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 IC50 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 pneumoniae*; *Neisseria meningitidis*; 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'-trihydroxyxystilbene) 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...
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 \textit{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.

\textit{Pneumococci} were also tested for susceptibility to ceftriaxone and penicillin using E-test method, and to chloramphenicol, clindamycin, erythromycin, ofloxacin, oxacillin, rifampicin, tetracycline, trimethoprim-sulfamethoxazole, 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, \textit{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].

| Susceptible | RSV MIC100=200 mg/L | RSV MIC100 ≤ 175 mg/L |
|------------|---------------------|----------------------|
| Strains    | 731/09; 148/10; 080/11; 84/11; 143/11; 149/11; 511/11; 43/12 | 317/08; 144/09; 345/11 |
| N          | 8                   | 5                    |
| Resistant or Intermediate | Strains | 883/07; 1079/07; 1180/07; 176/08; 305/08; 262/08; 619/08; 159/08; 620/09; 127/10; 421/10; 028/11; 120/11; 197/11; 380/12 | 295/08; 585/10; 124/11 |
| N          | 15                  | 3                    |

Table 1: Confusion matrix of trimethoprim-sulfamethoxazole resistance versus Resveratrol MIC100 of \textit{Streptococcus pneumoniae} strains. MIC: minimum inhibitory concentration.

**Results and Discussion**

Resveratrol inhibited all \textit{S. pneumoniae} and all \textit{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 \textit{N. meningitidis} (MIC range: 50 mg/ml-75 mg/L), and 200 mg/L for \textit{S. pneumoniae} (MIC range: 125 mg/L-200 mg/L). It is worth noting that the reference strain of \textit{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 \textit{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 \textit{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 pneumococcal isolates. In Germany, the overall \textit{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 1929 mammalian cell line and the MIC values. The SIs were 0.332 for \textit{meningococci} and 0.125 for \textit{pneumococci}, indicating low therapeutic selectivity.

In conclusion, Resveratrol inhibited the growth of all \textit{N. meningitidis} and \textit{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.
Table 2: Epidemiological data and antimicrobial susceptibility of *Streptococcus pneumoniae* strains. Epidemiological data and results of *Neisseria meningitidis* Strains Isolated in the State of Minas Gerais, Brazil, from 2007 to 2013. J Meningitis 1: 101. doi: 10.4172/2572-2050.1000101

| 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% CO₂ |
|-----------|------------------|-------------|--------|----------|-----|-----|----|-----|----|-----|-----|-----|----|----|-----|-----------------------------|----------------------------------|
| 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   | S  | R  | S   | 150                         | 150                              |
| 305/08    | 2008             | 9           | F      | 6A/C    | S   | S   | S  | S   | S  | S   | S   | S   | S  | R  | S   | 200                         | 200                              |
| 317/08    | 2008             | 57          | F      | 3        | 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   | S  | R  | S   | 200                         | 200                              |
| 619/08    | 2008             | 46          | M      | 6A/C    | S   | S   | S  | S   | S  | S   | S   | S   | S  | R  | S   | 200                         | 200                              |
| 159/09    | 2009             | 77          | M      | 23B      | S   | S   | S  | S   | S  | R   | R   | R   | S  | R  | 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      | 26A      | S   | S   | S  | S   | S  | S   | S   | S   | S  | S  | R   | 125                         | 125                              |
| 620/09    | 2009             | 22          | F      | 9N       | S   | S   | S  | S   | S  | S   | S   | S   | S  | I  | 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   | R   | S  | R  | 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   | S  | R  | S   | 125                         | 200                              |
| 585/10    | 2010             | 50          | F      | 9V       | S   | R   | S  | R   | S  | S   | S   | S   | S  | R  | S   | 175                         | 175                              |
| 029/11    | 2011             | 55          | M      | 23F      | S   | S   | R  | S   | S  | R   | R   | R   | S  | R  | R   | 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   | S  | I  | S   | 200                         | 200                              |
| 124/11    | 2011             | 13          | M      | 19A      | S   | S   | S  | S   | S  | R   | R   | R   | S  | R  | 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          | M      | 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   | R   | S  | R  | 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   | R  | R   | R   | R   | S  | R  | S   | 200                         | 200                              |
| 43/12     | 2012             | 48          | M      | 3        | S   | S   | S  | S   | S  | S   | S   | S   | S  | S  | S   | 150                         | 200                              |
chloramphenicol; ERI: erythromycin; OF: ofloxacin; OXA: oxacillin; PEN: penicillin; RIF: rifampicin; TT: tetracycline; ST: trimethoprim-sulfamethoxazole; VAN: vancomycin; 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% CO₂ |
|-----------|------------------|-------------|--------|------------|---------------------------------|----------------------------------------|
| 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                                     |
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.

| Year | 2012 | 2013 |
|------|------|------|
| No. | 18 | 39 |
| Sex | F | M |
| MIC | 75 | 75 |
| RSV | 75 | 75 |

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Conflict of interest

The authors have no conflict of interest to declare.

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