Journal of Global Antimicrobial Resistance 3 (2015) 184–189

Contents lists available at ScienceDirect

Journal of Global Antimicrobial Resistance

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

Characterisation of β-lactam resistance mediated by *blaZ* in staphylococci recovered from captive and free-ranging wallabies

Michelle M.S. Chen [a,*], Wayne S.J. Boardman [b,c], Ian Smith [d], Amanda E. Goodman [a], Melissa H. Brown [a]

[a] School of Biological Sciences, Flinders University, Bedford Park, SA 5042, Australia
[b] School of Animal and Veterinary Sciences, University of Adelaide, Roseworthy, SA 5371, Australia
[c] Barbara Hardy Institute, University of South Australia, Mawson Lakes, SA 5095, Australia
[d] Monarto Zoo, 3401 Old Princes Highway, Monarto, SA 5254, Australia

ARTICLE INFO

Article history:
Received 30 January 2015
Received in revised form 23 April 2015
Accepted 6 May 2015

Keywords:
Penicillin
blaZ
β-Lactamase
Wildlife
Wallaby
Staphylococcus

ABSTRACT

Staphylococci are commensal organisms of animals, but some species are opportunistic pathogens that are resistant to almost all antimicrobial agents in clinical use. Bacterial resistance to β-lactam antimicrobial agents is widespread and has been investigated in species isolated from humans in addition to food production and companion animals. However, minimal progress has been made towards identifying reservoirs of β-lactam-resistant staphylococci in wildlife. This study was aimed at investigating and characterising β-lactamase resistance from staphylococci of wallaby origin. Staphylococci from free-ranging and captive wallabies were assessed for their phenotypic susceptibility to β-lactam antimicrobial agents prior to sequence analysis of their *blaZ* and *blaR1* genes. Deduced amino acid sequences were classified according to the Ambler molecular characterisation method, assigned a protein signature type and compared with sequences generated from previous studies involving isolates from humans, cattle and companion animals. All BlaZ sequences identified in this study were assignable to a pre-existing β-lactamase class and protein signature type, including the more recently discovered protein signature type 12. Three major phylogenetic groups were resolved upon phylogenetic analysis against published BlaZ sequences. This study has found antibiotic-resistant staphylococci both in free-ranging and captive wallaby populations and these bacteria harbour *blaZ* variants that are different to those recovered from humans, cattle and companion animals. Further studies of staphylococci from non-traditional sources are required in order to enhance our knowledge of the epidemiology of antibiotic resistance genes.

1. Introduction

Staphylococci are commensal organisms that reside in the nasal cavities and on the skin of animals. Under immunocompromised host conditions, certain species such as *Staphylococcus aureus*, *Staphylococcus intermedius* and *Staphylococcus pseudintermedius* can become opportunistic pathogens in humans and other animals [1]. An ecological approach should be applied when furthering our understanding of infectious agents in humans. It has been estimated that almost 75% of emerging human pathogens originated in animals. Whilst there have been numerous studies investigating the incidence of staphylococci, in particular carriage of *S. aureus*, in humans, livestock and pets, the same information is lacking for native wildlife and animals in zoological park collections. Constraining epidemiological studies to animals that have the most direct contact with humans provides us with a narrow snapshot of the overall ecology of these pathogens. This was evident during the 1976 Nigerian brucellosis outbreak in the Ibarapa District, which resulted in >45% of cattle in nomadic herds testing positive for acute bovine brucellosis [2]. In contrast, all government and privately owned farms were *Brucella*-free. However, the flow-on effects of this outbreak, which effectively halved the number of cattle in the area, resulted in acute meat shortages, malnutrition in the general populace and over 100 human cases of brucellosis [2,3].

Since its introduction into clinical medicine in 1940, penicillin has been the drug of choice for the treatment of staphylococcal

* Corresponding author. Present address: School of Biological Sciences, Flinders University, GPO Box 2100, Adelaide, SA 5001, Australia. Tel.: +61 8 8201 2215.
E-mail address: michelle.chen@flinders.edu.au (Michelle M.S. Chen).

http://dx.doi.org/10.1016/j.jgar.2015.05.002

infections [4]. However, penicillin-resistant staphylococci were reported as early as 1942 [4] and ongoing studies have found the prevalence of penicillin-resistant *S. aureus* to be between 84% and 88% in Australia [5]. Penicillin resistance in staphylococci can be mediated by the production of an altered form of penicillin binding protein 2A (PBP2A), encoded by the *mecA* gene, or as a result of enzymatic inactivation of the drug by the β-lactamase enzyme. This enzyme is encoded by *blaZ*, which is part of the three-membered *bla* operon also containing a repressor (*blaI*) and signal transducer/sensor protein (*blaR1*) [4]. Under the simplest classification system, four classes (A, B, C and D) of the staphylococcal β-lactamase gene product, BlaZ, have been reported based on conserved and distinguishing amino acid motifs in the protein sequence [6]. Classes A, C and D hydrolyse their substrates through a serine active site, whereas class B β-lactamases function through an active-site zinc ion [6,7]. A more detailed typing scheme that groups different BlaZ together on the basis of their amino acid sequence into 1 of 12 signature types has been proposed [7]. Novel protein signature types are formed when three or more deviations from an existing type are detected [7].

As *blaZ* can be carried on the chromosome and on mobile genetic elements [8], to investigate its spread within a bacterial genus and between strains of the same species it is necessary to combine traditional microbiological techniques with bioinformatic tools. Numerous methods for the phenotypic detection of β-lactam resistance in staphylococci have been established; all had a sensitivity of <72%. This has led to the detection of *blaZ* by PCR to be the recommended gold standard [9]. The presence of *blaZ* has been well documented in staphylococci of human and cattle origin [7,10] as well as in cats and dogs [11]. However, to our knowledge, there are no reports describing *blaZ* in staphylococci of wallaby origin. This study aimed to investigate the natural diversity of the *blaZ* gene from staphylococci of captive Black-flanked Rock wallaby (*Petrogale lateralis*) (BFRW), captive Yellow-footed Rock wallaby (*Petrogale xanthopus*) (YFRW) and captive Mainland Tammar wallaby (*Macropus eugenii*) (TMW) in addition to free-ranging BFRW both by traditional and molecular techniques.

2. Materials and methods

2.1. Bacterial isolates

A total of 89 staphylococcal isolates (56 penicillin-susceptible and 33 penicillin-resistant) were used in the first phase of this study to detect the presence of the *blaZ* gene. These strains originated from a previously described collection of staphylococcal strains isolated from anterior nasal swabs obtained from 68 captive and 30 free-ranging wallabies in South Australia [12]. Staphylococci were preliminary identified by Gram staining and catalase and coagulase production. DNA was extracted using a HiYield™ Genomic DNA Mini Kit (Bio-Deal, Auckland, New Zealand) and isolates were identified to species level by 16S rRNA sequencing. All Gram-positive, catalase-positive isolates that were identified as staphylococci by 16S rRNA sequencing were added to the strain collection.

2.2. PCR detection of the bla operon

All 89 isolates were tested using primer pairs 486–488, 487–373 and 487–531 to amplify a 1.16 kb cumulative fragment of the *blaR1–blaZ* genes as previously described [7]. This fragment contained the first 209 bp of *blaR1*, a 106 bp non-coding intergenic region, and the complete 846 bp *blaZ* gene. As a supplement to primer pair 487–531, primers to amplify an 861 bp fragment (B861) of the *bla* region encompassing *blaZ* [11,13] were used on a limited number of isolates. The *blaI* and *blaR1* genes were amplified from strains demonstrating the presence of the *blaZ* gene in addition to selected controls with oligonucleotides *bla*IF (5′-CTAATTTAATAAGAGTCAAGC-3′) and *bla*IR (5′-TGTTTGGACTT-GACCGACAT-3′), and *blaR1*F (5′-TCCATGACATACGTGAATTTT-3′) and *blaR1*R (5′-ATAATCAAGCGCCCACAGTT-3′) to give products of 979 bp and 1033 bp, respectively. These oligonucleotides were designed based on the *bla* operon from *Staphylococcus epidermidis* (GenBank accession no. X52734).

Each PCR reaction contained 12.5 μL of GoTaq® Green Master Mix (Promega, Madison, WI), 25 pmol each of forward and reverse primer, 5 μg of DNA and 9.5 μL of sterile nuclease-free water (Promega); all reactions were performed with 1.5 mM $MgCl_2$. The PCR cycle conditions for these sets of primers included an initial denaturation of 95 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 54 °C for 1 min and 72 °C for 1 min, and a final extension at 72 °C for 5 min. PCR products were separated on 1% agarose gels (Promega) in 0.5 × TAE [Tris–acetate–ethylene diamine tetra-acetic acid (EDTA)] buffer. Resulting gels were stained with ethidium bromide and were visualised under ultraviolet light and the image was analysed on Gel Doc™ EZ Imager (Bio-Rad, Melbourne, VIC, Australia). PCR products resulting from primer pairs 486–488 and 487–531 as well as representative amplicons from *blaI* and *blaR1* were purified from reaction components using a Wizard® SV Gel and the PCR Clean-up System (Promega) by centrifugation according to the manufacturer's instructions and were sequenced by First Base Laboratories (Selangor, Malaysia) using the forward primers.

2.3. Nucleotide analysis of blaZ and protein signature typing

DNA sequences were assembled manually using BioEdit v.7.1.11 [14] (http://www.mbio.ncsu.edu/bioedit/bioedit.html), were subjected to homology analysis on National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/genbank/) and multiple alignments were performed with Clustal X2 with bootstrap 1000 [15] (http://www.clustal.org/clustal2/). The *bla* operon from *S. epidermidis* (GenBank accession no. X52734) was used as a reference in addition to other published *blaZ* sequences available in GenBank. From the 103 *blaZ* sequences available in the public database as of September 2013, 43 sequences with lengths >780 bp were selected for further analysis. Approximate likelihood ratio test analysis was performed by PhyML 3.0 including bootstrap 100 analysis (http://www.phylogeny.fr/one_task.cgi?task_type=phyml) using default settings. Dendrograms were constructed in Newick format using TreeDyn 198.3 (http://www.phylogeny.fr/one_task.cgi?task_type=treedyn). Nucleotide sequences of *blaZ* genes determined in this study were submitted to GenBank under accession nos. KM362524–KM362542 and KM368805–KM368812.

2.4. Antibiotic susceptibility testing

A total of 41 staphylococcal strains ($n = 25$ wallabies), comprising 33 β-lactam (ampicillin, penicillin and oxacillin)-resistant isolates from a previous study and 8 *blaZ*-positive, β-lactam-sensitive isolates identified in Section 2.2, were challenged with the β-lactam antimicrobial agents amoxicillin/clavulanic acid (AMC) (30 μg) to ascertain β-lactamase activity and cefoxitin (30 μg) to confirm oxacillin results by Kirby–Bauer disc diffusion according to Clinical and Laboratory Standards Institute (CLSI) guidelines [16]. *S. aureus* ATCC 6538, *S. epidermidis* ATCC 12228 and *Escherichia coli* DH5α were used as reference strains.

2.5. Iodometric detection of β-lactamase production

Production of β-lactamase was detected using a method based on previous publications [17,18]. Briefly, an overnight bacterial