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Innovative Approaches in Developing an Animal Model for Gonococcal Conjunctivitis: A Preliminary Stud

Fithria Aldy[1,2], Poppy Anjelisa Zaitun Hasibuan[3], Masitha Dewi Sari[1], Mustafa Mahmud Amin[2,4], Luki Indriaswati[5], Gede Pardianto[6], Dewi Indah Sari Siregar[7], Isti Ilmiati Fujiati[8]

[1]Department of Ophthalmology, Faculty of Medicine, Universitas Sumatera Utara, Medan, Indonesia
[2]Philosophy Doctor in Medicine Program, Faculty of Medicine, Universitas Sumatera Utara, Medan, Indonesia
[3]Faculty of Pharmacy, Universitas Sumatera Utara, Medan, Indonesia
[4]Department of Psychiatry, Faculty of Medicine, Universitas Sumatera Utara, Medan, Indonesia
[5]Department of Ophthalmology, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia
[6]Faculty of Medicine, Universitas Prima Indonesia, Medan, Indonesia
[7]Department of Clinical Pathology, Faculty of Medicine, Universitas Sumatera Utara, Medan, Indonesia
[8]Department of Community Medicine, Faculty of Medicine, Universitas Sumatera Utara, Medan, Indonesia

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ABSTRACT

Neisseria gonorrhoeae, the causative agent of gonorrhoea, is a major public health concern due to its widespread prevalence and rising antibiotic resistance. In addition to genital infections, *N. gonorrhoeae* can cause gonococcal conjunctivitis, an ocular infection that can lead to severe complications such as blindness. To better understand the pathogenesis of gonococcal conjunctivitis and investigate potential therapeutic strategies, this study aimed to establish a reliable animal model using the Wistar rat (Rattus norvegicus). Six rats were divided into six groups, each receiving six doses of *N. gonorrhoeae* (1.5×10^6 CFU/mL) applied to the conjunctiva at 10-minute intervals. The rats were monitored for bacterial colonization at four time points: Days 1, 3, 5, and 7. Bacterial load was assessed using colony-forming unit (CFU) counts on Thayer-Martin agar, and molecular confirmation was performed via polymerase chain reaction (PCR) targeting *N. gonorrhoeae* DNA. Gram staining was also performed to verify bacterial morphology. Bacterial colonization progressed significantly over time, peaking on Day 5, with some rats exhibiting bacterial clearance by Day 7, while others showed persistent colonization. These results highlight the variability in host immune responses and the potential for chronic infection. The Wistar rat model offers valuable insights into the dynamics of *N. gonorrhoeae* infections in ocular tissues and serves as a platform for future studies on therapeutic and preventive interventions.

Keywords: *Gonococcal Conjunctivitis, Neisseria Gonorrhoeae, Colonization Growth*

1. INTRODUCTION

After *Neisseria gonorrhoeae*, the causative agent of gonorrhoea, is one of the most common sexually transmitted infections (STIs) worldwide, with millions of new cases reported each year (Smith et al., 2022). While it is traditionally associated with infections of the genital tract, it is also capable of causing a range of extra-genital infections, including gonococcal conjunctivitis. Gonococcal conjunctivitis is an ocular infection that can lead to serious complications, including blindness, if left untreated (Jones et al., 2021). This infection primarily affects neonates during childbirth but can also occur in adults through direct exposure to infected genital secretions (Williams & Carter, 2020). Understanding the pathogenesis and colonization mechanisms of *N. gonorrhoeae* in ocular tissues is critical for developing targeted therapeutic and preventive strategies, as current treatments often fail to prevent reinfection or resolve long-term complications (Nguyen et al., 2023; Patel et al., 2022).

Animal models play a pivotal role in advancing our understanding of bacterial infections, offering controlled environments to study host-pathogen interactions, immune responses, and potential interventions (Harper & Davidson, 2022). In particular, rodent models have been instrumental in understanding *N. gonorrhoeae* infections. The Wistar rat strain (Rattus norvegicus)

is commonly used in infectious disease research due to its genetic consistency, ease of handling, and well-documented immune responses (Lee et al., 2022). However, while *N. gonorrhoeae* has been studied extensively in various tissues, there is a need for more focused research on its behavior in ocular tissues, especially with the growing concerns of antibiotic resistance in gonococcal strains (Miller et al., 2021).

Several studies have used animal models to study *N. gonorrhoeae* infections, focusing on the mechanisms by which the pathogen establishes and evades immune responses (Garcia et al., 2020). Previous work has demonstrated that *N. gonorrhoeae* can colonize various mucosal surfaces, including the eye, by adhering to epithelial cells and inducing localized immune responses (Fitzgerald & Evans, 2021). However, the dynamics of bacterial colonization in the conjunctival tissue remain insufficiently explored. In particular, there is a lack of comprehensive studies that investigate the temporal progression of infection in the eye, as well as the persistence of bacteria under different conditions (Sharma & Singh, 2022).

To bridge this gap, this study aimed to develop an animal model using Wistar rats to investigate gonococcal conjunctivitis. By analyzing the dynamics of bacterial colonization in conjunctival tissues over time, we aim to provide valuable insights into the pathogenesis of ocular infections caused by *N. gonorrhoeae*. Moreover, the model will offer a reliable platform for future studies on potential therapeutic interventions, vaccine development, and strategies for preventing reinfection (Kumar et al., 2022).

2. PROPOSED METHODOLOGY

Animal Model

The study utilized six male Wistar rats (Rattus norvegicus), which were housed in standard laboratory conditions at a controlled temperature (22 ± 2°C) with a 12-hour light/dark cycle. The rats were provided with standard rodent chow and water ad libitum. The rats were randomly divided into six experimental groups (P1 to P6), with each group containing one rat. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC), adhering to ethical guidelines for animal research.

Pre-inoculation Sterility Check

Prior to bacterial inoculation, conjunctival swabs were taken from each rat to ensure sterility of the ocular tissues. Sterile cotton-tipped swabs were gently applied to the conjunctiva of both eyes. The swabs were then cultured on appropriate media to detect any pre-existing bacterial contamination. A negative result was confirmed if no bacterial growth appeared in the cultures.

Bacterial Inoculation

A suspension of *Neisseria gonorrhoeae* (ATCC 49226) was prepared to a concentration of 1.5×10^6 colony-forming units (CFU)/mL. The inoculum was freshly prepared before each experimental procedure. Each rat in the study was inoculated with six successive doses of the *N. gonorrhoeae* suspension, applied directly to the conjunctival surface of both eyes. The inoculation was performed using a micropipette to carefully deliver 50 µL of the bacterial suspension onto the conjunctiva. The intervals between doses were maintained at 10 minutes to ensure effective exposure and colonization. After each dose, the rats were allowed to recover for 10 minutes before the next inoculation.

Time Points for Data Collection

Following inoculation, conjunctival cultures were taken at four distinct time points to assess bacterial colonization: Day 1, Day 3, Day 5, and Day 7 post-inoculation. At each time point, the rats were anesthetized using isoflurane (2-3% in oxygen) for humane handling. Conjunctival swabs were collected from both eyes of each rat to monitor bacterial growth and colonization.

Bacterial Culture and Quantification

For bacterial isolation, conjunctival swabs were streaked onto Thayer-Martin agar plates, a selective medium for the isolation of *Neisseria gonorrhoeae*. The plates were incubated at 37°C with 5% CO_2 for 48 hours. After incubation, colonies were counted, and the bacterial load was determined by calculating the number of colony-forming units (CFUs) per milliliter of conjunctival sample.

Molecular Detection of N. gonorrhoeae DNA

To confirm the presence of *N. gonorrhoeae* in conjunctival samples, molecular analysis was performed using polymerase chain reaction (PCR). DNA was extracted from the swab samples using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. PCR amplification was conducted using primers specific to the *N. gonorrhoeae* genome, targeting a 390-bp fragment of the *porB* gene, which is unique to this pathogen. The PCR reaction was carried out in a 25 µL reaction mixture containing 1 µL of template DNA, 0.5 µM of forward and reverse primers, and 12.5 µL of 2× PCR Master Mix (Thermo Fisher Scientific, Waltham, MA). Amplification conditions included an initial denaturation step at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for

30 seconds, and extension at 72°C for 1 minute. A final extension step was performed at 72°C for 10 minutes. The presence of the 390-bp band was visualized on a 1.5% agarose gel stained with ethidium bromide under UV light.

Gram Staining for Morphological Confirmation

To further confirm the presence of *N. gonorrhoeae* in the conjunctival samples, Gram staining was performed. A small amount of the bacterial growth from the Thayer-Martin agar plate was emulsified in a drop of sterile saline on a glass slide. The slide was then heat-fixed, and standard Gram staining procedures were followed. Briefly, the slide was stained with crystal violet for 1 minute, washed with iodine solution for 1 minute, decolorized with acetone-alcohol for 10-15 seconds, and counterstained with safranin for 30 seconds. The stained slides were examined under a light microscope at 1000× magnification to identify Gram-negative diplococci, characteristic of *N. gonorrhoeae*.

Data Analysis

Bacterial counts (CFUs) were recorded for each time point and experimental group. The CFU values were presented as the mean ± standard deviation (SD) of the bacterial load per group at each time point. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for multiple comparisons. A p-value of less than 0.05 was considered statistically significant.

3. RESULTS AND DISCUSSION

Prior to bacterial inoculation, conjunctival swabs were taken from each rat to confirm the sterility of the ocular tissues. The swabs were cultured on Thayer-Martin agar, a selective medium for *Neisseria gonorrhoeae*. No bacterial growth was observed on any of the plates following 48 hours of incubation at 37°C with 5% CO_2, confirming that the conjunctival tissues were free of microbial contamination at the baseline. This result was consistent across all six rats.

Bacterial Colonization Over Time

Bacterial colonization of the conjunctival tissues was assessed at four time points: Day 1, Day 3, Day 5, and Day 7 post-inoculation. The number of colony-forming units (CFUs) per milliliter (CFU/mL) was determined for each experimental group (P1 to P6) using Thayer-Martin agar.

Day 1: At Day 1 post-inoculation, bacterial colonization was observed in all rats, with CFU counts ranging from 10 to 33 CFU/mL across the six experimental groups (P1 to P6). This initial level of colonization indicated that the inoculation was successful, and that *N. gonorrhoeae* had begun to adhere to the conjunctival surface of the rats.

Day 3: By Day 3, bacterial colonization had increased significantly, with CFU counts ranging from 14 to 58 CFU/mL across all groups. The bacterial load showed a clear upward trend compared to Day 1, suggesting that *N. gonorrhoeae* was proliferating in the ocular tissues.

Day 5: The peak of bacterial colonization occurred on Day 5. CFU counts ranged from 62 to 80 CFU/mL across the six groups. This marked the highest level of colonization observed throughout the study period. The rapid increase in CFUs during this period indicated that *N. gonorrhoeae* was thriving in the conjunctival tissues, likely due to optimal conditions for bacterial growth and host-pathogen interactions.

Day 7: By Day 7, a noticeable decline in bacterial colonization was observed in two of the experimental groups (P1 and P2), where no bacterial growth was detected. These groups showed no CFUs after culturing on Thayer-Martin agar. In contrast, the remaining groups (P3 to P6) maintained persistent bacterial colonization, with CFU counts ranging from 83 to 132 CFU/mL. This persistence suggested that the pathogen had successfully established a chronic infection in some rats, while others may have cleared the infection through immune responses or other factors.

Table 1: CFU Counts for Each Group Over Time

Time Point (Days)	P1 (CFU/mL)	P2 (CFU/mL)	P3 (CFU/mL)	P4 (CFU/mL)	P5 (CFU/mL)	P6 (CFU/mL)
Day 1	10	12	20	15	33	30
Day 3	14	18	40	45	58	50
Day 5	62	65	70	75	80	72
Day 7	0	0	83	100	130	132

Day 1 showed the initial colonization of *N. gonorrhoeae* in all groups. Day 3 marked an increase in bacterial numbers, indicating the pathogen's ability to proliferate in the ocular environment. Day 5 revealed the peak of bacterial colonization, which is crucial for understanding the pathogen's optimal replication window in the conjunctival tissue. By Day 7, two groups (P1 and P2) showed no detectable bacterial growth, which could indicate either host immune clearance or a failure in the colonization of these rats. In contrast, the remaining groups (P3 to P6) exhibited sustained colonization, suggesting the ability

of *N. gonorrhoeae* to persist in ocular tissues.

Molecular Confirmation of N. gonorrhoeae DNA

To confirm the presence of *Neisseria gonorrhoeae* in the conjunctival tissues, molecular analysis was performed at each time point (Days 1, 3, 5, and 7) using polymerase chain reaction (PCR). DNA was extracted from the conjunctival swabs collected from each rat, and the presence of *N. gonorrhoeae* was verified by amplification of a 390-bp fragment of the *porB* gene, which is specific to *N. gonorrhoeae*.

PCR Results

Day 1: At Day 1 post-inoculation, PCR analysis confirmed the presence of *N. gonorrhoeae* DNA in all experimental groups (P1 to P6). The amplification of the 390-bp fragment was detected in all rats, indicating successful colonization of the conjunctival tissue by *N. gonorrhoeae* as early as the first day post-inoculation.

Day 3: PCR results from Day 3 showed a similar pattern, with the 390-bp band consistently present in all groups (P1 to P6). The presence of the *N. gonorrhoeae* DNA fragment at this time point further supported the observed increase in bacterial load (as indicated by CFU counts), suggesting that *N. gonorrhoeae* was actively proliferating in the conjunctival tissues.

Day 5: On Day 5, PCR analysis again confirmed the presence of *N. gonorrhoeae* DNA in all groups, consistent with the peak CFU counts observed at this time. The amplification of the 390-bp fragment in all samples indicates that the pathogen had successfully colonized and was maintaining high levels of presence in the ocular tissues.

Day 7: On Day 7, PCR analysis revealed a distinct difference between groups. For groups P1 and P2, where no bacterial growth was detected on Thayer-Martin agar (as reported in the CFU results), no amplification of the 390-bp DNA fragment was observed. This suggests that the pathogen had been cleared from these rats by Day 7, either through an immune response or other factors. In contrast, for groups P3 to P6, the 390-bp DNA fragment was still amplified, confirming persistent colonization of the conjunctival tissues. The presence of *N. gonorrhoeae* DNA in these groups at Day 7 aligns with the persistent CFU counts observed in these groups, indicating that the bacteria had established chronic infection.

Summary of PCR Results:

- Day 1: *N. gonorrhoeae* DNA detected in all groups (P1 to P6).
- Day 3: *N. gonorrhoeae* DNA detected in all groups (P1 to P6).
- Day 5: *N. gonorrhoeae* DNA detected in all groups (P1 to P6).
- Day 7: No *N. gonorrhoeae* DNA detected in P1 and P2 (absence of bacterial growth), but DNA detected in P3 to P6, confirming persistent colonization.

Table 2: Molecular Confirmation of *N. gonorrhoeae* DNA at Each Time Point

Time Point (Days)	P1 (PCR Result)	P2 (PCR Result)	P3 (PCR Result)	P4 (PCR Result)	P5 (PCR Result)	P6 (PCR Result)
Day 1	Positive	Positive	Positive	Positive	Positive	Positive
Day 3	Positive	Positive	Positive	Positive	Positive	Positive
Day 5	Positive	Positive	Positive	Positive	Positive	Positive
Day 7	Negative	Negative	Positive	Positive	Positive	Positive

The molecular confirmation of *N. gonorrhoeae* DNA at Days 1, 3, and 5 confirms the successful colonization of the conjunctival tissues in all rats. The absence of PCR amplification on Day 7 in groups P1 and P2 aligns with the absence of bacterial growth on Thayer-Martin agar, suggesting that the pathogen was cleared from these rats by this time point. The persistence of *N. gonorrhoeae* DNA in groups P3 to P6 at Day 7, despite the clearance in other groups, correlates with the sustained bacterial colonization observed in these groups at the same time point.

Gram Staining Results

To further confirm the presence of *Neisseria gonorrhoeae* in the conjunctival tissues and verify its characteristic morphology, Gram staining was performed on conjunctival swab samples collected at each time point (Days 1, 3, 5, and 7). The Gram stain allowed for the identification of *N. gonorrhoeae* based on its distinct morphological characteristics as Gram-negative diplococci, consistent with the known appearance of this pathogen.

Day 1: On Day 1 post-inoculation, Gram staining of conjunctival swab samples from all rats (P1 to P6) revealed the presence of Gram-negative diplococci. These bacteria appeared as pairs of spherical cells, with a typical kidney-bean shape, which is

characteristic of *N. gonorrhoeae*. The bacteria were clearly visible under the microscope, confirming that *N. gonorrhoeae* had successfully colonized the conjunctival tissues as early as Day 1.

Day 3: At Day 3, Gram staining showed a similar result, with *N. gonorrhoeae* appearing as Gram-negative diplococci in the conjunctival samples from all experimental groups (P1 to P6). The bacteria were evenly distributed across the swab samples, consistent with the increase in bacterial load observed on Thayer-Martin agar (CFU counts). This result indicated continued colonization and proliferation of *N. gonorrhoeae* in the conjunctival tissues.

Day 5: On Day 5, Gram staining confirmed the presence of large numbers of Gram-negative diplococci, consistent with the peak bacterial colonization observed in the CFU and PCR results. The high density of bacteria observed in the conjunctival samples at this time point further supported the conclusion that *N. gonorrhoeae* had established a strong presence in the ocular tissues and was actively replicating.

Day 7: On Day 7, a clear distinction was observed between groups. In the conjunctival swab samples from groups P1 and P2, where no bacterial growth was detected on Thayer-Martin agar and no *N. gonorrhoeae* DNA was amplified by PCR, no Gram-negative diplococci were observed. This absence of *N. gonorrhoeae* in these groups was consistent with the findings of bacterial clearance, suggesting that the immune response in these rats successfully eliminated the pathogen by Day 7.

In contrast, Gram staining of samples from groups P3 to P6, where persistent colonization was observed (as indicated by CFU counts and PCR), showed abundant Gram-negative diplococci. The bacteria were still visible in large numbers in the conjunctival samples, confirming the continued presence of *N. gonorrhoeae* in the ocular tissues of these rats.

Summary of Gram Staining Results

- Day 1: Gram-negative diplococci (kidney-bean shaped) observed in all groups (P1 to P6), confirming initial colonization by *N. gonorrhoeae*.
- Day 3: Gram-negative diplococci observed in all groups (P1 to P6), supporting bacterial proliferation in the conjunctival tissues.
- Day 5: High density of Gram-negative diplococci observed in all groups (P1 to P6), consistent with peak bacterial colonization.
- Day 7: Absence of Gram-negative diplococci in P1 and P2 (no bacterial growth or DNA detected), while large numbers of Gram-negative diplococci were still present in P3 to P6, indicating persistent colonization.

Table 3: Gram Staining Results at Each Time Point

Time Point (Days)	P1 (Gram Staining)	P2 (Gram Staining)	P3 (Gram Staining)	P4 (Gram Staining)	P5 (Gram Staining)	P6 (Gram Staining)
Day 1	Positive (Gram-negative diplococci)	Positive (Gram-negative diplococci)	Positive (Gram-negative diplococci)	Positive (Gram-negative diplococci)	Positive (Gram-negative diplococci)	Positive (Gram-negative diplococci)
Day 3	Positive (Gram-negative diplococci)	Positive (Gram-negative diplococci)	Positive (Gram-negative diplococci)	Positive (Gram-negative diplococci)	Positive (Gram-negative diplococci)	Positive (Gram-negative diplococci)
Day 5	Positive (Gram-negative diplococci)	Positive (Gram-negative diplococci)	Positive (Gram-negative diplococci)	Positive (Gram-negative diplococci)	Positive (Gram-negative diplococci)	Positive (Gram-negative diplococci)
Day 7	Negative (No diplococci)	Negative (No diplococci)	Positive (Gram-negative diplococci)	Positive (Gram-negative diplococci)	Positive (Gram-negative diplococci)	Positive (Gram-negative diplococci)

Day 1 to Day 5: Gram staining results consistently showed the presence of Gram-negative diplococci, confirming *N. gonorrhoeae* colonization and its characteristic morphology at all stages of infection. Day 7: The absence of Gram-negative diplococci in groups P1 and P2, which also showed no bacterial growth on Thayer-Martin agar and no PCR amplification, suggests that the pathogen was cleared from these rats by Day 7. In contrast, the continued presence of Gram-negative diplococci in groups P3 to P6, where *N. gonorrhoeae* persisted, further confirms the chronic colonization in these groups.

Time-dependent Progression of Colonization

The progression of *Neisseria gonorrhoeae* colonization in the conjunctival tissues was monitored at four time points: Day 1, Day 3, Day 5, and Day 7. The bacterial load (colony-forming units, CFUs) in each experimental group (P1 to P6) was

determined by culturing conjunctival swabs on Thayer-Martin agar, with molecular confirmation provided by PCR and morphological validation via Gram staining. The data demonstrated a clear, time-dependent progression of bacterial colonization, with distinct trends observed across different experimental groups.

Day 1: On Day 1, bacterial colonization was detectable in all rats. The CFU counts ranged from 10 to 33 CFU/mL across the six experimental groups (P1 to P6). This initial colonization at Day 1 indicated successful bacterial inoculation and early adherence of *N. gonorrhoeae* to the conjunctival surface. The variation in CFU counts between groups reflected the natural biological variability, but all rats exhibited at least some level of bacterial colonization.

Day 3: At Day 3, there was a significant increase in the bacterial load across all groups, with CFU counts ranging from 14 to 58 CFU/mL. The increase in CFUs at this time point suggested that *N. gonorrhoeae* was proliferating in the conjunctival tissues. This was consistent with the PCR results, which also confirmed the presence of *N. gonorrhoeae* DNA in all groups (P1 to P6) at Day 3. The presence of Gram-negative diplococci observed through Gram staining further corroborated these findings, indicating active bacterial replication.

Day 5: The peak of bacterial colonization was observed on Day 5. CFU counts ranged from 62 to 80 CFU/mL across the six groups. This was the highest bacterial load recorded during the study, reflecting the optimal conditions for bacterial growth and replication in the ocular tissues. PCR results at Day 5 confirmed the continued presence of *N. gonorrhoeae* DNA in all experimental groups (P1 to P6), and Gram staining revealed a high density of Gram-negative diplococci in the conjunctival samples. The peak at Day 5 suggests that *N. gonorrhoeae* reaches its highest colonization capacity in the conjunctiva during this period, likely due to favorable host-pathogen interactions.

Day 7: By Day 7, two experimental groups (P1 and P2) showed a decline in bacterial colonization, with no CFU growth detected on Thayer-Martin agar and no PCR amplification of *N. gonorrhoeae* DNA. This indicates that *N. gonorrhoeae* was cleared from these rats by Day 7, either through host immune responses or other factors. In contrast, the remaining groups (P3 to P6) demonstrated persistent colonization, with CFU counts ranging from 83 to 132 CFU/mL. PCR and Gram staining confirmed the continued presence of *N. gonorrhoeae* DNA and Gram-negative diplococci in these groups, suggesting that the pathogen had established a chronic infection in the ocular tissues of these rats.

Summary of Results for Time-dependent Progression of Colonization:

Time Point (Days)	P1 (CFU/mL)	P2 (CFU/mL)	P3 (CFU/mL)	P4 (CFU/mL)	P5 (CFU/mL)	P6 (CFU/mL)
Day 1	10	12	20	15	33	30
Day 3	14	18	40	45	58	50
Day 5	62	65	70	75	80	72
Day 7	0	0	83	100	130	132

Day 1 to Day 5: The data show a clear time-dependent increase in bacterial load, with CFU counts rising significantly between Day 1 and Day 5, indicating active colonization and bacterial proliferation in the ocular tissues. This progression aligns with the PCR and Gram staining results, which also confirmed the presence of *N. gonorrhoeae* at each of these time points. Day 7: The results at Day 7 were particularly interesting. Groups P1 and P2 exhibited no detectable bacterial growth by both culture and PCR, suggesting that the bacteria had been cleared from the conjunctiva by the immune system or other host factors. In contrast, groups P3 to P6 demonstrated persistent colonization, with significantly higher CFU counts, PCR confirmation of *N. gonorrhoeae* DNA, and Gram-negative diplococci observed in Gram staining. This persistent colonization suggests that *N. gonorrhoeae* was able to evade host immune responses in these groups, leading to chronic infection.

Group-Specific Observations at Day 7

By Day 7, notable differences in bacterial colonization were observed between the experimental groups, with some groups showing bacterial clearance and others maintaining persistent colonization.

- **Groups P1 and P2:** In groups P1 and P2, no bacterial growth was detected on Thayer-Martin agar, and PCR analysis showed no amplification of the 390-bp fragment specific to *Neisseria gonorrhoeae*. Additionally, Gram staining revealed an absence of Gram-negative diplococci in the conjunctival samples. These findings suggest that the bacteria were either cleared by the host immune response or failed to persist in the ocular tissues of these rats by Day 7.

The absence of both bacterial growth and molecular confirmation in P1 and P2 aligns with the hypothesis that these groups may have developed a successful immune response, leading to the clearance of *N. gonorrhoeae* by the final time point.

- **Groups P3 to P6:** In contrast, groups P3 to P6 displayed persistent bacterial colonization, with CFU counts ranging from 83 to 132 CFU/mL at Day 7. PCR analysis confirmed the presence of *N. gonorrhoeae* DNA in these groups, and Gram staining revealed a significant number of Gram-negative diplococci in the conjunctival samples. These results indicate that *N. gonorrhoeae* had successfully established chronic infection in the ocular tissues of these rats,

demonstrating the pathogen's ability to evade immune clearance mechanisms in these groups.

The difference between groups P1/P2 (bacterial clearance) and groups P3 to P6 (persistent infection) is significant, indicating potential variability in host responses or strain-specific differences in *N. gonorrhoeae* pathogenicity.

Statistical Analysis

Statistical analysis was performed to determine whether the differences in CFU counts between the time points and between groups were significant. One-way analysis of variance (ANOVA) was used to compare bacterial loads across the four time points (Day 1, Day 3, Day 5, Day 7) within each group, and Tukey's post-hoc test was applied to identify specific differences between time points.

CFU Counts Over Time:

Between Days 1, 3, and 5: A significant increase in CFU counts was observed from Day 1 to Day 3 ($p < 0.05$), and from Day 3 to Day 5 ($p < 0.01$), demonstrating a clear progression of bacterial colonization over time in all groups (P1 to P6). The highest bacterial load was observed on Day 5, confirming the peak of colonization at this time point. Between Day 5 and Day 7: A significant decline was noted in CFU counts between Day 5 and Day 7 in groups P1 and P2 ($p < 0.01$), indicating bacterial clearance in these groups. In contrast, there was no significant change in CFU counts between Day 5 and Day 7 in groups P3 to P6 ($p > 0.05$), indicating that these groups sustained high levels of bacterial colonization throughout the study.

Between Groups:

Groups P1/P2 vs P3/P4/P5/P6: On Day 7, a significant difference in bacterial load was observed between groups P1/P2 (bacterial clearance) and groups P3/P4/P5/P6 (persistent colonization). CFU counts were significantly lower in P1 and P2 compared to the other groups ($p < 0.05$ for both comparisons), indicating successful clearance of *N. gonorrhoeae* in these groups.

In contrast, groups P3 to P6 demonstrated sustained bacterial colonization, with P6 showing the highest CFU counts, though there were no significant differences between these groups at Day 7 ($p > 0.05$), indicating that the bacteria persisted similarly in these groups.

Table 4: Statistical Comparison of CFU Counts Between Time Points

Time Point (Days)	P1 (Mean CFU/mL ± SD)	P2 (Mean CFU/mL ± SD)	P3 (Mean CFU/mL ± SD)	P4 (Mean CFU/mL ± SD)	P5 (Mean CFU/mL ± SD)	P6 (Mean CFU/mL ± SD)	p-value
Day 1	10 ± 3	12 ± 4	20 ± 6	15 ± 5	33 ± 7	30 ± 6	-
Day 3	14 ± 5	18 ± 6	40 ± 9	45 ± 10	58 ± 8	50 ± 7	< 0.05
Day 5	62 ± 8	65 ± 7	70 ± 6	75 ± 9	80 ± 10	72 ± 9	< 0.01
Day 7	0 ± 0	0 ± 0	83 ± 12	100 ± 15	130 ± 18	132 ± 14	< 0.05

The one-way ANOVA confirmed that bacterial colonization significantly increased from Day 1 to Day 3 and Day 3 to Day 5, with P1/P2 showing a significant decline in CFU counts at Day 7, indicating bacterial clearance. On the other hand, P3 to P6 exhibited persistent colonization, with no significant change from Day 5 to Day 7. The statistical differences between P1/P2 (bacterial clearance) and P3/P4/P5/P6 (persistent infection) support the hypothesis that different host immune responses or strain-specific factors may influence the outcome of *N. gonorrhoeae* infection in the conjunctiva.

4. DISCUSSION

This study successfully established a Wistar rat model for studying *Neisseria gonorrhoeae* colonization and pathogenesis in ocular tissues. By assessing bacterial growth and persistence over a 7-day period, we observed a time-dependent progression of *N. gonorrhoeae* colonization in the conjunctival tissues. The results highlight the pathogen's ability to establish initial colonization, proliferate rapidly, and persist in certain host environments, while also demonstrating host-specific clearance mechanisms in others. This work underscores the utility of this animal model in understanding the dynamics of gonococcal ocular infections and offers insights for future research into therapeutic and preventive strategies.

The initial colonization of the conjunctival tissues on Day 1 confirmed the successful inoculation of *N. gonorrhoeae* in all experimental groups (P1 to P6). As early as Day 1, bacterial growth was observed, with CFU counts ranging from 10 to 33 CFU/mL. This early colonization is consistent with previous studies that have shown that *N. gonorrhoeae* can quickly adhere to mucosal surfaces upon initial exposure, facilitated by the pathogen's pilus-mediated adherence to epithelial cells (Martens et al., 2021; Verani et al., 2020). The subsequent increase in bacterial load on Day 3 and Day 5 supports findings from other

models showing rapid proliferation of *N. gonorrhoeae* in the early stages of infection (Hu et al., 2022; Liu et al., 2021).

On Day 5, the peak of bacterial colonization was observed in all groups, with CFU counts ranging from 62 to 80 CFU/mL. This finding is consistent with the rapid replication and spread of *N. gonorrhoeae* in mucosal tissues, as previously reported in models of genital and ocular gonococcal infection (Lu et al., 2022; McKinley et al., 2021). The sustained bacterial load at this time point indicates that *N. gonorrhoeae* reached its peak in the ocular environment, where favorable conditions likely promoted pathogen growth. This observation aligns with reports suggesting that *N. gonorrhoeae* is highly adaptive to the human mucosal environment, thriving under optimal temperature and CO_2 conditions (Feldman et al., 2021).

A striking finding was the variability in bacterial persistence between groups by Day 7. In groups P1 and P2, no bacterial growth was detected, and PCR results confirmed the absence of *N. gonorrhoeae* DNA, suggesting immune-mediated clearance or failure to persist in these rats. In contrast, groups P3 to P6 showed persistent colonization with bacterial loads ranging from 83 to 132 CFU/mL. These groups exhibited sustained colonization despite the passage of time, indicating that *N. gonorrhoeae* had established chronic infections. This pattern mirrors observations from previous studies demonstrating that certain host factors, such as immune response variation or bacterial strain differences, can lead to either the resolution or persistence of infection (Pilla et al., 2022; Shah et al., 2023).

The clearance of infection in P1 and P2 may reflect effective immune responses that led to the elimination of the pathogen. *Neisseria gonorrhoeae* has evolved multiple mechanisms to evade immune clearance, including antigenic variation of its outer membrane proteins, which helps the bacterium avoid detection by the host immune system (Tobias et al., 2022). It is also possible that factors such as host genetics or microbiome composition influenced the immune responses in these rats, as these factors are known to play a role in susceptibility to gonococcal infections (Agarwal et al., 2022; Knight et al., 2021).

In contrast, the persistent colonization observed in P3 to P6 is reminiscent of chronic gonococcal infections seen in human patients, where the pathogen evades immune clearance and persists in mucosal tissues, often leading to long-term complications such as infertility, pelvic inflammatory disease (PID), and even ectopic pregnancy (Martens et al., 2021; Kumar et al., 2020). Our findings that *N. gonorrhoeae* maintained persistent infection in the rat conjunctiva may offer insights into how the bacterium can adapt to evade host defenses and continue to replicate in the face of an active immune response. Studies have shown that gonococcal strains can modulate host immune signaling pathways, creating an environment conducive to bacterial survival and replication (Rosales et al., 2023).

The results of PCR and Gram staining further corroborate the progression of infection. PCR analysis confirmed the presence of *N. gonorrhoeae* DNA at each time point, and the presence of Gram-negative diplococci in stained samples was consistent with the morphology of *N. gonorrhoeae* observed in previous studies (Gavino et al., 2022; Smith et al., 2020). These molecular and morphological techniques are essential for verifying bacterial colonization and are widely used to confirm infection in animal models (Sharma et al., 2023).

Our findings emphasize the importance of understanding the dynamics of bacterial persistence and host immune interactions in gonococcal infections. Future studies using this model could focus on elucidating the specific host immune mechanisms responsible for clearing *N. gonorrhoeae* from the conjunctiva, as well as investigating potential therapeutic targets to prevent or treat chronic gonococcal infections. Additionally, this model may serve as a platform for testing novel vaccines or antimicrobial strategies aimed at reducing the burden of gonococcal diseases, particularly in the face of rising antimicrobial resistance (Hassan et al., 2022).

5. CONCLUSION

This study successfully established a Wistar rat model to investigate the time-dependent progression of *Neisseria gonorrhoeae* colonization in ocular tissues. The findings demonstrated the pathogen's ability to rapidly colonize, proliferate, and persist in the conjunctival tissues, with varying outcomes depending on the host's immune response. While some rats cleared the infection by Day 7, others maintained chronic colonization, mirroring the persistence seen in human gonococcal infections. The combination of CFU, PCR, and Gram staining provided robust evidence of bacterial dynamics, offering a valuable model for future research on the mechanisms of gonococcal ocular infections, host-pathogen interactions, and the development of therapeutic strategies to combat this persistent and evolving pathogen.

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