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Evidence that intra-amniotic infections are often the result of an ascending invasion – a molecular microbiological study

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Abstract

Background: Microbial invasion of the amniotic cavity resulting in intra-amniotic infection is associated with obstetrical complications such as preterm labor with intact or ruptured membranes, cervical insufficiency, as well as clinical and histological chorioamnionitis. The most widely accepted pathway for intra-amniotic infection is the ascension of microorganisms from the lower genital tract. However, hematogenous dissemination of microorganisms from the oral cavity or intestine, retrograde seeding from the peritoneal cavity through the fallopian tubes, and introduction through invasive medical procedures have also been suggested as potential pathways

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for intra-amniotic infection. The primary reason that an ascending pathway is viewed as most common is that the microorganisms most often detected in the amniotic fluid are those that are typical inhabitants of the vagina. However, thus far, no studies have shown that microorganisms in the amniotic cavity are simultaneously present in the vagina of the woman from which they were isolated. The objective of the study was to determine the frequency with which microorganisms isolated from women with intra-amniotic infection are also present in the lower genital tract.

Methods: This was a cross-sectional study of women with intra-amniotic infection with intact membranes. Intra-amniotic infection was defined as a positive culture and elevated concentrations of interleukin-6 (IL-6) (>2.6 ng/mL) in amniotic fluid and/or acute histologic chorioamnionitis and funisitis. Microorganisms isolated from bacterial cultures of amniotic fluid were taxonomically identified through matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF) and 16S ribosomal RNA (rRNA) gene sequencing. Vaginal swabs were obtained at the time of amniocentesis for the identification of microorganisms in the lower genital tract. The overall bacterial profiles of amniotic fluids and vaginal swabs were characterized through 16S rRNA gene sequencing. The bacterial profiles of vaginal swabs were interrogated for the presence of bacteria cultured from amniotic fluid and for the presence of prominent (>1% average relative abundance) operational taxonomic units (OTUs) within the overall 16S rRNA gene bacterial profiles of amniotic fluid.

Results: (1) A total of 75% (6/8) of women had bacteria cultured from their amniotic fluid that are typical residents of the vaginal ecosystem. (2) A total of 62.5% (5/8) of women with bacteria cultured from their amniotic fluid also had these bacteria present in their vagina. (3) The microorganisms cultured from amniotic fluid and also detected in the vagina were *Ureaplasma urealyticum*, *Escherichia coli*, and *Streptococcus agalactiae*. (4) 16S rRNA gene sequencing revealed that the amniotic fluid of women with intra-amniotic infection had bacterial profiles dominated by *Sneathia*, *Ureaplasma*, *Prevotella*, *Lactobacillus*, *Escherichia*, *Gardnerella*, *Peptostreptococcus*, *Peptoniphilus*, and *Streptococcus*, many of which had not been cultured from the amniotic fluid samples. (5) Seventy percent (7/10) of the prominent (>1% average relative abundance) OTUs found in amniotic fluid were also prominent in the vagina.

Conclusion: The majority of women with intra-amniotic infection had bacteria cultured from their amniotic fluid that were typical vaginal commensals, and these bacteria were detected within the vagina at the time of amniocentesis. Molecular microbiological interrogation of amniotic fluid from women with intra-amniotic infection revealed that the bacterial profiles of amniotic fluid were largely consistent with those of the vagina. These findings indicate that ascension from the lower genital tract is the primary pathway for intra-amniotic infection.

Keywords: 16S rRNA sequencing; amniotic cavity; amniotic fluid; bacteria; chorioamnionitis; culture; funisitis; *Gardnerella*; microbial invasion; microbiome; microbiota; pregnancy; preterm birth; *Sneathia*; *Ureaplasma*; vaginal flora.

Introduction

Microbial invasion of the amniotic cavity resulting in intra-amniotic infection has been associated with obstetrical complications [1–6], including spontaneous preterm labor [3, 7–17], preterm prelabor rupture of membranes (PPROM) [18, 19], cervical insufficiency [20–26], a sonographic short cervix [27], idiopathic vaginal bleeding [28, 29], and histological [30, 31] and clinical chorioamnionitis [32, 33]. Indeed, intra-amniotic infection has been detected in 6–35% of women with preterm labor and intact membranes [3, 8–17, 34], and in 40–50% of women with PPROM [18, 19]. Intra-amniotic infection has further been detected in 61% of cases of clinical chorioamnionitis at term [32]. Intra-amniotic infection has thus been associated with labor dysfunction [35–37], maternal morbidity [38–42], and neonatal morbidity and mortality [43–50]. Recent evidence indicates that intra-amniotic infection can be treated [51–56], highlighting the need for further investigation into its etiologies.

Multiple routes of invasion have been proposed for intra-amniotic infection [2, 5, 17, 57, 58], including (1) ascending infection from the lower genital tract through the cervix, (2) hematogenous dissemination from distant sites such as the intestine or the oral cavity through the placenta, (3) retrograde seeding from the peritoneal cavity through the fallopian tube, and (4) accidental introduction of microorganisms at the time of invasive medical procedures. However, ascending infection from the lower genital tract is widely viewed as the primary route by which microbial invasion of the amniotic cavity occurs [2, 5, 17, 57–60]. The principal evidence supporting ascending infection as the primary route for intra-amniotic infection is that the bacterial taxa most often identified in the amniotic cavity are typical members of the human vaginal microbiota [4, 59, 61–71], including *Ureaplasma* and

Mycoplasma spp. [1, 13, 18, 19, 32, 62, 72–77], *Gardnerella vaginalis* [1, 13, 19, 32, 62, 76, 77], *Streptococcus agalactiae* [13, 18, 32, 72, 75, 77, 78], *Escherichia coli* [1, 32], *Sneathia* and *Leptotrichia* spp. [13, 19, 32, 72, 73, 75, 76], and *Prevotella* spp. [62, 77].

Direct evidence of ascending infection as a primary cause of intra-amniotic infection, however, is lacking. Specifically, demonstration of ascending infection requires bacteria in the amniotic fluid to also be present in the vagina of the woman from whom the amniotic fluid was collected. Molecular surveys will be beneficial for this task as they provide greater insight into the diversity of microorganisms inhabiting body sites than does culture [4, 13, 18, 32]. Although molecular surveys have been used to characterize the composition of intra-amniotic [13, 18, 19, 32, 74, 79–82] and vaginal [68–71, 83–93] microbial communities in pregnant women, the concurrent presence of specific microorganisms in the amniotic fluid and the vagina has not been investigated. The objective of this study, therefore, was to characterize the microorganisms found in amniotic fluid of women with intra-amniotic infection and intact membranes, and to evaluate the frequency of ascending infection by determining whether the microorganisms cultured from, and molecularly identified in, amniotic fluid were also present in the vagina.

Materials and methods

Study population

This was a cross-sectional study of women who had an amniocentesis performed for the diagnosis of intra-amniotic inflammation and/or infection (see Clinical definitions). Patients with intra-amniotic infection were included in the study if they met the following criteria: (1) live intrauterine pregnancy, (2) intact chorioamniotic membranes, (3) availability of a stored and revived amniotic fluid cultivar, (4) if amniotic fluid was available for molecular microbial characterization, the cultivar was confirmed to be present within the fluid using 16S ribosomal RNA (rRNA) sequencing, and (5) availability of vaginal swab samples collected from the patient within 24 h of the amniocentesis. Patients were excluded from the study if chromosomal or fetal anomalies were present. The collection of samples and their use for research were approved by the Human Investigation Committee of Wayne State University and the Institutional Review Board of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development. All subjects provided written informed consent. Demographic characteristics of the study population are shown in Table 1.

Clinical definitions

Gestational age was determined by the date of the last menstrual period and confirmed by ultrasound examination. The gestational age derived from sonographic fetal biometry was used if the estimation was inconsistent with menstrual dating. Intra-amniotic inflammation was defined as an amniotic fluid interleukin-6 (IL-6) concentration ≥2.6 ng/mL [12, 33, 94–99] and/or the presence of acute histologic chorioamnionitis and funisitis (see Placental histopathological examination). Intra-amniotic infection was defined as a positive amniotic fluid culture, including genital mycoplasmas [2, 7, 8, 100, 101], and intra-amniotic inflammation [19, 76, 102–112]. Clinical chorioamnionitis was diagnosed by the presence of maternal fever (temperature >37.8°C) accompanied by two or more of the following criteria: (1) uterine tenderness, (2) foul-smelling amniotic fluid, (3) fetal tachycardia (heart rate >160 beats/min), (4) maternal tachycardia (heart rate >100 beats/min), and (5) maternal leukocytosis (leukocyte count >15,000 cells/mm^3) [32, 33, 98, 104–108, 113–119].

Placental histopathological examination

Placentas were examined histologically by perinatal pathologists blinded to clinical diagnoses and obstetrical outcomes according to standardized Perinatology Research Branch protocols [120, 121]. Briefly, three to nine sections of the placenta were examined, and at least one full-thickness section was taken from the center of the placenta; others were taken randomly from the placental disc. Acute inflammatory lesions of the placenta (maternal inflammatory

Table 1: Demographic characteristics of the patients in the study.

Subject	Age, years	Gestational age at amniocentesis, weeks	Gestational age at delivery, weeks	Mode of delivery	Birthweight, g
#1	26	20.1	20.7	Vaginal	296
#2	20	31.3	31.4	Vaginal	1525
#3	38	22.0	22.1	Vaginal	471
#4	17	37.9	37.9	Vaginal	3060
#5	27	23.0	23.1	Vaginal	640
#6	31	19.6	34.9	Vaginal	1965
#7	24	39.9	39.9	Vaginal	3085
#8	21	22.0	22.1	Vaginal	494

response and fetal inflammatory response) were diagnosed according to established criteria, including staging and grading [120, 122]. The results of placental histopathological examination are presented in Table 2.

Amniotic fluid sample collection

Amniotic fluid samples were obtained by transabdominal amniocentesis under antiseptic conditions and monitored by ultrasound in order to detect intra-amniotic inflammation and/or infection in patients with intact membranes. Samples of amniotic fluid were transported to the laboratory in a sterile, capped syringe for clinical determinations, including amniotic fluid white blood cell count [123], Gram stain examination [124], and glucose concentration [125] (Table 2). The rest of the sample was used for research purposes, which included the determination of IL-6.

Determination of IL-6 in amniotic fluid

Amniotic fluid concentrations of IL-6 were determined by using a sensitive and specific enzyme immunoassay obtained from R&D Systems (Minneapolis, MN, USA). The IL-6 concentrations were determined by interpolation from the standard curve. The inter- and intra-assay coefficients of variation for IL-6 were 8.7% and 4.6%, respectively. The detection limit of the IL-6 assay was 0.09 pg/mL. In some cases, the IL-6 concentration in amniotic fluid was used for clinical purposes.

Clinical microbiology

Amniotic fluid was cultured for aerobic and anaerobic bacteria. Specifically, amniotic fluid was inoculated onto chocolate agar, trypticase soy agar with 5% sheep blood, and MacConkey agar culture media. Aerobic plates were incubated at 35°C in an 8% CO_2 chamber. Anaerobic plates were incubated at 35°C in 5% CO_2, 10% hydrogen and 85% nitrogen. Plates were incubated for 4 days. A Mycofast test kit (MYCOFAST US; Logan, UT, USA) was used for the detection of *Mycoplasma* and *Ureaplasma* spp. [126]. Initial taxonomic characterization of cultivars was done via discriminatory biochemical tests and matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF) [127].

Vaginal swab collection

Vaginal Dacron swabs (Medical Packaging Swab-Pak, Camarillo, CA, USA) were obtained using a Pederson speculum in the absence of lubrication and turned at a 45° angle to enable the collection of posterior fornix fluid samples. All vaginal samples were collected within 24 h of amniocentesis and prior to any rupture of membranes. All swabs were stored at ≤–70°C until analysis.

Isolation of DNA from amniotic fluid and vaginal samples

Prior to DNA extraction, the order of all samples was randomized to avoid cross-contamination of DNA between samples based on body site or patient identity. Total DNA was extracted from vaginal swabs, 200 µL of amniotic fluid supernatant, and 200 µL of amniotic fluid pellet [all pellets were resuspended in 450 µL sterile phosphate-buffered saline (PBS)] using the DNeasy PowerLyzer PowerSoil Kit (QIAGEN, Valencia, CA, USA) with four modifications to the manufacturer's protocol: (1) vaginal swabs were immersed in 500 µL of the supplied bead solution and 200 µL of phenol:chloroform:isoamyl alcohol pH 7–8 solution for 10 min prior to cell lysis by mechanical disruption rather than being suspended in 700 µL of supplied bead solution alone; (2) 100 µL of solution C2, 100 µL of solution C3, and 1 µL of RNase A were added and samples were incubated at 4°C for 5 min prior to centrifugation rather than being added to samples over two steps; (3) lysates were combined with 650 µL of solution C4 and 650 µL of 100% ethanol prior to loading samples on the supplied spin column rather than being combined with 1200 µL of solution C4 alone; (4) DNA was eluted in 60 µL of solution C6 rather than 100 µL of solution C6. To assess potential background DNA contamination within the DNA extraction kit or reagents, three blank DNA extraction kit control samples were processed alongside biological samples and sequenced. Purified DNA was stored at –20°C.

Table 2: Clinical characteristics of the patients in the study.

Subject	Total amniotic fluid white blood cell count, cells/mm³	Amniotic fluid IL-6, pg/mL	Glucose, mg/dL	Microorganisms in amniotic fluid by culture	Acute histologic chorioamnionitis[a]	Acute funisitis[b]
#1	20	126,800	25	*Ureaplasma urealyticum*	Stage 3	Stage 2
#2	206	169,372	1	*Staphylococcus warneri*	Stage 3	Stage 2
#3	340	171,100	9	*Streptococcus anginosus*	Stage 3	Stage 1
#4	1650	29,300	1	*Escherichia coli*, *Ureaplasma urealyticum*	Stage 1	None
#5	110	148,500	1	*Escherichia coli*	Stage 2	Stage 2
#6	10	39,200	19	*Ureaplasma urealyticum*	None	None
#7	0	1485	1	*Streptococcus agalactiae*	Stage 2	Stage 2
#8	299	102,800	0	*Ureaplasma urealyticum*	Stage 3	Stage 1

[a]Acute histologic chorioamnionitis: Stage 1, early, acute subchorionitis/chorionitis; Stage 2, intermediate, acute chorioamnionitis; Stage 3, necrotizing chorioamnionitis. [b]Funisitis: Stage 1, early umbilical phlebitis/chorionic vasculitis; Stage 2, intermediate, umbilical arteritis.

Amplification and sequencing of bacterial 16S rRNA genes in amniotic fluid and vaginal swabs

The 16S ribosomal RNA (rRNA) gene is widely used as a phylogenetic marker gene for characterizing bacterial communities in environmental and clinical samples. To characterize and compare the bacterial communities in paired amniotic fluid and vaginal samples, the V4 region of the 16S rRNA gene was amplified (515F/806R primers) from sample DNA extracts and sequenced at the University of Michigan's Microbial Systems Molecular Biology Laboratory (https://microbe.med.umich.edu/services) using the dual indexing strategy developed by Kozich et al. [128]. Sequencing was done on the Illumina MiSeq platform, using a MiSeq V2 500 cycle Reagent Kit (San Diego, CA, USA; MS102-2003), according to the manufacturer's instructions with modifications found in Kozich et al. [128]. AccuPrime High Fidelity Taq (Life Technologies, Carlsbad, CA, USA; 12346094) was used instead of AccuPrime Pfx SuperMix. Each polymerase chain reaction (PCR) contained 1.0 μM of each primer, 3 μL template DNA, and 0.15 μL AccuPrime HiFi Polymerase and DNase-free water to produce a final volume of 20 μL. PCR was performed using the following conditions: 95°C for 2 min, followed by 30 cycles of 95°C for 20 s, 55°C for 30 s and 72°C for 5 min, with an additional elongation at 72°C for 10 min. To determine if bacteria cultured from amniotic fluid were also present in the vagina of respective patients (i.e. the 16S rRNA gene of the cultured bacterium was also detected in the vagina), the V4 region of the 16S rRNA gene was amplified (515F/806R primers) from vaginal DNA extracts and sequenced at Michigan State University's Research Technology Support Facility (https://rtsf.natsci.msu.edu/) using protocols established by Caporaso et al. [129].

16S rRNA gene sequence processing and bacterial community characterization

Mothur software (Ann Arbor, MI, USA; v 1.39.5) was used to assemble paired-read contigs from FASTQ files, to trim, filter, and align sequences, to identify chimeras, to assign sequences to taxonomies, and to assign sequences to operational taxonomic units (OTUs) based on their percent nucleotide similarity [130]. Briefly, quality-filtered sequences (maximum length 300 bp, with no ambiguous base calls and homopolymers of no more than 8 bp) were aligned to the SILVA 16S rRNA gene reference database (release 102) [131]. Chimeras were identified using the method of Edgar et al. [132] as implemented in mothur, and these sequences were subsequently removed. A preclustering step (diffs = 2) was performed to reduce the impact of sequencing errors. OTUs were defined by clustering 16S rRNA gene sequences at a nucleotide similarity level of 99%. The remaining sequences were classified against the SILVA 16S rRNA gene reference database using a k-nearest neighbor approach with a confidence threshold of 80%. Any reads derived from an unknown domain, Eukaryota, chloroplasts, mitochondria, or Archaea, were removed.

Good's coverage values (an indicator of sample coverage) for all amniotic fluid supernatant and pellet samples, vaginal swabs, and the three blank DNA extraction kit controls exceeded 98.0%. Raw OTU count data were converted to percentages within each subject's dataset. There was no difference between the structure of the bacterial profiles of the supernatant and pellet portions of amniotic fluid samples [N = 6; non-parametric multivariate analysis of variance (NPMANOVA); Bray-Curtis: F = 0.698, P = 0.590], yet they were highly patient-specific (F = 3.337, P = 0.004). Therefore, bacterial community data for amniotic fluid supernatants and pellets for individual patients were combined bioinformatically to generate a single amniotic fluid bacterial community profile per patient. The profiles of amniotic fluid and blank DNA extraction kits differed in both composition (Jaccard; $F = 1.30$, P = 0.014) and structure (Bray-Curtis; $F = 2.11$, P = 0.045). Forty OTUs were identified in the three blank DNA extraction kit samples. The two OTUs with the highest mean relative abundance in extraction kit samples (OTU 87: *Bacteroides* and OTU 8: *Escherichia*) were found in two of the three kit controls. Only two OTUs (OTU 5: *Lactobacillus* and OTU 38: *Streptococcus*) were present in all three kit controls, and they were present at lower mean relative abundances in kit controls than in amniotic fluid samples. Therefore, we did not remove any OTUs from the dataset.

16S rRNA gene sequencing and taxonomic identification of bacteria cultured from amniotic fluid

Amniotic fluid bacterial cultivars were recovered from frozen stocks on chocolate agar, Columbia CNA with sheep blood agar, MacConkey agar, or SP4 broth with urea media. Genomic DNA was extracted from pure cultures of the bacteria using an UltraClean Microbial DNA Isolation kit (MoBio, now Qiagen), following the manufacturer's protocol. The 16S rRNA gene was first amplified from purified DNA with the 8F/1492R primer set and then bidirectionally sequenced using the Sanger chain termination method with the 515F and 806R primers targeting the V4 hypervariable region of the gene. Forward and reverse reads were trimmed using DNA Baser software (http://www.dnabaser.com/) with default settings, and assembled using the contig assembly program (CAP) of BioEdit software (Carlsbad, CA, USA; v7.2.5) with default settings. The initial taxonomic identity of each bacterial cultivar as determined by MALDI-TOF was then confirmed by searching for similarity by BLAST against cultured bacterial type strain 16S rRNA gene sequences ≥1200 bp (12,736 sequences) contained within the Ribosomal Database Project (RDP) database (Release 11, Update 5) [133, 134]. BLAST results were consistent with those of MALDI-TOF for each of the bacterial cultivars.

Determining if bacteria cultured from amniotic fluid are also present in the vagina

To determine if bacterial cultivars from amniotic fluid were also present in the patient's vagina, the 16S rRNA gene sequences obtained from each patient's vaginal sample were screened for the presence of the 16S rRNA gene sequence of that patient's respective cultivar(s). Specifically, the V4 region of the 16S rRNA gene sequence for each cultivar was trimmed to exclude the 515F and 806R primer regions, and a matching sequence was identified by searching for similarity using BLAST [133] against individual databases containing each woman's vaginal 16S rRNA gene sequence library. Sequence libraries used to construct BLAST databases were generated prior to the preclustering step in the mothur protocol described earlier.

Statistical analysis

NPMANOVA with 10,000 permutations was conducted to evaluate variation in the composition and structure of the bacterial profiles

of amniotic fluid and vaginal samples. Bacterial community composition and structure were characterized using the Jaccard and Bray-Curtis similarity indices, respectively [135]. All analyses were conducted using PAST software (Oslo, Norway; v2.17) [136].

Results

Clinical characteristics of the study population

Table 1 describes the demographic characteristics of the patients in this study. Seven of the eight patients (87.5%) delivered within 1 week of amniocentesis and their amniotic fluid testing positive for bacterial cultures. Most of the patients included in this study underwent preterm labor (87.5%) and/or were diagnosed with clinical chorioamnionitis (50%). Table 2 describes the clinical laboratory determinations and the microorganisms cultured from amniotic fluid, as well as the results of placental histopathological examination.

Are the bacteria cultured from amniotic fluid also present in the vagina?

Six of the eight (75%) patients had bacteria cultured from their amniotic fluid that are typical vaginal commensals (Figure 1A), specifically *Ureaplasma urealyticum*, *E. coli*, and *S. agalactiae*. The other two patients (25%) had bacteria in their amniotic fluid that are not commonly associated with the vagina (Figure 1A), namely *Staphylococcus warneri* and *Streptococcus anginosus*. When vaginal swabs were interrogated for the presence of amniotic fluid isolates, matches were found in five of the eight (62.5%) patients. Precisely, in these five cases, bacteria cultured from amniotic fluid were detected in paired vaginal swabs based on matching 16S rRNA gene sequences (≥99.5% shared nucleotide identity) (Figures 1B, 2). The bacterial cultivars with matching 16S rRNA gene sequences in the vagina were identified as *U. urealyticum* (three cultivars), *E. coli* (two cultivars), and *S. agalactiae* (one cultivar) (Table 3). These bacteria were typically present at low relative abundances within their respective vaginal bacterial communities (Figure 2). These results show that most women with intra-amniotic infection are colonized by vaginal microorganisms.

Comparison of the molecular bacterial profiles of paired amniotic fluid and vaginal samples

Six of the eight women with intra-amniotic infection had a sufficient volume of amniotic fluid to generate 16S rRNA gene profiles of the fluid. Among these six women, there were 10 prominent (≥1% average relative abundance) OTUs among the amniotic fluid samples (Figure 3, taxa in red font). Their taxonomic identities at the genus level, in the order of most to least relative abundance in the

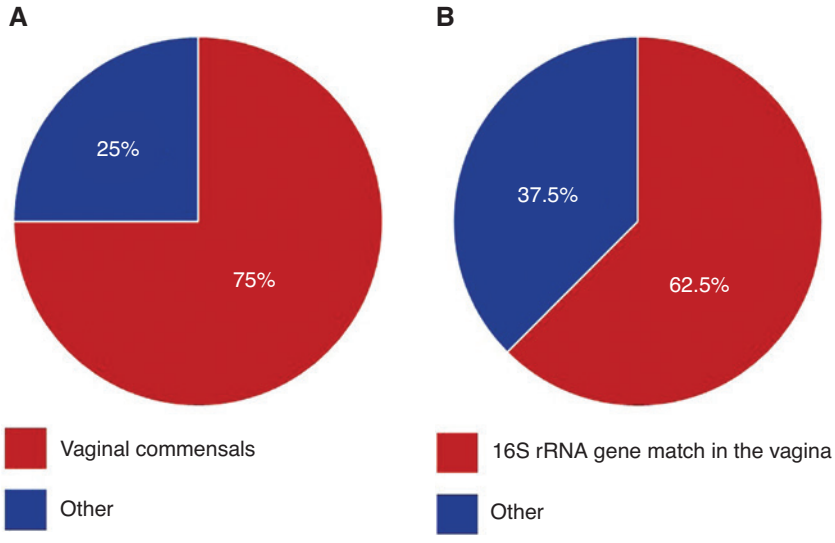

Figure 1: Pie charts illustrating the relationships between bacteria isolated from the amniotic fluid and vaginal microbiota.
The percentage of women for whom the bacteria isolated from their amniotic fluid samples (A) were typical commensals of the human vaginal ecosystem, and (B) had exact matches of their 16S rRNA genes also detected among the vaginal microbiota.

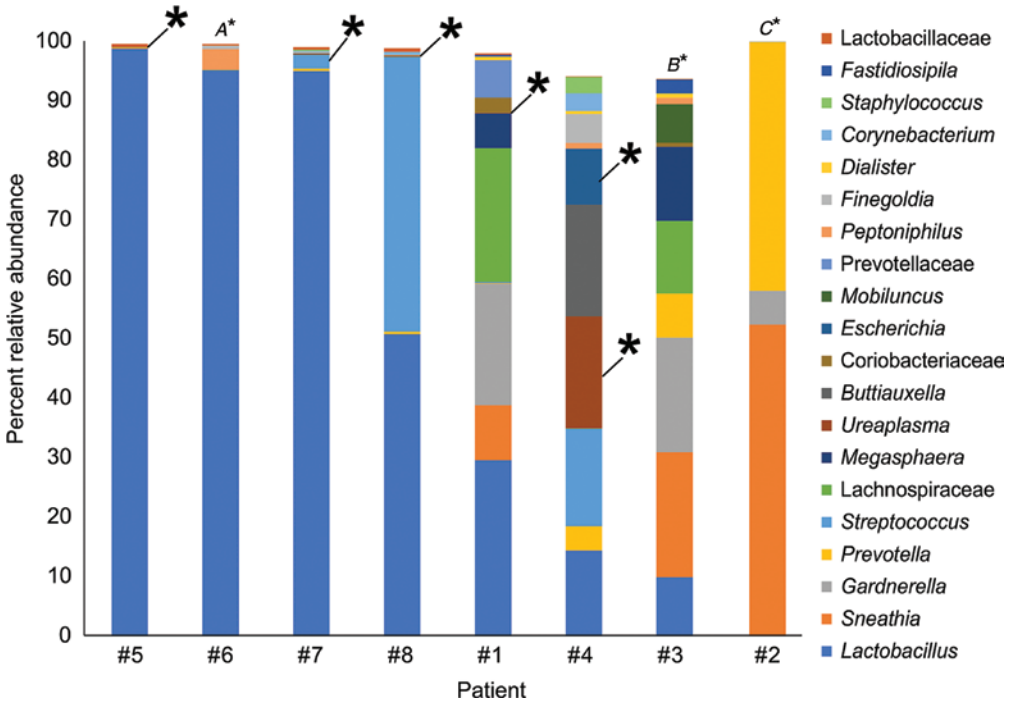

Figure 2: Percent relative abundance of bacterial taxa in the vaginal samples of eight patients with intra-amniotic infection confirmed through culture.
The top 20 bacterial taxa, based on average percent relative abundance among the vaginal samples, are displayed. Asterisks indicate a match between the 16S rRNA gene sequence of the bacterial cultivar obtained from amniotic fluid and 16S rRNA gene sequences in the same subject's vaginal sample. The taxonomic identities of intra-amniotic bacterial cultures without matching sequences in their subject's vaginal sample were (A) *Ureaplasma urealyticum*, (B) *Streptococcus anginosus*, and (C) *Staphylococcus warneri*.

Table 3: The frequency of detection of bacteria cultured from amniotic fluid within the microbiota of paired vaginal samples using 16S rRNA gene sequencing.

Microorganism	No. of amniotic fluid samples from which it was cultured	No. of paired vaginal samples in which it was detected
Ureaplasma urealyticum	4	3
Escherichia coli	2	2
Streptococcus agalactiae	1	1
Staphylococcus warneri	1	0
Streptococcus anginosus	1	0

amniotic fluid, were *Sneathia*, *Ureaplasma*, *Prevotella*, *Lactobacillus*, *Escherichia*, *Gardnerella*, *Peptostreptococcus*, *Peptoniphilus*, and *Streptococcus* (Figure 3). Seven of the 10 (70.0%) prominent OTUs in amniotic fluid were also prominent in vaginal bacterial communities (Figure 3, taxa in bold red font). At the genus level, the seven prominent OTUs shared between amniotic fluid and the vagina were *Lactobacillus*, *Sneathia*, *Gardnerella*, *Prevotella*, *Ureaplasma*, and *Escherichia* (Figure 3, taxa in bold red font). In four of six (66.7%) patients with paired amniotic fluid and vaginal samples, at least 50% of the prominent OTUs in the amniotic fluid were also present in the vagina (Figure 3, case #1, 2, 3, and 4). In 50.0% (3/6) of patients with paired amniotic fluid and vaginal samples, at least 88.9% of the prominent OTUs in the amniotic fluid were also present in the vagina (Figure 3, case #1, 2, and 4). The prominent OTUs most commonly shared between paired amniotic fluid and vaginal samples were *Sneathia* (OTUs 3 and 6), *Lactobacillus* (OTUs 5 and 14), *Gardnerella* (OTU 9), and *Prevotella* (OTU 10). In general, among women with intra-amniotic infection, there was overlap between the bacterial profiles of amniotic fluid and the vagina.

Discussion

Principal findings of the study

(1) Seventy-five percent (6/8) of women had bacteria cultured from their amniotic fluid that are typical residents of the vaginal ecosystem; (2) 62.5% (5/8) of women had bacteria cultured from their amniotic fluid also present in the

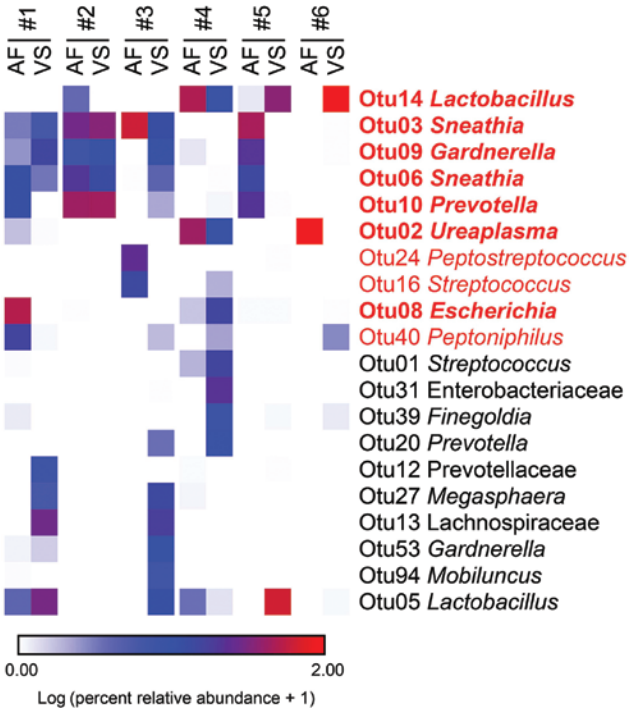

Figure 3: Heat map illustrating similarity in percent relative abundances of prominent (≥1% average relative abundance) operational taxonomic units (OTUs) among paired amniotic fluid (AF) and vaginal swab (VS) samples of six women with intra-amniotic infection confirmed through culture.
OTUs that were prominent among amniotic fluid samples are highlighted in red. OTUs that were prominent only among vaginal swab samples are in black. OTUs that were prominent in both amniotic fluid and vaginal swab samples are in bold red font.

bacterial communities of their paired vaginal sample; (3) the bacterial cultivars associated with ascending infection were *U. urealyticum*, *E. coli*, and *S. agalactiae*; (4) as assessed by 16S rRNA gene sequencing, the bacterial communities of amniotic fluids with positive bacterial cultures were dominated by *Sneathia*, *Ureaplasma*, *Prevotella*, *Lactobacillus*, *Escherichia*, *Gardnerella*, *Peptostreptococcus*, *Peptoniphilus*, and *Streptococcus*, indicating that microbial cultures do not detect many bacteria present in amniotic fluid; and (5) 70% (7/10) of prominent (>1% average relative abundance) OTUs in amniotic fluid bacterial communities were also prominent in bacterial communities of the vagina.

Evidence that ascending infection from the lower genital tract is responsible for intra-amniotic infection

Although several routes of invasion have been proposed for intra-amniotic infection, ascension of microorganisms from the vagina has been considered the most common [2, 5, 17, 57–60]. This hypothesis is based on indirect evidence [17]. First, microorganisms detected in amniotic fluid are often those associated with the human vagina [4, 59, 61–63, 67]. Second, in twin pregnancies, when there is intra-amniotic infection, the microorganisms are found in the presenting (rather than non-presenting) sac [100, 137]. Third, women with bacterial vaginosis, a condition in which there is a change in the microbial ecosystem of the vagina, are more likely to have intra-amniotic infection [1, 138, 139]. Fourth, in some cases of early neonatal sepsis or neonatal pneumonia, the microorganisms involved are similar to those found in the vagina [137, 140–142]. Lastly, in an experimental study of non-pregnant women, carbon nanoparticles were placed within the vagina prior to hysterectomy, and these particles were recovered from the fallopian tubes within 28–34 min [143]. The authors concluded that uterine contractions, induced by oxytocin administered prior to surgery, likely played a role in ascension of carbon particles from the vagina to fallopian tubes [143]. These data suggest that uterine contractions, particularly during prolonged labor, could promote ascending infection.

Mechanisms responsible for ascending infection

A fundamental question which remains unresolved is why some women develop intra-amniotic infection and others do not [5]. The uterine cervix, chorioamniotic membranes, and amniotic fluid, as well as microbial pathogenic factors, may play a role in the likelihood of ascending infection. During pregnancy, the uterine cervix produces a mucous plug, which has been shown to have antimicrobial properties [144–152]. Therefore, some women with cervical shortening and the loss of the mucous plug can develop intra-amniotic infection, and, even if the mucous plug is present, its anti-microbial properties may be inadequate, which can also result in ascending infection [149, 152–154]. The chorioamniotic membranes represent a physical and biochemical barrier to microorganisms, given that they produce antimicrobial peptides [155–157] and contain cells of the innate immune system capable of protecting the host against bacteria [158–163]. Therefore, when the membranes rupture, ascension of bacteria into the amniotic cavity can occur. This is supported by the high frequency of intra-amniotic infection in patients with PPROM [18, 34, 164, 165]. Indeed, patients with PPROM have higher frequencies of intra-amniotic infection than those with intact membranes [19, 102]. Amniotic fluid also contains multiple antimicrobial factors which could

control proliferation of bacteria gaining access to the amniotic cavity [97, 99, 166–174]. Similarly, amniotic fluid contains cells of the innate immune system, which represent another means of host defense [117, 175–185].

All women have microorganisms in the lower genital tract; however, changes in the microbial ecosystem, such as those observed in bacterial vaginosis, are associated with intra-amniotic infection [1, 138, 139, 186]. It is possible that the dysbiotic shift of the vaginal microbiota from communities dominated by *Lactobacillus* to those comprising primarily *Gardnerella*, *Prevotella*, *Porphyromonas*, *Bacteroides*, *Peptostreptococcus*, *Megasphaera*, and *Sneathia*, or related changes in bacterial load [187–189], predispose to ascending infection. Virulence factors of microorganisms may also explain why some gain access to the amniotic cavity while others do not – for example, genital mycoplasmas are the most common organisms found in amniotic fluid in cases of intra-amniotic infection [26, 75, 76, 190, 191]. Although these microorganisms are considered to have less pathogenic potential than others (e.g. *S. agalactiae*, or group B streptococcus (GBS) [192–195]), and are present in the vaginal ecosystems of many normal pregnant women [69, 70, 196], a consistent observation is that these organisms are the most frequent microorganisms responsible for intra-amniotic infection [26, 75, 76, 190, 191]. Virulence factors have been identified in *Ureaplasma* spp. [197, 198] and *Mycoplasma* spp. [199], which may explain their invasive potential.

What is the origin of intra-amniotic infections in which bacteria in amniotic fluid could not be detected in the lower genital tract?

Three patients in our study had microorganisms in the amniotic cavity that were not detected in vaginal swabs, suggesting that alternative sources for microbial invasion of the amniotic cavity should exist. Previous reports have identified microorganisms in amniotic fluid that had also been identified in the oral cavity [200–202]. Indeed, for animal models of periodontal disease, in which the microorganisms involved are found in the peripheral blood, hematogenous dissemination has been proposed as a route for intra-amniotic and fetal infection [203–209]. Other sources of microorganisms could be the gastrointestinal tract, as a leaky gut can result in bacteremia [210–213]. For example, in the current study, *S. warneri* and *S. anginosus* were cultured from amniotic fluid but were not present in the matching vaginal swabs. Strains of *S. anginosus* have been isolated from the sinus, mouth, throat, and feces, and they are associated with a number of infections [214]. In addition, *S. warneri*, a normal inhabitant of human epithelia and mucosal membranes, has also been associated with orthopedic infections [215, 216], meningitis [217], and endocarditis [218]. Notably, each has been previously reported as etiological agents of bacteremia [219, 220]. Therefore, the absence of each of these bacteria in paired vaginal samples may be due to the hematogenous spread of the microbe originating from a distant body site to the amniotic cavity, as opposed to ascending from the vagina. Alternatively, it is possible that the molecular microbiological methods used in this study have not identified all potential microorganisms present in the vagina; further studies with a large sample size and sampling of other body sites are required to address this question.

Clinical implications of ascending infection

Establishing that microorganisms present in the vagina are responsible for most cases of intra-amniotic infection increases the value of studying the vaginal microbiota. Although the issue is controversial, there is a growing body of evidence supporting that changes in the vaginal microbiota precede spontaneous preterm birth [84–93]. How such alterations translate to increased frequency of ascending intra-amniotic infection remains to be determined.

Research implications

Additional studies are required to determine why some microorganisms ascend and others do not. For example, why are *Lactobacillus* so frequent in the vagina and not found in the amniotic cavity? Why are *Sneathia* and *Ureaplasma*, which are rare in the vaginal ecosystem, so frequently found in the amniotic cavity? A possible explanation is that these microorganisms possess pathogenic factors that allow them to ascend and invade the intra-amniotic space [71, 199].

Strengths and limitations

This is the first sequencing-based study to compare the amniotic fluid and vaginal microbiota in patients with intra-amniotic infection confirmed through bacterial culture. It provides evidence of ascending infection as the primary cause of intra-amniotic infection. Nevertheless,

the observed associations between the vaginal microbiota and amniotic fluid infection are implied and not causally demonstrated. Future studies are required to comprehensively characterize the cervical, vaginal, oral, and intestinal microbiota in women susceptible to microbial invasion of the amniotic cavity in order to confirm the origins of intra-amniotic infection. This will be fundamental to furthering prevention and treatment of intra-amniotic infection.

Conclusion

In most women, the microorganisms causing intra-amniotic infection are members of the vaginal ecosystem, supporting the hypothesis that an ascending pathway is the most common route for microbial invasion of the amniotic cavity.

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