Integrated molecular, phenotypic and epidemiological surveillance of antimicrobial resistance in *Neisseria gonorrhoeae* in Germany

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ABSTRACT

Numbers of infections with *Neisseria gonorrhoeae* are among the top three sexually transmitted infections (STI) worldwide. In addition, the emergence and spread of antimicrobial resistance (AMR) in *Neisseria gonorrhoeae* pose an important public-health issue. The integration of genomic, phenotypic and epidemiological data to monitor *Neisseria gonorrhoeae* fosters our understanding of the emergence and spread of AMR in *Neisseria gonorrhoeae* and helps to inform therapy guidelines and intervention strategies. Thus, the Gonococcal resistance surveillance (Go-Surv-AMR) was implemented at the Robert Koch Institute in Germany in 2021 to obtain molecular, phenotypic and epidemiological data on *Neisseria gonorrhoeae* isolated in Germany. Here, we describe the structure and aims of Go-Surv-AMR. Furthermore, we point out future directions of Go-Surv-AMR to improve the integrated genomic surveillance of *Neisseria gonorrhoeae*. In this context we discuss current and prospective sequencing approaches and the information derived from their application. Moreover, we highlight the importance of combining phenotypic and WGS data to monitor the evolution of AMR in *Neisseria gonorrhoeae* in Germany. The implementation and constant development of techniques and tools to improve the genomic surveillance of *Neisseria gonorrhoeae* will be important in coming years.

1. Introduction

Sexually Transmitted Infections (STIs) continue to be a global health concern, affecting populations worldwide. Among the various pathogens responsible for STIs, *Neisseria gonorrhoeae* (NG) remains a significant public health challenge. NG is a human restricted pathogen causing the STI gonorrhea. After infection of mucosal surfaces, clinical presentation ranges from urethritis, cervicitis, proctitis and pharyngitis to disseminated infections. If left untreated ascending infections of the urogenital tract can cause chronic pelvic pain, infertility, miscarriages and ectopic pregnancies (Meyer and Buder, 2020; Unemo and Shafer, 2014).

According to the World Health Organization (WHO) NG is responsible for approximately 82.4 million infections annually. This alarming prevalence ranks it as the third most common STI on a global scale (WHO, 2023). However, the management and treatment of NG infections have become increasingly complex due to the emergence of antimicrobial resistance (AMR). NG has developed resistance to all classes of antibiotics traditionally used to treat NG infections. Clinical resistance against the first-line antibiotics cefixime, ceftriaxone and azithromycin raise concerns about the efficacy of available treatment options (Unemo et al., 2021; Unemo and Shafer, 2011). In recognition of the growing threat posed by NG and the challenges of AMR, the WHO designated NG as a high-priority pathogen in 2017 (WHO, 2017). Addressing the issue is crucial as it has the potential to undermine public health efforts.

Moreover, NG is naturally competent and has thus the ability to take up and integrate DNA from both related and divergent species, adding an additional layer of complexity to its evolution and adaptability (Cehovin and Lewis, 2017). In this context, the importance of phenotypic AMR testing is evident, as it allows monitoring the spread of relevant AMR as well as tailoring therapy to individual cases. The basis of phenotypic AMR testing is the isolation and cultivation of NG. However, success is limited due to the fastidious nature of NG with very specific nutrient, moisture and temperature requirements. In addition, depending on the location of infection, low bacterial loads and surrounding microbiota may further compromise successful isolation (Bissessor et al., 2011; Nadal-Baron et al., 2022). Hence, understanding the genetic mechanisms and evolution of this pathogen is crucial for the development of effective control and treatment strategies.

Molecular typing methods play a crucial role in these efforts. The use of standardized nomenclatures allows for international comparisons of sequence types. Over the years several typing schemes were developed for NG, that are commonly used today: NG multilocus sequence typing (MLST), which assigns sequence types based on seven housekeeping genes (Bennett et al., 2007); NG multiantigen sequence typing (NG-MAST) which relies on sequencing fragments of the outer membrane protein genes *porA* and *tbpB* and assigns sequence types based on the variants of these fragments (Martin, 2004); NG Sequence Typing for Antimicrobial Resistance (NG-STAR) uses the sequences of seven genes connected to AMR and assigns sequence types based on the combination of these fragments (Demczuk et al., 2017). With the advent of whole genome sequencing (WGS) a typing scheme based on the NG core genome (cgMLST) was developed and can now be used in conjunction with other typing schemes (Harrison et al., 2020).

In this context, this article examines the prevalence, impact, and challenges of NG infections and its AMR in Germany, and the need for innovative approaches to combat this persistent STI threat.

2. Establishment of an integrated genomic surveillance of AMR in NG in Germany – Go-Surv-AMR

Surveillance systems are the cornerstone of public health, monitoring and tracking infectious diseases within populations (Aiello et al., 2020). To address this critical information gap, the RKI assesses the magnitude of NG AMR on the basis of the mandatory notification of those events. As a central addition to the statutory notification system, the RKI initiated a comprehensive Gonococcal resistance surveillance (Go-Surv-AMR) program for NG infections on the basis of former and smaller-scaled structures initiated in 2014 and in collaboration with the consiliary laboratory for NG in Germany. In this framework, a nationwide network of clinical laboratories regularly provides cultivated NG isolates and their own susceptibility test data (Fig. 1). In parallel, the RKI collects essential epidemiological and clinical information connected to these isolates that is indispensable for understanding the dynamics of NG infections across Germany. To gain deeper insights into AMR trends, the RKI conducts follow-up AMR testing and employs state-of-the-art WGS analysis on the isolates submitted by these laboratories. These advanced techniques enable the exploration of genetic factors associated with resistance mechanisms. Facilitating collaboration and knowledge sharing, participating laboratories receive annual feedback.

3. New technologies improve the molecular epidemiology of NG

3.1. Genetic fingerprint

The high discriminatory power of WGS has revolutionized our ability to distinguish closely related strains with unparalleled precision. This capability is of immense value, particularly in outbreak investigations, as it allows us to pinpoint the source of infection and the pathways of transmission (Simar et al., 2021).

Gaining insights into the clonal connections among NG isolates is an essential approach for managing the expansion of troublesome genetic lineages (Costa-Lourenco et al., 2017). Starting from 2014, NG isolates collected in Germany have been subjected to NG-MAST, a process that not only facilitates international comparisons but also enables the tracking of global and national lineage prevalence (Pinto et al., 2021). Subsequently, in a study spanning from 2014 to 2017, various genotypes were identified, linking genogroups with distinct AMR profiles. This study offers compelling evidence of a genetically diverse and dynamically evolving gonococcal population within Germany. Furthermore, it sheds light on an association between genogroup G10557 and reduced susceptibility to cefixime, a finding that was undescribed until then (Banhart et al., 2020). Since the introduction of WGS in 2018 at the RKI, enhanced discriminatory properties allow us to gain even deeper insights into the genetic diversity of NG strains in Germany. This technological advancement has further improved our ability to distinguish between different NG strains, providing a more comprehensive understanding of their genetic variations and facilitating more precise genetic analyses.

3.2. Genomic resistance surveillance

In the case of NG, extensive investigations using WGS have offered deep insights into the genetic mechanisms associated with the observed susceptibility patterns for the primary antimicrobial classes. Thus, mutations in the *gyrA*, *parC* and *parE* genes have been associated with resistance to fluoroquinolone antibiotics such as ciprofloxacin (Ellington et al., 2017; Unemo and Shafer, 2011).

Mutations in genes encoding the MtrC-MtrD-MtrE efflux pump and its transcription regulator MtrR as well as in the 23 S rRNA were identified as the main drivers of resistance to the macrolide azithromycin. In 2018, WGS using short reads conclusively demonstrated that the observed increase in azithromycin resistance in Germany, from 4.3% in 2016 to 9.2% in 2018, was directly linked to the clonal spread of isolates
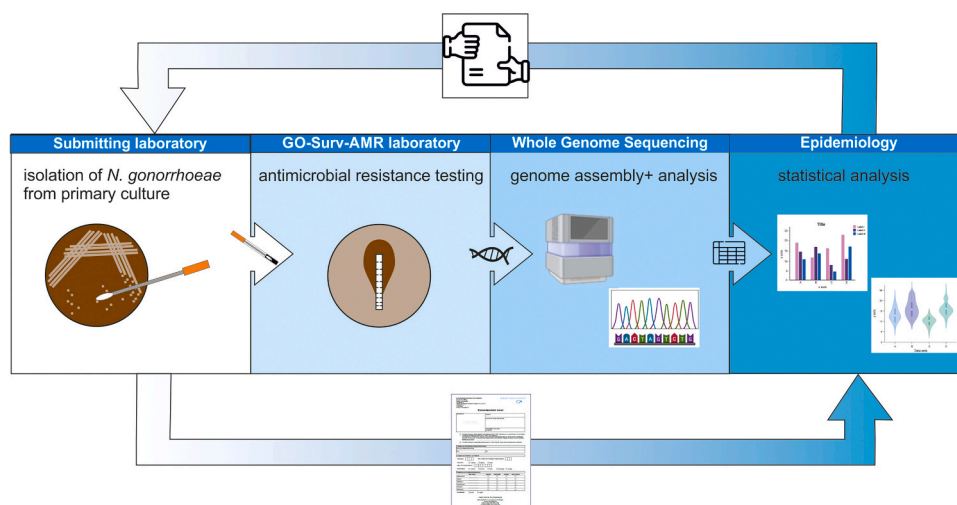

Fig. 1. Schematic representation of the data flow in the Go-Surv-AMR project. Submitting laboratories send NG isolates to the RKI Go-Surv-AMR laboratory, where follow-up AMR testing and WGS analyses are performed. In parallel, essential epidemiological data is collected and analyzed. Once a year participating laboratories receive feedback.

carrying the mosaic-like mtr locus. (Banhart et al., 2021). Recent data from 2022 reveal a continued trend. In the Go-Surv-AMR dataset, 22.2% of the collected isolates carried the *mtrD* mosaic 2 gene, while 22.5% carried the *mtrR* promoter mosaic 2 gene (Selb and Klaper et al., 2023).

Among the most notable resistance genes is *penA*, which, when mutated, confers resistance to the crucial first-line antibiotics cefixime and ceftriaxone, particularly in combination with specific mutations in the *rpoB*, *rpoD* and *mtrR/mtrCDE* genes. However, the exact mechanism of resistance to cefixime/ceftriaxone in NG has not been fully elucidated (Cameron et al., 2019). The latest surveillance data show that the prevalence of these strains in Germany remains consistently low (Selb and Klaper et al., 2023).

In addition to chromosomally encoded genes, plasmids can also mediate AMR in NG (Mlynarczyk-Bonikowska et al., 2022). The conjugative plasmid harboring a *tetM* gene and TEM β-lactamase expressing plasmids confer resistance to tetracycline and penicillin, respectively (Cehovin et al., 2020).

3.3. Pangenome analysis

While short read sequencing has revolutionized our ability to explore the genetic makeup of various organisms, it also has its own set of limitations, particularly when dealing with plasmids, complex genomic diversity, genetic rearrangements, and the study of evolutionary development (Romagnoli et al., 2023). NG is renowned for its capacity to engage in genetic recombination and horizontal gene transfer, leading to an extraordinary level of genetic diversity (Gibbs and Meyer, 1996). This diversity is a key factor contributing to the bacterium's adaptability and its ability to develop antibiotic resistance (Quillin and Seifert, 2018). Particularly pharyngeal NG isolates are known to play a significant role in the development and dissemination of AMR (Eyre et al., 2018; Ohnishi et al., 2011). Within the oropharynx, other commensal *Neisseria* species can host genetic antibiotic resistance elements that have emerged due to prior exposure to antibiotics (Furuya et al., 2007; Laumen et al., 2021). The coexistence of these related bacterial species in the oropharynx creates an optimal environment for the transfer of AMR through horizontal gene transfer (Unemo and Shafer, 2014). It is believed that horizontal gene transfer events have given rise to genetic mosaics (Boc and Makarenkov, 2011). The presence of mosaicism in genes associated with immune evasion, such as the *por* gene as well as within the *penA* and *mtrR/mtrCDE* loci responsible for resistance to key antibiotics, underscores the significance of recombination as a vital strategy employed by NG in its evolutionary adaptation and survival (Ameyama et al., 2002; Fudyk et al., 1999; Wadsworth et al., 2018). A mosaic gene is a gene with blocks of sequences having different evolutionary histories, often due to recombination events or partial gene transfer, including genetic material from other species (Boc and Makarenkov, 2011). Short read sequencing technologies often struggle to accurately capture repetitive elements and identify complex structural variations (SV) within genomes (Fig. 2) (Cameron et al., 2019; Chen et al., 2016). When the size of the genetic event is significantly larger than the read size, the flanking regions of the event may not be adequately covered, resulting in a fragmented assembly and incomplete representation. Thus, short read sequencing leads to challenges in accurately determining the orientation and structure of complex genetic variations like inversions and duplications (Mahmoud et al., 2019).

A fragmented assembly is also a challenge in plasmid identification and reconstruction. (Wick et al., 2021). In addition to SVs, plasmids and chromosomes can share highly similar genetic regions, making the assignment of reads to its origin difficult (Tang et al., 2023). Researchers often mitigate these limitations by using long read sequencing technologies to obtain a more detailed view of the genomic structure (Marx, 2023). The long reads are able to span SVs and thereby the technology provides more sequence information that facilitates de novo assembling of the chromosome and plasmids (Mahmoud et al., 2019). Nevertheless, short read sequencing is often considered more accurate than long read sequencing due to lower per-base error rates resulting from the sequencing technology and well-established base-calling algorithms. Thus, the trend is moving towards combining short and long reads (Amarasinghe et al., 2020; Xu et al., 2022). In NG AMR is determined by mutations in chromosomal genes, such as the mosaic *mtrR/mtrCDE* loci, as well as plasmid encoded resistance genes. Therefore, the combination of the sequence accuracy of short read sequencing and the structural overview provided by long read sequencing are needed to fully capture the extend of AMR within the NG population in Germany.

4. Conclusions and future perspectives

In conclusion, it is evident that improved molecular and phenotypic surveillance serves as an indispensable tool for monitoring the evolving landscape of NG, particularly in the context of AMR, in Germany. The ability to track the development and spread of NG is pivotal to guide public health efforts. Looking ahead, several future perspectives have to be considered:

Since 2022 notification of all laboratory diagnosed gonorrhea cases irrespective of the AMR status of the pathogen is mandatory which represents a further significant step. This enables the collection of more comprehensive data and extends our understanding of the epidemiology of NG infections. Simultaneously, the incorporation of pangenome analysis can provide valuable insights into the genetic transfer dynamics
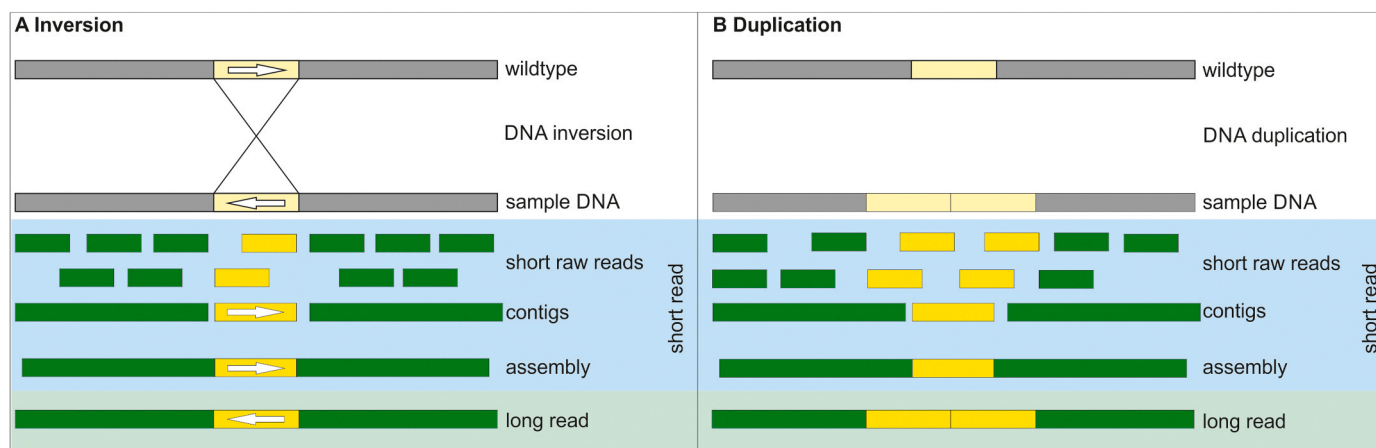

Fig. 2. De novo assembly of DNA inversion (A) and duplication (B) based on short read and long read sequencing. Short read sequencing fails to correctly assign the orientation (A) or genetic structure (B) as the read size does not span the genetic event. Long-read sequencing correctly assigns orientation (A) and genetic structure (B). Yellow: reads of genetic event; green: reads of flanking regions.

in this context.

The advances in molecular analysis constantly improve our understanding of the mechanisms contributing to AMR and help in monitoring the spread of AMR-related NG populations. Still, AMR predictions solely based on molecular methods are not always accurate. Mutations or combinations of mutations in the DNA of NG that lead to AMR are currently not fully understood. Furthermore, the genetic variability of NG allows for the uptake of novel genes or determinants that could confer a new, currently unknown AMR mechanism in NG. Consequently, phenotypic AMR testing is still necessary to further elucidate the genetic determinants contributing to AMR and to identify new mutations leading to AMR. Thus, the combination of both approaches is needed to fully track the development of AMR in NG. The resistance surveillance program Go-Surv-AMR employs exactly this course of action to generate data on the genetic background of AMR within the NG population in Germany.

Additionally, the investigation of the pharynx as a potential reservoir for AMR is a critical avenue to explore. This can be due to factors such as limited accessibility of antibiotics in the pharynx or the possibility of horizontal gene transfer from commensal *Neisseria* species. Therefore, the isolation of NG from the pharynx is crucial to determine the extent of NG resistance to antibiotics and to unravel the genetic underpinnings contributing to this resistance. In order to approach these essential questions, the RKI initiated a study to assess the AMR of NG isolated from the pharynx (Go-Pharynx).

In summary, these perspectives underscore the importance of proactive and adaptive strategies for addressing the complex challenges posed by STIs and AMR in Germany. This multi-faceted approach will not only enhance our understanding of these issues but also support the development of more effective prevention and treatment strategies.

CRediT authorship contribution statement

Heuer Dagmar: Conceptualization, Project administration, Supervision, Writing – original draft, Writing – review & editing. **Jansen Klaus:** Project administration, Writing – original draft, Writing – review & editing. **Selb Regina:** Writing – original draft, Writing – review & editing. **Tlapák Hana:** Conceptualization, Visualization, Writing – original draft, Writing – review & editing. **Klaper Kathleen:** Conceptualization, Visualization, Writing – original draft, Writing – review & editing.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used ChatGPT in order to improve readability and language. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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Declarations of interest

None.

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