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First evidence for the existence of *Simkania negevensis* in the genitalia of human females

Raneem A. Abu-Saif[a], Asma S. Al-Basha[b], Hesham M. Al-Younes[a,*]

[a] *Department of Biological Sciences, School of Science, the University of Jordan, Amman, Jordan*
[b] *Department of Obstetrics and Gynecology, School of Medicine, the University of Jordan, Amman, Jordan*

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ABSTRACT

Introduction: *Simkania negevensis* has been linked to some respiratory and non-respiratory diseases. However, there is still a serious lack of clinical investigations that attempt to determine possible body sites that could be inhabited by this microorganism and evaluate its true pathogenic capacity. The goal of this study was to examine the potential presence of *Simkania* and its prevalence in the genital tract of human adult females.
Methods: Lower vaginal swabs from 169 Jordanian adult females who attended Obstetrics and Gynecology clinic were collected and tested for *Simkania* DNA by PCR method.
Results: The presence of bacterial nucleic acids was confirmed in the genital system of adult females with an overall prevalence of 24.26% (41/169). Interestingly, the positivity of *Simkania* DNA was significantly higher in women of reproductive age than females of non-reproductive age (28.03% versus 10.81%; $p \leq 0.05$). Moreover, the presence of *S. negevensis* was evident in approximately 43% of females suffering from vaginal itching and/or abnormal discharge, exhibiting about two-fold increase in the positivity rate compared to detection rates assessed for women who attended the clinic for routine checkup or menstruation problems. However, the current work failed to find any link between the bacterial agent and spontaneous abortion (miscarriage).
Conclusions: This study showed for the first time the presence of *S. negevensis* in the genitalia of human females. These novel data could provide a basis to clarify the exact role of *S. negevensis* in the female genitalia and its potential involvement in genital system disorders.

Primera evidencia de la existencia de *Simkania negevensis* en los genitales femeninos humanos

Palabras clave:
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RESUMEN

Introducción: *Simkania negevensis* se ha asociado a algunas enfermedades respiratorias y no respiratorias. Sin embargo, todavía existe una importante carencia de investigaciones clínicas para intentar determinar posibles lugares del cuerpo que puedan ser colonizados por este microorganismo, y evaluar su auténtica capacidad patogénica. El objetivo de este estudio era examinar la potencial presencia de *Simkania* y su prevalencia en el tracto genital de mujeres adultas.
Métodos: Se recogieron hisopos vaginales de 169 mujeres adultas de Jordania que acudieron a una clínica de obstetricia y ginecología y se analizaron para detectar ADN de *Simkania* mediante PCR.
Resultados: Se confirmó la presencia de ácidos nucleicos bacterianos en el aparato genital femenino adulto, con una prevalencia global del 24,26% (41/169). Una observación interesante fue que la positividad a ADN de *Simkania* fue significativamente superior en mujeres en edad reproductiva en comparación

Abbreviations: CAP, community-acquired pneumonia; PCR, polymerase chain reaction; Cpn 60, chaperonin-60; IUD, intrauterine device; CI, confidence interval; WHO, World Health Organization; NGS, next generation sequencing; IVF, *in vitro* fertilization; IUI, intrauterine insemination.

* Corresponding author.
E-mail address: alyounes@ju.edu.jo (H.M. Al-Younes).

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con las mujeres en edad no reproductiva (28,03 frente al 10,81%; $p \leq 0,05$). Además, la presencia de *S. negevensis* era evidente en aproximadamente el 43% de las mujeres que sufrían picor vaginal y/o flujo vaginal anormal, mostrando un incremento cercano al doble en la tasa de positividad en comparación con las tasas de detección evaluadas para mujeres que acudían a la clínica para revisiones de rutina o por problemas menstruales. Sin embargo, la investigación no logró encontrar una asociación entre el agente bacteriano y el aborto espontáneo (aborto natural).

Conclusiones: Este estudio mostró por primera vez la presencia de *S. negevensis* en los genitales femeninos humanos. Estos nuevos datos pueden sentar una base para clarificar con precisión el papel de *S. negevensis* en los genitales femeninos humanos y su potencial implicación en trastornos del aparato genital.

Introduction

Simkania negevensis, originally characterized as a *Chlamydia*-like microorganism, belongs now to family *Simkaniaceae* within the *Chlamydiales* order.[1] Similar to other *Chlamydiales* members, *S. negevensis* is an obligate intracellular Gram-negative bacterium and possesses a developmental cycle with two distinct phases: the large, non-infectious, intracellular, replicative reticulate body and the small intracellular, infectious elementary body.[1]

Seroepidemiological studies from different countries on apparently healthy individuals detected seropositivity rates ranging from 4.3% to 80%.[2,3] Besides, this agent is considered as a potential emerging pathogen in the respiratory system, causing community-acquired pneumonia (CAP) and bronchiolitis, as confirmed by different techniques.[1,3,4] However, other reports revealed no link between *Simkania* and chronic obstructive pulmonary disease, chronic cough, asthma or even CAP and bronchiolitis.[5–9] Interestingly, because of the remarkable high and comparable prevalences of *Simkania* in cases suffering from CAP or bronchitis and in healthy controls (57.1% versus 62.5%), it was tempting to hypothesize that *S. negevensis* is not a causative agent of respiratory diseases and may exist as a commensal agent in the respiratory system.[5] On the other hand, sporadic studies suggested a role of *Simkania* in nonrespiratory system conditions. For instance, DNA of *Simkania* has been amplified from an aortic aneurysm and intestinal biopsies from patients of Crohn's disease.[10,11]

In general, microorganisms belonging to the order *Chlamydiales* are medically important. For instance, the very well-known *Chlamydia trachomatis* and *Chlamydia pneumoniae* are mainly implicated in human genital and respiratory diseases, respectively.[12,13] Moreover, recently discovered *Chlamydia*-like bacteria, including *S. negevensis, Waddlia chondrophila* and *Parachlamydia acanthamoebae* are associated with clinical conditions. *W. chondrophila* has been documented as an agent of adverse pregnancy outcomes, whereas, *P. acanthamoebae* is an emerging respiratory pathogen and may also be linked to adverse pregnancy outcomes in ruminants and humans.[4,12,13]

In addition to the biological characteristics of *Simkania* shared with other *Chlamydiales* members, tropism to inhabit certain body organs and disease potential of *Simkania* could be also similar to these related microbes. Thus, it is attractive to hypothesize that *Simkania* could be implicated in human urogenital infections, in addition to its being a respiratory microbe. Thus, this study aims at investigating the existence of *Simkania* in genital system of human adult females.

Materials and methods

Study subjects and study design

This study was carried out during the period from July 2018 to August 2019. Here, 169 Jordanian women, who attended for different reasons the Obstetrics and Gynecology clinic of the University of Jordan Hospital, Amman, Jordan, participated in this study and provided genital samples. Females aged between 20 and 91 years with a mean age of 39.5 ± 11.5 years. Subjects who had received antibiotics within the last 3 months prior to enrollment or were on menstrual cycle were excluded from the study. Demographic features, available clinical data and laboratory and diagnostic information were recorded for participating females. Moreover, an informed signed consent was obtained from each of them.

Sample collection and transport

Lower vaginal specimens were collected using sterile cotton-tipped swabs, immediately placed into a sterile tube containing 1 ml sterile transport (2-sucrose-phosphate) medium, which consisted of 17.1 g sucrose, 0.5 g dibasic potassium phosphate, 0.25 g monobasic potassium phosphate dissolved in 200 ml distilled water, followed by the addition of 20% fetal bovine serum and adjustment of pH to 7.2. Tubes containing the swabs were vortexed for 30 sec to dislodge any material into the liquid medium and the swabs were then removed and discarded. Next, the medium was transferred into a sterile 1.8-ml cryovial and stored at −72 °C until use.

DNA isolation

DNA from 200 μl of each specimen was harvested according to a protocol specific for isolating nucleic acids from body fluids, provided with the G-spin™ Total DNA Extraction kit (iNtRON Biotechnology, South Korea). Extracted DNA was eluted in a final volume of 50 μl of a buffer provided with the kit, aliquoted and stored at −20 °C until tested. DNA amounts were estimated spectrophotometrically.

PCR assay

To detect the bacterial DNA, a polymerase chain reaction (PCR) assay, amplifying a 311 bp fragment of the *S. negevensis chaperonin*-60 (*cpn* 60) gene, was performed according to a protocol of Donati et al.[14] with modification. Forward and reverse primers were 5′-GATGGAACGATGACTGTTGAA-3′ and 5′-GCACAAACTTTTAGTCCTGC-3′, respectively. Conditions for the PCR amplification were: denaturation for 4 min at 94 °C and 35 cycles, each of which consisted of denaturation step for 1 min at 94 °C, annealing for 1 min at 57 °C, extension for 1 min at 72 °C and a final elongation step for 7 min at 72 °C. In PCR runs, negative controls were included and contained all components of the amplification mixture and nuclease-free water instead of the purified DNA. Purified *Simkania* nucleic acids, generously provided by Thomas Rudel and Jo-Ana Herweg, Department of Microbiology, University of Würzburg, Würzburg, Germany, and the bacterial DNA isolated

previously from *Simkania*-positive nasopharyngeal samples, served as positive controls.[5] For sequencing purposes, some PCR amplicons were shipped with the required primers to MACROGEN (South Korea).

Statistical analysis

Statistical analysis of the results was calculated using chi-square test. A probability value (p) ≤0.05 was considered statistically significant.

Ethical statement

This work has been approved by the Institutional Review Board (IRB) of the University of Jordan Hospital. The individuals who participated in this work gave signed and informed consent. Anonymity and confidentiality were guaranteed at all times, as well as data protection.

Results

The majority of participating women were married at the time of enrollment (165/169; 97.63%), whereas 4 women were widowed or divorced (2.37%). Ninety seven females (57.4%) were middle-aged women (30-49 years). Sixty one females (36.09%) were apparently healthy subjects with no symptoms, among which 42 women visited the clinic for routine checkup and 19 women were seeking fertility, while the remainder (63.91%) suffered from various symptoms at attendance. About 84% of participants were pregnant for at least one time. In addition, more than half of the enrolled females (58%) did not utilize any birth control method (intrauterine device (IUD), contraceptive pills or condoms by their husbands). More sociodemographic and clinical profiles of the entire female population involved in the study are shown in Table 1. Here, PCR detected *Simkania* DNA in the genitalia of 41 out of 169 females, suggesting an overall prevalence of 24.26% among individuals examined (Table 2). Sequencing of some PCR amplicons confirmed the amplification of the desired domain within the bacterial gene *cpn* 60 (data not shown). To analyze the age-specific detection rates, subjects were divided into five age groups: 20–29 years, 30–39 years, 40–49 years, 50–59 years and ≥60 years. Only 7 cases were DNA positive out of 35 females in the youngest group aged 20–29 years, resulting in a prevalence of 20.00% (95% CI: 25.8 ± 0.7). Clearly, the DNA positivity reached a peak (33.96%) in the age group of 30–39 years (95% CI: 34.6 ± 0.896; 18/53) and gradually decreased in the subsequent groups, reaching 27.27% in the cohort of 40–49 years (95% CI: 43.62 ± 0.912; 12/44) and 13.33% in the group of 50–59 years (95% CI: 53.5 ± 1.103; 4/30). Remarkably, none of the participants older than 59 years was DNA positive (95% CI: 68 ± 10.311; 0/7). Further analysis of the results showed the absence of an inverse or a proportional correlation between bacterial nucleic acid positivity and specific ages of females (data not shown). Interestingly, DNA of *Simkania* was evident in females with ages ≤55 years only, while all females older than 55 years (12 individuals) were found negative. Apparently, more than 50% of positive cases are clustered within the age brackets 29–35 years and 46–50 years. These relatively narrow age intervals account for approximately 29.27% (12/41) and 21.95% (9/41) of all PCR-positive cases, respectively. Sociodemographic and clinical information of the 41 PCR-positive women are summarized in Table 1.

To demonstrate the presence of any relation between the female reproductive age and some of its correlates with the positivity of *S. negevensis* in genital system, women were divided into two groups based on criteria set by WHO,[15] which defined the reproductive age of females to be from 15 to 49 years. Accordingly, one group consisted of 132 reproductive age females (20–49 years old), while the second group comprised only 37 non-reproductive age females (with ages 50 years or older). Remarkably, about 2.5-fold significant increase in the prevalence of *Simkania* was obtained among women of the reproductive age compared to non-reproductive age individuals (28.03% versus 10.81%; $p < 0.05$). Interestingly, *Simkania* DNA was detected in 30.61% (15/49) of females who experienced spontaneous abortion (miscarriage), compared to about 22.5% (21/93) of females with no history of miscarriage, suggesting possibly a correlation between the bacterium and this adverse pregnancy outcome ($p < 0.29$).

In an attempt to correlate *S. negevensis* to clinical manifestations, detection rates of the bacterium in the females were analyzed based on the reasons for attending the Obstetrics and Gynecology clinic. Reasons were categorized into (1) vaginal inflammation (itching and/or discharge), (2) menstrual and bleeding complaints, (3) routine checkup, (4) multiple reasons, (5) seeking pregnancy and (6) other reasons. As illustrated in Table 2, 23.81% (10/42) of symptomless women, visiting the clinic for a regular checkup (pap smear screening, IUD checking, antenatal visit and postpartum follow-up), were positive for *S. negevensis*. A slightly less DNA positivity rate, which remained above 20% (4/19; 21.05%), was reported for women who were planning to conceive and with no detectable symptoms. However, among other pregnancy-seeking females with coincidental complaints (18 cases), only one case was DNA positive (Table 2). In females who attended for reasons related to menstrual cycle abnormalities, the prevalence rate was almost the same and did not exceed 25% (13/57). Surprisingly, the bacterial DNA was evident in 13 out of 30 females with symptoms of vaginal inflammation (itching and/or abnormal discharge), yielding about 2-fold increase in the nucleic acid positivity (43.33%), compared to rates reported for females who attended for checkup or menstrual cycle complaints (Table 2). Collectively, these data clearly demonstrate that *S. negevensis* may be linked to vaginal inflammation and its related signs, such as itching and abnormal secretions.

Discussion

To the best of our knowledge, this is the first investigation that confirmed the presence of *S. negevensis* in the female genitalia. During conducting the present research, Greub and his colleagues could not detect this microorganism in 22 urethral samples from both men and women and in 135 cervicovaginal swabs using their newly developed quantitative real-time PCR.[16] Contradictory results using cell culture, PCR and serological methods were also noticed in studies that investigated the prevalence and the association of *Simkania* with pulmonary conditions.[1,3,5] The obvious discrepancy between our findings and those of Vouga et al.[16] may reflect variations in the DNA amplification techniques, types of clinical samples and their processing in addition to differences in the populations examined. Many in-house PCR methods are available in the literature for the detection of *Simkania* and other *Chlamydiales* members in clinical and environmental samples. In spite of that, the sensitivity and specificity of the majority of these tests remain unknown. Among these protocols are real-time PCR, nested-PCR, multiplex PCR and standard PCR.[5,9,14,17] Here, we used a standard PCR method to target *S. negevensis cpn* 60 gene, using outer primers of a previously described nested PCR.[14] This modified test was successfully used to detect *Simkania* nucleic acids in nasopharyngeal samples.[5] The specificity of this assay was confirmed by sequence analyses of some randomly chosen amplicons produced in the present study, which showed more than 97% homology with the desired target in *Simkania* genome (data not shown). Generally, standard PCR test has most likely the advantage of a decreased risk of contamination, compared to nested PCR that is highly sensitive to this drawback by PCR product carryover. To corroborate

Table 1
Demographic and clinical features of the entire study population and those females who tested positive for *S. negevensis*.

Characteristics	*Simkania* DNA-positive females (total number of females in the category)	(%)
Age (years)		
Range	20–55	
Mean	37.78 ± 9.79	
Median	37.5	
Age groups (years)		
20–29	7 (35)	20.00
30–39	18 (53)	33.96
40–49	12 (44)	27.27
50–59	4 (30)	13.33
≥60	0 (7)	0.0
Marital status		
Married	41 (165)	24.85
Widowed or divorced	0 (4)	0.0
Birth control methods		
IUD[a]	11 (44)	25.00
Oral contraception	6 (15)	40.00
Condoms	4 (12)	33.33
None	20 (98)	20.41
Previous pregnancies (births)		
Yes	36 (142)	25.35
No	5 (27)	18.52
Type of previous pregnancies		
No pregnancies at all	5 (27)	18.52
Full-term delivery and no abortion history	21 (92)	22.83
Spontaneous abortion (miscarriage)	15 (49)	30.61
Ectopic pregnancy	0 (1)	0.00
Stillbirth	0 (0)	0.00
Number of previous pregnancies		
Zero	5 (27)	18.52
One	4 (20)	20.00
Two	7 (24)	29.17
Three	8 (28)	28.57
Four	6 (19)	31.58
≥five	11 (51)	21.57
Self-reported symptoms at attendance		
Vaginal itching and/or discharge	13 (44)	29.54
Menstrual and bleeding complaints:		
Bleeding between periods and after intercourse	2 (17)	11.76
Heavy cycles	7 (18)	38.89
Irregular cycles	3 (13)	23.08
Missed (infrequent) cycles	2 (11)	18.18
Mixed symptoms[b]	0 (2)	0.00
Other symptoms[c]	0 (4)	0.00
None	14 (60)	23.33

[a] Intrauterine device.
[b] Vaginal itching and/or discharge with menstrual complaints.
[c] Painful intercourse, blood in urine or vaginal dryness.

Table 2
Correlation of DNA positivity in genital swabs collected from adult females with reasons for visiting the Obstetrics and Gynecology clinic.

Reasons for visit	No. examined	No. positive	Positivity %	95% CI[a]
Vaginal inflammation: itching and/or discharge	30	13	43.33	0.254–0.625
Menstrual and bleeding complaints	57	13	22.81	0.127–0.358
Routine checkup (without symptoms)	42	10	23.81	0.120–0.394
Multiple reasons (vaginal itching and/or discharge with menstrual complaint)	2	0	0.0	
Seeking pregnancy (n = 37) (*IVF*[b], *IUI*[c])				
Without symptoms	19	4	21.05	0.060–0.455
With coincidental vaginal discharge	11	0	0.0	
With coincidental menstrual and bleeding complaints	5	1	20.00	0.005–0.716
With coincidental painful intercourse	1	0	0.0	
With coincidental blood in urine	1	0	0.0	
Other reasons[d]	1	0	0.0	
Total	169	41	**24.26**	

[a] Confidence interval.
[b] *In vitro* fertilization.
[c] Intrauterine insemination.
[d] Vaginal dryness.

our novel PCR-based finding that *Simkania* inhabits the female genitalia, the use of the cell culture approach for isolation and subsequent characterization of the microbe in addition to one of the modern culture-independent molecular techniques, such as next generation sequencing (NGS), is recommended.

Evaluation of age-related positivity rates clearly showed that about three quarters (73.17%) of *Simkania*-positive females aged between 30 and 49 years. Importantly, about half of the *Simkania* DNA-positive females aged between 29–35 years (12/41; 29.27%) and 46–50 years (9/41; 21.95%). Deeper analysis of age-specific prevalences demonstrated that DNA positivity reached the peak in females within the age range 30–39 years, slightly decreased in the subsequent age group of 40–49 years and then sharply decreased in females aged 50–59 years. In contrast, none of the females older than 60 years was positive for *Simkania* and this may be due to the low number of women investigated in this elderly cohort (only 7 subjects). Overall, based on these interesting novel findings, further studies with larger female populations are needed to gain a better understanding of the relatedness between the age and the incidence of *S. negevensis* in genitalia.

Many *Simkania*-like bacteria have been associated with diseases relevant to the urogenital system and/or respiratory system. Globally, *C. trachomatis* is considered the most common sexually transmitted pathogen, leading to cervicitis, urethritis, ectopic pregnancy, miscarriage, epididymitis, prostatitis and male infertility.[12,13,18] The prevalence of this pathogen was estimated at about 100 million new cases in 2005.[12] *Chlamydia abortus* is abortigenic in mammals and is involved in miscarriage and stillbirths.[12,13] Additionally, *W. chondrophila* has been associated with miscarriage and ectopic pregnancy in humans and animals.[4,12,13] *P. acanthamoebae* and *C. pneumoniae* were initially identified as an emerging respiratory pathogens and they are now linked to adverse pregnancy aspects.[4,12,13] Based on the literature and the novel results of this work, *Simkania* appears to exist in other body parts in addition to the respiratory system. Very recently, *S. negevensis* DNA was significantly detected in intestinal biopsies taken from patients diagnosed with Crohn's disease.[11] Consistently, the present study revealed the bacterial nucleic acids in another nonrespiratory site, namely, the female genitalia. Intriguingly, a significant link between the presence of *S. negevensis* and the reproductive age of women was found. The current work also provides evidence for a possible association of *S. negevensis* with the vaginal inflammation. However, these results are still immature and need to be backed up by further investigations to verify the ability of this organism to exhibit tropism to the female genital tract and its pathogenic potential in the genitalia.

As mentioned above, this study clearly demonstrated significant higher prevalence of *Simkania* DNA in vaginal swabs from women of the reproductive age. One of the important correlates of the reproductive age is miscarriage. Among risk factors known to associate with this spontaneous abortion are advancing maternal and paternal age, genetic abnormalities, immunological and implantation disorders, uterine and endocrine abnormalities and lifestyle factors.[19] Another risk factor linked to increasing chance of miscarriage is the infection with certain microbes.[18] For instance, a significant correlation between *Waddlia* seropositivity in women who experienced miscarriage was demonstrated, compared to those who had no history of miscarriage.[4,12,13] Comparably, a correlation between *C. trachomatis* infection and miscarriage was also documented.[12,13,18,20] In addition, many studies have linked *P. acanthamoebae* with miscarriage in ruminants and humans.[12,13,21] Consistent with these studies, the present research shows a possible involvement of *S. negevensis* in miscarriage. This result might be inconclusive due to the relatively small size of population examined. Therefore, future research is required to explore if *Simkania* is able to adversely affect the success of pregnancy in humans.

Possible sources of infection and modes of transmission for *Simkania*-like agents have been proposed. *P. acanthamoebae* and *C. abortus* may have zoonotic nature, leading to disorders in humans after contact with infected cattle.[12,13,22,23] Cattle may contract *P. acanthamoebae* from the animal drinking water.[4,21] Moreover, maternal-fetal transmission of this microbe has also been demonstrated and was postulated to be the result of zoonotic transmission.[22] *W. chondrophila* could be acquired from well water or following contact with infected animals and may be transmitted by sexual intercourse.[13,24] Similar to *Chlamydiales* members, postulations on the source of infection/reservoir of *Simkania* and its mode of transmission have been reported. Different types of water, such as drinking and environmental water and reclaimed wastewater, were suggested to be a major source of the bacterium.[3,14,25–29] Ticks were also proposed as a reservoir and a source of infection.[30] Additional modes of transmission could be human-to-human transmission through aerosols or droplets, because of *Simkania* association with the respiratory system.[3] With respect to the source of infection with *Simkania* in females tested positive in the current study, zoonotic transmission is very unlikely, as participants inhabit urban areas with no farms and no domestic animals are kept in or between houses. The use of bloodstream for *Simkania* dissemination from potentially infected or colonized respiratory system or other body organs to the genitalia of females could also be excluded at this point, as blood samples collected from 100 adult males and females were all negative for the bacterium (data not shown). It might be argued that the tested blood samples did not belong to the cohort of women tested here. Nevertheless, to resolve any dispute on this issue, further work is required to conclusively clarify the exact role of blood and its cells in the spread of *Simkania* in the human body. The possibility that the household water used for body washing was the source of infection is also ruled out. This seems reasonable because none of the females older than 55 years was found positive. It is, therefore, tempting to hypothesize that sexual transmission might be the source of microbial infection. This could be supported by the fact that all females with confirmed *Simkania* DNA positivity were married at the time of the study and the incidence of *S. negevensis* was found significantly associated with reproductive age. This attractive speculation can be explored in future work by detecting and characterizing the bacterium in females along with their sexual partners.

In summary, the present work provides the first evidence for the presence of *S. negevensis* in the human female genitalia, as confirmed by PCR technique. This study serves as a foundation to verify the causal role of *Simkania*, if present, in genital system disorders and its association with adverse pregnancy outcomes in addition to its potential being a sexually transmitted agent.

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Conflicts of interest

The authors have no conflicts of interest in relation to this work.

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