

REAL-TIME PCR ANALYSIS OF AMNIOTIC FLUID IN UNCOMPLICATED TERM PREGNANCIES: EVIDENCE FOR LOW-BIOMASS ENVIRONMENT

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Abstract

Amniotic fluid (AF) plays a critical role in fetal development by providing mechanical protection, thermoregulation, and a medium for nutrient exchange. Traditionally considered sterile in healthy pregnancies, recent molecular studies have suggested the presence of microbial DNA in AF, although these findings remain controversial due to the challenges of contamination in low-biomass samples.

In this cross-sectional study, we analyzed 73 AF samples from uncomplicated term pregnancies using a controlled real-time PCR approach targeting common bacterial and fungal taxa. Samples were collected under strict sterile conditions at a private general hospital in Skopje, with informed consent obtained from all participants under ethical approval from the Faculty of Medicine, University in Skopje, North Macedonia.

No microbial DNA was detected above negative controls, supporting the notion that AF in healthy term gestations represents a sterile or extremely low-biomass environment.

Our study is, to our knowledge, the first molecular study of this type conducted in the Republic of North Macedonia, providing original molecular evidence regarding the microbial status of AF.

Our results highlight the importance of rigorous contamination-aware methodologies when studying low-biomass intrauterine environments and contribute to understanding the timing of initial microbial colonization and neonatal microbiome establishment.

Key words: Amniotic fluid, Real-time PCR, Low-biomass environment, Neonatal microbiome, Sterile womb hypothesis, Microbial DNA, Term pregnancy.

Introduction

Amniotic fluid (AF) surrounds the developing fetus and is essential for normal growth, providing mechanical protection, thermal stability, and biochemical support throughout gestation [1,2]. In addition to these functions, AF represents a unique biological compartment that reflects fetal physiology and maternal–fetal interactions. From a morphological and developmental perspective, the composition of AF is closely linked to fetal skin maturation, swallowing activity, and renal function, all of which evolve dynamically during pregnancy [16,18].

Consequently, any microbial presence within this compartment could have implications not only for immunological priming but also for fetal tissue development and inflammatory signaling pathways.

The traditional view of AF as a sterile environment under physiological conditions has been largely based on culture-dependent microbiological methods, which consistently yield negative results in uncomplicated term pregnancies [4,5]. Historically, microbial detection in AF has been associated primarily with pathological states, including chorioamnionitis, preterm premature rupture of membranes (PPROM), and preterm labor [5,12].

With the advent of highly sensitive molecular techniques, such as polymerase chain reaction (PCR) and next-generation sequencing (NGS), several studies have reported the detection of microbial DNA in AF even in pregnancies with uncomplicated outcomes [3,6,7]. However, these observations remain highly debated, as investigations of low-biomass biological compartments are particularly vulnerable to

background contamination [8,13]. Trace amounts of microbial DNA originating from laboratory reagents, extraction kits, water sources, or the processing environment may be sufficient to generate false-positive signals when amplified by sensitive molecular assays [6–8,14].

This issue is especially critical in AF research, as AF is considered a low-biomass environment. Multiple studies have demonstrated that contaminant DNA frequently overlaps with bacterial taxa previously reported in AF and placental samples, underscoring the substantial risk of misinterpretation in the absence of rigorous methodological safeguards [8,9,13].

Without appropriate negative controls and carefully designed analytical frameworks, such signals may be mistakenly interpreted as evidence of a resident microbiome [6–8].

Recent well-controlled metagenomic studies employing stringent contamination controls have found no reliably detectable microbial nucleic acids in AF from healthy pregnancies, findings that support the “sterile womb” hypothesis [6,7,9,15].

Understanding the microbial composition of AF is critically important, as in utero microbial exposure may affect fetal immune development, perinatal outcomes, and the establishment of the neonatal microbiome. [10,11].

Despite extensive investigation, the existence and clinical relevance of a physiological AF microbiome in healthy term pregnancies remain unresolved, particularly when examined using highly sensitive molecular methodologies. From a morphological sciences perspective, establishing whether AF harbors a true microbial signal is essential for accurate interpretation of fetal tissue–microbe interactions and developmental immune programming. Understanding normal AF microbial status allows comparison with abnormal conditions.

The present study aimed to assess the presence of microbial DNA in AF from healthy term pregnancies using a controlled real-time PCR approach targeting a panel of representative vaginal and perinatal microorganisms. By implementing strict sterile sampling procedures and incorporating appropriate negative controls, we intended to determine whether microbial DNA can be detected in AF above background contamination levels.

Materials and Methods

Study Design and Participants

This cross-sectional study included 73 women with uncomplicated term pregnancies (≥ 37 weeks of gestation) who delivered at a private general hospital in Skopje. The study cohort included 38 spontaneous vaginal deliveries and 35 elective cesarean sections. Inclusion criteria were healthy term pregnancies without clinical or laboratory evidence of infection and routine normal microbiological screening during pregnancy.

Exclusion criteria included preterm premature rupture of membranes (PPROM), clinically confirmed chorioamnionitis, fetal anomalies, and emergency cesarean sections.

Special attention was paid to minimizing environmental and reagent-derived contamination, in accordance with established recommendations for low-biomass microbiome research. Sample handling, DNA extraction, and PCR preparation were conducted in physically separated laboratory areas using sterile, DNA-free consumables. Negative controls were incorporated at each analytical step to monitor potential contamination.

Ethical approval was obtained from the Ethics Committee of the Faculty of Medicine, Skopje, and written informed consent was obtained from all participants prior to sample collection.

To the best of our knowledge, this represents the first study conducted in the Republic of North Macedonia to evaluate the presence of microbial DNA in amniotic fluid from uncomplicated term pregnancies using a real-time PCR approach.

Sample Collection

Amniotic fluid was collected under strict sterile conditions. In cesarean deliveries, AF was aspirated immediately following surgical opening of the uterine cavity. In vaginal deliveries, samples were obtained after amniotomy during labor. Sterile swabs were placed in PBW transport medium (Fisher BioReagents) and transported to the laboratory under controlled temperature conditions for molecular analysis.

DNA Extraction and Real-Time PCR

Extraction of DNA was carried out employing the STARMag 96×4 Universal Cartridge Kit on the automated Seegene Microlab Nimbus system. Amplification and detection of microbial DNA were carried out via real-time PCR employing the Allplex™ Vaginitis Screening Assay (Seegene Inc.), targeting *Candida spp.*, *Trichomonas vaginalis*, *Lactobacillus spp.*, *Gardnerella vaginalis*, *Atopobium vaginae*, *Mobiluncus spp.*, and *Candida albicans*. Amplification was carried out on a CFX96™ Real-Time PCR System (Bio-Rad), and data were analyzed using Seegene Viewer v3 software. Both negative and positive controls were included in each PCR run to assess assay performance and to monitor potential contamination.

Statistical Analysis

Detection rates of target microorganisms were compared between delivery modes. A p-value of <0.05 was considered statistically significant.

Results

A total of 73 AF samples were analyzed (38 from vaginal deliveries; 35 from cesarean sections). Across all samples, no microbial DNA was detected for any of the targeted taxa above the negative control thresholds. This included *Candida spp.*, *Trichomonas vaginalis*, *Lactobacillus spp.*, *Gardnerella vaginalis*, *Atopobium vaginae*, *Mobiluncus spp.*, and *Candida albicans*. The absence of microbial detection was consistent across both delivery modes, and no statistically significant differences were observed (all comparisons $p > 0.05$). Table 1

Table 1. Detection of microbial DNA in AF by delivery type

Target Organism	Vaginal Delivery (n=38)	Cesarean Section (n=35)
<i>Candida spp.</i>	Not detected	Not detected
<i>Trichomonas vaginalis</i>	Not detected	Not detected
<i>Lactobacillus spp.</i>	Not detected	Not detected
<i>Gardnerella vaginalis</i>	Not detected	Not detected
<i>Atopobium vaginae</i>	Not detected	Not detected
<i>Mobiluncus spp.</i>	Not detected	Not detected
<i>Candida albicans</i>	Not detected	Not detected

Source: Authors' research

Discussion

Our findings demonstrate that, under rigorously controlled sterile conditions, amniotic fluid from healthy term pregnancies does not contain detectable microbial DNA for commonly targeted organisms when analyzed using established real-time PCR methods. These results are consistent with recent metagenomic studies that incorporated strict contamination controls and similarly reported no reliable microbial nucleic acids in AF from uncomplicated pregnancies [6–9].

In contrast, previous PCR-based studies in high-risk or pathological pregnancies have reported microbial DNA more frequently; however, these studies often involved preterm labor or premature rupture of membranes, where infection actively contributes to microbial presence [12]. Studies strictly limited to

healthy pregnancies with intact membranes and appropriate negative controls have generally failed to detect microbial DNA above background levels [3,4,6].

Although some earlier reports proposed the existence of an intrauterine microbiome based solely on molecular detection [11,19], subsequent analyses with stringent contamination controls have largely been unable to replicate these findings [6–9].

This emphasizes the critical importance of distinguishing true biological signals from background contamination in low-biomass samples. Trace microbial DNA originating from reagents, laboratory water, buffers, or the laboratory environment may be amplified by sensitive PCR assays, resulting in false-positive signals [8,13]. Rigorous methodological safeguards, including negative controls and spatial separation of sample processing areas, are therefore essential for reliable interpretation of molecular data from AF [6–8].

The absence of detectable microbial DNA in our cohort supports the sterile womb hypothesis for healthy term pregnancies, suggesting that fetal exposure to microbes before birth is minimal [6,9].

This has important implications for neonatal microbiome establishment, indicating that initial microbial colonization is likely to occur predominantly during and after birth [10,11]. Furthermore, the microbial status of AF may influence fetal immune development, tolerance induction, and the timing of early postnatal colonization [10,11,20].

From a morphological perspective, the lack of microbial exposure within AF during normal term pregnancies suggests that fetal tissues develop in an environment largely free from microbial stimuli. Such a sterile or low-microbial environment may be essential for normal organogenesis and for preventing premature inflammatory activation during critical stages of fetal development [10,11,17].

Overall, our results support the interpretation that molecular signals previously detected in AF under non-pathological conditions are more likely attributable to technical artifacts than to genuine microbial colonization.

Limitations and Future Directions

While real-time PCR provides high sensitivity for pre-defined target organisms, it inherently cannot detect unknown or unexpected microbial taxa that are not included in the assay panel [4,5]. Consequently, rare, novel, or low-abundance microorganisms may remain undetected using this methodology.

Future investigations employing comprehensive metagenomic or shotgun sequencing approaches, combined with rigorous contamination controls and negative controls at all analytical steps, could further investigate the potential presence of rare or previously uncharacterized microbial taxa in amniotic fluid [6,7,15].

Such studies would expand our understanding of the microbial landscape in AF and refine insights into fetal microbial exposure under physiological conditions.

Conclusion

This controlled real-time PCR study detected no microbial DNA in amniotic fluid from healthy term pregnancies, reinforcing the concept that AF constitutes a sterile or extremely low-biomass environment under normal physiological conditions. To our knowledge, this represents the first molecular analysis of AF using real-time PCR in a cohort from a private general hospital in Skopje, providing original data for the Republic of North Macedonia.

Collectively, these findings emphasize the importance of rigorous, contamination-aware methodological approaches when investigating intrauterine environments characterized by very low microbial biomass. By establishing a robust contamination-controlled molecular baseline, this study contributes to the morphological and developmental understanding of fetal environments, supporting the conclusion that initial microbial colonization primarily occurs during and after birth rather than in utero.

Overall, our data provide strong molecular evidence consistent with the sterile womb hypothesis in healthy term pregnancies, offering a reference framework for future research in fetal development, neonatal microbiome establishment, and related immunological and morphological studies.

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