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Author for correspondence:
José Givanildo da Silva,
Email: givanildojgs@gmail.com

Antimicrobial activity of polypyrrole nanoparticles and aqueous extract of *Moringa oleifera* against *Staphylococcus* spp. carriers of multi-drug efflux system genes isolated from dairy farms

José Givanildo da Silva[1], Mariana de Barros[2], Nataly Diniz de Lima Santos[3], Patrícia Maria Guedes Paiva[3], Thiago Henrique Napoleão[3], Maria José de Sena[1], Mateus Matiuzzi da Costa[4], Helinando Pequeno de Oliveira[4], Maria Aparecida Scatamburlo Moreira[2] and Rinaldo Aparecido Mota[1]

[1]Laboratório de Doenças Infectocontagiosas dos Animais Domésticos, Universidade Federal Rural de Pernambuco, Av. Dom Manoel de Medeiros, s/n, Dois Irmãos, CP 52171-900, Recife, Pernambuco, Brazil; [2]Laboratório de Doenças Bacterianas, Departamento de Veterinária, Universidade Federal de Viçosa, Campus UFV, Centro, CP 36570-900, Viçosa, MG, Brazil; [3]Laboratório de Bioquímica de Proteínas, Departamento de Bioquímica, Universidade Federal de Pernambuco, Av. Professor Moraes Rego, 1235, Cidade Universitária, CP 50670-901, Recife, Pernambuco, Brazil and [4]Laboratório de Microbiologia e Imunologia Animal, Universidade Federal do Vale do São Francisco, Rodovia BR-407 Km 12, Lote 543, C1, CP 56300-000, Petrolina, Pernambuco, Brazil

Abstract

Our objectives were to identify genes of the multi-drug efflux system and to evaluate the antimicrobial activities of polypyrrole nanoparticles (PPy-NPs) and aqueous extract of *Moringa oleifera* against *Staphylococcus* spp. isolated from dairy farms in Northeast Brazil. Initially, 162 *Staphylococcus* spp. isolates were subjected to *in vitro* antimicrobial sensitivity tests. Of these, 35 presented antimicrobial multi-drug resistance phenotypes. These 35 isolates were then referred for the detection of *norA, norB, norC, msrA, mgrA, tet-38,* and *lmrS* genes, all of which feature in multi-drug efflux systems. In the isolates carrying the genes, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of PPy-NPs and *Moringa oleifera* aqueous extract were determined. In the molecular analysis of the 35 isolates *norA, norC, tet-38,* and *msrA* genes were detected and for the other genes *norB, lmrS* and *mgrA* there was no amplification. Antimicrobial activity was verified of PPy-NPs and aqueous extract of *Moringa oleifera* in *Staphylococcus* spp. carrying multi-drug efflux system genes. We concluded that there are multi-drug efflux system genes present in the *Staphylococcus* spp. from the agricultural environment in Northeast Brazil, and that aqueous extract of *Moringa oleifera* and PPy-NPs show bactericidal activity against these isolates.

Staphylococcus spp. are the biggest cause of contagious mastitis in bovine species, and antibiotic therapy is considered the main form of treatment. However, the indiscriminate use of antimicrobials has encouraged the emergence of multi-drug-resistant isolates (Lin *et al.*, 2015). Mechanisms of *Staphylococcus* spp. resistance include enzyme production, modification of the drug target and multi-drug efflux systems (Costa *et al.*, 2013; Kumar *et al.*, 2013). Multi-drug efflux systems comprise of cytoplasmic proteins that are involved in the extrusion of toxic agents from inside the bacteria to the external environment. They are encoded in chromosomes or plasmids and perform active transport of the antimicrobial agent out of the cell (Truong-Bolduc *et al.*, 2005; Micas, 2008). Moreover, they prevent the drug from reaching inhibitory concentrations inside the bacterial cell. In *Staphylococcus* spp., more than 20 efflux systems classified into five families of membrane proteins have already been identified: the major facilitator superfamily (MFS), the small multidrug resistance family, the multidrug and toxin extrusion family, the ATP-binding cassette (ABC) superfamily, and the resistance-nodulation-division superfamily (Truong-Bolduc *et al.*, 2006; Kumar *et al.*, 2013; Jang, 2016).

For the pathogen, the effectiveness of its multi-drug efflux systems is related to three mechanisms: elimination of endogenous metabolites harmful to the bacterium, secretion of virulence determinants, and responses to cellular stress (Poole, 2008). As such, the drugs can be defined as 'accidental substrates' of these transporters. However, the ability of multi-drug efflux systems to expel antimicrobials, both synthetic and natural, presents problems

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for treatment of infections, especially those caused by the highly pathogenic *Staphylococcus* spp. (Truong-Bolduc *et al.*, 2005).

The emergence of antimicrobial resistance has stimulated research into possible alternatives for the affected drugs, such as polymers and herbal medicines (Peixoto *et al.*, 2016; Sanchez Ramirez *et al.*, 2019). Bacteriophages, lactic acid bacteria, nanoparticles of chemical compounds and extracts or essential oils from plants are some of the alternative therapies reported against *Staphylococcus* spp. isolated from the agricultural environment (Quintero *et al.*, 2011; Costa Junior *et al.*, 2018; Berguenmaier de Olanda *et al.*, 2019; Leite *et al.*, 2019; Sperandio *et al.*, 2019).

The objective of this study was to detect multi-drug efflux system genes in *Staphylococcus aureus* and non-*aureus* spp. present in bovine mastitis, milkers, and the milking environment and evaluate the antimicrobial activity of polypyrrole nanoparticles (PPy-NPs) and aqueous extract of *Moringa oleifera* seed oil against these *Staphylococcus* spp.

Materials and methods

Ethical approval

This study was approved by the Animal Use Ethics Committee of the Universidade Federal Rural de Pernambuco, Recife, Brazil (license number 106/2017), the Ethics Committee on Animal Use of the Universidade Federal de Pernambuco (process number 23076.033782/2015-70) and the Research Ethics Committee of the Universidade de Pernambuco, Recife, Brazil (CAAE number: 28833619.7.0000.5207).

Staphylococcus *spp. isolates*

Five dairy farms (A, B, C, D and E) located in different regions of the state of Pernambuco, Northeastern Brazil, were included in the present study. Milk samples collected from 676 udder quarters were subjected to the California Mastitis Tests (CMT) and those that were equal to or greater than one cross (+) (319 samples) or found positive in the routine strip cup test (16 samples) were selected for microbiological testing. Also, samples from the dairy environment (mechanical milking equipment and milking buckets) and milkers (hands and nasal cavities) were obtained using swabs and subjected to microbiological analysis. A total of 15 samples of milk and hand swabs from milkers, 14 samples from teat taps and nine samples of milking buckets were obtained. The distribution by farm of the collected samples is described in the online Supplementary Table S1.

Isolation and preliminary identification of Staphylococcus *spp.*

Isolation of *Staphylococcus* spp. was performed according to the methodology of Carter (1998) as described in the online Supplementary File.

Susceptibility to antimicrobials

Staphylococcus spp. underwent *in vitro* antimicrobial sensitivity tests using the disk diffusion technique on Mueller-Hinton agar (CLSI, 2018). The following antibiotic-impregnated disks were used oxacillin (1 µg), cefoxitin (30 µg) and penicillin G (10 U, vancomycin (30 µg), sulfamethoxazole (25 µg) + trimethoprim (25 µg), tetracycline (30 µg), gentamycin (10 µg) and neomycin (30 µg).

Molecular analysis

Isolates of *S. aureus* and non-*aureus* that showed resistance to at least two drugs were selected for molecular analysis. Genomic DNA was extracted by thermal extraction; method as described by Fan *et al.* (1995).

Identification of S. aureus *and of efflux system genes in* S. aureus *and non-aureus*

To identify *S. aureus,* all isolates underwent amplification of the *nuc* gene region according to the methodology described by Brakstad *et al.* (1992). For detection of *norA, norB, norC, msrA, mgrA, tet-38,* and *lmrS* genes, all *Staphylococcus* spp. underwent PCR following the methodologies described by Martineau *et al.* (2000), Truong-Bolduc *et al.* (2003), Truong-Bolduc *et al.* (2005), Truong-Bolduc *et al.* (2006) Details are provided in the online Supplementary Table S2.

Determination of minimum inhibitory concentration

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of PPy-NPs in water and *Moringa oleifera* aqueous extract were determined using the broth microdilution methodology following recommendations of the Clinical Laboratory Standards Institute (CLSI, 2018). For PPy-NPs in water the method used was that of da Silva *et al.* (2016) and the concentrations of PPy-NPs tested were 2, 1, 0.5, 0.250, 0.125, and 0.062 mg/ml. The protein content of aqueous *Moringa oleifera* extract was determined according to Lowry *et al.* (1951). The presence of lectins was monitored according to Paiva and Coelho (1992) and Bing *et al.* (1967) (see online Supplementary File). Aqueous *Moringa oleifera* seed extract was used at initial concentrations of 6.1, 3.05, 1.525, 0.7625, 0.3812, and 0.1906 mg/ml.

Results

In this study, 162 *Staphylococcus* spp. were isolated from samples collected from dairy farms in the state of Pernambuco, Brazil. The results of isolation, molecular identification of *Staphylococcus aureus* and non-*aureus* samples from milk, milkers, and milking utensils are shown in the online Supplementary Table S3.

In the antimicrobial susceptibility test, 35/162 (21.6%) of *Staphylococcus* spp. isolates comprising 23/35 (65.7%) *S. aureus* and 12/35 (34.3%) *S.* non-*aureus*, were resistant to at least two antimicrobials. In the molecular analysis of these isolates, *norA, norC, tet-38,* and *msrA* genes were all observed. Table 1 details the efflux system genes in *Staphylococcus aureus* and non-*aureus* according to origin.

The *Moringa oleifera* extract showed a specific hemagglutination assay value of 32, indicating the presence of lectins. Previous assays revealed the presence of flavonoids, tannins, saponins, phenylpropanoids, alkaloids, and reducing sugars in the extract (unpublished data). The results of the antimicrobial activity of aqueous *Moringa oleifera* seed extracts and of polypyrrole nanoparticles against *Staphylococcus* spp. carrying multi-drug efflux system genes are presented in Table 2.

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Table 1. Frequency of the efflux system genes in *S. aureus* and *S.* non-*aureus* according to origin

Gene	Total	*S. aureus*	*S.* non-*aureus*	Milk		Milkers nasal swab		Milkers Hands swab		Teat cup		Milking buckets	
				SA	SNA	SA	SNA	SA	SNA	SA	SNA	SA	SNA
norA	74.3% (26/35)	65.4% (17/26)	34.6% (9/26)	68.8% (11/16)	31.2% (5/16)	100% (1/1)	0% (0/1)	33.3% (1/3)	77.7% (2/3)	50% (2/4)	50% (2/4)	100% (2/2)	–
norC	74.3% (26/35)	69.2% (18/26)	30.8% (8/26)	64.7% (11/17)	35.3% (6/17)	66.6% (2/3)	33.3% (1/3)	100% (1/1)	0% (0/1)	66.6% (2/3)	33.3% (1/3)	100% (2/2)	0% (0/2)
tet-38	51.4% (18/35)	72.2% (13/18)	27.8% (5/18)	77.8% (7/9)	22.2% (2/9)	66.6% (2/3)	33.3% (1/3)	–	–	60% (3/5)	40% (2/5)	100% (1/1)	–
msrA	40% (14/35)	35.7% (5/14)	64.3% (9/14)	0% (0/2)	100% (2/2)	25% (1/4)	75% (3/4)	33.3% (1/3)	66.6% (2/3)	50% (3/6)	50% (3/6)	–	–

SA, *Staphylococcus aureus*; SNA, *Staphylococcus* non-aureus; –, not identified.

Discussion

In this study *norA, norC, tet-38,* and *msrA* were detected in our samples. The first three belong to MFS, the family with the largest number of studies in the context of staphylococci (Jang, 2016); *MsrA* belongs to the ABC family (Martineau *et al.*, 2000). In *Staphylococcus aureus* the reported identification frequencies of the efflux system genes ranges widely from 2 to 98.8% (Hassanzadeh *et al.*, 2020).

These results indicate the circulation of isolates with molecular mechanisms of resistance to quinolones, tetracyclines, erythromycin, and macrolides in the studied farms. The detected genes confer resistance to these antimicrobials and so, if they are expressed, multidrug resistance is observed (Truong-Bolduc *et al.*, 2003; Deng *et al.*, 2012; Duran *et al.*, 2012; Kumar *et al.*, 2013; Phillips-Jones and Harding, 2018). In our case the most frequently observed genes were *norA* and *norC* (Table 1), both of which are responsible for quinolone resistance, followed by *tet-38,* which confers resistance to tetracyclines. These findings might reflect the frequent use of these antimicrobials to treat mastitis at the studied farms. Their use has likely contributed to the selection pressure for the emergence of resistant isolates (Hawkey, 2008; Holko *et al.*, 2019).

Regarding isolate origin, milk samples gave the highest percentages of detected genes. This indicates a role for isolates with an efflux system in the etiology of bovine mastitis in the study region (Northeast Brazil). A similar situation has previously been reported in Egypt (Elsayed *et al.*, 2019). The exception was *msrA*, which was more prevalent in the liner samples and milkers (Table 1), although given the small number of samples, these results must be interpreted with considerable caution. The *msrA* gene supports resistance to erythromycin and macrolides, which are usually not antimicrobials of choice for mastitis in Brazil (unlike Argentina, where these drugs are commonly used: Srednik *et al.*, 2018). This probably explains why it was detected more frequently in isolates from liners (utensil manipulated by humans), nasal swabs, and milkers' hands at the studied farms (Truong-Bolduc *et al.*, 2005; Jang, 2016; Pereira and Scussel, 2017).

Our detection of multi-drug efflux system genes in *Staphylococcus* spp. demonstrates that there is variability in the genetic components of resistance in the studied region. It is, therefore, pertinent to research alternatives to the affected drugs, such as plant compounds with antibacterial activity such as *Moringa oleifera,* and chemical compounds such as PPy.

At all concentrations except 6.1 mg/ml, aqueous *Moringa oleifera* extract showed activity for at least one isolate. Greatest inhibition was found at concentrations of 1.525 and 0.7625 mg/mL. This inhibitory effect was due to aqueous *Moringa oleifera* extract's bactericidal activity at these concentrations; this was verified by MBC testing. However, only *msrA*-carrying isolates were 100% inhibited, which indicates better bactericidal activity against *Staphylococcus* carriers of this gene.

Moringa oleifera (family *Moringaceae*) is a tree grown in tropical and subtropical regions (Pontual *et al.*, 2012*a*). Its seeds are used to treat and reduce the turbidity of water intended for human consumption. In addition, its flowers are consumed and have hypoglycemic, tonic, and diuretic therapeutic actions (Moura *et al.*, 2011; Santos *et al.*, 2012). It also has larvicidal activity against *Aedes aegypti* and is antiparasitic against *Trypanosoma cruzi* (Pontual *et al.*, 2012*a*, 2018). Further, it has technological potential in the food industry, due to its caseinolytic activity and milk-coagulating effects (Pontual, *et al.*, 2012*b*). No previous

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Table 2. Distribution by MIC of the aqueous extract of *Moringa oleifera* and PPy-NPs against *Staphylococcus* spp. carriers of *norA, norC, tet-38* and *msrA* isolated from milk cows, Milkers and dairy farm environment

MIC of the aqueous extract of *Moringa oleifera*	*norA*					*norC*					*Tet-38*					*msrA*				
	M	HS	SN	TC	MB	M	HS	SN	TC	MB	M	HS	SN	TC	MB	M	HS	SN	TC	MB
6.1 mg/ml	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
3.05 mg/ml	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1/1 (100%)	–	2/3 (66.6%)	–	–	–
1.525 mg/ml	4/16 (25%)	1/1 (100%)	1/3 (33.3%)	1/4 (25%)	1/2 (50%)	3/16 (18.7%)	–	–	2/4 (50%)	2/2 (100%)	1/9 (11.1%)	–	2/3 (66.6%)	2/5 (40%)	–	–	1/3 (33.3%)	1/4 (25%)	2/6 (33.3%)	–
0.7625 mg/ml	5/16 (31.2%)	–	1/3 (33.3%)	3/4 (75%)	–	5/16 (31.2%)	–	3/3 (100%)	–	–	2/9 (222%)	–	–	1/5 (20%)	–	(50%)	–	–	2/6 (33.3%)	–
0.3812 mg/ml	–	–	–	–	–	–	–	–	–	–	–	–	1/3 (33.3%)	–	–	–	–	1/4 (25%)	–	–
0.1906 mg/ml	–	–	1/3 (33.3%)	–	–	–	–	–	–	–	–	–	–	–	–	1/2 (50%)	–	2/4 (50%)	2/6 (33.3%)	–
Not inhibited	7/16 (43.75%)	–	–	–	1/2 (50%)	8/16 (50%)	1/1 (100%)	–	2/4 (50%)	–	6/9 (66.7%)	–	–	2/5 (40%)	–	–	–	–	–	–
MICof PPy-NPs																				
0.125 mg/ml	7/16 (43.7%)	1/1 (100%)	3/3 (100%)	4/4 (100%)	1/2 (50%)	7/16 (43,7%)	–	3/3 (100%)	2/4 (50%)	2/2 (100%)	2/9 (222%)	–	3/3 (100%)	3/5 (60%)	1/1 (100%)	1/2 (50%)	3/3 (100%)	2/4 (50%)	3/6 (50%)	–
0.062 mg/ml	1/16 (6.2%)	–	–	–	1/2 (50%)	1/16 (6,2%)	–	–	–	–	1/9 (111%)	–	–	–	–	–	–	–	–	–
Not inhibited	8/16 (50%)	–	–	–	–	8/16 (50%)	1/1 (100%)	–	2/4 (50%)	–	6/9 (66,7%)	–	–	2/5 (40%)	–	1/2 (50%)	–	2/4 (50%)	3/6 (50%)	–

MIC, minimum inhibitory concentration; M, milk; HS, hands swab; SN, nasal swab; TC, Teat cup; MB, Milking buckets.

studies have evaluated the antimicrobial activity of aqueous *Moringa oleifera* extract against *Staphylococcus* spp. isolated from the agricultural environment. This makes it difficult to establish comparative parameters for the results we obtained but also indicates this study's novelty. However, there are reports of *Moringa oleifera* activity against *Staphylococcus aureus* and *Staphylococcus epidermidis* (Viera *et al*., 2010; Moura *et al*., 2011; Marrufo *et al*., 2013; Zaffer *et al*., 2014; Fayemi *et al*., 2018; Fouad *et al*., 2019), which corroborate this study.

These results with aqueous *Moringa oleifera* extract are promising, however, further studies are needed to identify the active components and develop effective therapies. Although there is no clear mechanism, there is evidence that the plant's antimicrobial activity is related to a wide spectrum of bactericidal substances produced by the plant (Kostova and Dinchev, 2005; Viera *et al*., 2010). However, the seeds of *M. oleifera* also contain a water-soluble lectin called (WSMoL), which was previously reported to have antibacterial effects (Moura *et al*., 2015, 2017; Coriolano *et al*., 2019).

For PPy-NPs, it was possible to determine MIC at two concentrations, 0.125 and 0.062 mg/ml (Table 2), the first of which delivered the greatest inhibition. The bactericidal activity of PPy-NPs at these concentrations (0.125 and 0.062 mg/ml) was responsible for the inhibitory effect found in the tests to determine MBC. The results differ from those obtained with the aqueous *Moringa oleifera* extract, where there was variability in inhibition across the concentrations. However, PPy-NPs did not inhibit all isolates with the four detected genes, indicating a narrower range of activity as compared with aqueous *Moringa oleifera* extract.

PPy is a polymer obtained through the oxidative chemical or electrochemical polymerization of monomer solutions (Sajesh *et al*., 2013). It is used in engineering and biomedical sciences (de Oliveira and de Oliveira, 2014; Xue *et al*., 2014). The antimicrobial activity of PPy against *Staphylococcus aureus*, as well as other gram-positive and negative bacteria has already been reported (Sayyah *et al*., 2014; da Silva *et al*., 2017). No other reports have investigated *Staphylococcus* spp. isolated from the agricultural environment. Unlike aqueous *Moringa oleifera* extract, in PPy the mechanism responsible for antimicrobial activity has been elucidated. The polymer causes collapse of the cytoplasmic membrane resulting in bacterial death (Varesano *et al*., 2013; Sanchez Ramirez *et al*., 2019). This study demonstrates that PPy-NPs could be used as an alternative to traditional antimicrobials. For this to occur, it is necessary to carry out further studies aimed at drug development.

In conclusion, we have shown that there are genes from multi-drug efflux systems in *Staphylococcus* spp. isolated from the agricultural environment in Northeast Brazil. Aqueous *Moringa oleifera* extract and PPy-NPs both showed bactericidal activity against these isolates carrying multi-drug efflux system genes.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0022029920000874.

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