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

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SUPPLEMENTARY FILE

Supplementary Materials and Methods

Staphylococcus *spp. isolates*

Of the five farms studied, two used mechanical milking; in these farms, swabs were collected from the teat sets (forming pools of each equipment). In the three farms that performed manual milking, swabs were collected from the buckets used for milking.

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 Table S1.

Supplementary Table S1. Distribution by dairy farm of samples collected.

Dairy farms	Milk	Milkers		Environment	
		Hands	Nasal	Teat cup	Milking buckets
A	37	3	3	-	2
B	4	2	2	-	2
C	116	3	3	4	-
D	104	4	4	-	5
E	74	3	3	10	-
Total	**335**	**15**	**15**	**14**	**9**

Isolation and preliminary identification of Staphylococcus *spp.*

Isolation of *Staphylococcus* spp. was performed by direct plating of all samples on manitol salt agar, followed by incubation at 37°C for 24-48 h. All swabs were inoculated into 9mL Mueller Hinton broth (Difco Laboratories Inc., Detroit, USA) containing 6.5% NaCl, after incubation at 37ºC for overnight and plating on manitol salt agar. Then, isolated colonies were subjected to Gram staining technique to check morphology, catalase test, coagulase, and sugar fermentation (Carter, 1998).

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). The DNA obtained was quantified using a spectrophotometer with readings at 260 nm absorbance.

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

For detection of *norA, norB, norC, msrA, mgrA, tet-38,* and *lmrS* genes, all *Staphylococcus* spp. underwent PCR following the methodologies described in Table S2.

Table S2. Genes, family, oligonucleotide sequences and size of amplified fragments of *efflux system genes*.

Gene	Family	Sequence	Fragment Size (pb)	References
norA	MFS[1]	TGCAATTTCATATGATCAATCCC AGATTGCAATTCATGCTAAATATT	150	(Truong-Bolduc et al., 2003)
norB	MFS[1]	ATAAGGTAAGATAACTAGCA ATCTCTATTTGCCTCCCTATA	150	(Truong-Bolduc et al., 2006)
norC	MFS[1]	ATAAATACCTGAAGCAACGCCAAC AAATGGTTCTAAGCGACCAA	200	(Truong-Bolduc et al., 2006)
Tet-38	MFS[1]	TTCAGTTTGGTTATAGACAA CGTAGAAATAAATCCACCTG	200	(Truong-Bolduc et al., 2005)
mgrA	n.c. [2]	CGAATTCATTCATGATT AAAGTTGATTGTTTATTAA	200	(Truong-Bolduc et al., 2005)
msrA	ABC[3]	TCCAATCATTGCACAAAATC AATTCCCTCTATTTGGTGGT	890	(Martineau et al., 2000)

[1]MFS: Major facilitator superfamily (Superfamília dos principais facilitadores); [2]n.c.: not classified in family ; [3]ABC: ATP-binding cassette (Casete ligador de ATP).

The reactions for all genes contained a final volume of 12.5 µL arranged as follows: 100 ng of isolate DNA, 0.5 µL of each primer at 10 pmol (table 1), 6.25 µL of Go Taq Green Master Mix (Promega Corporation, Madison, United States), and 2.5 µL of Milli-Q ultrapure water. To check the amplification, 10 µL of each reaction was submitted to electrophoresis in 2% agarose gel stained with BlueGreen, for 60 min at 100 V. Gels were then visualized under ultraviolet light and photo-documented.

As a positive control, *S. aureus* strains 5EUFV and SE21D, used by the Laboratory of Bacterial Diseases of the Universidade Federal de Viçosa, Minas Gerais, Brazil, were also used in the PCR reactions.

PCR products were purified after amplification and submitted and sequenced bidirectionally using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, USA). After complete editing, contigs were submitted to BLAST of the GenBank database through the NCBI website in order to identify species.

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, 1.08 g of sodium dodecyl sulfate was solubilized in 100 mL Milli-Q water, and then added to 500 μL of pyrrole (0.483 g). The resulting suspension was kept under intense stirring for 45 min. Following this, an aqueous suspension (50 ml) of ammonium persulfate (0.256 g) was slowly added dropwise and stirred for a further 35 min. The resulting suspension of PPy-NPs with a concentration of 2 mg/mL of solute was maintained at 4 °C for 24 h.

PPy-NPs in water (Da Silva Junior *et al.*, 2016) (2 mg/mL) was diluted 1:2 with Mueller-Hinton broth (SIGMA-ALDRICH) that had been inoculated with isolate. The concentrations of PPy-NPs tested were 2 mg/mL, 1 mg/mL, 0.5 mg/mL, 0.250 mg/mL, 0.125 mg/mL, and 0.062 mg/mL. The microdilutions were incubated on plates kept at 35 °C for 20 h. OD readings were performed at 600 nm using the ELISA microplate reader (Multiskan Go Thermo Scientific) for two time points (0 h and 20 h). MIC was defined as the lowest concentration of PPy-NPs in water that inhibited more than 75% of bacterial growth. MBC was defined as the minimum concentration that resulted in inactivation of 99% of bacterial cells. MBC was determined using the plate-plating method with Mueller-Hinton agar, not observing visible bacterial growth.

Moringa oleifera seeds were collected at Recife (Pernambuco, Brazil), powdered using a blender, and stored at -20 °C. A voucher specimen is deposited under number 73,345 at the herbarium Dárdano de Andrade Lima from the Instituto Agronômico de Pernambuco (Recife, Brazil). The plant collection was authorized (number 38690) by the *Instituto Chico Mendes de* Conservação da Biodiversidade (**ICMBio**) from the Brazilian Ministry of Environment.

The extract was prepared by homogenization of seed flour (10 g) with distilled water (100 mL) for 16 h at 4 °C using a magnetic stirrer. The mixture was filtered through a gauze and centrifuged (3000 × *g*, 15 min). The supernatant corresponded to the extract. Protein

concentration was determined according to Lowry *et al*. (1951), using a standard curve of bovine serum albumin (31.25–500 µg/mL). The extract was evaluated for hemagglutinating activity as described in the next section.

The presence of lectins was monitored through the HA assay, which evaluates the functionality of the carbohydrate-binding sites of these proteins. The assay was performed in 96-well microtiter plates (Kartell S.P.A., Italy) according to Paiva & Coelho (1992), using a suspension (2.5%, v/v) in 0.15 M NaCl of rabbit erythrocytes treated with glutaraldehyde (Bing et al., 1967). Finally, HA was quantified as the reciprocal of the highest dilution of sample that promoted full agglutination of erythrocytes. Specific hemagglutinating activity was defined as the ratio between HA and protein concentration (mg/mL).

Aqueous *Moringa oleífera* seed extract was used at initial concentrations of 6.1 mg/mL, 3.05 mg/mL, 1.525 mg/mL, 0.7625 mg/mL, 0.3812 mg/mL, and 0.1906 mg/mL. The conditions for incubation, reading, and determination of MIC and MBC are as described in the previous paragraph.

To prepare the inoculum, *S. aureus* isolates were cultured in plates with a non-selective solid medium Mueller-Hinton Agar (SIGMA-ALDRICH) and incubated at 37 °C for 24 h. The isolated colonies were suspended in 0.9% saline solution until reaching a turbidity equivalent to the 0.5 McFarland standard; contains approximately 1×10^8 CFU/mL (equivalent to an optical density of 0.08-0.13 at 625 nm). Subsequently, MGB was inoculated with the bacterial suspension at a final bacterial concentration of approximately 5×10^5 CFU/mL. The samples were processed in triplicate, with 100 µL deposited in each well of a 96-well microplate. *S. aureus* ATCC 25938, *S. aureus* N315 and *S. epidermidis* ATCC 12228 strains were used as controls.

Results

In this study, 162 *Staphylococcus* spp. were isolated from a sample 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 Supplementary Table S3.

Supplementary Table S3. Isolation, molecular identification of *Staphylococcus* spp. samples from milk,

Sample	n[1]	Isolation of *Staphylococcus* spp.[2]	Molecular identification of *Staphylococcus* spp.[3]	
			S. aureus	**Sna[4]**
Milk	335	115/335 (34,3%)	43/115 (37,4 %)	72/115 (62,6 %)
Milkers hand swab	15	12/ 15 (80%)	8/12 (66.6%)	4/12 (33.3%)
Milkers nasal swab	15	13/15 (86.6%)	2/13 (15.3%)	11/13 (84.7%)
Teat cup swab	14	14/14 (100%)	4/14 (28.5%)	10/14 (71.5%)
Milking bucket swab	9	8/9 (88.8%)	7/8 (87.5%)	1/8 (12.5%)

[1]Samples collected; [2]Based on phenotypical characteristics; [3]Based on PCR for *nuc*. [4]*Staphylococcus* non-*aureus.*

References

Bing, D.H., Weyand, J.G., Stavinsky, A.B. 1967. Hemagglutination with aldehyde-fixed erythrocytes for assay of antigens and antibodies. Proc Soc Exp Biol Med. 124,1166–1170.

Carter, G.R., 1998. Fundamentos de bacteriologia e micologia veterinária.

CLSI, 2018. PerforCLSI. (2018). Performance Standards for Antimicrobial Susceptibility Testing. Clinical and Laboratory Standards Institute. Retrieved from www.clsi.orgmance Standards for Antimicrobial Susceptibility Testing., Performance Standards for Antimicrobial Susceptibility Testing.

da Silva Jr ., F.A.G., Queiroz, J.C., Macedo, E.R., Fernandes, A.W.C., Freire, N.B., da Costa, M.M., de Oliveira, H.P., 2016. Antibacterial behavior of polypyrrole: The influence of morphology and additives incorporation. Mater. Sci. Eng. C 62, 317–322. https://doi.org/10.1016/j.msec.2016.01.067

Fan, A.H.H., Kleven, S.H., Jackwood, M.W., 1995. Application of Polymerase Chain Reaction with Arbitrary Primers to Strain Identification of *Mycoplasma gallisepticum*. Avian Dis. 39, 729–735.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. The J of Biolog Chemistry 193, 265–275.

Martineau, F., Lansac, N., Me, C., Roy, P.H., Ouellette, M., Bergeron, M.G., 2000. Correlation between the resistance genotype determined by multiplex PCR assays and the antibiotic susceptibility patterns of. antimicrob. Agents Chemother. 44, 231–238.

Paiva, P.M.G., Coelho, L.C.B.B., 1992. Purification and partial characterization of two lectin isoforms from *Cratylia mollis Mart*. (*camaratu bean*). Appl Biochem Biotechnol 36, 113–118.

Truong-Bolduc, Q.C., Dunman, P.M., Strahilevitz, J., Projan, S.J., Hooper, D.C., 2005.

MgrA is a multiple regulator of two new efflux pumps in *Staphylococcus aureus*. J. Bacteriol. 187, 2395–2405. https://doi.org/10.1128/JB.187.7.2395-2405.2005

Truong-Bolduc, Q.C., Strahilevitz, J., Hooper, D.C., 2006. *NorC*, a new efflux pump regulated by *MgrA* of *Staphylococcus aureus*. Antimicrob. Agents Chemother. 50, 1104–1107. https://doi.org/10.1128/AAC.50.3.1104-1107.2006

Truong-Bolduc, Q.C., Zhang, X., Hooper, D.C., 2003. Characterization of *NorR* protein, a multifunctional regulator of *norA* Expression in *Staphylococcus aureus*. J. Bacteriol. 185, 3127–3138. https://doi.org/10.1128/JB.185.10.3127-3138.2003