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Resveratrol Susceptibility of *Streptococcus pneumoniae* and *Neisseria meningitidis* Strains Isolated in the State of Minas Gerais, Brazil, from 2007 to 2013

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Abstract

Objective: Evaluation of the *in vitro* susceptibility to Resveratrol of a bacterial collection representing the *S. pneumoniae* and *N. meningitidis* strains prevalent in the Brazilian state of Minas Gerais from 2007 to 2013.

Methods: One reference strain of *S. pneumoniae* (ATCC 49619), and sixty-three strains (31 *S. pneumoniae*, and 32 *N. meningitidis*) isolated from patients with meningitis and available at the certified strains collection of Ezequiel Dias Foundation were tested. The susceptibility to Resveratrol was tested on blood agar containing this drug at eight concentrations ranging from 25 mg/L to 200 mg/L diluted in 0.5% ethanol, and control plates with blood agar with 0.5% ethanol. *Pneumococci* were also tested for susceptibility to currently available antimicrobials used to treat meningitis using E-test and disc diffusion methods. The association between pneumococcal susceptibility to Resveratrol and to any other antibiotic tested was assessed with chi-square test, and the toxic doses of Resveratrol were determined upon L929 mammalian cells.

Results: The MIC100 for Resveratrol was 75 mg/L for *meningococci* (range: 50-75 mg/L), and 200 mg/L for *pneumococci* (range: 125-200 mg/L). There was no association between pneumococcal susceptibility to Resveratrol and to any currently available antimicrobials tested suggesting different modes of action. However, low selectivity indices (SI) calculated as the ratio between the IC_{100} in L929 cells and the MIC values were found for *meningococci* (0.332) and *pneumococci* (0.125).

Conclusion: Resveratrol inhibited the growth of all *N. meningitidis* and *S. pneumoniae* strains causing meningitis in the state of Minas Gerais, Brazil, from 2007 to 2013. Our results, despite the low selectivity indices observed, may warrant further studies to assess the potential of Resveratrol derivates as antimicrobial alternatives to treat meningococcal and pneumococcal infections.

Keywords: *Streptococcus pneumonia*; *Neisseria meningitides*; Resveratrol; 3,5,4'-trihydroxylstilbene; Bacterial meningitis; Antibacterials; Natural Compounds

Introduction

Neisseria meningitidis and *Streptococcus pneumoniae* are the most prevalent etiological agents of bacterial meningitis in Brazil [1]. *S. pneumoniae* resistance to penicillin has become a major global concern, and resistance has been linked to worse clinical outcomes in patients with pneumococcal meningitis [2]. In a recently published report, the World Health Organization warned that its six surveillance regions had national reports of 25% resistance or more for *pneumococci*, in some cases exceeding 50% resistance or non-susceptibility to penicillin [3]. Despite the urgent need for new antimicrobials, the high cost and complexity of the process of new antibiotics discovery has hindered the release of new drugs. Therefore the repurposing of existing drugs with known pharmacokinetics and safety profiles turns out to be an attractive approach to fight against multi resistant bacteria. Aligned with this purpose, Docherty and collaborators [4] reported that Resveratrol (3,5,4'-trihydroxylstilbene) selectively inhibited one reference strain of *N. meningitidis* (ATCC 13090). Resveratrol is a natural compound produced by grapevines, peanuts and other plants in response to interactions with pathogens. Antifungal, antibacterial, antiviral, and antiparasitic activities of Resveratrol have been reported [5-8].

In the present study we investigated the *in vitro* susceptibility to Resveratrol of a bacterial collection representing the *S. pneumoniae* and *N. meningitidis* strains prevalent in the Brazilian state of Minas Gerais from 2007 to 2013.

Materials and Methods

The bacterial collection tested in this study comprised sixty-three strains (31 *S. pneumoniae*, and 32 *N. meningitidis*) isolated from

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patients with meningitis, which have been identified using standard methods and maintained in the certified strains collection of Ezequiel Dias Foundation. In addition, one reference strain of *S. pneumoniae* (ATCC 49619) was also included in this study. The susceptibility to Resveratrol (Sigma-Aldrich, St Louis, MO), expressed as the minimum inhibitory concentration (MIC), was determined on blood agar containing this drug at eight concentrations ranging from 25 to 200 mg/L diluted in 0.5% ethanol, and control plates with blood agar with 0.5% ethanol [4]. All strains were tested in duplicates and incubated at 37°C, for 24 h, under ambient air or atmosphere with 5% CO_2. MIC100 was defined as the lowest concentration of Resveratrol that completely inhibited any visible growth.

Pneumococci were also tested for susceptibility to ceftriaxone and penicillin using E-test method, and to chloramphenicol, clindamycin, erythromycin, ofloxacin, oxacylin, rifampicin, tetracycline, trimethoprim-sulfametoxazol, and vancomycin using the disc diffusion method (Probac, São Paulo, Brazil). Experimental procedures and interpretation of results were performed according to clinical and laboratory standards institute (CLSI) clinical breakpoints [9]. In order to shed light on the mode of action of Resveratrol, we tested the association between the susceptibility to this drug and to the antibiotics mentioned above. Briefly, *pneumococci* were divided into two groups according to their MIC100 to Resveratrol: a) 200 mg/L; b) ≤ 175 mg/L. Then, a confusion matrix was built for each antibiotic tested distributing the strains into the groups "a" and "b" above according to their susceptibility or resistance to the respective antibiotic (Table 1 exemplifies a confusion matrix). Associations were assessed with chi-square test using GraphPad 5.0 (GraphPad Software, San Diego, CA).

Aiming to determine the toxic doses of Resveratrol upon L929 mammalian cells (mouse C3H/An connective tissue-ATCC CCL-1; cultured in Roswell Park Memorial Institute medium (RPMI)+2 mM Glutamine+10% foetal bovine serum (FBS)), after 4 days of compound exposure, 10% Alamarblue dye (Invitrogen, San Diego, CA) was added and the absorbance at 570 and 600 nm was measured after 4-6 h. The cell viability was expressed as the percentage of difference in the reduction between treated and untreated cells [10].

		RSV MIC100=200 mg/L	RSV MIC100 ≤ 175 mg/L
Susceptible	Strains	731/09; 148/10; 080/11; 84/11; 143/11; 149/11; 511/11; 43/12	317/08; 144/09; 435/09; 779/09; 345/11
	N	8	5
Resistant or Intermediate	Strains	883/07; 1070/07; 1180/07; 176/08; 305/08; 262/08; 619/08; 159/08; 620/09; 127/10; 421/10; 029/11; 120/11; 197/11; 380/12	295/08; 585/10; 124/11
	N	15	3

Table 1: Confusion matrix of trimethoprim-sulfametoxazol resistance versus Resveratrol MIC100 of *Streptococcus pneumoniae* strains. MIC: minimum inhibitory concentration.

Results and Discussion

Resveratrol inhibited all *S. pneumoniae* and all *N. meningitidis* strains tested (Tables 2 and 3). Some strains showed higher MICs when incubated under atmosphere with 5% CO_2 than in ambient air. The MIC100 was 75 mg/L for *N. meningitidis* (MIC range: 50 mg/ml-75 mg/L), and 200 mg/L for *S. pneumoniae* (MIC range: 125 mg/L-200 mg/L). It is worth noting that the reference strain of *N. meningitidis* tested by Docherty and collaborators [4] had MIC of 125 mg/L.

Although the antimicrobial activity of Resveratrol and some of its derivatives and natural oligomers has been demonstrated against various pathogenic bacteria, the mode of action of this drug has not been elucidated yet [11,12]. Preliminary studies suggest that the antibacterial activity of Resveratrol involves "quorum sensing" proteins, hyperpolarization of the bacterial membrane potential, inhibition of macromolecules biosynthesis, and inhibition of the virulence factor type III secretion system (T3SS) [13-15]. For the 32 *pneumococci* tested, there was no association between high (200 mg/L) or low (≤ 175 mg/L) levels of Resveratrol susceptibility and the susceptibility to any of the antibiotics tested. These findings suggest that Resveratrol and the antibiotics often used to treat pneumococcal infections do not share the same mode of action.

Furthermore, 58.1% of *pneumococci* clinical isolates, representing the strains occurring in Minas Gerais from 2007 to 2013, were nonsusceptible (resistant or intermediate) to trimethoprim-sulfamethoxazole, while only a low percentage were resistant to erythromycin (6.4%), or clindamycin (3.2%). All isolates tested in the present study were susceptible to ceftriaxone. Similar results had been previously reported by Mantese and collaborators [16] who found 79.5% nonsusceptibility to trimethoprim-sulfamethoxazole, 11.3% resistance to erythromycin, 11.3% to clindamycin, and 5.6% to ceftriaxone (5.6%) among Brazilian *pneumococci* isolates. In Germany, the overall *pneumococci* non-susceptibility rates were 11.0% for trimethoprim-sulfamethoxazole, 5.5% for clindamycin, 0.7% for levofloxacin, and 8.5% for tetracycline [17].

Resveratrol at 25 mg/L induced 100% cellular death (IC_{100}) in L929. The Selectivity Index (SI), a parameter used to estimate the therapeutic dose window of a candidate drug, was calculated as the ratio between the IC_{100} in L929 mammalian cell line and the MIC values. The SIs were 0.332 for *meningococci* and 0.125 for *pneumococci*, indicating low therapeutic selectivity.

In conclusion, Resveratrol inhibited the growth of all *N. meningitidis* and *S. pneumoniae* strains causing meningitis in the state of Minas Gerais, Brazil, from 2007 to 2013. To our knowledge, this is the first report of pneumococcal susceptibility to Resveratrol, and the first study to assess the antibacterial effect of this drug against a strain collection with epidemiological relevance for meningitis.

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Strain ID	Year of isolation	Age (years)	Gender	Serotype	CEF	CLI	CO	ERI	OF	OXA	PEN	RIF	ST	TT	VAN	MIC100 RSV (mg/L)- ambient air	MIC100 RSV (mg/L) - atmosphere with 5% CO_2
883/07	2007	41	F	11A	S	S	S	S	S	S	S	S	R	S	S	200	200
1070/07	2007	57	M	11F	S	S	S	S	S	S	S	S	R	S	S	200	200
1180/07	2007	29	M	6A/C	S	S	S	S	S	S	S	S	S	S	S	200	200
176/08	2008	33	M	7C	S	S	S	S	S	S	S	S	R	S	S	200	200
295/08	2008	2	F	1	S	S	S	S	S	S	S	S	R	S	S	150	150
305/08	2008	9	F	6A/C	S	S	S	S	S	S	S	S	R	S	S	200	200
317/08	2008	57	F	34	S	S	S	S	S	S	S	S	S	S	S	125	125
262/08	2008	9	F	6A/C	S	S	S	S	S	S	S	S	R	S	S	200	200
619/08	2008	46	M	6A/C	S	S	S	S	S	S	S	S	R	S	S	200	200
159/09	2009	77	M	23B	S	S	S	S	S	R	R	S	R	S	S	200	200
144/09	2009	77	M	35B	S	S	S	S	S	S	S	S	S	S	S	150	175
435/09	2009	71	M	28A	S	S	S	S	S	S	S	S	S	R	S	125	125
620/09	2009	22	F	9N	S	S	S	S	S	S	S	S	I	S	S	200	200
731/09	2009	1	M	7F	S	S	S	S	S	S	S	S	S	S	S	125	200
779/09	2009	58	M	15C	S	S	S	S	S	S	S	S	S	S	S	150	175
127/10	2010	41	M	6C	S	S	S	S	S	R	R	S	R	S	S	200	200
148/10	2010	12	M	13	S	S	S	S	S	S	S	S	S	S	S	150	200
421/10	2010	75	F	10A	S	S	S	S	S	S	S	S	R	S	S	125	200
585/10	2010	50	F	9V	S	R	S	R	S	S	S	S	R	S	S	175	175
029/11	2011	55	M	23F	S	S	R	S	S	R	R	S	R	R	S	175	200
080/11	2011	12	M	24F	S	S	S	S	S	S	S	S	S	S	S	200	200
84/11	2011	3	F	12F	S	S	S	S	S	S	S	S	S	S	S	200	200
120/11	2011	4	F	18C	S	S	S	S	S	S	S	S	I	S	S	200	200
124/11	2011	13	M	19A	S	S	S	S	S	S	R	S	R	S	S	150	175
143/11	2011	3	M	6B	S	S	S	S	S	S	S	S	S	S	S	200	200
149/11	2011	<1	F	9N	S	S	S	S	S	S	S	S	S	S	S	200	200
345/11	2011	<1	M	4	S	S	S	S	S	S	S	S	S	S	S	175	175
197/11	2011	4	F	3	S	S	S	S	S	R	R	S	R	S	S	175	200
511/11	2011	45	M	9V	S	S	S	S	S	S	S	S	S	S	S	200	200
380/12	2012	32	M	14	S	S	S	R	S	R	R	S	R	S	S	200	200
43/12	2012	48	M	3	S	S	S	S	S	S	S	S	S	S	S	150	200

Table 2: Epidemiological data and antimicrobial susceptibility of *Streptococcus pneumoniae* strains. Epidemiological data and results of susceptibility tests to antibiotics and Resveratrol of *pneumococci* strains causing meningitis in Minas Gerais, Brazil, from 2007 to 2013. M: male; F: female; S: susceptible; I: intermediate; R: resistant; MIC: minimum inhibitory concentration; CEF: ceftriaxone; CLI: clindamycin; CO:

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chloramphenicol; ERI: erythromycin; OF: ofloxacin; OXA: oxacylin; PEN: penicillin; RIF: rifampicin; TT: tetracycline; ST: trimethoprim-sulfametoxazol; VAN: vancomicin; RSV: Resveratrol

The lack of association between pneumococcal susceptibility to Resveratrol and the antibiotics tested herein suggests that the former does not share the same mode of action with the currently available antimicrobials used to treat meningitis. Our results, despite the low selectivity indices observed, may warrant further studies to assess the potential of Resveratrol derivates as antimicrobial alternatives to treat meningococcal and pneumococcal infections.

Strain ID	Year of isolation	Age (years)	Gender	Sero group	MIC100 RSV (mg/L) - ambient air	MIC100 RSV (mg/L) - atmosphere with 5% CO_2
78/08	2008	7	F	Y	50	50
92/08	2008	73	M	W135	75	75
264/08	2008	14	F	B	75	75
563/08	2008	58	F	W135	75	75
100/09	2009	NA	NA	W135	75	75
119/09	2009	NA	NA	W135	75	50
281/09	2009	15	M	Y	75	75
24/10	2010	12	F	C	50	75
35/10	2010	50	M	C	50	75
57/10	2010	57	F	C	50	50
56/10	2010	11	M	C	50	50
108/10	2010	44	F	C	75	75
131/10	2010	5	F	B	75	75
132/10	2010	1	F	Y	50	50
138/10	2010	64	M	C	50	50
177/10	2010	5	F	C	75	75
201/10	2010	6	F	B	50	75
215/10	2010	10	M	C	50	50
42167	2012	21	F	C	50	75
42320	2012	6	F	C	75	75
15/12	2012	4	M	B	75	75
78/12	2012	30	M	C	50	75
86/12	2012	15	F	C	75	75
96/12	2012	22	M	C	50	75
101/12	2012	40	M	C	50	75
170/12	2012	NA	NA	NA	50	50
175/12	2012	<1	M	B	50	50
326/12	2012	73	F	W135	75	75
345/12	2012	18	M	B	75	75
616/12	2012	<1	F	B	50	75

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843/12	2012	18	F	B	75	75
17/13	2013	39	M	C	75	75

Table 3: Epidemiological data and Resveratrol susceptibility of *Neisseria meningitidis* strains. Epidemiological data and results of susceptibility test to Resveratrol of *meningococci* strains causing meningitis in Minas Gerais, Brazil, from 2007 to 2013. M: male; F: female; NA: non available; MIC: minimum inhibitory concentration; RSV: Resveratrol

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Conflict of interest

The authors have no conflict of interest to declare.

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