Molecular characterization of the human microbiome from a reproductive perspective

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The process of reproduction inherently poses unique microbial challenges because it requires the transfer of gametes from one individual to the other, erstwhile preserving the integrity of the gametes and individuals from harmful microbes during the process. Advances in molecular biology techniques have expanded our understanding of the natural organisms living on and in our bodies, including those inhabiting the reproductive tract. Over the past two decades accumulating evidence has shown that the human microbiome is tightly related to health in disease states involving the different body systems, including the reproductive system. Here we introduce the science involved in the study of the human microbiome. We examine common methods currently used to characterize the human microbiome as an inseparable part of the reproductive system. Finally, we consider a few limitations, clinical implications, and the critical need for additional research in the field of human fertility. (Fertil Steril® 2015;■:■–■. ©2015 by American Society for Reproductive Medicine.)

Key Words: DNA sequencing, fertility, microbes, molecular biology, reproductive system

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The inter-relationship between DNA and microbiology began in 1869 with the discovery of nucleic acids by Johannes Friedrich Miescher, a young Swiss physician and biochemist (1). Dr. Miescher studied pus on fresh surgical bandages, which he collected from the nearby surgical clinic. In pus, Dr. Miescher found the ideal and sufficient base material for his analyses. His discovery of the nucleotides that constitute DNA was made possible thanks to a large aggregate of bacteria together with human leukocytes. Following this discovery, for more than 100 years culture-based methods were used for isolation of microbes. Interestingly, nowadays cultivation-independent DNA sequencing methods are being used to detect colonization by microbes. Thus, evolution of molecular approaches fostered by sequencing of the genome led to a paradigm shift in understanding about microbes, the human body, DNA, and the human microbiome.

The human microbiome was defined in 2001 by Joshua Lederberg, an American molecular biologist. The human microbiome may be defined as the totality of micro-organisms and their collective genetic material present in or on the human body. The introduction of cultivation-independent techniques, such as DNA sequencing, with the former knowledge derived from the classic cultivation-dependent techniques, has revealed surprising information that oftentimes contradicts what was considered dogma only a decade ago. For example, Steel et al. (2) have shown that placental tissues derived from elective, term cesarean deliveries contained organisms in 70% of placental membranes. This finding indicates that the sole presence of micro-organisms does not induce preterm labor. However, intra-amniotic culture-independent (uncultivated) bacteria were recovered from pregnant women who had confirmed histologic intra-amniotic inflammation, and subsequently, preterm birth (3). Colonization at birth is a normal process, and van Nimwegen et al. (4) have shown that the neonatal microbiome can differ according to the mode of delivery, and the mode of delivery can be correlated to atopic diseases later in childhood.

Cultivation-dependent and -independent techniques have also broadened understanding of the normal human microbiome at different anatomic sites and how microbes may spread and colonize the reproductive tract. For instance, the upper genital tract was previously considered to be

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sterile, but endometrial cultures obtained surgically at hysterectomy have demonstrated the presence of one or more micro-organisms in the uterus in nearly one-quarter of asymptomatic women examined (5). Furthermore, with the usage of advanced molecular biology techniques, Mitchell et al. (6) have recently provided additional evidence that the upper genital tract in asymptomatic women is not a sterile environment. In their study, the vast majority of women (55 of 58 [95%]) tested positive for at least one species of bacteria. Using similar techniques, Aagaard et al. (7) have demonstrated that the placenta harbors a unique low-abundance microbiome. Additionally, a recent review by Payne and Bayatibojakhi (8) summarized the evidence regarding the relationship between oral cavity bacteria and preterm birth through hematogenous spread to the placenta. These examples beg the question: what constitutes a balanced (symbiotic or commensal) microbiome, and what makes it a diseased, a parasitic, or harmful microbiome?

Advanced technological tools in molecular biology have allowed researchers to "look deeper" into the microbiome world and have revealed an enormous amount of information that was not previously accessible. Technological breakthroughs, such as high-throughput methods for DNA sequencing, enabled examination of the same sources studied by Miescher, and others, with more powerful tools that reveal a deeper level of understanding and new conclusions. For example, it is now accepted that the human body contains 10^{13}–10^{14} symbiotic microbial cells (9), which outnumber our own body cells. Thanks to worldwide human genome and microbiome projects, we now know that there are 3.3 million microbial genes in the human gut microbiome alone (10), as compared with only 20,000–25,000 protein-coding genes present in the entire human genome (11).

The fact that the human body harbors bacteria was first described by Antonie van Leeuwenhoek in the 17th century (12), and as a point of fact, the human cells themselves harbor ancient bacteria, which are the mitochondria with their mitochondrial (bacterial) DNA (13). Within the past decade, with the newly gathered information provided by high-throughput analyses, we begin to question what was previously considered impossible. Does the uterus have its own microbiome? Is the normal healthy fetus growing in a nonsterile environment? Is there a chance that the most common bacteria associated with chorioamnionitis are not isolable by culture? Are certain types of lactobacilli necessary for normal fecundity? These are only a few of the questions that have arisen in the field of reproduction.

One of the main goals of The Human Microbiome Project, a 5-year project launched by the National Institutes of Health in 2007, was to explore the relationship between disease and the changes in the human microbiome. A central tenet is that most of the microbiome cannot be easily cultured, and therefore information collected through bacterial cultures is very limited and does not represent the actual human microbiome repertoire in normal or disease states. A relatively new way to obtain information on the microbiome is by conducting high-throughput DNA sequencing and analyses. Samples obtained from the skin, gastrointestinal tract, and vagina naturally contain human and bacterial DNA. In actual biological samples, multiple DNA strands can be sequenced simultaneously (see below). Prior knowledge about the origin or function of the DNA sequences found can provide an incredible amount of information, such as disease outbreaks, bacterial virulence, and pathogenic strains, within a relatively very short period.

THE NATURE OF SAMPLES REQUIRED AND DNA EXTRACTION

For the purpose of DNA sequencing, biological specimens can be simply collected with a swab. There is no need for a culture medium because there is no need to keep the microbes alive. There are multiple protocols for DNA extraction. Most of the protocols contain the following steps. [1] Cell lysis to expose the DNA within the bacteria. This is done by chemical and physical methods such as detergents, blending, grinding, or sonication. [2] Removal of the membrane lipids by adding a detergent or surfactants. [3] Removal of proteins by adding a protease. [4] Removal of the RNA by adding an RNase. The microbial DNA is now free and has to be purified. Commonly used procedures for DNA purification are ethanol precipitation or phenol–chloroform extraction or silica-based strategies.

BASIC BIOLOGY OF THE VARIOUS MOLECULAR TECHNIQUES

The first bacterial genome was sequenced in 1995 (14). Since then, bacterial DNA databases are growing rapidly (15), and today's technology enables the analyses of millions of different DNA sequences obtained from a single sample. Large amounts of information can be obtained, but conducting even a targeted sequencing of specific genes in the sample may be a complex and costly endeavor. There are four commonly used techniques: fingerprinting, DNA microarrays, targeted sequencing, and whole-genome sequencing (WGS, using the Sanger and pyrosequencing techniques principles).

The fingerprinting technique relies on the amplification of a specific gene, typically the bacterial ribosomal 16S ribosomal RNA (rRNA) gene (see below), or the amplification of Q3 variable number tandem repeats by polymerase chain reaction (Fig. 1). The different variants in the sample are then separated by gel electrophoresis (16). The differentiation among the variants is based on the different gel electrophoresis band patterns rather than the actual sequencing of the gene. Therefore, the fingerprinting technique is significantly cheaper than sequencing-based techniques, and it is useful for clustering bacteria communities according to changes in the dominant members across different samples (17). However, on the basis of the gel electrophoresis band patterns, only the few most abundant members of the community are detected, and therefore there is a limited range of detection (dynamic range). The advantage of sequencing over fingerprinting is a greater dynamic range. Gene sequencing, not limited to 16S rRNA, provides information with a higher resolution that enables answering questions, such as this: which specific bacterial genes or species contribute to differences among communities (including functional differences)? However, this advantage comes at a higher cost and requires more complex analyses.

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