Circulating isolates of *Neisseria mucosa* do not inhibit the growth of *Neisseria gonorrhoeae*

Saïd Abdellati[1$]

Jolein Laumen[1$]

Natalia Gonzalez[1]

Sheeba S. Manoharan-Basil[1]

Christophe Van Dijck[1]

Tessa De Block[1]

Irith De Baetselier[1]

Delphine Martiny[2,3]

Chris Kenyon[1,4*]

[$] *These two authors contributed equally to this publication*

[1]*HIV/STI Unit, Institute of Tropical Medicine, Antwerp, Belgium*

[2]*Department of Microbiology, Laboratoire Hospitalier Universitaire de Bruxelles, Pôle Hospitalier Universitaire de Bruxelles, Université Libre de Bruxelles, Brussels, Belgium,*

[3]*Faculté de Médecine et Pharmacie, Université de Mons, Mons, Belgium*

[4]*Division of Infectious Diseases and HIV Medicine, University of Cape Town, Anzio Road, Observatory 7700, South Africa*

*Corresponding author. HIV/STI Unit, Institute of Tropical Medicine, Antwerp, 2000, Belgium. Tel: +32 3 2480796; Fax: +32 3 2480831; E-mail: ckenyon@itg.be

 doi:10.20944/preprints202108.0468.v1

Running title: *Neisseria mucosa* does not inhibit *Neisseria gonorrhoeae*

Keywords: *Neisseria mucosa*, *Neisseria gonorrhoeae*, inhibition

Abstract:

We used agar overlay assays to assess if 24 circulating and historical isolates of *Neisseria mucosa* could inhibit the growth of 28 circulating and historical isolates of *N. gonorrhoeae.* We found no evidence of inhibition by *N. mucosa* (n=24). Positive controls *Streptococcus pneumoniae* and *Escherichia coli* demonstrated a strong inhibitory effect against the growth of *N. gonorrhoeae*.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 24 August 2021 doi:10.20944/preprints202108.0468.v1

Introduction

Does *Neisseria mucosa* inhibit the growth of *Neisseria gonorrhoeae,* as recently reported by Aho et al. [1]? This is an important question as it may help explain the paradoxical findings of our recently published PReGo Study [2]. This placebo-controlled trial randomized high-risk men who have sex with men to intensive use of Listerine® mouthwash and gargle or placebo to try to reduce the incidence of bacterial STIs in this population, where oral sex plays an important role in STI transmission [3-5]. The study found that Listerine increased rather than decreased the incidence of oropharyngeal *N. gonorrhoeae*. Listerine® had a similar though statistically non-significant effect in the OMEGA study [6]. *N. mucosa* is a healthy core component of the oropharyngeal microbiome and even low concentrations of Listerine® have been shown to be bacteriocidal to *Neisseria spp* [7]. If the use of the Listerine® mouthwash reduced the prevalence/abundance of *N. mucosa* and *N. mucosa* inhibits the growth of *N. gonorrhoeae*, then Listerine® could increase the susceptibility for *N. gonorrhoeae* infection [2]. In a similar vein, a randomized controlled trial established that nasal inoculation with *N. lactamica* reduced the incidence of colonization with *N. meningitidis* [8]. If the in-vitro anti-gonococcal effect of *N. mucosa* could be confirmed, *N. mucosa* might be evaluated as a probiotic to prevent gonococcal infection.

As a first step in evaluating this hypothesis, we tested if our locally circulating isolates of *N. mucosa* and other commensal *Neisseria*, including those circulating in the PReGo participants, were able to inhibit *N. gonorrhoeae.* We found that *N. mucosa* did not exert this inhibitory effect. The inhibitory effect of a number of bacterial species such as various streptococcal species on *N. gonorrhoeae* is well established [9-13]. We included a number of these species as positive controls in our experiments.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 24 August 2021 doi:10.20944/preprints202108.0468.v1

We also obtained one of the *N. mucosa* strains used by Aho et al. and confirmed that, in our laboratory, this strain did not inhibit the growth of *N. gonorrhoeae*. We did, however, note that *N. mucosa* exerted a repellant effect on the top agar layer in the agar overlay experiments. This created an optical illusion of reduced growth of *N. gonorrhoeae*, which may explain the discordant findings between Aho et al., and ourselves.

Methods

Inhibitory ("producer") bacterial isolates

We obtained *Neisseria* isolates from two clinical studies conducted at our centre:

PReGo (Preventing Resistance in Gonorrhoea Study) isolates

This was a single center randomized controlled trial at the Institute of Tropical Medicine in Antwerp, Belgium, between 2019 and 2020 that assessed the efficacy of an antiseptic mouthwash to prevent STIs among 343 MSM using PrEP [2]. A subgroup of 64 participants were selected to participate in a survey of their oropharyngeal microbiomes [2].

ComCom (Commensals in the Community Study) isolates

In June 2020, Institute of Tropical Medicine (ITM) employees were invited to participate in a survey of their oropharyngeal microbiomes [14]. The first 32 eligible employees (male or female) presenting to the study team were included in this survey.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 24 August 2021 doi:10.20944/preprints202108.0468.v1

Sample processing

Culture

The details of how the samples were taken and processed has been described elsewhere [2]. In brief, for each individual, an oropharyngeal swab (ESwab™ COPAN Diagnostics Inc., Italy) was taken and inoculated onto blood and Modified Thayer-Martin agar plates using the streak plate technique and incubated at 35-37°C and 5% CO_2. Plates were examined after 48 hours and *Neisseria*-like colonies were selected based on a positive Oxidase test and a Gram stain. *Neisseria*-like colonies were enriched on blood agar plates and stored in skim milk at -80°C.

Identification

Cultures of *Neisseria*-like colonies were shipped to Centre Hospitalier Universitaire Saint-Pierre where species were identified using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS).

Agar overlay inhibition testing

We tested all *N. mucosa* isolates obtained during these two studies (n=14) as well as a random selection of *N. meningitidis* (n=3) and other commensal *Neisseria* obtained from these two studies – *N. subflava* (n=4), *N. cinerea* (n=2), *N. lactamica* (n=1), *N. oralis* (n=3), *N. elongata* (n=1) and *Neisseria* spp. (n=1; Table 1). We also included 6 *N. mucosa* isolates from our ITM historical collection. Five of these were ATCC strains and one was a historical clinical specimen obtained from a patient in 1977. The DSM4631/ATCC 25996 isolate used by Aho et al., was obtained from the DSMZ (https://www.dsmz.de/collection/catalogue/details/culture/DSM-46).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 24 August 2021 doi:10.20944/preprints202108.0468.v1

As positive controls, we used ATCC strains of *Streptococcus pneumoniae* (n=1) and *Escherichia coli* (n=1). We also assessed the inhibitory effects of three other bacterial species obtained from ATCC: *Staphylococcus aureus* (n=2), *Streptococcus pyogenes* (n=1) and *Lactobacillus crispatus* (n=1).

N. gonorrhoeae target strains

Three strains of *N. gonorrhoeae* were used as target strains for all experiments (WHO-F, WHO-X and MoNg003 – a clinical isolate obtained from an individual with asymptomatic pharyngeal *N. gonorrhoeae* infection attending our STI clinic in 2020. In addition, one ATCC strain of *N. gonorrhoeae*, WHO-W and 23 other circulating strains of *N. gonorrhoeae* were tested against some of the putative inhibitory bacteria (Table 1).

Agar overlay assay

The details of the agar overlay assay have been described elsewhere [1]. Briefly, all strains used in the experiment were propagated on blood agar plates for 18-24h. The cultures were suspended in 10 µl of phosphate-buffered saline (PBS) containing 10^9 CFU/ml of producer strains. These were spotted onto GC agar and incubated for 24h. 10 ml of melted GCB agar containing 10^6 CFU/ml of a target strain was added to each spotted plate. The plates were then re-incubated for 24 to 48 hours. The diameter of the zone of inhibition surrounding each producer strain was assessed at 24 hours.

Results

None of the commensal *Neisseria* or *N. meningitidis* exhibited any activity against *N. gonorrhoeae (Table 1).*

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 24 August 2021 doi:10.20944/preprints202108.0468.v1

The colonies of *N. mucosa* exhibited a consistently repellant effect on the layer of agar poured over them (Fig. 1). This created 'pitting colonies' or a convex slope between the top of the second layer of agar and the edge of each *N. mucosa* colony, which created an illusion of reduced *N. gonorrhoeae* growth around each *N. mucosa* colony [15]. Closer inspection, however, confirmed that *N. gonorrhoeae* growth over this convex slope around the *N. mucosa* colonies was not macroscopically distinguishable from that elsewhere (Fig. 1).

The isolate of *Streptococcus pneumoniae* demonstrated clear evidence of inhibition against all 9 strains of *N. gonorrhoeae* (median diameter of inhibition = 21 mm). The inhibitory effect of *E. coli* was less pronounced. Inhibition was evident in 3 out of 9 *N. gonorrhoeae* strains tested – median diameter of inhibition 11mm.

Discussion

Unlike Aho et al., we could find no evidence that *N. mucosa* or any other commensal *Neisseria* was able to inhibit the growth of *N. gonorrhoeae [1].* This was despite using a large number of clinical and reference strains of *N. gonorrhoeae* as target strains, and the largest collection of commensal *Neisseria* tested to date as inhibitory bacteria.

How can these discordant findings be explained? Aho found this inhibitory effect in 3 out of 5 *N. mucosa* isolates. The isolates were all taken from ATCC collections and the inhibition assays were performed by undergraduates as part of a microbiology course.

No photos were provided of the agar overlay assays showing that *N. mucosa* inhibited the growth of *N. gonorrhoeae*. However, one image of *N. mucosa* inhibiting the growth of *N. flavescens* was provided.

In our study, we followed an identical agar overlay protocol utilizing a larger panel of isolates of *N. mucosa* and *N. gonorrhoeae*. The experiments were performed by a laboratory technician with over 25 years of experience culturing *Neisseria* species (SA). The plates were examined by this person and two others with extensive experience in culturing *Neisseria* species (CK and JL). All three concurred that pitting around each colony of *N. mucosa* created an optical illusion of decreased growth around the colony. Close inspection confirmed that there was no inhibition of growth.

We consider this a parsimonious explanation for the different findings between the two studies. It could be possible that only certain strains of *N. mucosa* are able to inhibit specific strains of *N. gonorrhoeae* and that we did not include any of these combinations in our experiments. We did, however, test one of the three isolates of *N. mucosa* shown to have an inhibitory effect by Aho et al. This isolate (ATCC 25996) had no effect on the growth of 23 contemporarily circulating strains of *N. gonorrhoeae* in our laboratory. We did not have access to, and therefore did not include any of the same strains of *N. gonorrhoeae* used by Aho et al. As a result, we cannot exclude the possibility that our *N. mucosa* strains would have had an inhibitory effect on the *N. gonorrhoeae* strains used by Aho et al.

We cannot completely exclude the possibility that an unevaluated different experimental condition such as the source of the agar used was responsible for the differences in the results between the two studies.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 24 August 2021 doi:10.20944/preprints202108.0468.v1

Our study, unlike that of Aho et al., did include positive controls. These showed clear and consistent evidence of inhibition. Taken together, these findings suggest that *N. mucosa* is unlikely to have a significant inhibitory effect on the growth of *N. gonorrhoeae* – at least in the agar overlay assays evaluated here. More importantly for our current research, we consider it unlikely that a broad range of *N. mucosa* isolates contains a sufficiently potent compound against our currently circulating strains of *N. gonorrhoeae* to be able to explain the findings of the PReGo and OMEGA studies.

We concur with Aho et al., that commensal microbes represent a possible source of antimicrobial compounds that could play an important role in reducing the emergence of AMR in *N. gonorrhoeae* and other bacteria. Based on our findings, we consider it more likely that such anti-gonococcal compounds will be discovered from organisms such as *S. pneumoniae* than *N. mucosa* [12, 16].

Acknowledgements

We would like to thank the PReGo and ComCom study participants for providing the samples used in this study.

Authors' contributions

SA conceptualized the study. SA, NG and CK were responsible for the acquisition, analysis and interpretation of data. CK and SA wrote the first draft. All authors revised the manuscript and approved the final version.

 doi:10.20944/preprints202108.0468.v1

Consent for publication

Not applicable

Data availability

The data we used is provided in the article

Funding

No specific funding was received for this work.

Competing interests

None to declare. All the authors declare that they have no conflicts of interest.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 24 August 2021 doi:10.20944/preprints202108.0468.v1

Table 1. Inhibitory activity of various commensal *Neisseria* and other species in agar overlay assay

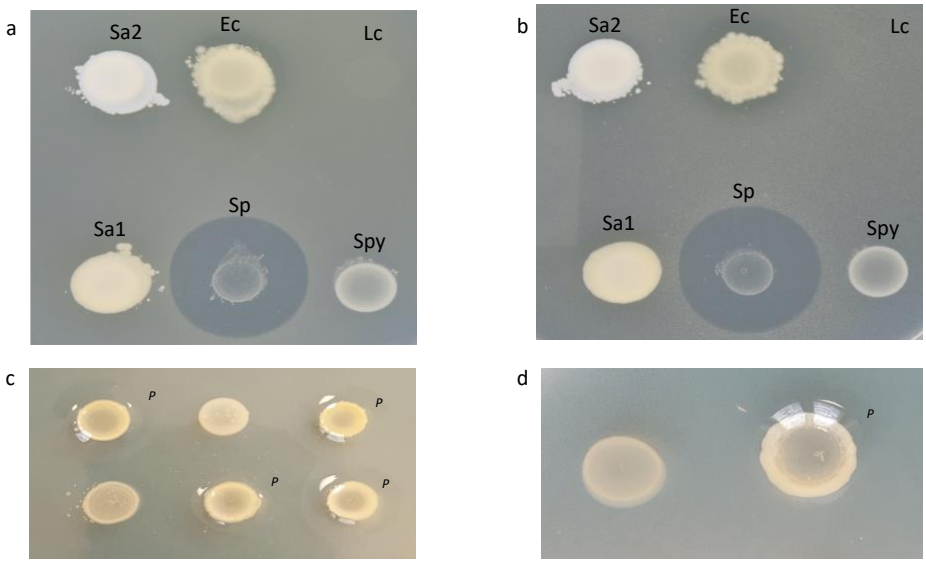

Figure 1. Agar overlay assay demonstrating strong inhibititory effect that colonies *Streptococcus pneumoniae* (Sp - ATCC 49619) and to lessor extent *Escherichia coli* (Ec – ATCC 25922) have on the growth of a lawn of *Neisseria gonorrhoeaea* strain RL1 in figure (1a) and strain 21.189 in figure (1b). Strains of *Staphylococcus aureus* (Sa1- ATCC 29213; Sa2 – ATCC 25913), *Streptococcus pyogenes* (Spy – LMG 14238) and Lactobacillus crispatus (LMG 9479) do not exhit any inhibitory effect in these figures. Figure 1c shows that none of 6 strains of *Neisseria mucosa* inhibit the growth of a lawn of *N. gonorrhoeae* strain 21.163. Four of these colonies do however show evidence of 'pitting' (*P*) - a convex slope between the top of the second layer of agar and the edge of the *N. mucosa* colony. Figure 1d is a close up of two colonies of *N. mucosa* with an overgrowth of *N. gonorrhoeae* WHO-W illustrating a colony without (left) and with 'pitting' (*P* – right).

References

1. Aho EL, Ogle JM, Finck AM. The Human Microbiome as a Focus of Antibiotic Discovery: Neisseria mucosa Displays Activity Against Neisseria gonorrhoeae. Front Microbiol. 2020;11. doi: ARTN 577762 10.3389/fmicb.2020.577762. PubMed PMID: WOS:000599309800001.
2. Van Dijck C, Tsoumanis A, Rotsaert A, Vuylsteke B, Van den Bossche D, Paeleman E, et al. Antibacterial mouthwash to prevent sexually transmitted infections in men who have sex with men taking HIV pre-exposure prophylaxis (PReGo): a randomised, placebo-controlled, crossover trial. The Lancet Infectious Diseases. 2021;21(5):657-67.
3. Wong ML, Chan RK, Koh D, Wee S. Increase in oral sex and pharyngeal gonorrhoea: an unintended effect of a successful condom promotion programme for vaginal sex. AIDS. 1999;13(14):1981-2. PubMed PMID: 10513660.
4. Centers for Disease C, Prevention. Transmission of primary and secondary syphilis by oral sex--Chicago, Illinois, 1998-2002. MMWR Morb Mortal Wkly Rep. 2004;53(41):966-8. Epub 2004/10/22. PubMed PMID: 15496825.
5. Barbee LA, Khosropour CM, Dombrowski JC, Manhart LE, Golden MR. An estimate of the proportion of symptomatic gonococcal, chlamydial and non-gonococcal non-chlamydial urethritis attributable to oral sex among men who have sex with men: a case-control study. Sex Transm Infect. 2016;92(2):155-60. Epub 2015/08/25. doi: 10.1136/sextrans-2015-052214. PubMed PMID: 26297719; PubMed Central PMCID: PMCPMC4861816.
6. Chow EP, Williamson DA, Hocking JS, Law MG, Maddaford K, Bradshaw CS, et al. Antiseptic mouthwash for gonorrhoea prevention (OMEGA): a randomised, double-blind, parallel-group, multicentre trial. The Lancet Infectious Diseases. 2021;21(5):647-56.
7. Laumen J.G.E., Abedllati S, De Baetselier I, Crucitti T, Xavier B.B., Chapelle S., et al. Mechanisms of increased resistance to chlorhexidine following exposure of Neisseria gonorrhoeae isolates to chlorhexidine. ECCMID 2021. 2021.
8. Deasy AM, Guccione E, Dale AP, Andrews N, Evans CM, Bennett JS, et al. Nasal Inoculation of the Commensal Neisseria lactamica Inhibits Carriage of Neisseria meningitidis by Young Adults: A Controlled Human Infection Study. Clin Infect Dis. 2015;60(10):1512-20. Epub 2015/03/31. doi: 10.1093/cid/civ098. PubMed PMID: 25814628.
9. Graver MA, Wade JJ. The role of acidification in the inhibition of Neisseria gonorrhoeae by vaginal lactobacilli during anaerobic growth. Ann Clin Microb Anti. 2011;10. doi: Artn 8 10.1186/1476-0711-10-8. PubMed PMID: WOS:000208655000008.
10. Bouttier S, Yeo A, Any-Grah AASA, Geiger S, Huang N, Nicolas V, et al. Characterization and in vitro evaluation of a vaginal gel containing Lactobacillus crispatus for the prevention of gonorrhea. Int J Pharmaceut. 2020;588. doi: ARTN 119733 10.1016/j.ijpharm.2020.119733. PubMed PMID: WOS:000577558900001.
11. Bisaillon JG, Beaudet R, Saheb SA, Morisset R. Interference of Neisseria gonorrhoeae growth by aerobic bacterial representatives of the urogenital flora. Rev Can Biol. 1980;39(4):201-8. Epub 1980/12/01. PubMed PMID: 6785845.
12. McBride ME, Duncan WC, Knox JM. Bacterial interference of Neisseria gonorrhoeae by alpha-haemolytic streptococci. Br J Vener Dis. 1980;56(4):235-8. Epub 1980/08/01. doi: 10.1136/sti.56.4.235. PubMed PMID: 6775764; PubMed Central PMCID: PMCPMC1045785.
13. Kraus SJ, Geller RC, Perkins GH, Rhoden DL. Interference by Neisseria gonorrhoeae growth by other bacterial species. J Clin Microbiol. 1976;4(3):288-95. Epub 1976/09/01. doi:

10.1128/jcm.4.3.288-295.1976. PubMed PMID: 823175; PubMed Central PMCID: PMCPMC274452.
14. Laumen JGE, Van Dijck C, Abdellati S, De Baetselier I, Manoharan-Basil SS, Van den Bossche D, et al. High cephalosporin, fluoroquinolone and macrolide MIC distributions in commensal Neisseria in the Belgian general population and STI clinic attendees: a cross sectional survey. . BioRxiv. 2021; ( ): .
15. Kandi V. Bacterial Colony Characters: Pitting Colonies. Journal of Medical Microbiology & Diagnosis. 2015;4(2):1.
16. Lux T, Nuhn M, Hakenbeck R, Reichmann P. Diversity of bacteriocins and activity spectrum in Streptococcus pneumoniae. J Bacteriol. 2007;189(21):7741-51. Epub 2007/08/21. doi: 10.1128/JB.00474-07. PubMed PMID: 17704229; PubMed Central PMCID: PMCPMC2168751.