Biofilm formation and invasive ability contribute to CC17 serotype III group B *Streptococcus* virulence

Lijun Wang[1], Zhi Tao[2], Binghuai Lu[3]

[1]Department of Clinical Laboratory, Beijing Tsinghua Changgung Hospital, School of Clinical Medicine, Tsinghua University, Beijing 102218, China;
[2]Department of Obstetrics and Gynecology, Beijing Tsinghua Changgung Hospital, School of Clinical Medicine, Tsinghua University, Beijing 102218, China;
[3]Department of Pulmonary and Critical Care Medicine, Laboratory of Clinical Microbiology and Infectious Diseases, China-Japan Friendship Hospital, Beijing 100029, China.

To the Editor: Group B *Streptococcus* (GBS) colonizes the vagina and rectum of 7–30% of pregnant women and contributes to materno-neonatal invasive infections. To date, the pathogenesis of clinical GBS strains has remained elusive.

Our previous study revealed that the clinical GBS isolates representing hypervirulent clonal complex 17 (CC17) with enhanced invasiveness would disproportionately affect neonates and cause severe invasive diseases, including bacteriemia or meningitis, frequently than other strains of clonal complexes (CCs), for example, CC23.[1] There is a stable carriage of serotype III in CC17, whereas most CC23 GBS isolates harbor serotype Ia.

Herein, we investigate the mechanisms by which CC17 serotype III exerts potential invasions. Fourteen clinical GBS isolates were involved, including seven CC17 serotype III isolates from the blood or cerebral spinal fluid of neonates (invasive isolates) and seven CC23 serotype Ia isolates from vaginal and rectal swabs of asymptomatic pregnant women (colonizing isolates as the control group). The Ethics Committee of Beijing Tsinghua Changgung Hospital approved the study procedure (No.19200-1-01). The written informed consent from participants was exempted because the privacy of subjects was not affected.

GBS persistence in the host niches is a precondition for invasion, whereas the biofilm mode of GBS cells in vaginal environments is pivotal for such persistence. Since genotypic characteristics of GBS are associated with biofilm formation abilities, we performed a microtiter plate assay to determine the biofilm formation of the above GBS isolates. Briefly, overnight cultures of GBS were inoculated at 1000-fold dilution in Todd Hewitt Broth (Oxoid Ltd., London, UK) with 1% glucose. A total of 200 μL aliquots of each culture were added into sterile 96-well flat-bottom plates and incubated at 37°C with 5% CO_2 for 48 hours. The biofilms were stained with 0.1% crystal violet for 10 minutes and destained with 30% acetic acid for 10 minutes, then measured at 550 nm in a Synergy H Microplate Reader (Biotek, VT, USA). Each assay was performed in duplicate wells and repeated three times. Our study defined isolates with $A_{550\,nm} > 0.5$ as biofilm producers and those having <0.5 absorbances as weak biofilm producers.[2]

The GBS invasion into epithelial cells in colonization niches is considered as a primary step to cause infections. It might invade vaginal epithelial cells and survive within the intracellular environment. Therefore, we detected the cytotoxicity of GBS to human VK2 vaginal epithelial cells to address its ability of invasion. Briefly, human vaginal epithelial cells VK2 (ATCC CRL-2616) were grown in a keratinocyte serum-free medium (Life Technologies, Carlsbad, CA, USA). VK2 cells at a concentration of 10^6 cells/mL were infected with approximately 10^6 CFU/mL of exponential phase GBS (initial Multiplicity of Infection MOI:1) and incubated for 1 hour at 37°C with 5% CO_2. The toxicity on VK2 cells induced by GBS isolates was determined using the Cytotoxicity LDH Assay kit (Dojindo, Kumamoto, Japan) following the manufacturer's instructions.

Furthermore, the host immunity will initiate attacking against GBS after it breaks through mucosal barriers. For instance, GBS is internalized by the dendritic cells (DCs). Furthermore, it stimulates pro-inflammatory cytokines' secretion from DCs.[3] In this study, we analyzed phagocytosis and pivotal cytokines secretion of DCs to address the pathogenesis of hypervirulent GBS CC17 as described previously.[4] Interleukin (IL)-12, mainly

Access this article online

Quick Response Code:

Website: www.cmj.org

DOI: 10.1097/CM9.0000000000001861

Correspondence to: Prof. Binghuai Lu, Department of Pulmonary and Critical Care Medicine, Laboratory of Clinical Microbiology and Infectious Diseases, China-Japan Friendship Hospital, Beijing 100029, China
E-Mail: zs25041@126.com

Chinese Medical Journal 2022;135(7)

Received: 29-08-2021; **Online:** 02-11-2021 **Edited by:** Peng Lyu

released by DCs in response to microbial pathogens, is an inductor for T helper type 1 cell (Th1) polarization. The active form interleukin 12 p70 (IL-12p70) of IL-12 works as a robust response to invasive pathogens. At the same time, transforming growth factor-β1 (TGF-β1), the predominant isoform of TGF-β, which is most expressed in immune cells, might repress Th1 polarization, down-regulate differentiation of Th2 cells, and induce immunological tolerance in DCs. Briefly, DC2.4 cells at a concentration of 10^6 cells/mL were infected with 10^6 CFU/mL of exponential phase GBS isolates (initial MOI:1) and incubated for 2 hours at 37°C with 5% CO_2. After incubation, 100 μg/mL of gentamycin and 5 μg/mL of penicillin G (Solarbio, China) were added to kill extracellular bacteria for 1 hour. Sequentially, intracellular GBS isolates were enumerated. The GBS phagocytosis was expressed as CFU/mL of intracellular bacteria. The cytokines of TGF-β1 and IL-12p70 secreted by DC2.4 cells were measured by ELISA kits (ExCell Bio, Shanghai, China) after 20 hours of interaction between GBS and DC2.4 cells.

All data were expressed as means ± standard error of the mean (SEM). Statistical analyses were conducted using

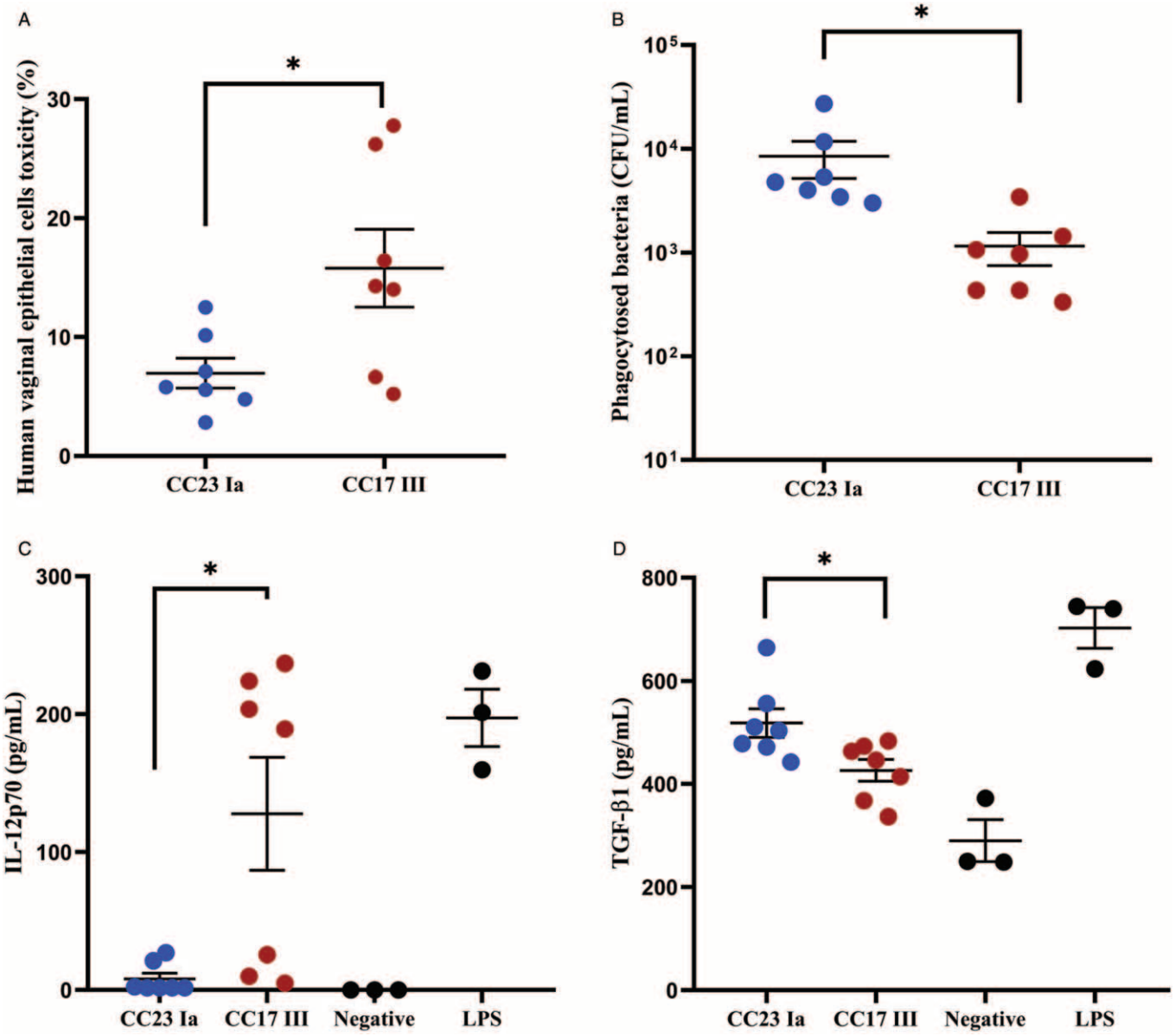

Figure 1: The differences of virulence ability between CC17 serotype III and CC23 serotype Ia GBS Isolates. (A) CC17 serotype III Isolates Induced higher cytotoxicity to human vaginal epithelial cells VK2 (ATCC CRL-2616) than CC23 serotype Ia isolates. CC17 serotype III isolates induced 15.79 ± 3.28% cytotoxicity, and CC23 serotype Ia isolates induced 6.96 ± 1.25% cytotoxicity ($P = 0.03$). (B) CC23 serotype Ia isolates were phagocytosed by DCs DC2.4 more than CC17 serotype III. The mean number of phagocytosed bacteria of CC23 serotype Ia and CC17 serotype III was 8505 CFU/mL and 1157 CFU/mL, respectively ($P = 0.04$). (C) CC17 isolates stimulated DC2.4 to release much more IL-12p70 compared with colonizing isolates ($P = 0.01$). (D) Colonizing CC23 isolates stimulated DC2.4 to release much more TGF-β1($P = 0.02$). Non-stimulated cells served as negative control and 1 μg/mL LPS stimulation as the positive control. Results are means ± SEM measured in triplicates. Data were analyzed using the two-way unpaired *t* test. CC17: Clonal complex 17; DCs: Dendritic cells; GBS: Group B *Streptococcus;* IL-12p70: Interleukin 12p70; LPS: Lipopolysaccharide; TGF-β1: Transforming growth factor-β1.

GraphPad Prism version 8.0.1 (GraphPad Software, CA, USA). A P value of < 0.05 was considered statistically significant.

According to the criteria mentioned above of biofilm production, all CC23 colonizing isolates were weak biofilm producers, whereas six (85.71%) CC17 isolates were biofilm producers, revealing the potentiality of forming biofilms of CC17 sublineage was higher than that of CC23 ($P = 0.03$). This result demonstrated a strong correlation between the hypervirulent CC17 clone and biofilm-forming and helped interpret the previous epidemiological findings that CC17 persisted better in women.[5]

Cytotoxicity LDH results showed CC17 serotype III and CC23 serotype Ia isolates induced $15.79 \pm 3.28\%$ and $6.96 \pm 1.25\%$ cytotoxicity to human vaginal epithelial cells, respectively ($P = 0.03$; Figure 1A). The GBS sublineage, as a key genotypic characteristic, was associate with the invasive ability.

Moreover, DC2.4 cells can effectively internalize CC23 serotype Ia and CC17 serotype III isolates 2 hours post-phagocytosis. The number of phagocytosed bacteria of CC23 serotype Ia and CC17 serotype III was 8505 ± 3327 CFU/mL and 1157 ± 409 CFU/mL, respectively. Therefore, CC23 serotype Ia isolates were phagocytosed more than CC17 serotype III ($P = 0.04$) [Figure 1B]. The results were consistent with a previous observation that serotypes III and V GBS differed in bacterial internalization and intracellular survival.[4] On the other hand, significant differences in the level of both IL-12p70 and TGF-β1 between CC17 serotype III and CC23 serotype Ia isolates were observed. Invasive CC17 isolates stimulated DC2.4 releasing much more IL-12p70 (127.80 ± 40.88 pg/mL) compared with colonizing CC23 isolates (8.10 ± 4.15 pg/ mL; $P = 0.01$) [Figure 1C]. Nevertheless, colonizing CC23 isolates stimulated DC2.4 releasing more TGF-β1 (518.10 ± 27.83 pg/mL) than CC17 isolates (426.30 ± 21.18 pg/mL; $P = 0.02$) [Figure 1D]. Accordingly, CC17 serotype III isolates were more resistant to internalization by DCs than colonizing isolates and stimulated DCs to release much more IL-12p70. It is most likely that CC17 strains tend to initiate a cascade of pro-inflammatory responses and result in invasive infections after breaking through mucosal barriers, whereas CC23 strains would be inclined to maintain the colonization status due to the induction of TGF-β1 production.

In summary, CC17 serotype III tends to possess the potential for invasive infections. Future studies are needed to clarify the downstream mechanisms of hypervirulent CC17 strains' pathogenicity.

Funding

This study was partially supported by the Beijing Municipal Science and Technology Commission, China (No. Z141107002514036).

Conflicts of interest

None.

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How to cite this article: Wang L, Tao Z, Lu B. Biofilm formation and invasive ability contribute to CC17 serotype III group B *Streptococcus* virulence. Chin Med J 2022;135:869–871. doi: 10.1097/CM9.0000000000001861