Journal of Global Antimicrobial Resistance 1 (2013) 195–199

Contents lists available at SciVerse ScienceDirect

Journal of Global Antimicrobial Resistance

journal homepage: www.elsevier.com/locate/jgar

Activity of the antiseptic polyhexanide against meticillin-susceptible and meticillin-resistant *Staphylococcus aureus*

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ARTICLE INFO

Article history:
Received 26 February 2013
Received in revised form 3 May 2013
Accepted 8 May 2013

Keywords:
Polyhexanide
Antiseptic
Minimum inhibitory concentration
Minimum bactericidal concentration
Staphylococcus aureus
Meticillin-resistant *Staphylococcus aureus*

ABSTRACT

Staphylococcus aureus is one of the most important pathogens, with increasing emergence of meticillin-resistant *S. aureus* (MRSA) strains. This is associated not only with multiresistance to antibiotics but also with increasing resistance to topical antibiotics and antiseptics. As the antiseptic polyhexanide has only a low risk of emergence of resistant strains, the aim of the study was to obtain data on the sensitivity of *S. aureus* towards polyhexanide. The effect of polyhexanide was tested against 80 meticillin-susceptible *S. aureus* (MSSA) and 80 MRSA strains from sporadic cases as well as against 6 MRSA outbreak strains. The clonal diversity of the 166 strains was proven by pulsed-field gel electrophoresis (PFGE). Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) were determined by the serial broth microdilution technique according to DIN 58940. Time–kill studies were performed for reference strains MSSA ATCC 29213 and MRSA ATCC 33591. MICs and MBCs in the range of 0.5–2 mg/L were found. According to a created epidemiological cut-off (ECOFF) value of 4 mg/L, all strains were regarded as susceptible to polyhexanide, including MRSA epidemic strains and MSSA and MRSA sporadic strains with various antibiotic susceptibility patterns. Addition of up to 4% albumin to the test medium did not change the MICs and MBCs. Time–kill studies showed reduction rates of 4 $\log_{10}$ CFU/mL for 200 mg/L and 5 $\log_{10}$ CFU/mL for 400 mg/L polyhexanide within 5–30 min. It is concluded that polyhexanide is suitable for topical eradication of *S. aureus*.

1. Introduction

Staphylococcus aureus has been recognised as a pathogen of great importance for a long time. Patients colonised by *S. aureus* have a potential source of bacteraemia and severe systemic infections [1]. The emergence of meticillin-resistant *S. aureus* (MRSA) strains is also associated with multiresistance to antibiotics [2]. Some strains also showed resistance to topical antibiotics such as mupirocin [3–5] or reduced susceptibility to antiseptics such as chlorhexidine [6] or triclosan [7]. This underlines the need for investigations of other reliable and effective topical eradication agents.

In vitro investigations and animal studies have shown the toxicological safety and tissue compatibility of fractionated polyhexamethylene biguanide (polyhexanide) and polyethylene glycol 4000 (Lavasept®, Lavasorb®, Lavanid®) [8,9]. In tests of selected strains, polyhexanide showed activity against bacteria including *S. aureus* [10–12]. In a prospective, randomised controlled, double-blind study, it led to a significant reduction in micro-organisms on soft-tissue wound surfaces; this also included wounds colonised by *S. aureus* [13].

To obtain more detailed information about the activity of polyhexanide against *S. aureus*, 160 isolates from sporadic cases and 6 epidemic strains were tested quantitatively. The susceptibility of the strains to various antibiotic agents was tested to assess a possible correlation between polyhexanide activity and particular antibiotic resistance patterns. All strains were typed by pulsed-field gel electrophoresis (PFGE) to prevent testing of multi-copy strains. These data allow the determination of whether polyhexanide is suitable for topical eradication of *S. aureus*, in particular MRSA.

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2213-7165/$ – see front matter
http://dx.doi.org/10.1016/j.jgar.2013.05.007

2. Materials and methods

2.1. Strains

In total, 160 clonally different isolates [80 each of MRSA and meticillin-susceptible *S. aureus* (MSSA)] were collected consecutively for testing. They derived from different infected patients of the university hospitals of Essen and Rostock and synlab laboratories Kassel (Germany). In addition, the epidemic MRSA strains RKI 134/93, 635/93, 994/93, 1000/93, 1150/93 and 131/98 (Robert-Koch-Institut, Wernigerode, Germany) were tested.

The strains were identified by standard techniques. Isolates with a clinical background or resistant to fusidic acid were tested for expression of the Panton–Valentine leukocidin (PVL) by real-time PCR targeting the *lukS-PV* gene [14]. All strains were stored at −70 °C using the Protect system (Technical Service Consultants Ltd., Heywood, UK). An aliquot from each isolate was thawed and subcultured on sheep blood agar (Oxoid, Wesel, Germany) before testing.

2.2. Pulsed-field gel electrophoresis typing

PFGE was carried out with a modification of the method of Sambrook et al. [15] using a Gene Navigator System apparatus (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) as described below. DNA was digested with *Sma*I (Boehringer Mannheim, Mannheim, Germany). Separation of fragments was performed for 24 h in a 1% low-melting-point agarose gel and a 0.05 M Tris–borate–ethylene diamine tetra-acetic acid (TBE) buffer (pH 8.5) at 10 °C and 180–185 V with a pulse time of 0.5–40 s. Gels were stained with ethidium bromide, washed with electrophoresis buffer and photographed under ultraviolet light. Lambda DNA concatemers (New England BioLabs, Ipswich, MA) were used as molecular size standards. PFGE patterns were interpreted according to Tenover et al. [16]. Isolates were considered as genetically different if the patterns differed by three or more bands.

2.3. Determination of the activity of polyhexanide

Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) were determined by serial broth microdilution testing according to Deutsches Institut für Normung (DIN) [17,18] using Mueller–Hinton broth (MHB) (E. Merck, Darmstadt, Germany) supplemented with $CaCl_2$ (final Ca^{2+} concentration 50 mg/L) and $MgCl_2$ (final Mg^{2+} concentration 25 mg/L). Polyhexanide was obtained from Fresenius Kabi AG (Bad Homburg, Germany). The stock solution contained 200 mg polyhexanide and 10 mg polyethylene glycol (polyethylene glycol 4000) per millilitre.

The stock solution was diluted in the test broth by serial doubling dilution to final concentrations of 0.06–128 mg/L polyhexanide. Furthermore, five MSSA and fie MRSA were tested in broth supplemented with 0.2% and 4% bovine serum albumin (BSA) (Sigma–Aldrich, St. Louis, MO). In these experiments, the final concentrations of polyhexanide were 0.06–128 mg/L. The final inoculum of the strains was 5×10^5 CFU/mL.

The MIC was read after 24 h of incubation at 35 °C as the lowest concentration of antiseptic preventing visible growth. For the determination of MBCs, a 10 μL aliquot from wells near the threshold for turbidity of the MIC plate was transferred onto blood agar plates in duplicate. Incubation was performed for 6 h at 35 °C. The MBC was defined as the lowest antiseptic concentration preventing visible re-growth. In addition, the MBCs of five MRSA and five MSSA each were determined after incubation of the MIC plates for 3, 24 and 48 h.

The MRSA type strain ATCC 33591 and the MSSA type strains ATCC 25923 and ATCC 29213 were tested repeatedly as quality controls.

2.4. Time–kill studies

Killing rates of polyhexanide against the reference strains MSSA ATCC 29213 and MRSA ATCC 33591 using supplemented MHB were determined in duplicate, so values represent the mean of two experiments. Stock solutions were diluted in Ca- and Mg-supplemented MHB with addition of 3% Tween 80, 0.1% cysteine, 0.1% histidine and 3% saponin to inactivate polyhexanide, thereby preventing a lasting effect of antiseptic carry-over.

According to the concentration in therapeutic preparations, the final concentration of polyhexanide was 400 mg/L and 200 mg/L. The final inoculum of strains was ca. 10^8 CFU/mL. Briefly, 100 μL of the bacterial suspensions was transferred to 9.9 mL of MHB (1:100); from this, 1 mL was transferred to 9 mL of MHB (1:1000); and from this, 1 mL was transferred to 9 mL of MHB (1:10,000) after 1, 3, 5, 10, 15 and 30 min and 1, 2, 6, 24 and 48 h exposure time. From each dilution, 100 μL was transferred to plates with Mueller–Hinton agar (E. Merck). After 24 h of incubation at 35 °C, plates containing 30–300 colonies were counted and the CFU/mL was calculated.

2.5. Antibiotic susceptibility testing

Susceptibility of the strains to oxacillin, penicillin G, ampicillin, cefazolin, imipenem, gentamicin, trimethoprim/sulfamethoxazole, tetracycline, levofloxacin, clindamycin, erythromycin, rifampicin, fosfomycin, fusidic acid, tigecycline, teicoplanin, vancomycin, daptomycin, quinupristin/dalfopristin, linezolid and mupirocin was determined by the VITEK® 2 System (bioMérieux, Hazelwood, MO). In the presence of resistance to penicillin G, ampicillin was also regarded as resistant. Resistance to oxacillin was regarded as resistance to all tested β-lactams.

2.6. Statistical analysis

The influence of resistance to antibiotics on the MICs and MBCs of polyhexanide was tested by Kruskal–Wallis exact test (SPSS v. 19.0; SPSS Inc., Chicago, IL) for 80 MRSA with 21 antibiotic resistance patterns and 80 MSSA with 15 antibiotic resistance patterns.

3. Results

The PFGE profiles of the 166 strains exhibited differences in three or more bands (data not shown). Thus, it was ascertained that a broad range of genetically different isolates was investigated, and a bias by testing isolates of the same clone was excluded.

As a median of 13 determinations, the type strains MSSA ATCC 25923 (PVL-expressing) and ATCC 29213 had MICs and MBCs of 1 mg/L, and the type strain MRSA ATCC 33591 had an MIC and MBC of 2 mg/L. The maximum deviation between tests was one dilution step. The activity of polyhexanide against sporadic MSSA and MRSA as well as against epidemic MRSA, which had caused several outbreaks, ranged between 0.5 mg/L and 2 mg/L (Table 1). The distributions of the MICs and MBCs are shown in Figs. 1 and 2.

According to the frequency of MICs and MBCs, an epidemiological cut-off (ECOFF) concentration of 4 mg/L was regarded as the lowest possible value for a clinical breakpoint. All isolates were regarded as susceptible to polyhexanide.

Of the 80 MSSA, 67 (84%) were resistant to penicillin G. Considering the other antibiotics tested, 15 different patterns of resistance were found (Table 2). However, no link between

Table 1
Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of polyhexanide against *S. aureus*.

Strain	*n*	MIC (mg/L)			MBC (mg/L)		
		Range	MIC_{50}	MIC_{90}	Range	MBC_{50}	MBC_{90}
MSSA, sporadic	80	0.5–2	1	1	0.5–2	1	2
MRSA, sporadic	80	0.5–2	1	2	0.5–2	1	2
MRSA, epidemic	6	0.5–2	1	2	1–2	1	2

$MIC_{50/90}$, MIC reached by 50% and 90% of the strains, respectively; $MBC_{50/90}$, MBC reached by 50% and 90% of the strains, respectively; MSSA, meticillin-susceptible *S. aureus*; MRSA, meticillin-resistant *S. aureus*.

resistance to antibiotics and the MICs and MBCs of polyhexanide was observed (Kruskal–Wallis test; for MIC, $\chi^2 = 9.89$, df = 14, $P = 0.895$, for MBC, $\chi^2 = 13.47$, df = 14, $P = 0.533$). In the 80 MRSA, 21 different patterns were found; 66 strains (83%) were resistant to gentamicin. Three MRSA strains exhibited mupirocin resistance, one of them high-level resistance (MIC $\geq$ 512 mg/L). One fusidic acid-intermediate MRSA expressed the PVL. No resistance was found to teicoplanin, vancomycin, tigecycline, daptomycin, quinupristin/dalfopristin and linezolid. As with MSSA, the resistance patterns of MRSA (Table 3) did not influence the MICs and MBCs of polyhexanide (Kruskal–Wallis test; for MIC, $\chi^2 = 9.43$, df = 20, $P = 0.991$, for MBC, $\chi^2 = 12.39$, df = 20, $P = 0.956$). In inactivation experiments with 10 strains, BSA did not influence the MICs and MBCs of polyhexanide.

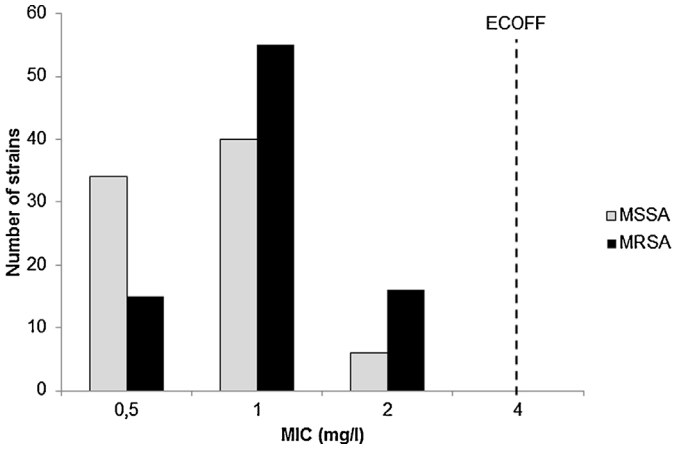

Fig. 1. Minimum inhibitory concentration (MIC) distribution of meticillin-susceptible *S. aureus* (MSSA) and meticillin-resistant *S. aureus* (MRSA) (sporadic and epidemic). ECOFF, epidemiological cut-off.

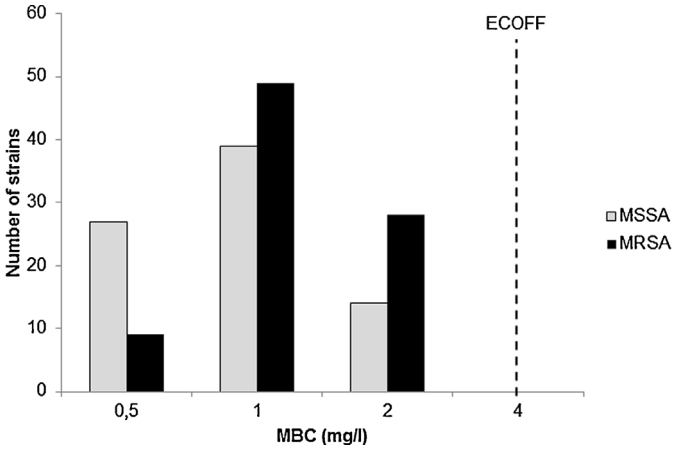

Fig. 2. Minimum bactericidal concentration (MBC) distribution of meticillin-susceptible *S. aureus* (MSSA) and meticillin-resistant *S. aureus* (MRSA) (sporadic and epidemic). ECOFF, epidemiological cut-off.

Table 2
Resistance patterns of meticillin-susceptible *S. aureus* (MSSA) strains (*n* = 80).

Agents to which the strain is resistant or intermediately susceptible (I)	No. of strains
PNG[a]	39
PNG[a], TET	14
PNG[a], ERY	9
No resistance	4
ERY	3
TET	2
PNG[a], IPM, LFX, SXT, TET	1
PNG[a], SXT, TET, CLI, ERY	1
GEN, CLI, ERY, LFX	1
PNG[a], GEN	1
PNG[a], CLI, ERY	1
PNG[a], SXT (I)	1
GEN, SXT	1
TET, ERY	1
TET, ERY (I)	1
Total	80

PNG, penicillin G; TET, tetracycline; ERY, erythromycin; IPM, imipenem; LFX, levofloxacin; SXT, trimethoprim/sulfamethoxazole; CLI, clindamycin; GEN, gentamicin.
[a] Strains resistant to penicillin G were also resistant to ampicillin.

In 10 strains, the MBC was determined after 3, 24 and 48 h of exposure to polyhexanide. There was no change of MBCs in comparison with the exposure of 6 h that was performed in the standardised MBC determinations.

The killing curves of polyhexanide in the first hour are shown in Fig. 3 for MSSA ATCC 29213 and in Fig. 4 for MRSA ATCC 33591. MSSA and MRSA showed similar CFU reduction rates. The reduction rate of 400 mg/L polyhexanide was slightly higher in the first hour. The detection limit was reached after 60 min with 400 mg/L polyhexanide and before 2 h with 200 mg/L polyhexanide. Within 5–30 min, reduction rates of 4 $\log_{10}$ CFU/mL for

Table 3
Resistance patterns of meticillin-resistant *S. aureus* (MRSA) strains (*n* = 80).

Agents to which the strain is resistant or intermediately susceptible (I)[a]	No. of strains
GEN, LFX, CLI, ERY	27
GEN, SXT, TET, LFX, CLI, ERY	18
GEN, LFX, CLI, ERY	8
LFX	8
GEN, TET, LFX, CLI, ERY	3
GEN, SXT, TET, LFX, CLI, ERY, RIF	1
GEN, SXT, TET, LFX, CLI, ERY, MUP (hl)	1
GEN, SXT, TET, LFX, CLI, ERY, MUP	1
GEN, SXT, TET, LFX, CLI, ERY	1
GEN, SXT, LFX, CLI, ERY, FOS	1
GEN, SXT, LFX, CLI, ERY, MUP	1
GEN, SXT, LFX, CLI, ERY	1
GEN, SXT, TET, LFX	1
SXT (I), TET, LFX, CLI, ERY	1
GEN, TET (I), LFX, CLI, ERY	1
GEN, LFX	1
SXT, CLI, ERY, FUS (I)	1
LFX, CLI, ERY	1
TET (I), CLI, ERY	1
TET, ERY	1
ERY	1
Total	80

GEN, gentamicin; LFX, levofloxacin; CLI, clindamycin; ERY, erythromycin; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline; RIF, rifampicin; MUP, mupirocin; hl, high-level resistance; FOS, fosfomycin; FUS, fusidic acid.
[a] All strains were also resistant to oxacillin, penicillin G, ampicillin, cefazolin and imipenem.

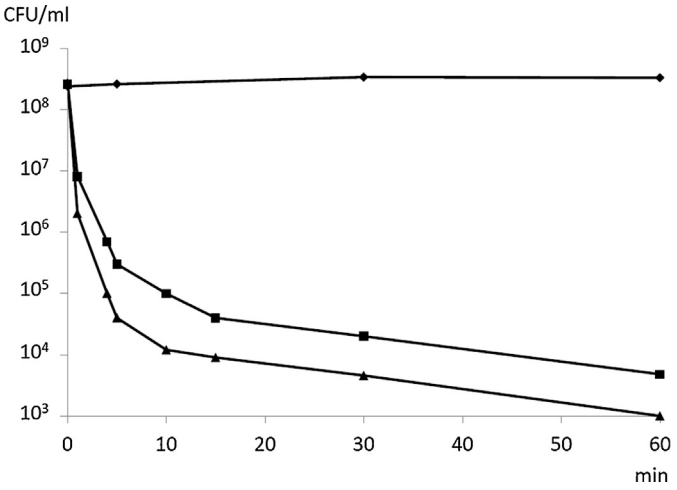

Fig. 3. Killing curves of polyhexanide against meticillin-susceptible *S. aureus* (MSSA) ATCC 29213. ◆, control; ■, 200 mg/L polyhexanide; ▲, 400 mg/L polyhexanide.

200 mg/L polyhexanide and 5 $\log_{10}$ CFU/mL for 400 mg/L polyhexanide occurred. No re-growth was observed after a longer time of exposure.

4. Discussion

Biguanides of the polyhexamethylene biguanide hydrochloride type were originally developed as surface disinfectants in the food and beverages industry. The antimicrobial action is based on the binding of the cationic molecules to the anionically charged bacteria, leading to membrane rupture and denaturation of proteins [19]. Therapeutic polyhexanide preparations (Lavasept®, Lavasorb®, Lavanid®) also contain polyethylene glycol as a surfactant.

According to the manufacturer, in therapeutic preparations the concentrations of polyhexanide (e.g. solutions in 0.9% NaCl or gels with hydroxyethyl cellulose) should be 0.1–0.2% of the stock solution. This corresponds to final concentrations of 200–400 mg/L polyhexanide.

In the present study, the MICs and MBCs of polyhexanide for MRSA and MSSA, determined with a standardised susceptibility test, did not exceed 2 mg/L, so an ECOFF of 4 mg/L was created.

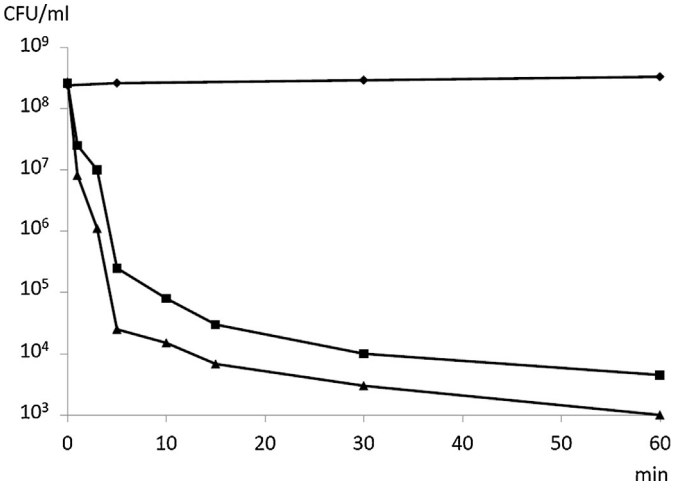

Fig. 4. Killing curves of polyhexanide against meticillin-resistant *S. aureus* (MRSA) ATCC 33591. ◆, control; ■, 200 mg/L polyhexanide; ▲, 400 mg/L polyhexanide.

A possible occurrence of reduced susceptibility should not be linked with a failure to eradicate *S. aureus*. If changes in the wild-type population lead to higher MBCs, they still might be lower than the applied concentrations in clinical use. Therapeutic preparations possess concentrations in the order of 100 times higher than needed for bactericidal action against *S. aureus*. Of course, one might argue that administered as a wound rinse the local concentration of the polyhexanide could be much lower, so that a higher concentrated solution is needed for effective and antiseptic wound rinsing. However, the presence of albumin does not change the activity of the antiseptic.

The bactericidal action of 0.5–2 mg/L polyhexanide against *S. aureus* is effective in 3 h and was not increased by further exposure, as there was no significant difference in MBCs determined after exposure of 3, 24 or 48 h.

The findings of the time–kill studies for MSSA ATCC 29213 (Fig. 3) and MRSA ATCC 33591 (Fig. 4) showed similar results to previously published data. Using therapeutic concentrations, reduction rates of 4–5 $\log_{10}$ CFU/mL within 5–30 min were determined. This is a little more rapid than observed by Werner [10] for the MSSA reference strain ATCC 6538.

As observed in the current experiments, polyhexanide showed a similar activity against MRSA and MSSA, which is interesting as no in vitro studies have been performed for a larger number of MRSA strains to our knowledge. Statistical analysis proved the absence of correlation between MICs and MBCs to polyhexanide and antibiotic resistance. In contrast to the current findings, a good correlation between chlorhexidine and antibiotic susceptibility in both MIC and MBC among Gram-negative bacteria, and mainly in MBC among Gram-positive bacteria, was detected [20].

When a topical antibiotic is applied repeatedly over several days, it acts at various local concentrations for different exposure times, which enhances the possibility of resistance development [21]. In vitro studies showed that the development of resistance of *S. aureus* to mupirocin could be induced relatively easily in contrast to antiseptics such as polyhexanide [22]. But for chlorhexidine the development of reduced susceptibility in staphylococci could be noted [6]. Nevertheless, no MRSA strains with reduced susceptibility to polyhexanide were found in this study, although it is chemically related to chlorhexidine. In fact, successful attempts at MRSA eradication were reported [23]. However, the inhibition of polyhexanide action by mucin was demonstrated in previous investigations, so that clinical use of polyhexanide for eradication of *S. aureus* in the nasal vestibule does not appear promising [24,25]. In this study, expression of PVL toxin did not affect the action of polyhexanide.

In conclusion, the tissue compatibility and antibacterial activity of polyhexanide as well as the low risk of emergence of strains with reduced susceptibility suggest the antiseptic to be suitable for the treatment of wound infections and skin colonisation with *S. aureus*, including MRSA. Since, in addition to the hospital environment, MRSA strains are increasingly found in the community setting, efficient and safe eradication of such strains becomes even more important.

Funding

None.

Competing interests

None declared.

Ethical approval

Not required.

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