

Research Article

Distinct Patterns of Host Adherence by *Neisseria gonorrhoeae* Isolated from Experimental Gonorrhea

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Received 6 September 2019; Revised 30 March 2021; Accepted 3 April 2021; Published 15 May 2021

Academic Editor: Louis Detolla

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Neisseria gonorrhoeae (*N. gonorrhoeae*, gonococci, or GC), the etiologic agent of gonorrhea, is a human-obligate bacterial pathogen. The GC surface contains pili that mediate the adherence to host cells. Studies have shown that GC pili, coded by pilin genes, undergo remarkable changes during human experimental gonorrhea, possibly generated by DNA phase variation during infection. The question that arises is whether the changes in pilins can alter the adherence capacity of *N. gonorrhoeae* to host cells. In this study, six variants initially isolated from male volunteers infected with one single clone of GC were examined for their adherence patterns with human Chang conjunctiva cells. In this study, we showed that the variants showed distinct adherence patterns to this cell line under light microscopy and scanning electron microscopy. Moreover, two reisolates showed higher adherence capacities than that of the input strain. The results provide an additional example as to how the pilus variation may play a role in the pathogenesis of *N. gonorrhoeae*.

1. Introduction

The Gram-negative bacterium *N. gonorrhoeae* (*N. gonorrhoeae*, gonococci, or GC) is the etiologic agent of sexually transmitted disease, gonorrhea, the second most common sexually transmitted infection after chlamydia infection [1]. Studies have suggested that GC has the ability to facilitate the human immunodeficiency virus infection [2–5]. With more than 106 million cases per year, the emergence of an antimicrobial resistance gonococcus (GC), especially a “Super Bug” GC [6–9], has become a worldwide concern [10, 11]. Without a highly effective vaccine to

prevent gonorrhea, it presents a substantial challenge to public health [12, 13].

GC is an obligate human pathogen that possesses the tremendous ability to change its surface components, most notably the pili [14, 15], opacity-associated (Opa) proteins [16, 17], and lipooligosaccharides (LOSs) [18, 19]. It is expected that such a remarkable capacity to change may have a significant impact as an immune escape mechanism [20–22]. A series of very controversial human experiments were initially carried out by Swanson et al. to understand the roles of pili, Opa proteins, and LOSs in the pathogenesis of GC infection [15, 19, 23–25]. The results confirmed that all three

major components varied significantly during the human experimental gonorrhoea, indicating that these three major GC components are virulence factors [26–28].

The antigenic repertoire of GC surface pili is encoded by *pilE* and *pilS* genes [29]. However, the genes are subject to DNA phase and antigenic variations, which produce millions of different antigen variants during gonococcal infection [30, 31]. The *recA* gene controls the DNA phase variation in *N. gonorrhoeae*. As a result, in a *recA*-deficient strain, the phase variation by pili occurs at a 100–1000-fold reduced rate [32].

GC pili play an essential role in the mediation of adherence to hosts [33] and changes in the pilin amino acids may alter the adherent capacity of *N. gonorrhoeae* [23]. Studies by Swanson et al. showed that the pilin sequences of almost all of the reisolates were changed during infection. However, no studies on the biological functions were conducted with these reisolates. In this study, the adherence patterns of host-pathogen interaction were examined with scanning electron microscopy (SEM) in *N. gonorrhoeae* MS11_{mk} isolated from human experimental gonorrhoea.

2. Materials and Methods

2.1. Ethics Statement. All experimental procedures were specifically approved for this study by the Medical Ethics Committee of Tongji Hospital and were conducted in accordance with institutional guidelines (IRB: TJC20140113). All volunteers involved in the experiment signed informed consent.

2.2. Bacterial Strains. The *N. gonorrhoeae* MS11_{mk} used in this study was reisolated from human experimental gonorrhoea by Swanson et al. in the 1980s [23]. Briefly, two male volunteers were challenged with GC suspension and urine samples of the subjects were collected for the isolation of GC 2 days after inoculation. All strains were cultured and maintained as previously described [34]. The input GC are the pilus⁺ (*P*⁺) and LOS_b phenotype and express no outer membrane protein II (*Opa*[−]) [19, 34]. Both the input and reisolated GC were constructed and designated to be *recA* mutants, following the methods by Koomey et al. [35]. A cosmid clone containing the *recA* gene of MS11 was isolated by complementation of the *E. coli* HB101 *recA* mutation. Defined mutations in and near the cloned gonococcal *recA* gene were constructed in vitro and concurrently associated with a selectable genetic marker for *N. gonorrhoeae* and the mutated alleles were then reintroduced into the gonococcal chromosome by transformation-mediated marker rescue [35]. All GC were grown overnight for 18 h on solid GC agar at 37°C in a humidified 5% CO₂ atmosphere, unless otherwise stated.

2.3. Immunoblotting. GC was suspended in phosphate-buffered saline (PBS) to the same optical density at 540 nm and immersed in 20 µl solubilizing solution (containing 4% sodium dodecyl sulfate and 8% 2-mercaptoethanol). After heating in a boiling water bath for 10 min, 10 µl of each lysate

was applied to the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) slab gel. After electrophoresis, the SDS-PAGE-separated components were electrophoretically transferred to a sheet of nitrocellulose, which, after blocking with a solution containing bovine serum albumin, was incubated with rabbit monoclonal antiserum MCO2 for 2 h with a dilution of 1 : 500. The visualization of pilin antigen-bound antibodies through reaction with ¹²⁵I-protein A was followed by autoradiography [28, 34]. The LOS was isolated from each of the strains, and approximately 1 mg of the LOS was analyzed on a 13% acrylamide gel. The LOS phenotypes were confirmed with rabbit anti-LOS MAbs (1 : 200) specific for LOS_a and LOS_b, and the primary antibodies were detected with a goat anti-rabbit IgG [36]. *Opa* expression was confirmed by SDS-PAGE and immunoblotting with monoclonal antibody 4B12 with a dilution of 1 : 200, which recognizes all *Opa* proteins from *N. gonorrhoeae* MS11_{mk} [37].

2.4. Adherence and Internalization Assays. The human conjunctiva cells, which were used for all adhesion and invasion assays, were cultured in RPMI 1640 medium (GIBCO/BRL) with 10% fetal calf serum (HyClone). For adherence assays, the cells were grown to confluence ($\approx 2 \times 10^5$ cells per well) in 24-well culture plates (Falcon) and washed twice with serum-free RPMI 1640. GC was suspended in RPMI 1640 at an OD₅₄₀ of 0.04, and 0.5 ml of the bacterial suspensions was added to each well. The plates were incubated at 37°C in the presence of 5% CO₂ in a humidified atmosphere for 1.5 to 6 h. Experiments were terminated by washing three times with 2 ml of serum-free RPMI 1640. Adherent bacteria were counted by suspending the cells in sterile PBS containing 0.5% saponin (Calbiochem) for 5 min at 37°C and plating dilutions on GC agar plates. The level of adherence was calculated by determining the CFU associated with the host cell monolayers after a 24 h incubation period. Internalization assays were similarly conducted like the adherence assays, but after the period of the bacterial interaction with cell lines, the monolayers were washed twice and then incubated for 90 min with 1.5 ml of RPMI 1640 supplemented with 100 µg/ml gentamycin (GIBCO/BRL).

2.5. Scanning Electron Microscopy (SEM). The microscopy assay followed the protocol by Stephens et al. with some modifications [38]. The infection process has been described above. After a coinoculation of 5 h at 37°C in the presence of 5% CO₂ in a humidified atmosphere, the wells were moderately washed with filtered-sterilized PBS to remove the unbound bacteria. Thereafter, the culture was fixed in a 3% glutaraldehyde solution buffered to pH 7.3 with 0.1 M sodium cacodylate. After postfixation in 1% osmium tetroxide in the same buffer, samples were dehydrated in increasing concentrations of ethanol and = critical point dried with CO₂. The samples were coated with gold-palladium before examination in a scanning microscope (JSM-35CF; JEOL USA, Peabody, MA), as described elsewhere in detail [39].

2.6. Normal Human Serum (NHS) and Serum Killing. Serum samples were collected intravenously from volunteers with no history of GC infection. The sera were aliquoted and stored at -70°C . The NHS was immediately thawed before use. The inactivated serum was achieved by heating the NHS at 56°C for 30 min. The method used to evaluate the serum sensitivity of bacterial strains was described previously [36]. The GC were propagated on GC HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid)-agarose medium (GCHA) in case they formed into clusters. After culturing for 14 to 16 h, bacteria were suspended in PBS to an OD_{540} of 0.5 and diluted to 1:10 in RPMI medium. Diluted bacterial suspensions ($50\ \mu\text{l}$) were mixed with an equal volume of dilutions of 10% NHS in RPMI medium and incubated at 37°C in 5% CO_2 for 60 min. Control assays were done in a manner that was identical to the heat-inactivated serum (56°C for 30 min). After performing serial 10-fold dilutions of the mixtures, the number of viable bacteria was determined by plating on GC agar. Serum resistance levels were determined by comparing the number of surviving bacteria treated with fresh NHS with that of the survivors treated with heat-inactivated NHS (defined as 100%).

2.7. Statistical Analyses. All statistical analyses were completed using Prism software, version 6 (Graph Pad, San Diego, CA, USA). Statistical significance was assessed using Student's *t*-test for the univariate analysis of two sets of data and two-way ANOVA was used for multiple comparisons; *** $p < 0.001$.

3. Results

3.1. Construction of *recA*-Deficient Mutants. The *recA* gene accounts for the generation of pilus antigenic diversity and piliation phase [32]. To reduce the rates of pili changes during *in vitro* experiments, both input and reisolated GC [23] were constructed to be *recA* mutants, following the methods by Koomey and Falkow et al. [35]. SDS-PAGE and immunoblotting were carried out to determine the phenotype of LOS, Opa protein, and pili of the reisolates and input strain (Figure 1). All of the 6 reisolates expressed pilin subunits that exhibited the same electrophoretic mobility as the input strain (Figure 1(a)), while previous studies by Swanson et al. indicated that the pilin mRNA sequencing of these strains exhibited multiple sequence changes in pilin mRNAs compared to the input GC [23]. The reisolates expressed no outer membrane protein (Opa) (Figure 1(b)). Interestingly, out of the six reisolates, two appeared to be LOSa phenotype (Figure 1(c)), which was probably generated from the parental strains that were LOSb phenotype. This phenomenon was in accordance with studies by other researchers [18, 19], as the GC LOS transition frequently occurs *in vivo*.

3.2. Reisolates Exhibit Distinct Adherence Patterns to Chang Conjunctiva Cells. Light microscopy and SEM were used to examine the adherence patterns of the input strain and reisolates to Chang conjunctiva cells, which represent tissues

that are natural sites for infection by GC [40, 41]. As illustrated in Figure 2, the reisolate G29, being identical to the input strain MS11, accumulated together to form clusters between the interface of bacteria and Chang cells (Figures 2(a) and 2(b)). The reisolates 4R1 and SA were noticeably distributed on the cell surfaces (Figures 2(c) and 2(d)), while 6uF possessed small parts that formed bacterial clusters similar to the MS11 strain (Figure 2(f)). The 7uB formed clusters to facilitate higher adherence to Chang cells as compared with the other reisolates (Figure 2(e)). For strain V3, only a few organisms were visualized on the cells, indicating that the V3 strain was less adherent than the other reisolates (Figure 2(g)).

Under the SEM (Figure 3), the reisolate G29 formed morphologically similar ball-like bacterial clusters as the input strain MS11. This finding was consistent with observations of light microscopy (Figures 3(a) and 3(b)). Although the SA and 4R1 reisolates exhibited similarly constituted morphologies, SA appeared to establish more noticeably sufficient linkages with one another than 4R1 (Figures 3(c) and 3(d)). Furthermore, the 7uB displayed remarkable adherent junctions between the GC and conjunctiva cells including intra-GC, followed by 6uF, 4R1, and SA. The interactions between abundant pili of 7uB were able to promote the formation of a complex network on the surface of conjunctiva cells (Figure 3(e)). Moreover, the flattened 6uF was distinguishable from MS11 and 7uB (Figure 3(f)). However, the V3 showed relatively incompact connections and were distributed separately at the interface of the epithelial cells (Figure 3(g)). Interestingly, a spherical bacterial cluster distributed on the surface of an unknown material (beads or dust) was inadvertently observed. The strain 7uB was able to accumulate with tight conjunctions to form a golf ball-like structure (Figure 3(h)). The observations made with SEM were similar to the findings of light microscopy. Taken together, the results indicated that with regard to the adherence to host cells, the GC underwent tremendous modulations during experimental gonorrhoea.

3.3. Reisolates Adhered to and Internalized by Chang Conjunctiva Cells. The input strain MS11 and six reisolates with the Opa⁻ phenotype were incubated with human Chang conjunctiva cells. The reisolate 7uB exhibited higher adherence compared with the other strains, followed by 6uF, G29, MS11, and SA (Figure 4(a)). The V3 showed relatively lower adherence capacity as compared to the input strain (Figure 4(a)). G29 exhibited almost the same level of adherence with that of input strain MS11. It is demonstrated in Figure 4(a) that the adherence capacities of these bacteria increase with a prolonged period of incubation. The gentamycin protection assays for bacterial internalization revealed that the reisolate 4R1 significantly invaded the cells (Figure 4(b)). Interestingly, the 7uB, 6uF, and SA displayed moderately lower internalization levels, which were inconsistent with the findings of adherence. The disparity in the data of the adherence and internalization may be predominantly attributed to alterations in bacterial components and their varying degrees of functional aspects during host-pathogen interaction.

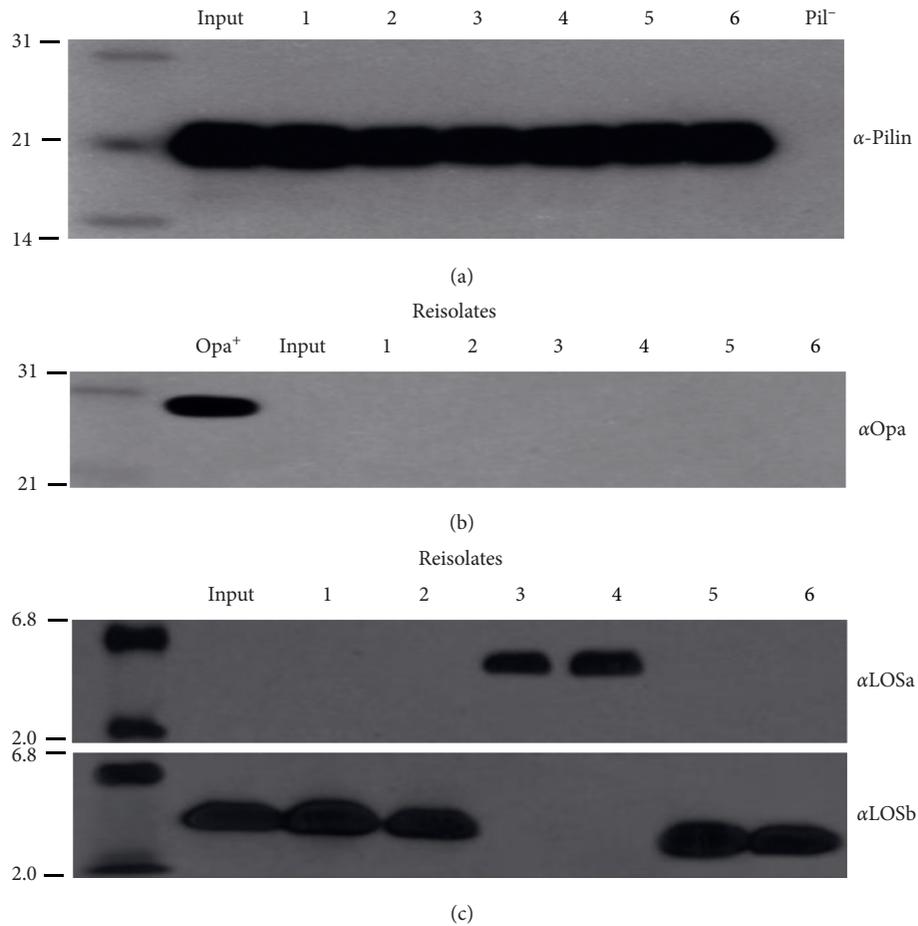


FIGURE 1: Analysis of the pili, Opa protein, and LOS phenotypes of reisolates. (a) Whole cell lysates of reisolates were subjected to SDS-PAGE and were immunoblotted with anti-pilus monoclonal antibody MCO2. (b) The expression of the Opa protein was detected with monoclonal antibody 4B12, which reacts with all of the Opa proteins expressed by the strain MS11mk. (c) The LOS preparations were analyzed by SDS-PAGE and then immunoblotting with α LOSa and α LOSb. Autoradiography was used to detect the LOS labelled in vitro. Lanes 1 to 6 represent G29, 4R1, SA, 7uB, 6uF, and V3, respectively. The molecular mass markers (in kilodaltons) are indicated on the left side of the panels.

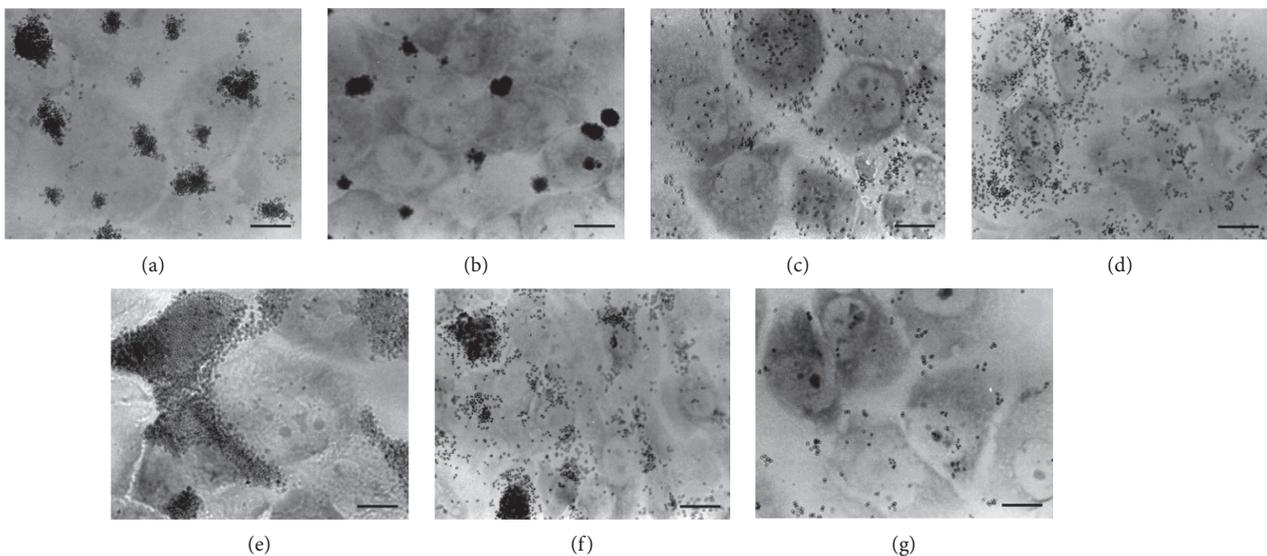


FIGURE 2: Light micrograph showing the patterns of adherence of reisolates to human conjunctiva cells ((a–g) represent MS11, G29, 4R1, SA, 7uB, 6uF, and V3, resp.). After the 5 h adherence assay, nonadherent bacteria were removed by washing three times with sterilized PBS. Cells were stained with a modified Wright stain and viewed under light microscopy. Scale bar: 10 μ m.

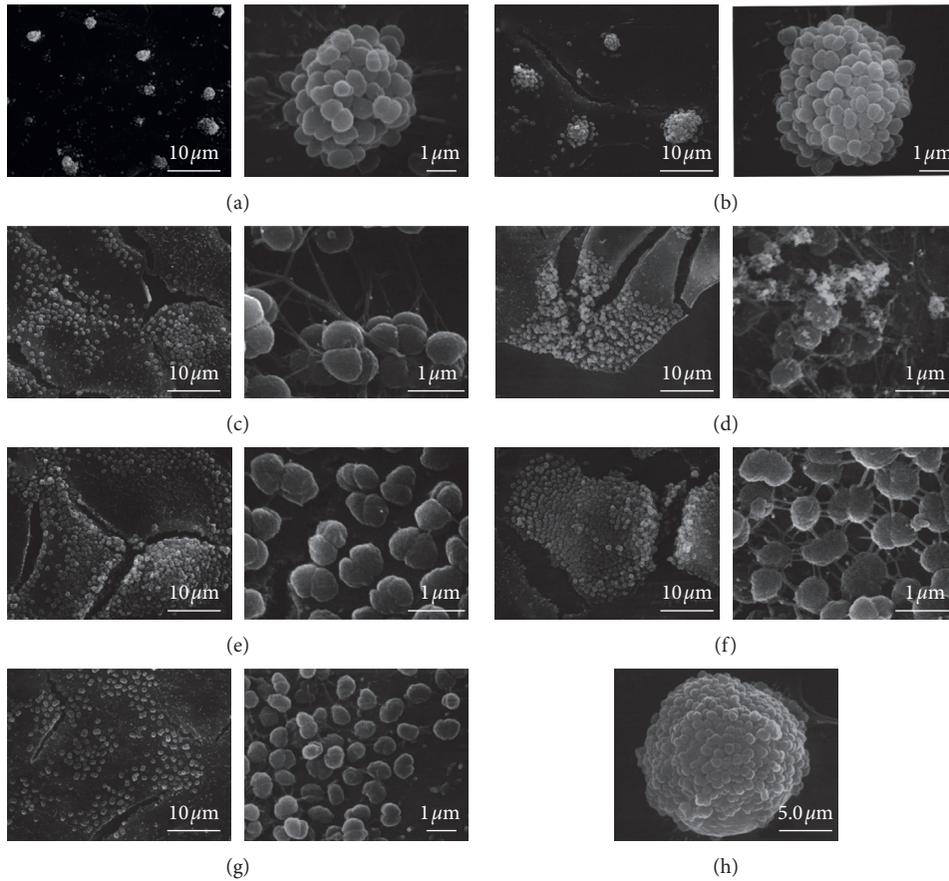


FIGURE 3: Scanning electron micrograph showing adherence of bacterial reisolates to host cells. Each panel depicts a representative group of bacteria ((a–h) represent MS11, G29, 4R1, SA, 7uB, 6uF, V3, and 7uB, resp.). The left panel indicates the low power magnification and the right panel indicates the higher magnification. Scale bars are mentioned at the right bottom of each image. Figure 3(h) depicts the interaction of 7uB to dust or unknown bead particles.

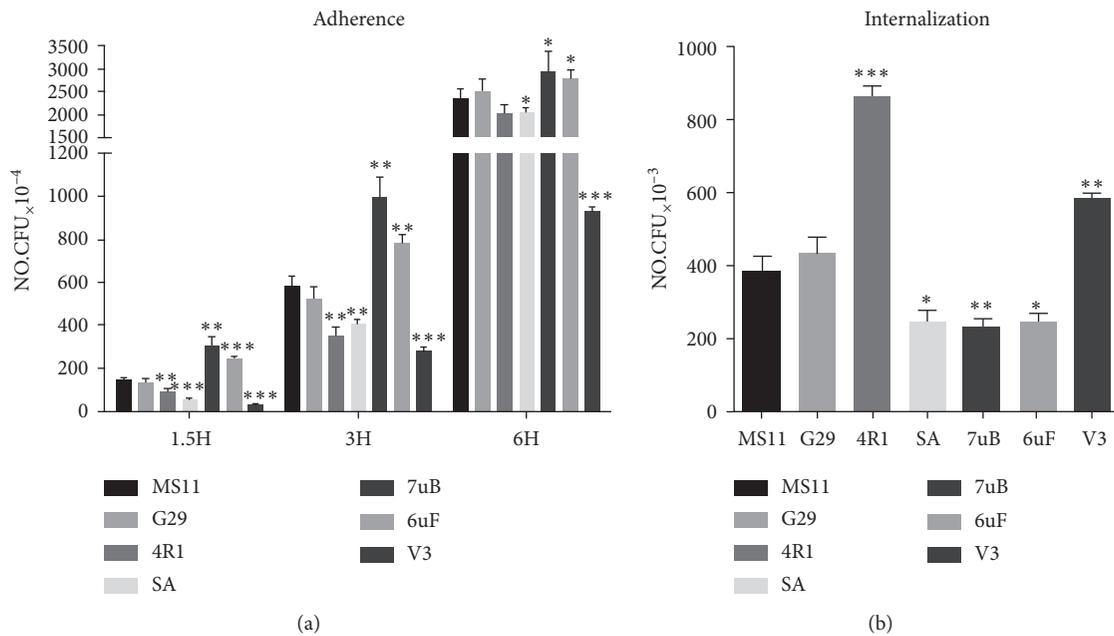


FIGURE 4: The (a) adherence and (b) internalization of reisolates and input strain (MS11) to human Chang conjunctiva cells. The level of adherence to cells was calculated by determining the cell-associated CFUs of the host cell monolayers (coincubation for 1.5 to 6 h after an incubation period of 24 h). For internalization, an approach similar to adherence assays by killing the extracellular bacteria with 100 µg/ml (final concentration) gentamicin as previously described. The number of internalized bacteria was determined by counting CFUs recovered following gentamicin treatment. ****p* < 0.001. ***p* < 0.01. **p* < 0.05.

3.4. *The Sensitivity of Reisolates to Serum Killing.* Reisolates SA and 7uB exhibited markedly higher resistance to the effect of serum killing compared to the input strain MS11 and the remaining reisolates in the presence of 10% NHS (Figure 5(a)). The strains MS11, G29, and SA were further examined at different concentrations of NHS, and the sensitivity of SA attenuated by 50% upon incubation with 75% NHS (Figure 5(b)). The resistance to NHS results from the specific masking of the LOS epitopes that react with the nonsialylated molecules among bactericidal antibodies in human serum [42, 43]. The LOSb variants (which can be sialylated) were much more sensitive to the killing effect of NHS than the LOSa variants (which cannot be sialylated) [36]. All strains used in this study were Opa⁻ GC; the SA and 7uB were LOSa phenotype. This observation was identical to that of the previous study. These results indicated that the input GC retained various components that are involved in the bacterial resistance to serum killing during infection.

4. Discussion

Neisseria gonorrhoeae (*N. gonorrhoeae*, gonococci, or GC), the etiologic agent of gonorrhea, is a human-obligate bacterial pathogen, and therefore, there still is no animal model. Dr. John Swanson's group led studies by using human volunteers as the models to study the molecular pathogenesis of GC (human experimental gonorrhea) [23, 28, 32, 37, 44, 45]. One of the studies was to challenge human volunteers through the urethra with a single, live, and virulent clone of GC MS11_{mk}. After showing signs of gonorrhea, the discharged GC were collected and the reisolates were stored for further studies. The major finding of this 1987 study was that the pilin sequences of almost all reisolates were changed during infection [23], indicating that pili play a major role during gonorrhea. Because no studies on biological functions were then conducted with these reisolates, the study was to understand in part the changes of these reisolates for the adherence to host cells.

"Seeing is believing." This study focused on the distinct colony morphologies, adherent patterns of six GC reisolates from human experimental gonorrhea to Chang human conjunctiva cells. The results obtained from the presented study provide photographic adherent patterns of GC isolates to host cells, which are consistent with other reports, indicating the possibilities of surface component variations of GC during infection. As shown in Figure 1(a), both the input strain and reisolates expressed pilin. However, the pili of some strains are not visible when observed with SEM. This is consistent with the results reported by others [46]. Studies by Swanson et al. showed that pili exhibit highly variable characteristics both in the laboratory and in vivo through frequently programmed recombination events, including expression of different silent, partial pilin genes after their incorporation into the expressed complete pilin gene via gene conversion. Gonococcal pilus phase variation is involved in switches from piliation (*P*+) to nonpiliation (*P*-) and vice versa. The pilin gene recombination generates antigenically distinct pilins with variant epitopes, which can be detected by antibodies [23, 44, 47]. The recombination

events might occur in the process of these experiments, generating nonpiliation (*P*-) GC strains where the pili are not visible.

The internalization of bacteria was not in accordance with adherence (Figure 4), and it should be noted that the levels of adherence and internalization were determined by counting the CFUs recovered from cell lysis. The SEM observations showed the GC tend to form clusters easily on epithelial cells. It was assumed that the bundled GC clusters might not be easily separated in the lysis buffers. Moreover, the input strain MS11 and re isolate G29 formed ball-like structures on the interface of epithelial cells. Such formation might block the contact between the GC and host cells, leading to poor internalization, while the V3 with loose colonies easily interacted with the host cells. The re isolate SA and 7uB, the most bundled strains, exhibited efficient resistance to serum killing (Figure 5) compared with parental MS11 and the other reisolates, which might be attributable to the LOS types. The bactericidal effect of the NHS results is mainly from the recognition and interaction between the bacteria-impelled antibody IgM and LOS, which elicits complement-mediated killing actions [48, 49]. The variation between the LOS types has been demonstrated in human experimental infections [19]. A previous study has illustrated that the LOSb variants were much more sensitive to the killing effect of NHS than the LOSa variants [36, 50]. Moreover, studies by Ward et al. suggested that *N. gonorrhoeae* harvested directly from a urethral exudate displayed unstable serum resistance and in vivo resistant strains may shift to sensitive ones when subcultured in a laboratory [51].

The 1987 study uncovered the possible pathogenic roles of gonococcal pili [23, 28, 32, 37, 44, 45]. Since then, progress in our understanding of gonococcal pilus-mediated pathogenesis and other pathogens with this model has been made. 1. The study has given an understanding of why GC is a human-obligate pathogen. This conclusion was based on the identification of pilC and its relationship with pilE. It appeared that the pilC piled on the tip of pilE mediates the human-specific interactions [52–54]. Furthermore, the CD46, a human-specific receptor, may also participate in the pilus-host interactions [55]. 2. The same approaches from the 1987 study have been applied in studying the other components such as Opa and LOS of GC, indicating that the human experimental gonorrhea has proven to be a very valuable tool to study the pathogenesis of gonorrhea [23, 28, 32, 37, 44, 45] and other pathogens. *Haemophilus ducreyi*, a Gram-negative pathogen and the cause of the sexually transmitted disease chancroid, was also injected into human skins to mimic infection processes of chancroid in later nineties by Dr. Spinola's Group [56, 57]. 3. They demonstrated that the main biological function of pili is to mediate host-pathogen interactions. The mouse model developed by Jerse's et al. group showed that they were still able to be infected by pilus negative GC, when the GC was coexpressed with other adherent molecules such as the Opa proteins [58, 59].

In summary, there are still no promising animal models, which fully mimic the pathogenic processes during GC infection. On the horizon, the results generated from the

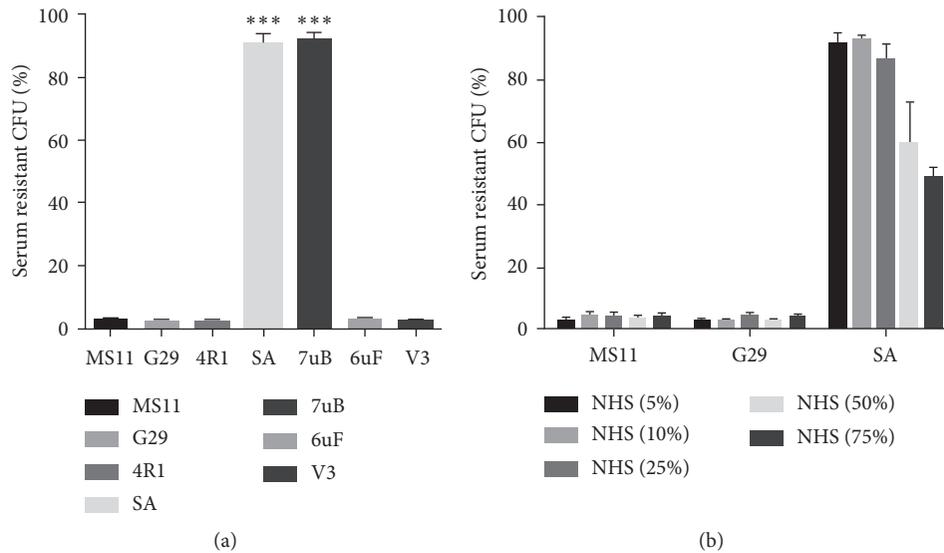


FIGURE 5: Serum resistance levels of the input and reisolated strains were assayed at different concentrations of the NHS. Bacterial suspensions were allowed to coinoculate with the NHS for 60 min, and control assays were performed identically with heat-inactivated serum (56°C for 30 min). The serum resistance of the input strain and reisolates were assayed for their resistance levels at different concentrations of NHS (10% for (a); 5%–75% for (b)). All of the experiments were performed in triplicate. *** $p < 0.001$.

1987 study on human experimental gonorrhoea were the only way to ascertain the representative of pilus pathogenesis for gonorrhoea. This enclosed study provides additional data as to why different pilus variants affect the pathogenesis for experimental gonorrhoea. Knowledge of this may help in the development of an effective gonococcal vaccine.

Data Availability

The experimental data used to support the findings of this study are included in the article.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors' Contributions

Yingxia He and Song Zhang contributed equally to this article.

Acknowledgments

The work was in part done twenty-nine years ago, while the PI (Tie Chen) was pursuing postdoctoral training at the Rocky Mountain Laboratories (RMLs), Hamilton, Montana, USA. The PI would therefore like to take this opportunity to express his highest appreciation to Dr. John Swanson, the big boss, who passed away seven years ago, and Dr. Robert Belland, the small boss, who also passed away last year, for their daily supervision, guidance, and help during my tenure at RMLs from 1998 to 2009. Either of them could be the corresponding author for this study. Finally, the PI would like to acknowledge the Tongji Hospital, HUST, China, for

their support, which allowed for the continuation of this project and the submission of this manuscript. This work was supported by grants from the National Natural Science Foundation of China (NSFC 81271780 and 81471915) and by two local grants from Tongji Hospital, Tongji Medical College, to Tie Chen.

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