The Journal of Antibiotics (2012) 65, 631–633

www.nature.com/ja

NOTE

Role of EDTA and CSE1034 in curli formation and biofilm eradication of *Klebsiella pneumoniae*: a comparison with other drugs

Manu Chaudhary and Anurag Payasi

The Journal of Antibiotics (2012) **65,** 631–633; doi:10.1038/ja.2012.82; published online 24 October 2012

Keywords: biofilm; CSE1034; extended spectrum β-lactamases; *Klebsiella pneumoniae*; minimum inhibitory concentration

A wide variety of microbial infections in the body (~80%) are caused by bacterial biofilm,[1,2] which are hardly treated because of the development of resistance against antimicrobial agents through various mechanisms.[3] *Klebsiella pneumoniae* is the causative agent of ~14–20% of hospital-acquired urinary tract infections, respiratory tract infections and septicemia.[4] It has been observed that the extended spectrum β-lactamases producing *K. pneumoniae* strain is not susceptible to third-generation cephalosporins viz., ceftizoxime, cefotaxime, ceftriaxone and ceftazidime etc.[5] Analysis performed in Europe with 5000 *Enterobacteriaceae* isolates showed a high degree of resistance to many antibiotics[6] due to bacterial biofilm formation.[7] It has earlier been reported that curli fibers are a major factor in adhesion to surfaces and biofilm formation in many enterobacteria.[8]

The increasing rate of the biofilm problem and its impact on antibiotic resistance triggered us to think a new means, which could disrupt the biofilm formation by inhibiting bacterial adhesion and curli formation. In view of the above-mentioned antibiotic resistance because of the bacterial biofilm, we introduced a nonantibiotic adjuvant EDTA along with β-lactam and β-lactamase inhibitor, which altogether termed as CSE1034, has the potential to break bacterial biofilms significantly. Therefore, current study is focused on the role of EDTA and CSE1034 in curli formation, bacterial adhesion and biofilm eradication of *K. pneumoniae* and its comparison with other drugs.

For the current study, a total of fifteen clinical isolates of *K. pneumoniae* were obtained from four different medical colleges of North India including Postgraduate Institute of Medical Science, Lucknow, UP., India, Vijayanagara Institute of Medical Sciences, Bariely, UP, India, Aligarh Muslim University, Aligarh, UP, India and Government Medical College and Hospital, Chandigarh, India. The isolates were then tested for extended spectrum β-lactamases production using the screening criteria described by Clinical and Laboratory Standards Institute 2009.[9]

The drugs, meropenem (1 g), imipenem plus cilastatin (500 mg), cefoperazone plus sulbactam (2 g), piperacillin plus tazobactam (4.5 g), amoxycillin plus clavulanic acid (1.2 g) and CSE1034 (ceftriaxone 1 g plus sulbactam 500 mg plus 37 mg EDTA) injections were included in the study. EDTA disodium was used as a nonantibiotic adjuvant to CSE1034 and was tested separately. Susceptibility testing of each drug on all of the isolates was performed on planktonic culture using Clinical and Laboratory Standards Institute guidelines.[9] Curli production was measured using a congo red agar plate as described elsewhere.[10] To assess the effect of EDTA disodium and drugs on curli formation, different concentrations of EDTA including 1.25, 2.5, 4.0, 5.0, 10.0 and 20 mM and half of minimum inhibitory concentration (MIC) of drugs were used. Adhesion study was carried out by taking 150 µl of 10^6 cfu ml^{-1} of *K. pneumoniae* in 10 ml of Mueller-Hinton broth (Himedia, Mumbai, India) containing the same concentration of EDTA and drugs as used in curli study. Following incubation for 4 days at 37 °C, the tubes were washed twice with phosphate-buffered saline (7 mM Na_2HPO_4, 3 mM NaH_2PO_4 and 130 mM NaCl at pH 7.4) to remove nonadherent cells. Adherent bacteria were fixed with 95% (v/v) ethanol and stained with 1% (w/v) crystal violet (Merck, Paris, France) for 5 min. The excess stain was washed with sterile distilled water and then the tubes were air-dried. Bacterial adhesion prevention values represents the lowest concentration of EDTA and drugs at which bacteria failed to adhere and hence stained purple color were not found following staining.

Next, we examined the role of EDTA and drugs on biofilm eradication. *In vitro* biofilm model of five selected clinical isolates was developed using calgary biofilm device as described elsewhere.[11] Minimum biofilm eradication concentration (MBEC) was determined of all of the isolates as reported earlier.[12] To assess the effects of EDTA and drugs on preformed biofilm, the EDTA concentration was the same as mentioned above, however, four times of MIC of different drugs were used in this experiment. Scanning electron

Department of Cell Culture and Molecular Biology, Venus Medicine Research Centre, Baddi, India
Correspondence: Dr M Chaudhary, Department of Cell Culture and Molecular Biology, Venus Medicine Research Centre, Hill Top Industrial Estate, Bhatoli Kalan, Baddi, Himachal Pradesh 173205, India.
E-mail: research@venusremedies.com
Received 31 May 2012; revised 11 September 2012; accepted 13 September 2012; published online 24 October 2012

microscope was also carried out of some of the treated samples of drugs, as well as EDTA alone, using Hitachi S3700-N SEM (Hitachi high Technology, Tokyo, Japan) as described elsewhere.[13]

Our results revealed that all of the strains were extended spectrum β-lactamases positive. MIC values for extended spectrum β-lactamases producing *K. pneumoniae* was 64–256 and 32–128 μg ml^{-1} for meropenem and CSE1034, but rest of the drugs exhibited eight times higher MIC of CSE1034. MIC of EDTA disodium was 2048 μg ml^{-1}. The MBEC value for CSE1034 was four times of its MIC, whereas other comparative drugs exhibited several folds higher MBEC values (Table 1). EDTA is a nonantibiotic component and showed MBEC value of 8192 μg ml^{-1}. These results suggest microorganisms in planktonic cultures and biofilms behave differentially toward antibiotic. The clinical isolates which developed bright red colonies on CRA plates, were found to be curli producer whereas the clinical isolate exhibited mild pink color considered to be non-curli producer. Among all the 15 clinical isolates, only 11 (73%) clinical isolates were found to be curli positive. Further results showed the curli formation was inhibited at 5.0 mM and higher concentration of EDTA disodium. Similarly, when various drugs were evaluated on the curli formation, interestingly, only CSE1034 could inhibit curli formation and gave mild-pink color, whereas rest of the drugs showed red color indicating failure to inhibit curli formation. Adhesion of the bacterial cells onto the glass surface was inhibited with increasing concentration of EDTA and complete adhesion was noted at 5.0 mM and higher concentration of EDTA. Similarly, when various drugs were evaluated on the bacterial adhesion, only CSE1034 was able to prevent bacterial adhesion, this suggest that the biofilm formation can be controlled by preventing the adhesion of bacterial cells onto any surface, as earlier it has been demonstrated that adhesion is the necessary step while biofilm is developed.[14]

Table 1 MICs and MBECs of antibacterial agents for *K. pneumoniae* clinical isolates

Name of drugs	*MIC in (μg ml^{-1})*	*MBEC in (μg ml^{-1})*
Piperacillin plus tazobactam	512–1024	>4096
Amoxycillin plus clavulanic acid	>512	>4096
Meropenem	64–256	2048–4096
Imipenem plus cilastatin	512–1024	2048–4096
CSE1034	32–128	256–512
Cefperazone plus sulbactam	>512	>4096
EDTA disodium	1024–2048	4096–8192

Abbreviations: MBEC, minimum biofilm eradication concentration; MIC, minimum inhibitory concentration.
MICs and MBECs of all antibacterial agents were performed on all of the clinical isolates.

Biofilm formation is dependent on cell-to-cell adhesion and cations, particularly calcium, are thought to have an important role in bonding of polymer molecules in the biofilms, leading the cohesion of the polymer layer.[15] Earlier studies have shown that EDTA at 50 mM is useful in disrupting the biofilm.[16,17] Contrary to this, we noted that EDTA at 10 mM was found to be effective in disruption of bacterial biofilm when used alone. A large number of antibiotics and their combinations are available in the market.[18,19] However, none of these found to be effective in biofilms eradication as evident in this study. Current studies demonstrated that CSE1034 at four times of its MIC effectively eradicates the *K. pneumoniae* biofilm compared with other drugs, including meropenem, imipenem plus cilastatin, cefoperazone plus sulbactam, piperacillin plus tazobactam and amoxycillin plus clavulanic acid.

In this study, we successfully demonstrated through the scanning electron microscopy images that exposure of preformed biofilm for 24 h to 10 mM EDTA disodium caused little disorganization of biofilm, as well as made the cell wall more porous probably by chelating the divalent ions, thus enhancing the entry of drug into the

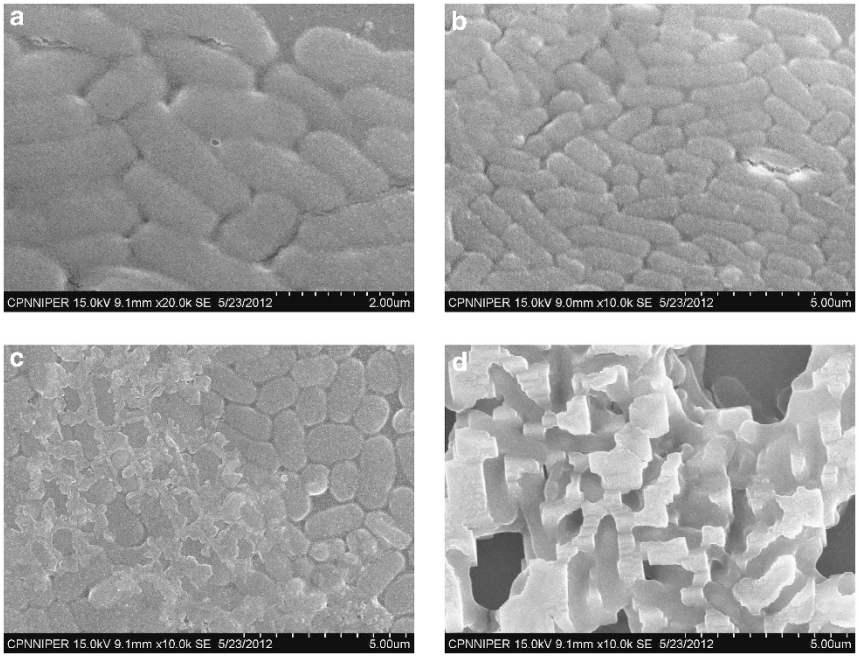

Figure 1 Scanning electron microscopy of *K. pneumoniae* biofilm on the surface of pegs of the microplate lid. (**a**) Control. (**b**) *K. pneumoniae* biofilm exposed to meropenem. (**c**) *K. pneumoniae* biofilm exposed to EDTA (little disorganization of the biofilm appeared). (**d**) *K. pneumoniae* biofilm exposed to CSE1034 (significant disorganization of biofilm; a honeycomb like structure of disrupted biofilm).

bacterial cells; CSE1034 treatment led to significant disorganization of biofilm and formed a honeycomb like structure of disrupted biofilm, whereas meropenem failed to break bacterial biofilm as observed by scanning electron microscope analysis (Figure 1). Other drugs including imipenem plus cilastatin, cefoperazone plus sulbactam, piperacillin plus tazobactam and amoxycillin plus clavulanic acid were not evaluated by scanning electron microscope because of very high MIC and MBEC values.

Our result clearly demonstrates that EDTA as a nonantibiotic adjuvant effectively inhibits curli formation and bacterial adhesion at $\geqslant 5$ mM concentration when used alone and also reacts with divalent ions of extracellular polysaccharides and bacterial cells thus damaging the microbial biofilm. When the same concentration of EDTA is incorporated in CSE1034, the antibacterial activity of CSE1034 is enhanced synergistically and hence it can be concluded that CSE1034 shows good antibacterial activity and can be considered of choice in biofilm infections caused by *K. pneumoniae*.

ACKNOWLEDGEMENTS

We are thankful to the sponsor, Venus Pharma GmbH, AM Bahnhof 1-3, D-59368, Werne, Germany, for providing financial assistance to carry out this study. We also thank to all institutes which provided strains.

1 Banin, E., Brady, M. K. & Greenberg, E. P. Chelator-induced dispersal and killing of Pseudomonas aeruginosa cells in a Biofilm. *Appl. Environ. Microbiol.* **18,** 2064–2069 (2006).
2 Surman, J. S. & Walker, J. T. *Medical Biofilms Detection, Prevention and Control.* Jass, J., Surman, S. & Walker, J. (eds) Ch. 1 1–28. (John Wiley & Sons, Chichester, England, 2003).
3 Jeff, G. L. *et al.* The exopolysaccharide alginate protects Pseudomonas aeruginosa biofilm bacteria from IFN-γ-mediated macrophage killing. *The J. Immunol* **175,** 7512–7518 (2005).
4 Jennifer, D. B., Rebecca, A. A., Jennifer, J. & Steven, C. Signature-tagged mutagenesis of Klebsiella pneumoniae to identify genes that influence biofilm formation on extracellular matrix material. *Infect. Immun.* **74,** 4590–4597 (2006).
5 Paterson, D. L. & Bonomo, R. A. Extended-spectrum β-lactamases: a clinical update. *Clin. Microbiol. Rev.* **18,** 657–686 (2005).
6 Nijssen, S. *et al.* β-lactam susceptibilities and prevalence of ESBL-producing isolates among more than 5000 European Enterobacteriaceae isolates. *Int. J. Antimicrob* **24,** 585–591 (2004).
7 Smith, K., Perez, A., Ramage, G., Gemmell, C. G. & Sue, L. Comparison of biofilm-associated cell survival following *in vitro* exposure of meticillin resistant Staphylococcus aureus biofilms to the antibiotics clindamycin, daptomycin, linezolid, tigecycline and vancomycin. *Int. J. Antimicro. Agents* **33,** 374–378 (2009).
8 Gualdi, L. *et al.* Cellulose modulates biofilm formation by counteracting curli-mediated colonization of solid surfaces in Escherichia coli. *Microbiol* **154,** 2017–2024 (2008).
9 Clinical and Laboratory Standards Institute (CLSI). *Performance Standards for Antimicrobial Susceptibility Testing, 19th Informational Supplement, CLSI Document M100-S19* (CLSI, Wayne, PA29, 2009).
10 Reisner, A., Krogfelt, K. A., Klein, B. M., Zechner, E. L. & Molin, S. *In vitro* biofilm formation of commensal and pathogenic Escherichia coli strains: impact of environmental and genetic factors. *J. Bacteriol.* **188,** 3572–3581 (2006).
11 Ceri, H. *et al.* The calgary biofilm device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. *J. Clin. Microbiol.* **37,** 1771–1776 (1999).
12 Chaudhary, M. & Payasi, A. Alternative approach to increase antibiotic sensitivity of coagulase +ve and −ve Staphylococcus spp in planktonic and sessile cells. *J. Phar. Res.* **5,** 316–320 (2012).
13 Prosser, B. L. T., Taylor, D., Dix, B. A. & Cleeland, R. Method of evaluating effects of antibiotics on bacterial biofilm. *Antimicrob. Agents Chemother.* **31,** 1502–1506 (1987).
14 Gottenbos, B., van der mei, H. C. & Busscher, H. J. Models for studing initial adhesion and surface growth in biofilm formation on surface. *Methods Enzymol* **310,** 523–553 (1999).
15 Turakhia, M. H., Cooksey, K. E. & Characklis, W. G. Influence of a calci unspecific chelatant on biofilm removal. *Appl. Environ. Microbiol.* **46,** 1236–1238 (1983).
16 Lambert, R. J., Hanlon, G. W. & Denyer, S. P. The synergistic effect of EDTA/antimicrobial combinations on Pseudomonas aeruginosa. *J. Appl. Microbiol.* **96,** 244–253 (2004).
17 Raad, I. I. *et al.* The role of chelators in preventing biofilm formation and catheter-related bloodstream infections. *Curr. Opin. infect. Dis* **21,** 385–392 (2008).
18 Kunin, C. M. & Steele, C. Culture of the surfaces of urinary catheters to sample urethral flora and study the effect of antimicrobial therapy. *J. Clin. Microbiol* **21,** 902–908 (1985).
19 Warren, J. W., Muncie, Jr H. L., Berquist, E. J. & Hoopes, J. M. Sequelae and management of urinary infection in the patient requiring chronic catheterization. *J. Urol.* **125,** 1–8 (1981).