International Journal of Antimicrobial Agents 33 (2009) 360–363

Contents lists available at ScienceDirect

International Journal of Antimicrobial Agents

journal homepage: http://www.elsevier.com/locate/ijantimicag

Short communication

Inability of a reserpine-based screen to identify strains overexpressing efflux pump genes in clinical isolates of *Staphylococcus aureus*

Emmanuel Frempong-Manso[a], Jose L. Raygada[a], Carmen E. DeMarco[a], Susan M. Seo[a], Glenn W. Kaatz[a,b,*]

[a] *Department of Medicine, Division of Infectious Diseases, Wayne State University School of Medicine, Detroit, MI 48201, USA*
[b] *John D. Dingell Department of Veterans Affairs Medical Centre, Detroit, MI 48201, USA*

ARTICLE INFO

Article history:
Received 18 September 2008
Accepted 16 October 2008

Keywords:
Staphylococcus aureus
Efflux
Multidrug resistance
Reserpine

ABSTRACT

Overexpression of efflux pump genes conferring multidrug resistance (MDR) in *Staphylococcus aureus* results in reduced susceptibility to select biocides, dyes and fluoroquinolones. Reserpine is commonly used as an inhibitor of MDR efflux pumps and previous work from our laboratory using a reserpine-based screen to identify clinical isolates with an efflux phenotype revealed that nearly one-half overexpressed *norA-B-C*, *mepA* or *mdeA*. The accuracy of reserpine in predicting efflux pump gene overexpression in clinical strains was examined in detail. Bloodstream isolates of *S. aureus* previously classified as non-effluxing strains by the reserpine screen underwent gene expression analysis using quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). The reserpine screen failed to identify many strains shown by qRT-PCR to overexpress one or more MDR efflux pump genes. Microdilution susceptibility testing with and without reserpine also failed to predict efflux pump activity. Although gene expression does not always correlate with protein translation, our results indicate that in clinical *S. aureus* isolates the use of reserpine to predict the contribution of efflux to reduced susceptibility is not dependable. All strains used in studies designed to assess MDR efflux pump gene expression in clinical isolates should be evaluated by a method independent of in vitro susceptibility testing.
Published by Elsevier B.V. on behalf of International Society of Chemotherapy

1. Introduction

The genome of *Staphylococcus aureus* N315 contains ca. 30 open reading frames (ORFs) encoding proteins having homology with multidrug resistance (MDR) efflux pumps (http://www.membranetransport.org) [1]. Members of each of the five drug transport protein families are represented, but major facilitator superfamily (MFS) proteins predominate. Mutations resulting in increased expression of MDR efflux pump genes have been associated with reduced susceptibility to a variety of compounds including antimicrobial agents, biocides and dyes [2,3]. Several chromosomally encoded *S. aureus* MDR pump proteins have been described but few have been studied in detail. Proteins identified as MDR efflux pumps include MepA, MdeA, NorA, NorB, NorC, SepA, SdrM and Sav1866 (*S. aureus* N315 ORFs 0323, 2203, 0650, 1269, 0099, 1971, 1972 and 1683, respectively) [1,4]. The most extensively studied of these is NorA (MFS) followed by MepA, the first-described *S. aureus* multidrug and toxin extrusion family efflux protein [3,5]. Common substrates for these MDR efflux pumps include select fluoroquinolones, dyes such as ethidium bromide and acriflavine, and biocides such as benzalkonium chloride, chlorhexidine and dequalinium [1].

Increased expression of *norA* and *mepA* results in moderate to high-level increases in the minimum inhibitory concentrations (MICs) of biocides and dyes and in low to moderate increases in those of fluoroquinolones [3,6]. However, in the case of NorA this low-level MIC increase has been shown to predispose to the appearance of high-level target-based resistance in vitro [7]. The clinical relevance of this observation has not been established but seems likely to be important in infections where drug delivery is inefficient or improper dosages are employed. In a similar fashion, the level of resistance conferred against biocides does not exceed concentrations deployed in practice but may provide a survival advantage to organisms existing in relatively protected environments where biocide delivery is compromised.

The clinical impact of MDR efflux pump overexpression in human *S. aureus* infections may be significant but has not been studied. Reduced susceptibility to substrate antimicrobial agents such as fluoroquinolones may adversely affect the therapeutic outcome of an infection for which these agents are used. Additionally, the natural function of MDR efflux pumps may include processes other

* Corresponding author. Present address: Department of Internal Medicine, Division of Infectious Diseases, Wayne State University School of Medicine, B4333 John D. Dingell VA Medical Centre, 4646 John R, Detroit, MI 48201, USA.
Tel.: +1 313 576 4491; fax: +1 313 576 1112.
E-mail address: gkaatz@juno.com (G.W. Kaatz).

0924-8579/$ – see front matter. Published by Elsevier B.V. on behalf of International Society of Chemotherapy
doi:10.1016/j.ijantimicag.2008.10.016

than drug resistance. Recently it has been shown that NorB may contribute in a positive fashion to survival of *S. aureus* in a mouse abscess model, suggesting a role of this MDR pump in bacterial fitness [8]. Clearly this is an area worthy of further study.

Reserpine functions as an inhibitor of many MDR efflux pumps, although the exact mechanism of this activity has not been clarified. It has been demonstrated that reserpine binding to membranes enriched for the *Bacillus subtilis* MDR efflux pump Bmr is reduced in the presence of a single amino acid substitution (Val286Leu) in Bmr, suggesting a diminished interaction with the transporter [9]. Whilst no data exist that verify a physical interaction between pump protein and reserpine, the Bmr data indicate that such an association is likely.

The effect of reserpine on efflux activity is often used as the benchmark to which the activities of novel efflux pump inhibitors are compared. Using a reserpine-based screening procedure, we recently demonstrated that efflux pump gene overexpression among clinical bloodstream isolates of *S. aureus* was quite common, with nearly one-half of screen-positive strains overexpressing at least one MDR pump gene [10]. In the present investigation, we evaluated the accuracy of the use of reserpine in the identification of pump gene-overexpressing strains.

2. Materials and methods

2.1. Bacterial strains, media and reagents

Bloodstream isolates (one per patient) and nafcillin susceptibility data were collected from the microbiology laboratory at the Detroit Medical Center in 2005 ($N = 232$). All media and reagents were the highest grade available and were obtained from Sigma Chemical Co. (St Louis, MO) or BD Biosciences (Sparks, MD). *Staphylococcus aureus* SH1000, which is a derivative of *S. aureus* NCTC 8325-4 in which the *rsbU* mutation has been repaired, was used as the control strain for quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) and Northern slot-blots [11].

2.2. Screening for an efflux phenotype and microdilution susceptibility testing

Screening for a potential efflux phenotype was accomplished by determining agar dilution MICs of chlorhexidine, dequalinium, ethidium bromide and norfloxacin in the presence and absence of reserpine (20 mg/L). A four-fold reduction in the MIC of at least three compounds, or two if the strain was resistant to norfloxacin, in the presence of reserpine was considered a positive screen. Microdilution MIC testing, with and without reserpine (20 mg/L), was also performed on all strains with the four compounds used in the initial screen following Clinical and Laboratory Standards Institute guidelines [12].

2.3. qRT-PCR

The expression level of *norA-B-C*, *mepA* and *mdeA* relative to that of *S. aureus* SH1000 was determined in all strains by qRT-PCR using TaqMan chemistry as described previously [10]. A four-fold increase in expression compared with that of *S. aureus* SH1000 was considered significant. PCR was used to evaluate strains for the presence of *qacA/B* employing GenBank sequence X56628 for primer design. Quantitation of *qacA/B* expression was not done as these genes are absent in SH1000.

2.4. Confirmation of gene expression data

Confirmation of qRT-PCR results for at least 12 strains demonstrating increased expression of each pump gene was performed in two ways. The primary approach employed Northern blotting using the Bio-Rad Bio-Dot SF apparatus exactly as described previously, with a two-fold or greater increase in expression compared with SH1000 considered significant [10]. Band intensity was determined using a scanned image of the Northern blot followed by pixel enumeration using TotalLab TL120 software (Nonlinear Dynamics Ltd., Newcastle upon Tyne, UK). If qRT-PCR and Northern slot-blots gave incongruous results, a new RNA sample was obtained from the strain in question and qRT-PCR was repeated. Consistency between the two independently determined qRT-PCR results was accepted as confirmatory evidence of gene overexpression.

3. Results and discussion

3.1. Gene expression analyses

The number of strains overexpressing each evaluated gene and the fold increase in expression in efflux screen-positive and -negative strains compared with *S. aureus* SH1000 is presented in Table 1. Previously published data for screen-positive strains are provided for comparative purposes [10]. Corrections to those earlier data include the identification of two additional *norA*-overexpressing strains and repeat analyses of some strains that altered the mean gene expression data slightly. Some strains in each category overexpressed more than one gene and others overexpressed none, resulting in a total greater than the number of strains in that category. For example, among screen-positive strains, 58 did not overexpress any gene; and of the 28 strains with increased *norA* expression, 17 overexpressed this gene only, whereas the remaining 11 overexpressed one or more additional genes in combination with *norA*.

A total of 56 screen-positive (49.1%) and 72 screen-negative (61.0%) strains overexpressed at least one of the pump genes included in our analysis, resulting in an overall frequency of 55.2% for the entire strain collection (128/232). No significant differences in expression of any gene were observed between screen-positive and screen-negative strains. Examination of strains overexpressing only a single gene also revealed no differences between categories (data not shown). Thus, quantitative differences in gene expression between strains in each category do not provide an explanation as to why the reserpine screen failed to identify significant numbers of pump gene-overexpressing organisms.

Slot-blots verified qRT-PCR data in the majority of cases and the remainder gave consistent qRT-PCR results using two independent RNA preparations. The lack of complete concurrence between qRT-PCR and Northern blot results is not very surprising as the latter method is inherently less sensitive than the former. Inaccuracies in band intensity calculations may occur if there is a high background signal or if the band in question is saturated. qRT-PCR suffers from neither of these limitations. Thus, Northern blotting is at best only moderately useful in confirming qRT-PCR results, and the reproducibility of data using independently generated RNA samples and another cycle of qRT-PCR is an easier, faster and more accurate method.

The reserpine-based screening method misclassified many pump gene-overexpressing strains and failed to detect more such strains than it identified (72 versus 56 strains, respectively). It is likely that a contributing factor to our results is that overexpression of a pump gene may not correlate with increased production of protein, resulting in a screen-negative classification despite increased transcription. Quantitation of pump proteins, which was beyond the scope of this study, would be required to determine the impact, if any, of this possibility on our data.

Overexpression of one, two, three, four or five of the evaluated pump genes occurred in 34.1%, 12.9%, 3.9%, 3.9% and 0.4% of the 232 strains, respectively. Of great interest was the observation that 21%