test bacteria to during use concentration of test biocide or biocidal products, and (3) determination of antimicrobial susceptibility profile of biocide-exposed bacteria and stability profile of any change in antimicrobial susceptibility. During use exposure reflects the worst-case scenario during product usage by customers, notably dilution of product and lengthy contact time. It differs from the term low concentration, which usually reflects a concentration below the minimal inhibitory concentration (MIC), but not necessarily a concentration that occurs in practice. The manufacturer guidelines for during use exposure conditions of the biocidal product were used.

Suspension testing and exposure to microbicide

Bacterial exposure to biocides and biocidal products was carried out in suspension using the British Standards Institute suspension test protocol.[18] Briefly, bacterial suspensions in diH20 produced from overnight cultures were standardized to 1×10^8 colony forming units/mL through optical density measurement. Suspensions were used within 15 minutes of preparation. One milliliter of standardized suspension was added to 9 mL of the appropriate concentration of a biocide-product (diluted in diH20) at 1.25 times the required concentration for a 30-second, 5-minute, and 24-hour exposure. Then 1 mL of this suspension was removed and added to 9 mL of neutralizer. After neutralization, suspensions were centrifuged at 5,000 g for 10 minutes, and the supernatant was discarded. The remaining cells were then used in further antimicrobial susceptibility testing experiments. Concentrations of biocide tested were as follows: Oxy BAC F31 RO 1331 1% and 0.001%, unformulated H_2O_2 0.001%, TRI 0.0004%, and CHG 0.00005%. The 1% concentration of the formulated product corresponded to the during use concentration, whereas the lower concentrations for the oxidizing agents and the cationic biocides corresponded to a concentration that resulted in a 1 $\log_{10}$ reduction in colony forming units per milliliter, leaving sufficient survivors for further antimicrobial susceptibility testing.

MIC and minimal bactericidal concentration

The MIC of each biocide was determined before and after biocide exposure with the British Standards Institute protocol.[19] To determine the minimal bactericidal concentration (MBC), 20 µL of suspension was removed from each well of the MIC microtiter plate where no bacterial growth was observed and the 2 lowest biocide concentrations at which growth was observed, and they were plated onto a tryptone soy agar plate containing 10% neutralizer. After 24 hours of incubation at 37°C, the MBC was defined as the lowest biocide concentration where no bacterial growth was observed.[11]

Antibiotic susceptibility testing

The susceptibility of both bacteria to the following antibiotics was determined before and after biocide exposure using the European Committee on Antimicrobial Susceptibility Testing disk diffusion protocol:[20] ampicillin (10 µg), ciprofloxacin (1 µg), ceftazidime (30 µg), tobramycin (10 µg), ticarcillin–clavulanic acid (75:10 µg), and gentamicin (10 µg). These antibiotics were selected because of their use as therapeutic agents in the treatment of infections with the organisms chosen for this study.

Phenotype stability testing

The stability of observed changes in antimicrobial susceptibility profile was investigated by 24 hours subculturing of surviving bacteria in TSB with or without a biocide; the exposure concentrations previously described were used.[11] Changes in the antimicrobial susceptibility profile were measured using the protocol previously described following 1, 5, and 10 subcultures. A check of culture purity was performed at each stage.

Reproducibility

Tests were carried out in triplicate on 3 separate occasions. No statistical analysis was conducted on antibiotic breakpoints because only the clinical resistance breakpoint given by European Committee on Antimicrobial Susceptibility Testing[20] was of interest. Likewise, no statistical analysis was performed on the MIC-MBC data. Here a significant change in the susceptibility profile corresponding to