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Antibacterial activity of violacein against *Staphylococcus aureus* isolated from Bovine Mastitis

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Bovine mastitis is the most important disease of dairy farms and is typically caused by bacterial infection.[1] This disease constitutes a serious problem in dairy herds incurring considerable economic losses due to reduced milk production and discarded milk.[2] In the United States, economic losses due to mastitis are estimated at US$2 billion per year, whereas in the United Kingdom and Northern Ireland, the annual losses are £300 million and £14 million, respectively. Mammary gland infections implicate an additional cost of €693 per cow per year in the Republic of Ireland, whereas in the Netherlands, these additional costs vary between €164 and €235.[3] *Staphylococcus aureus* and *Escherichia coli* are the most common etiological agents of subclinical and clinical bovine mastitis.[4]

Penicillin and its derivatives are recommended for the treatment of bovine mastitis caused by Gram-positive pathogens. However, a worldwide increase in *S. aureus* that are resistant to these antibiotics has been described. In the United States, >70% of isolates obtained from mastitis are penicillin resistant, whereas in Brazil and Ireland, the level is about 85%.[5] Multiple drug resistance to streptomycin, sulphametoxazole, ampicillin or tetracycline for coliform mastitis strains has been described elsewhere.[6] Therefore, new antibacterial compounds are being investigated as an alternative to treat infections of human and animal origin.

Violacein is a purple pigment produced by free environmental bacterial species, especially by *Chromobacterium violaceum*. This compound has several biological activities, including antitumoral and apoptosis-inducing properties in cancer cells, antioxidant, leishmanicidal, trypanocidal, antifungal, weak antiviral[7] and antimalarial effect.[8] As an antibacterial agent, violacein showed activity against *Mycobacterium tuberculosis*.[9]

In this study, we investigated the antibiotic activity of violacein against *S. aureus* and *E. coli* field strains isolated from bovine mastitis and its possible additive or synergistic effects with other antimicrobial drugs.

All of the isolates of *S. aureus* (15) and *E. coli* (15) were obtained from subclinical and clinical bovine mastitis, respectively, and characterized by morphology of colonies, staining and biochemical as well as antibiotic resistance, according to standard procedures.[10,11]

The *S. aureus* isolates MBSA 13 and MBSA 19 displayed intermediary resistance to erythromycin, while twelve of the fifteen *S. aureus* isolates analyzed (80%) were resistant to penicillin (Table 1). Moreover, the *S. aureus* isolates MBSA 10 and MBSA 55 were simultaneously resistant to penicillin and showed intermediary resistance to erythromycin, whereas *S. aureus* isolates MBSA 33 and MBSA 35 showed intermediary resistance to erythromycin and complete resistance to penicillin and ampicillin. The presence of these antimicrobial resistance profiles in *S. aureus* isolates is recognized as a worldwide problem and demands the search for new drugs and combinations of antimicrobials to increase the success of antibacterial therapy.

The sensitivity of the bacterial isolates to violacein was tested in Mueller–Hinton media by determining the MIC, according to the guidelines of the Clinical Laboratory Standards Institute.[11] *E. coli* isolates were not inhibited by violacein at concentrations up to 200 μM (data not shown). In contrast, all of the *S. aureus* isolates displayed sensitivity to violacein with MIC varying between 6.25 and 25 μM (Table 1) depending on the analyzed isolate. Therefore, violacein has the potential to be used as an antibacterial compound in bovine mastitis caused by *S. aureus*.

To determine whether violacein displays drug interactions with other antimicrobials, a double antimicrobial gradient assay was used. In this assay, several concentrations of violacein were combined with different concentrations of the selected antimicrobials. We determined the MIC of the combination, which is the lowest concentration of violacein, that when combined with the lowest concentration of another antimicrobial, inhibits growth. To evaluate the type of interaction between both antimicrobials, the fractionated inhibitory concentration (FIC) index was used as described by Chin *et al.*[12] FIC indexes were interpreted as follows: FIC $\leqslant$0.5=synergistic interaction; 0.5<FIC $\leqslant$1.0=additive interaction; 1.0<FIC $\leqslant$4.0=no interaction and FIC >4.0=antagonist interaction. For this assay, three penicillin-resistant isolates (*S. aureus* isolates MBSA 24, 35 and 63) were used and the combined effects of violacein with penicillin G procaine were analyzed (Table 2a). The *S. aureus* isolates MBSA 19, MBSA 24, MBSA 35 and MBSA 63 were selected for the evaluation of violacein–streptomycin combined effects (Table 2b).

Violacein displays a synergistic effect when combined with penicillin in the three selected isolates (Table 2a). Furthermore, violacein did not show any interaction with streptomycin (Table 2b). No antagonistic effects were observed when violacein was tested in combination with other antimicrobial compounds (for example, chloramphenicol and vancomycin; data not shown). Thus, in addition to the potential application of violacein as an antibiotic, this compound could be used further in the combined treatment with other antimicrobial compounds against multidrug-resistant isolates from bovine mastitis. We also observed synergistic effects of violacein combined with either chloramphenicol or vancomycin against these isolates (data not shown). However, as chloramphenicol is forbidden for treatment of food-producing animals in many countries and as vancomycin is one of the last resorts for treatment of methicillin-resistant

Table 1 *S. aureus* isolates used in this work, their antibiotic resistance profile and MICs for violacein

Isolate	*Source*	*Clinical or subclinical mastitis*	*Antimicrobial resistance*	*MIC for violacein (μM)*
S. aureus MBSA 4	UNESP–Botucatu*	Subclinical	Pn	25.00
S. aureus MBSA 6	UNESP–Botucatu*	Subclinical	Pn	12.50
S. aureus MBSA 10	UNESP–Botucatu*	Subclinical	Pn, Er^{IR}	12.50
S. aureus MBSA 13	UNESP–Botucatu*	Subclinical	Er^{IR}	6.25
S. aureus MBSA 19	UNESP–Botucatu*	Subclinical	Er^{IR}	6.25
S. aureus MBSA 24	UNESP–Botucatu*	Subclinical	Pn	12.50
S. aureus MBSA 30	UNESP–Botucatu*	Subclinical	Pn	6.25
S. aureus MBSA 31	UNESP–Botucatu*	Subclinical	Pn	12.50
S. aureus MBSA 32	UNESP–Botucatu*	Subclinical	Pn	6.25
S. aureus MBSA 33	UNESP–Botucatu*	Subclinical	Pn, Ap, Er^{IR}	12.50
S. aureus MBSA 35	UNESP–Botucatu*	Subclinical	Pn, Ap, Er^{IR}	25.00
S. aureus MBSA 55	UNESP–Botucatu*	Subclinical	Pn, Er^{IR}	25.00
S. aureus MBSA 58	UNESP–Botucatu*	Subclinical	None	6.25
S. aureus MBSA 62	UNESP–Botucatu*	Subclinical	Pn	12.50
S. aureus MBSA 63	UNESP–Botucatu*	Subclinical	Pn	25.00

Abbreviations: Ap, ampicillin; Er, erythromycin; IR, intermediary resistant; Pn, penicillin.
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Table 2 Combined activities of (a) penicillin G procaine–violacein and (b) streptomycin–violacein against *S. aureus* isolates

Isolate	*MIC (μM) Pn alone*	*MIC (μM) Violacein alone*	*MIC (μM) Pn+violacein (combination)*	*FIC*	*Combined effect*
(a)					
S. aureus MBSA 24	2.40	12.50	0.01+3.12	0.25	Synergism
S. aureus MBSA 35	1.20	25.00	0.02+6.25	0.27	Synergism
S. aureus MBSA 63	4.80	25.00	0.04+3.12	0.13	Synergism
Isolate	*MIC (μM) Sm alone*	*MIC (μM) Violacein alone*	*MIC (μM) Sm+violacein (combination)*	*FIC*	*Combined effect*
(*b*)					
S. aureus MBSA 19	3.45	6.25	0.06+6.25	1.02	No interaction
S. aureus MBSA 24	1.73	12.50	0.03+25.00	2.02	No interaction
S. aureus MBSA 35	3.45	25.00	2.15+12.50	1.12	No interaction
S. aureus MBSA 63	0.87	25.00	0.35+40.00	2.00	No interaction

Abbreviations: FIC, fractionated inhibitory concentration; Pn, penicillin; Sm, streptomycin.
In all the combined activities, the calculations were done according to Chin *et al.*[12]

Staphylococcus aureus (MRSA) infections in humans, the combination of violacein with these antimicrobials is not an interesting strategy for the treatment of bovine mastitis.

One of the main issues regarding the therapeutic uses of violacein is its toxicity *in vivo*, as this compound is cytotoxic to several tumor models, such as leukemic cells, colorectal tumors, human intestinal epithelial cells and ascite tumor models.[7] Very recently, Bromberg *et al.*[13] have shown that violacein causes oxidative stress and cell death by apoptosis in Ehrlich ascites tumor cells. In this work, the authors evaluated the toxicity of daily doses of this compound to major organs over 35 days. It was found that intraperitoneal doses of violacein up to $1\,mg\,kg^{-1}$ did not cause toxicity in blood, kidneys or liver of mice under these conditions, which provide good support for the *in vivo* use of violacein as a therapeutic compound with low side effects. Thus, the use of violacein as an antibiotic compound is a reasonable prospect, and further studies regarding the *in vivo* antibacterial activity of violacein and its accumulation in tissues of food-producing animals could reinforce the therapeutic potential of this drug against mastitis isolates of *S. aureus*.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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