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Polymicrobial urine cultures: reconciling contamination with the urobiome while recognizing the pathogens

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Polymicrobial or mixed urine cultures of more than one predominant microbe confound clinical urinary tract infection diagnosis. The current College of American Pathologists clinical laboratory standard states that a urine sample cultured with more than two isolates with >10,000 colony forming units/ml is to be considered contaminated. However, the presence of urinary sample bacteria in individuals without urinary symptoms (referred to as asymptomatic bacteriuria) is common especially in older people and in pregnant individuals. Furthermore, the discovery of an indigenous urinary microbiome (urobiome) in healthy humans throughout life from shortly after birth to death conflicts with the long-standing notion that urine derived from sterile filtered blood should be sterile above the urethral sphincter. Polymicrobial infections are not consistent with Koch's postulates that a single pathogen is causal for disease. In this review, we will discuss current standards of contamination, how to reconcile the sterility of urine with the existence of the urobiome, a history of polymicrobial infections, and why re-examining current practices is essential for the practice of medicine, improving quality of life, and potentially saving lives.

KEYWORDS

contamination, mixed culture, polymicrobial, urine culture, urinary tract infection, urogynecology, urology

1 Introduction

The College of American Pathologists guidance states “a contaminated urine culture was defined as the presence of more than 2 isolates at greater than or equal to 10–000 CFU/mL” (Valenstein and Meier, 1998; Bekeris et al., 2008). This includes commensal microbes thought to be of skin origin or, in adult females, vulvo-vaginal contaminants (Brubaker et al., 2021a). Standard practice is to resample although often the same result is obtained. Then, the predominant microbe is reported (often *E. coli*), while the other detected microbes are often ignored (Sfeir and Hooton, 2018). Examples include reports of “contamination” or “mixed flora” in asymptomatic bacteriuria of pregnant individuals

(46.7%) (O'Leary et al., 2020), in general practice (54.9%) (Hansen et al., 2022), and in outpatient clinics (46.2%) (Whelan et al., 2022). This is comparable to results obtained with a multiplex polymerase chain reaction (M-PCR) panel, where 56.1% of an older cohort (≥ 65 years old) with diagnosed urinary tract infection (UTI) had polymicrobial (more than one microbe) results (Vollstedt et al., 2020).

While the method of urine collection can sample different aspects of the urinary tract (e.g., suprapubic aspirate and transurethral catheter: bladder and upper urinary tract; midstream void: entire urinary tract including urethra and periurethral skin plus urogenital regions), there are subtle differences in microbes detected depending on sampling technique (Chen et al., 2020; Pohl et al., 2020; Brubaker et al., 2021a; Wang et al., 2023; Du et al., 2024; Shafik et al., 2005; McFadyen and Eykyn, 1968; Wolfe et al., 2012) Table 1. Nevertheless, midstream void or the so-called “clean catch” is most often used in clinical practice (LaRocco et al., 2015; Moreland et al., 2024). A recent meta-analysis of urine collection methods and contamination concluded that methods to decrease contamination (e.g. cleansing, boric acid preservative, and refrigerating urine sample to prevent nonspecific growth) were of limited value (LaRocco et al., 2015). The possibility remains that many microbes reported as contamination or mixed flora could represent potential polymicrobial infection that, in some cases, may breach the renal urine blood barrier and progress to urosepsis (Siegman-Igra, 1994; Siegman-IgraY, 1994; Peach et al., 2016; Akhtar et al., 2021; Collaborators, GBD 2021 Antimicrobial Resistance, 2024). Distinguishing contamination from the presence of clinically relevant microbes is essential to test interpretation and reporting. It is helpful to understand the history of urine culture contamination.

2 Origins of urine sample contamination

The belief that urine is sterile above the urethral sphincter is attributed to Pasteur and his studies proposing germ theory in the 1860s (Asscher et al., 1966; Roll-Hansen, 1979; Brubaker et al., 2023).

The seminal fact omitted in many contemporary accounts is that Pasteur boiled the urine, vacuum sealed the flasks and observed no growth. Indeed, Pasteur considered urine alone to be an excellent bacterial growth media (Asscher et al., 1966). The point of his famous experiment was to demonstrate that the growth he observed in urine open to the environment resulted from microbes and not spontaneous generation from miasma (Roll-Hansen, 1979). Later publications by Roberts reported no detectable microbes in fresh urine of healthy subjects using the techniques of the day (e.g., microscopy) (Roberts, 1881). However, all samples left open to the air and at room temperature developed cloudiness and an ammonia odor in two to three days. This was attributed to microbes that could metabolize urea (Roberts, 1881) and later shown to be due to urease expressing facultative anaerobes such as *Proteus* (Armbruster et al., 2017).

The success of Koch's postulates in identifying single microbes as causes of mortal diseases led to a quantum advance in the diagnosis of infectious disease in the late nineteenth century (Blevins and Bronze, 2010). As culture methods evolved for the detection of UTI-associated pathogens, protocols that detected the most common pathogen, *Bacterium coli commune* (later *Escherichia coli*), became standard clinical laboratory practice (Friedmann, 2014). Although multiple publications during the twentieth century reported microbes in the “sterile” urine of healthy individuals without symptoms (Hort, 1914; Marple, 1941; Philpot, 1956; McFadyen and Eykyn, 1968; Maskell, 1986; Maskell, 1988), special methods were necessary to culture these presumably “uncultivable” microbes (Maskell, 1988; Khasriya et al., 2013; Hilt et al., 2014; Price et al., 2016; Legaria et al., 2022), and the dogma remained that urine was sterile in the absence of clinical conditions, such as UTI. Thus, contamination as it is currently defined is thought to arise from extravascular (outside the bladder) sources and be unrelated to the cause of symptoms or infection; contamination is an artifact of urine sample collection (Valenstein and Meier, 1998; Bekeris et al., 2008; LaRocco et al., 2015). The prevailing view is that urine specimens can easily become contaminated with periurethral, epidermal, perianal, and vaginal flora (LaRocco et al., 2015). One report defined contamination as “bacteria that are found in normal vaginal or skin flora and do not cause UTI” (Blake and Doherty, 2006).

TABLE 1 Methods of urine collection for clinical laboratory analysis.

Method	Regions sampled	Advantages	Disadvantages	References
Midstream Void Clean Catch	Kidney, Ureter, Bladder, Urethra (Prostate), Urethral Meatus, Urogenital Regions	Least invasive	Includes urethral and urogenital flora, risk of “contamination”	(Brubaker et al., 2021b; Pohl et al., 2020; Wang et al., 2023)
Transurethral Catheterization	Kidney, Ureter, Bladder	Bladder and upper urinary tract only. Excludes urethral and urogenital flora	Invasive. Catheterization	(Pohl et al., 2020; Wang et al., 2023; Du et al., 2024; Wolfe et al., 2012)
Suprapubic Aspirate	Kidney, Ureter, Bladder	Bladder and upper urinary tract only. Excludes urethral and urogenital flora	Invasive (Suprapubic needle into the bladder)	(McFadyen and Eykyn, 1968; Wolfe et al., 2012)
Ureterscopy	Kidney, Ureter	Renal and ureteral flora only	Invasive, rarely used except under certain circumstances	(Shafik et al., 2005)

Urine culture contaminants attributed to dermal origin (skin) are usually identified as Gram-positive diphtheroids (*Corynebacteria*, club-like), Gram-positive clustered cocci (*Staphylococcus*), and Gram-positive cocci (*Streptococcus*, *Micrococcus*). A survey of the human skin microbiome identifying the ten most abundant taxa in four different regions of skin (dry, moist, sebaceous, and feet) found 22 different microbes. Of these, 21 are Gram-positive aerobes, microaerophiles or facultative anaerobes (Byrd et al., 2018) (Supplementary Table 1). Three are within the Viridians group streptococci (VGS) (Doern and Burnham, 2010). Only one is Gram-negative; the anaerobic coccus *Veillonella parvula* (found only in the dry region sample). Thus, skin contamination would be consistent with Gram-positive commensals.

The vaginal microbiome changes with age and reproductive status (menarche, reproductive age and post-menopausal) (Ravel et al., 2011; Nunn and Forney, 2016; Saraf et al., 2021; Park et al., 2023). Consequently, vaginal microbiota can vary with the patient. In general, the healthy vaginal microbiome is predominated by *Lactobacilli*, although other taxa have been observed (Ravel et al., 2011; Nunn and Forney, 2016; Saraf et al., 2021) (Supplementary Table 2). Vaginal contaminants are usually identified as Gram-positive bacilli and are attributed to commensal flora. These include *L. iners*, *L. crispatus*, *L. gasseri* and *L. jensenii* but can also include *Gardnerella vaginalis* (Gram-variable) and Gram-positive anaerobe *Atopobium vaginae* (now known as *Fannyhessea vaginae*) (Nouioui et al., 2018).

The vulvar microbiome has recently been investigated and includes representative taxa from both skin and vagina, including the genera *Corynebacterium*, *Lactobacillus*, *Staphylococcus*, *Prevotella*, *Propionibacterium* (*Cutibacterium*) and *Fingoldia* (Pagan et al., 2021). These microbes are Gram-positive diphtheroids, rods, and cocci except for the Gram-negative anaerobe *Prevotella*. Assessing gastrointestinal contamination including perianal and perineal regions becomes problematic as these microbes are facultative anaerobes that have also been identified as uropathogens. These include the genera *Escherichia*, *Pseudomonas*, *Klebsiella*, *Proteus*, and Gram positive *Enterococcus* commonly reported in UTI and are the microbes most often detected using current diagnostic techniques (urine dipstick and SUC) (Flores-Mireles et al., 2015; Moreland et al., 2023) Tables 2 and 3. Consequently, they are diagnosed as uropathogens rather than perineal contaminants.

Guidelines for common microbial contaminants in blood culture are available (Palavecino et al., 2024) and the Centers for Disease Control maintains a list of microbes that have been detected as commensals in UTI and blood infections (CDC, 2022). However, a list of urinary tract microbial contaminants is much more nebulous beyond listing niches (periurethral, epidermal, perianal, and vaginal), or assuming that microbes that are commensals in one niche are commensals in another (Blake and Doherty, 2006; LaRocco et al., 2015) (Supplementary Tables 1, 2).

Thus, in current clinical diagnostic practice, if a sample contains mixed flora, it is the number of different microbes (≥ 2 or more at 10^4 CFU/ml) that determines contamination and not the actual microbe unless it is a commonly recognized urinary pathogen

(Bekeris et al., 2008; LaRocco et al., 2015; Sfeir and Hooton, 2018; Mancuso et al., 2023).

3 Limitations of diagnostic standard urine culture

Today, diagnosis of UTIs typically relies on patient symptoms and urinalysis. The latter uses urine dipstick testing and, in some cases, the standard urine culture (SUC) method (Chambliss and Van, 2022; Moreland et al., 2024; Werneburg et al., 2023) Table 2. Urine dipstick testing, frequently used to determine further testing, such as urine cultures is reviewed elsewhere (Moreland et al., 2024; Chambliss and Van, 2022). The limitations of SUC have been identified and are starting to impact current diagnostics (Price et al., 2017; Dixon et al., 2020; Wojno et al., 2020; Brubaker et al., 2023; Gleicher et al., 2024; Werneburg and Hsieh, 2024).

It is now well recognized that SUC under aerobic conditions (Gillespie et al., 1960), or even under 5% CO₂, detects a limited number of microbes, almost all facultative anaerobes (Price et al., 2016, 2017; Wojno et al., 2020; Brubaker et al., 2023; Festa et al., 2023). As a result, reports based on SUC, including almost all literature to date, repeatedly document a constellation of the same microbes from the genera *Escherichia*, *Pseudomonas*, *Klebsiella*, *Proteus*, *Staphylococcus*, and *Enterococcus*, with *Escherichia coli* by far considered the predominant cause of UTI (Table 3) (Flores-Mireles et al., 2015; Kline and Lewis, 2016; Mancuso et al., 2023; Moreland et al., 2023; Werneburg et al., 2023; Timm et al., 2025). However, these results have been obtained because SUC was designed to detect fast growing, non-fastidious, facultative anaerobes, and thus fails to detect many other microbes. For example, a recent study directly compared SUC results to those of a multiplex polymerase chain reaction (M-PCR) panel for a cohort of 1,132 diagnosed UTI patients. M-PCR detected microbes in 823 of these patients, who also exhibited elevated infection-associated urine biomarkers (Haley et al., 2024). Of the 10 microbes most detected by M-PCR, only 4 were detected by SUC with 2 of those often not detected (Haley et al., 2024). Most striking was the failure of SUC to detect 3 of the 5 microbes most detected by M-PCR. These were the genera *Aerococcus* and *Actinotignum* and the Viridians group *Streptococcus* (including *S. anginosus*, *S. oralis*, and *S. gallolyticus* subsp. *pasteurianus* (formerly known as *Streptococcus pasteurianus*) (Doern and Burnham, 2010). Thus, except for *E. coli*, all known uropathogens ("the usual suspects") represented only 13% or less of UTIs diagnosed with symptoms (Haley et al., 2024) (Table 3).

One outstanding issue with SUC has been the diagnosis of sterile pyuria, defined as positive for white blood cells but negative urine cultures in patients that report UTI symptoms (Wise and Schlegel, 2015; Horton et al., 2018; Cohen et al., 2019; Xu et al., 2024). Yet, a recent report suggests that *Actinotignum* (which SUC cannot detect but M-PCR diagnostics finds to be quite common) may be an underlying cause of sterile pyuria (Horton et al., 2018). Until recently, however, few had questioned the standard diagnostic

TABLE 2 Assays for urinary tract infection diagnosis.

Method	Basis	Detection	Advantages	Disadvantages	References
Urine Dipstick	Detects nitrite (bacteria), leukocyte esterase (infection, WBC) and pH	Colorimetric, Time to result, minutes.	Quick, accessible, Rapid diagnosis as a first read of UTI	False negatives, false positives, half of known and emerging uropathogens do not make nitrite.	(Kavuru et al., 2020; Mundt and Shanahan, 2010; Moreland et al., 2024)
Standard Urine Culture (SUC)	Plating urine on MacConkey and sheep blood agar plates, incubate aerobically 30-37C.	Bacterial plates, 12-36h,	Current clinical Gold standard.	Aerobic culture misses up to 70% of known and emerging uropathogens, negative cultures, Favors the rapid growth of facultative anaerobes	(Gillespie et al., 1960; Brubaker et al., 2023; Price et al., 2017; Moreland et al., 2024)
Expanded Quantitative Urine Culture (EQUC)	Variety of plates and atmospheric conditions	Bacterial plates, 24-48h	Detects 70% of normal urinary bladder flora, distinguishes live from dead microbes	Time to result, great research tool	(Price et al., 2016; Deen et al., 2023; Du et al., 2024)
16S rRNA/18S rDNA Gene amplification/sequencing	PCR amplification of bacterial (16S) and fungi (18S) DNA in sample	Extraction of DNA followed by PCR using universal 16S and 18S primers. Hours	Detects bacterial and fungal DNA.	List of microbes without context. Cannot distinguish live or dead. Relative abundance in each sample.	(Hilt and Ferrieri, 2022)
M-PCR	PCR, specific primers identify a panel of microbes	Amplified DNA, Hours to result.	Rapid, sensitive	List of microbes without context of infection, Only detects microbes specific to primer sets.	(Wojno et al., 2020; Hilt and Ferrieri, 2022)
M-PCR with Immune Markers of Infection	PCR, specific primers identify a panel of microbes coupled with immunoassays of markers of infection	MPCR panel coupled with immunoassays for immune markers.	Rapid, sensitive, links results to immune response, distinguishes urobiome from uropathogens	Only detects microbes specific to primer sets	(Akhlaghpour et al., 2024; Haley et al., 2024)
Shotgun Next Generation Metagenomic Sequencing	Extracts and sequences all DNA in clinical sample. Requires host DNA depletion to amplify signal.	DNA extraction from clinical sample, host depletion of DNA followed by shotgun metagenomic DNA sequencing	Detects all DNA: viruses, bacteriophages, eukaryotic microbes, bacteria, and human. Targeted metagenomics uses specific sequencing primers.	Expensive, time limited. Recent targeted approaches may revolutionize this approach	(Chang et al., 2025; Hilt and Ferrieri, 2022; Neugent et al., 2022)

method as flawed. As this standard method supports the established dogma that UTIs are caused by microbes arising from the gastrointestinal tract and, in females, vulvo-vagina reservoirs, it has, for the most part, gone unchallenged (Timm et al., 2025).

4 The urobiome “complication”

With the advent of DNA-based techniques (metagenomics) and enhanced culture methods (metaculturomics), the existence of female urethral and bladder microbiota has been confirmed with subtle differences existing between the two (Chen et al., 2020; Wang et al., 2023). Thus, we now know that the typical human urinary tract above the urinary sphincter is not sterile; instead, it contains an indigenous urinary microbiome (also known as the urobiome) (Price et al., 2020; Brubaker et al., 2021b; Du et al., 2024). We also know that the urobiome can have multiple healthy states (Pearce et al., 2014; Price et al., 2020; Jayalath and Magana-Arachchi, 2022; Joos et al., 2024). Moreover, many adult males and most adult females have a detectable urobiome without

experiencing relevant urinary symptoms. Clearly, this does not mean we all have a UTI (Finucane, 2017). Consistent with clinical diagnosis of UTI, in the absence of relevant urinary symptoms, there is no “infection.” Diagnosis of a UTI requires that the patient exhibits at host response, and typically experiences symptoms (including urgency, frequency, urinary incontinence, and/or pain) (Anger et al., 2019).

Within any microbiome, microbes can be classified into 6 categories: non-pathogen (i.e., those that do not cause disease), pathogen (i.e., those that cause disease), commensal (i.e., those resident within the tissue and benefiting the host), symbiont (i.e., those resident within the tissue and benefiting both the host and the microbe), colonizer (i.e. resident within the tissue and may or may not cause disease), and pathobiont (i.e., resident within the tissue and generally beneficial but disease-causing under certain conditions) (Dey and Ray Chaudhuri, 2022). The urobiome has the full range of the 6 categories described above, including pathogens and pathobionts (Thomas-White et al., 2018; Du et al., 2024). Yet, most human beings do not have a clinical infection (i.e., UTI) even though pathogens or pathobionts are “citizens” of their

urobiome community. An informative study enrolled healthy volunteers ≥ 65 years old without urinary symptoms as a comparison group for patients diagnosed with UTIs (Akhlaghpour et al., 2024). In that study, an M-PCR panel consistently detected the known uropathogens *E. coli* and *Enterococcus faecalis*, as well as the emerging uropathogens *Aerococcus urinae*, *Actinotignum schaalii*, and members of the Viridians group *Streptococcus* in healthy volunteers without urinary tract symptoms. In addition, few of these volunteers experienced an increase in infection-related immune markers, a hallmark of infection (Akhlaghpour et al., 2024). Furthermore, many adult females do not get UTIs. Finally, it is well known that UTIs can resolve spontaneously (Hoffmann et al., 2020; Barnes et al., 2021; Midby and Miesner, 2024). This implies that the indigenous urobiome together with both innate and adaptive immune responses can often restore urinary health and resolve infection.

Biomass influences microbial communities. The gastrointestinal tract is the best-known example of a high biomass microbial niche. In contrast, the urobiome has relatively low microbial biomass and thus, in some cases, urine samples yield culture-negative and DNA-based-negative test results (Hilt et al., 2014; Pearce et al., 2015; Neugent et al., 2020). An analogy would be a city block in the Bronx with 35,000 inhabitants versus a high plains plateau in Wyoming with sparse settlements. Within both types of communities, however, interactions occur among the residents. The same is true for ecological microcosms within the human microbiome.

If most contamination arises from periurethral, epidermal, perianal, and vaginal flora as suggested (LaRocco et al., 2015), it would be helpful to compare the bladder urobiome to these other niches. A comparison of gastrointestinal, vaginal and bladder microbiomes revealed that while all three niches were distinct from each other, there were similarities between the vagina and bladder microbiomes (Thomas-White et al., 2018; Du et al., 2024) (Figure 1). Furthermore, a recent survey of the skin microbiome lists the top ten taxa from four different niches (Byrd et al., 2018). Of the 22 different microbes identified among the ten most abundant taxa in four different regions of skin (dry, moist, sebaceous, and feet), 18 (82%) are also found in urine obtained directly from the bladder by transurethral catheterization (Thomas-White et al., 2018; Du et al., 2024) (Supplementary Table 1). Therefore, mere taxonomic identity (via Gram stain, oxygen tolerance, metabolic panel, and/or MALDI-TOF MS) cannot distinguish bladder residents from skin periurethral, perianal, and vaginal residents. To make such a distinction requires genome sequencing and complex bioinformatic analysis that determines whether two isolates are of the same lineage or not.

5 Polymicrobial infections

The history of polymicrobial infections dates to the late nineteenth century and W.D. Miller, a microbiologist and dentist who had worked in Robert Koch's lab (Murray et al., 2014; Sedgley, 2004). By microscopy and characterization of pus from oral

abscesses, he reported that the application of Koch's postulates was not consistent with cultured microbes isolated from murine models. In fact, Miller obtained a more virulent response from the abscess pus than he did the cultured, recovered microbes. Armed with only culture conditions, a microscope and staining, Miller concluded that uncultivable microbes worsened the infection (Murray et al., 2014). It was not until the availability of DNA sequence-based methods in the late twentieth and early twenty-first century that microbial ecology and polymicrobial infections were confirmed and appreciated in some human niches.

Thus, the concept of polymicrobial UTIs is not new but their existence has been confirmed with many examples reported in the late twentieth century (Siegman-Igra, 1994; Siegman-Igra et al., 1988, 1993; Siegman-IgraY, 1994). Yet, it may be useful to divide the studies on mixed cultures/polymicrobial infections into two groups: before and after the advent of DNA molecular techniques. The former was limited to urine sediment, urine culture, microscopy, Gram staining, and metabolic panels. Urine culture limited to SUC (most often aerobic) identified mostly facultative anaerobes (Tables 3, 4). Consequently, this literature identified mostly Gram-negative rods as pathogens and excluded most Gram-positive rods and cocci as contaminants (with a few exceptions such as *Enterococcus* and coagulase negative *Staphylococcus* (CoNS), especially *S. saprophyticus*. Early studies on urosepsis and mixed (polymicrobial) cultures from patients revealed that matching cultures from urine and blood of the same microbe suggested that upper urinary tract infection had transitioned into the bloodstream. In one study, 716 bacteremic episodes were observed in 692 patients out of 52,012 admissions over 5 years (Siegman-Igra, 1994). Of these, 307 episodes in 303 patients were due to UTI. In this group, 198 had at least one microbe that was detected in both blood and urine culture (Table 4). These 198 urosepsis cases represented 194 patients (96 male, 98 female) with a mean age of 68 years. The most common microbe in monomicrobial infections was *Escherichia coli*; however, in polymicrobial infections, *Pseudomonas aeruginosa* was most predominant. *P. aeruginosa* also was among the microbes associated with fatal outcomes. In an earlier study of polymicrobial bacteremia, of 67 cases across multiple organ systems and causes, 46 percent were diagnosed with UTI (Siegman-Igra et al., 1988). Of the 67 cases, 28 died, with 42 percent diagnosed with UTI. While urosepsis is considered rare among the population, in neonates and patients 65 and older, it is a significant morbidity (Peach et al., 2016; Akhtar et al., 2021; Collaborators, GBD 2021 Antimicrobial Resistance, 2024).

While traditional urinary pathogens are predominantly Gram-negative facultative anaerobes, Gram-positive microbes are also detected in patients diagnosed with UTI, particularly in polymicrobial infections (Kline and Lewis, 2016). While usually regarded as commensals, Gram-positive microbes' pathogenic potential have been questioned (Clarke et al., 2010; Kline and Lewis, 2016; Leal et al., 2016). Yet, Gram-positive CoNS and *Enterococcus*, as well as emerging urinary pathogens such as *Aerococcus*, *Actinotignum*, *Gardnerella*, and *Corynebacteria* are found prevalently and abundantly in patients with UTI symptoms

TABLE 3 Incidence of known uropathogens using standard diagnostics and M-PCR.

Microbe	Gram stain	Oxygen tolerance	Uncomplicated UTI (% of cases)	Complicated UTI (% of cases)	M-PCR (% of cases)
<i>Escherichia coli</i>	Negative	Facultative Anaerobe	75 ^a 70 ^b 75 ^c 72.5 ^d	65 ^a 65 ^b 65 ^c 55.4 ^d	41 ^e
<i>Klebsiella pneumoniae</i>	Negative	Aerobe	11 ^a 9.8 (<i>Klebsiella</i> spp) ^b 6 (<i>Klebsiella</i> spp) ^c 5.1 ^d	16 ^a 9.7 (<i>Klebsiella</i> spp) ^b 8 (<i>Klebsiella</i> spp) ^c 8.9 ^d	13 ^e
<i>Enterococcus</i> spp	Positive	Facultative Anaerobe, Microaerophile	5.5 ^a 5.8 ^b 5.0 ^c 5.1 ^d	10.5 ^a 4.7 ^b 11.0 ^c 8.9 ^d	22 ^e
<i>Staphylococcus saprophyticus</i>	Positive	Facultative Anaerobe, Microaerophile	5.5 ^a 5.5 (<i>Staphylococcus</i> spp) ^b 6.0 ^c 3.8 (CoNS) ^d	NR ^a 7.3 (<i>Staphylococcus</i> spp) ^b NR ^c 2.5 (CoNS) ^d	6 (CoNS) ^e
<i>Proteus mirabilis</i>	Negative	Facultative Anaerobe	4.0 ^a 2.1 ^b 2.0 ^c 3.0 ^d	4.0 ^a 2.2 ^b 2.0 ^c 4.4 ^d	4 ^e
<i>Pseudomonas aeruginosa</i>	Negative	Obligate Aerobe	2.5 ^a 1.0 ^b 1.0 ^c 2.9 ^d	4.0 ^a 0.8 ^b 2.0 ^c 6.5 ^d	4 ^e
<i>Streptococcus</i> spp including GBS	Positive	Facultative Anaerobe	NR ^a 1.9 ^b 3.0 ^c 4.7 ^d	NR ^a 3.9 ^b 2.0 ^c 4.2 ^d	3 ^e
<i>Candida</i> spp (Yeasts)	NA	Facultative Anaerobe	NR ^a 1.3 ^b 1.0 ^c 1.6 ^d	NR ^a 2.9 ^b 3.0 ^c 2.8 ^d	5 ^e
<i>Staphylococcus aureus</i>	Positive	Facultative Anaerobe	NR ^a 5.5 (<i>Staphylococcus</i> spp) ^b 1.0 ^c 1.6 ^d	NR ^a 7.3 (<i>Staphylococcus</i> spp) ^b 3.0 ^c 2.3 ^d	1 ^e
<i>Citrobacter</i> spp	Negative	Facultative Anaerobe	NR ^a 1.1 ^b NR ^c 1.6 ^d	NR ^a 1.5 ^b NR ^c 2.8 ^d	1.4 ^e
<i>Enterobacter</i> spp	Negative	Facultative Anaerobe	NR ^a 1.0 ^b NR ^c 1.6 ^d	NR ^a 1.4 ^b NR ^c 3.7 ^d	3 ^e
<i>Pseudomonas aeruginosa</i>	Negative	Obligate Aerobe	1.0 ^a 1.0 ^b 1.0 ^c 2.9 ^d	2.9 ^a 0.8 ^b 2.0 ^c 6.5 ^d	4 ^e

^aAdapted from Figure 2 (Mancuso et al., 2023).^bAdapted from Figure 1 (Werneburg et al., 2023).^cAdapted from Figure 1 (Flores-Mireles et al., 2015).^dAdapted from Figure 1 (Gaston et al., 2021).^eAdapted from Figure 1 (Haley et al., 2024).

NR, Not reported.

CoNS, Collectively known as coagulase negative staphylococcus (CoNS) and comprising *S. epidermidis*, *S. haemolyticus*, *S. lugdunensis*, *S. saprophyticus* (Moreland et al., 2023).GBS, Group B *Streptococcus* (*Streptococcus agalactiae*) (Kline and Lewis, 2016).

NA, Not applicable.

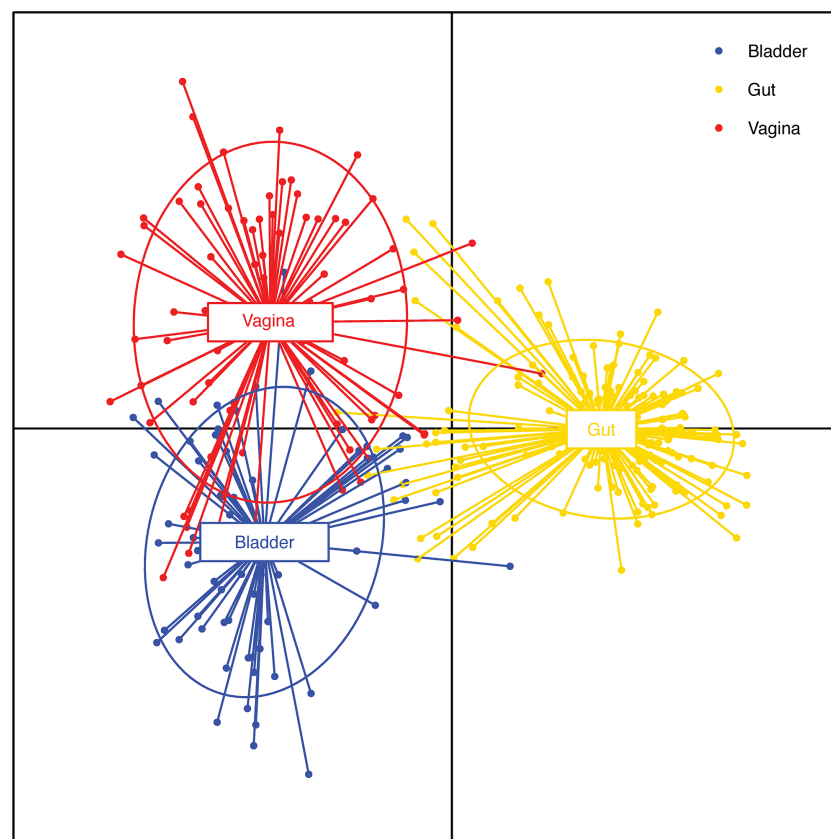


FIGURE 1

Comparison of bladder, vaginal and gut isolate functions. Discriminant analysis of principal components analysis of the functions of bacterial species isolated from asymptomatic individuals shared among 3 different niches: bladder (blue; $n=68$), vagina (red; $n=74$) and gut (yellow; $n=175$). The dots represent individual functions. The lines show the extent of those functions. Thus, a line that begins in the bladder centroid and ends in the vaginal centroid represents a function that is expressed by species that inhabit both the bladder and the vagina. Note the extensive overlap of functions in the bladder and vagina in contrast to the limited overlap between functions characteristic of the bladder or vagina and those of the gut. Adapted from (Du et al., 2024) (published under a Creative Commons license).

(Kline and Lewis, 2016; Moreland et al., 2023). Microaerophiles and anaerobes also have been observed (Legaria et al., 2022; Maskell, 1986) and cultures to rule out these microbes was suggested as part of diagnosis seventy years ago (Jawetz, 1953).

With the advent of metaculturomic methods (approaches designed to permit growth of typically uncultivated microbes) and metagenomic approaches (DNA-dependent methods that do not require growth), attempts (e.g., the human microbiome project) have sought to define the microbiota of various human niches, notably skin, respiratory tract, and the gastrointestinal tract, as well as the urogenital and reproductive tracts (Lloyd-Price et al., 2016; Joos et al., 2024). Lessons learned from the last two decades of research have taught us that there are multiple healthy states within niches that vary with sex, age and reproductive status (Lloyd-Price et al., 2016; Joos et al., 2024). Also, disease states are more complicated than originally anticipated by Koch and his postulated approach (Blevins and Bronze, 2010; Murray et al., 2014). A recent opinion paper questioned whether the urobiome has any impact on UTI, in part because Koch's postulates have not been performed to determine whether any of the members of the newly identified urobiome cause disease symptoms (Werneburg

and Southgate, 2024). Yet, Koch's postulates (one organism, one infection, one cause of disease) cannot be applied to polymicrobial infections including UTIs (Ronald et al., 2008; Murray et al., 2014; Short et al., 2014). The request to use Koch's postulates to validate the role of the urobiome (Werneburg and Southgate, 2024) is further complicated by the currently accepted tools that may no longer apply: a detection system (urine dipsticks, SUC) that misses many uropathogens (Moreland et al., 2024) and standards that define polymicrobial infections as contamination (Siegman-Igra et al., 1993; Siegman-IgraY, 1994; Bekeris et al., 2008).

One DNA-dependent method, multiplex PCR (M-PCR), allows quantitative, real-time detection of microbial DNA as long as a primer set is present to detect them (Moreland et al., 2024). Using M-PCR, a surprisingly high rate of polymicrobial specimens have been detected in older patients (≥ 65) with diagnosed UTI symptoms, ranging from 45 to 65 percent depending on simple or complex UTI, sex, and comorbidities (Vollstedt et al., 2020; Wojno et al., 2020; Korman et al., 2023; Wang et al., 2023; Akhlaghpour et al., 2024; Haley et al., 2024; Kardjadj et al., 2025). For example, in one study examining the differences of microbes based on catheterized (bladder) urine versus midstream void (entire

TABLE 4 Characteristics of microbes from UTI with matching blood infections^a.

Microbe	Blood Culture ^b			Urine Culture ^c		
	Monomicrobial n (%)	Polymicrobial n (%)	Total n (%)	Monomicrobial n (%)	Polymicrobial n (%)	Total n (%)
<i>Escherichia coli</i>	77 (59)	32 (33)	109 (48)	77 (59)	43 (28)	120 (43)
<i>Klebsiella</i> spp ^d	21 (16)	19 (20)	40 (18)	21 (16)	29 (19)	50 (18)
<i>Proteus</i> spp ^e	15 (12)	18 (19)	33 (15)	15 (12)	30 (20)	45 (16)
<i>Pseudomonas</i> spp	8 (6)	16 (16)	24 (11)	8 (6)	25 (17)	33 (12)
<i>Enterococcus</i>	4 (3)	7 (7)	11 (5)	4 (3)	14 (9)	18 (6)
Other	5 (4)	5 (5)	10 (4)	5 (4)	11 (7)	16 (5)
Total	130 (100)	97 (100)	227 (100)	130 (100)	152 (100)	282 (100)

^aAdapted from Table IV (Siegman-Igra, 1994) Used by permission.
^bBlood culture: Bottles with tryptic soy broth, 5% CO₂, 37C. Checked daily for growth, subcultured, stained after 24-48h.
^cUrine culture: 5% sheep blood and MacConkey agar plates (loops with 0.01-0.001ml). Incubated 18-24h (aerobic).
^dIncluding *Enterobacter* spp.
^eIncluding *Morganella* spp and *Providencia* spp.

urinary tract including urogenital areas), more polymicrobial infections were detected in midstream voided compared to catheter-collected samples (64.4% vs 45.7%, $p < 0.0001$) in females but the opposite in males (35.6% vs 47.0%, $p = 0.002$ (Wang et al., 2023). While M-PCR detected microbes that are not detected by SUC, the use of inflammatory markers of infection allowed the distinction of volunteers without relevant clinical symptoms from symptom-diagnosed UTI patients and stratifying those microbes into tiers based on abundance of occurrence (Akhlaghpour et al., 2024; Haley et al., 2024).

In the laboratory, specific microbes are studied in isolation using a reductionist approach. In the real world, communities of microbes make up an ecosystem that changes based on the predominating species and interactions between them (Murray et al., 2014; Short et al., 2014; Jayalath and Magana-Arachchi, 2022). These communities interact in synergy with residents by providing nutrients, soluble signaling factors, cell contact through adhesins, or in some cases facilitating antibiotic resistance (Ryan and Dow, 2008; Short et al., 2014; Murray et al., 2014; Gaston et al., 2021). Likewise, some microbes secrete molecules that eliminate their competition such as *Pseudomonas* (Gaston et al., 2021) or kill uropathogens such as commensal *Lactobacillus* (Abdul-Rahim et al., 2021; Johnson et al., 2022; Szczerbiec et al., 2022).

While polymicrobial results in the studies described above were reported as a group, the individual microbes within the subsets of polymicrobial infections were not identified. Instead, individual microbes were reported by taxon (Vollstedt et al., 2020; Wojno et al., 2020; Korman et al., 2023; Wang et al., 2023; Akhlaghpour et al., 2024; Haley et al., 2024). A reanalysis of the data from these studies could reveal associations of specific microbes that as a group or community are more pathogenic than as single isolates. In an effort to understand microbial ecology of polymicrobial isolates, one study examined 72 bacteria isolates collected from 23 patients diagnosed with polymicrobial UTI (de Vos et al., 2017). An ecological network analysis was developed, finding that most

interactions clustered based on evolutionary relatedness. Eight complete communities of four different microbes were found, four of which were predicted to be stable and four not stable (de Vos et al., 2017). The isolates used for this study were originally collected using standard culture techniques (Croxall et al., 2011), which may explain the predominance of classical uropathogens. Expanding and extending this concept, another study examined pathogens and bladder commensal isolates grown under urine-like conditions and the effects different microbes had on the community (Zandbergen et al., 2021). Artificial urine media conditioned by commensals had effects on growth of uropathogens and vice versa. This early attempt at gaining insights into the complexities of urobiome ecology shows that while a microbe in isolation may have certain growth characteristics, the community - through direct physical interaction, direct interaction with the host, or secretion/excretion of signaling molecules or metabolites, - may exhibit a different response than a single microbe in monoculture (Heidrich et al., 2022; Short et al., 2014; Murray et al., 2014).

6 Future directions: a call to action

To advance diagnosis, we suggest the following:

1. Reexamine standards for urine collection/culture contamination to ensure accurate sampling, taking into consideration the new knowledge concerning the urobiome. A recent scoping review highlights the issues, especially the lack of consensus among guidelines concerning urine culture thresholds for UTI and the reliance upon dated and sparse evidence for current standards (Hilt et al., 2023). To achieve accuracy, we should recognize that urine samples must be processed immediately or be stored under conditions that do not permit microbial growth (LaRocco et al., 2015).

2. Recognize the shortcomings of SUC. There is now sufficient data in the literature to adjudicate the accuracy of this method. It should be examined, weighing its strengths and acknowledging its weaknesses (Price et al., 2017; Wojno et al., 2020; Xu et al., 2021; Festa et al., 2023; Brubaker et al., 2023; Gleicher et al., 2024). Besides its ability to detect only a limited group of facultative anaerobes, time is also a consideration as re-sampling and re-culturing requires time that may allow an infection to progress.
3. Recognize the presence and importance of polymicrobial UTI. The literature provides evidence that these infections are more prevalent than previously believed and misdiagnosis as contamination should be considered and re-evaluated. Ultimately, clinical diagnostic pathways might be modified to diagnose these infections before they can progress to upper tract UTI and potentially urosepsis (Siegmán-Igra, 1994; Peach et al., 2016; Akhtar et al., 2021).
4. Recognize the importance of redefining contamination, acknowledging the existence of polymicrobial infection. Critical for antibiotic stewardship is the development of accurate and rapid diagnostics that not only detect microbes but also determine if they are involved in a host immune response. The increase in antibiotic resistance and future predictions are sobering (Collaborators, GBD 2021 Antimicrobial Resistance, 2024). Proper and appropriate use of antibiotics to treat UTI not only affects the health of patients but preserves critical antibiotics for those who need them (Simoni et al., 2024).
5. Take a lesson from other organ systems and diseases (Murray et al., 2014; Short et al., 2014), and ask how diagnostic tools can be improved and new tools developed or implemented to characterize microbial communities in polymicrobial infections/mixed cultures. One way to accomplish this would be to reanalyze data from polymicrobial samples to identify interaction networks and determine if any groups of microbes are more commonly associated with symptoms or the lack thereof (Vollstedt et al., 2020; Wang et al., 2023; Akhlaghpour et al., 2024; Haley et al., 2024).
6. Finally, the concept of contamination must be reevaluated in the context of the new knowledge that the urobiome exists. Urine is not sterile above the urethral sphincter and the existence of communities containing both commensal, non-pathogenic microbes and potential uropathogens should be acknowledged. While dysbiosis is generally a concept foreign to the UTI literature, it needs to be recognized, and diagnostics updated to reflect the current science (Price et al., 2017; Jayalath and Magana-Arachchi, 2022; Simoni et al., 2024). Koch's postulates are invalid in polymicrobial systems (Murray et al., 2014; Short et al., 2014) but unfortunately continue to persist as a consequence of the dogma that single microbes are causative of disease (Blevins and Bronze, 2010; Werneburg and Southgate, 2024).

The danger of dismissing species considered to be routine contaminants, such as members of the genus *Corynebacterium*, can lead to dismissing pathogens like *C. urealyticum*, which is both nitrate negative by urine dipstick and grows slowly under SUC conditions. Yet, while PCR testing is much more rapid than culture (Dixon et al., 2020; Gleicher et al., 2024; Zering and Stohs, 2024), without some reference to host immune response (indicative of infection), the practicing clinician is left with 10–20 names on a page with little clue what to do next (Zering and Stohs, 2024; Xu et al., 2021). Fortunately, one test correlates an M-PCR panel of 30 microbes with biomarkers of inflammation (Akhlaghpour et al., 2024; Haley et al., 2024). While this is but one study with older patients, it opens the opportunity for researchers to examine other groups (e.g., different age groups, males versus females, or pregnant versus non-pregnant individuals). It is likely the differences encountered will require tailored treatments depending on the type of patient.

So, what should we do with this additional information? We foresee both scientific and clinical paths. As scientists broaden the view from “identify the pathogen and kill it” to “understand the ecology,” new insights into therapies should arise that parallel those of the oncological research and clinical community that has spent decades working to better understand, diagnose, and treat cancers. Their recognition that the old diagnostics and treatment algorithms were insufficient has led to precision diagnostics, targeted treatments, and better outcomes for patients. We should do the same for our patients. Teams of scientists and clinicians have begun this process; others should join the effort.

Where does this leave clinical microbiologists and clinicians who deal daily with the quandary of accurate diagnosis balanced by microbial stewardship? Even though the human microbiome project began two decades ago, the translation of that research into clinical practice is just beginning (Gilbert et al., 2025). In other niches, where the research is more advanced, there have been three stages of translations into the clinic. The first stage involves identification of microbiota and normal (healthy) microbial ecology. This process leads to understanding the mechanisms underlying dysbiosis and symptoms/disease. The second stage develops metrics associating taxa, host response, and disease characteristics that lead to biomarkers and new diagnostics. Finally, armed with this information, strategies are developed to modify the microbiota to restore a normal, healthy state (Gilbert et al., 2025).

Our community is still in the earlier stages of this process, having shown that the urobiome exists and obtained evidence that it is associated with health and disease. Progress on the second stage has begun but there is much to do. To be consistent with developing science, clinical microbiologists must challenge current standards concerning urine culture contamination (Bekeris et al., 2008). Also, the incidence of polymicrobial infection must be established across diverse populations (e.g. age, sex, nosocomial versus outpatient status). For their part, clinicians should be aware of the older diagnostics flaws and help progress new diagnostic developments with the goal of a rapid, accurate test that identifies both microbes and host response. For diagnosis of UTI, the host response could be monitored by inflammatory biomarkers (Akhlaghpour et al., 2024). In the meantime, clinicians can be open to emerging new knowledge and

new diagnostics. Their intellectual preparation to use that knowledge to make their best clinical decision will be critical for improved patient care. Finally, all should recognize that better diagnostics and better understanding of their results are key to antibiotic stewardship as the convenience of empirical treatment, or the use of flawed culture methods ultimately will deprive those who need antibiotics to survive in an increasingly antibiotic-resistant clinical environment (Collaborators, GBD 2021 Antimicrobial Resistance, 2024; Simoni et al., 2024).

7 Conclusions

In this review, we have presented the current diagnostic state of urine contamination and discussed the limitations of current diagnostic techniques, such as SUC. We have reviewed the evidence for polymicrobial UTI, raising doubt concerning the appropriateness of applying Koch's postulates and recognizing the consequences of potential missed diagnosis. The challenges for clinical microbiologists, clinicians and research scientists are to question the current dogmas, address polymicrobial infections, and work to define the microbial ecology of the urinary tract. The future is bright as multidisciplinary collaboration offers cross-pollinating efforts to improve patient care and quality of life.

Author contributions

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Conflict of interest

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcimb.2025.1562687/full#supplementary-material>

SUPPLEMENTARY TABLE 1

Most abundant skin microbes by region/niche^a and incidence in urobiome^b. ^a From the top ten most abundant microbes in four different skin regions (Byrd et al., 2018). Note that some microbes are abundant in multiple regions (for ex., *Corynebacterium tuberculo*steaicum and *Staphylococcus epidermidis*). Across four regions with ten microbes each, there are 22 unique taxa. ^b Microbes in bold type were found in the human female urobiome (Du et al., 2024) as determined by sequencing of genomes from isolates from catheterized urinary bladder urine. ^c Skin regions/niches are defined as follows: Dry: Hypothenar palm and volar forearm. Moist: Nare, antecubital fossa, inguinal crease, interdigital web, and popliteal fossa. Sebaceous: Alar crease, cheek, glabella, external auditory canal, manubrium, retroauricular crease, occiput, back. Feet: Toe web space, toenail, and plantar heel. ^d Bacterial Diversity Database. See (Schober et al., 2025). ^e Also known as *Cutibacterium acnes*.

SUPPLEMENTARY TABLE 2

Most abundant vaginal microbes^a and incidence in urobiome^b. ^a Ravel et al., 2011 evaluated vaginal microflora and vaginal pH in 396 asymptomatic sexually active women representing four ethnic groups (white, black, Hispanic, and Asian) by pyrosequencing of 16S rRNA genes. *Lactobacillus* species dominated four groups, but one group accounting for 27% of women (termed as group IV community state type) lacked significant lactobacilli and was heterogeneous with higher proportion of strictly anaerobic bacteria including genera *Gardnerella*, *Prevotella*, *Atopobium*, as well as *Dialister*, *Megasphaera*, *Peptoniphilus*, *Sneathia*, *Eggerthella*, *Aerococcus*, *Fingoldia*, and *Mobiluncus*. This latter group was over-represented in black and Hispanic women (Ravel et al., 2011). ^b Microbes in bold type were found in the human female urobiome (Du et al., 2024) as determined by sequencing of genomes from isolates from catheterized urinary bladder urine. ^c For bacterial vaginosis, see (Saraf et al., 2021) ^d Bacterial Diversity Database. See (Schober et al., 2025). ^e *Atopobium vaginae* is now known as *Fannyhessea vaginae* (Nouioui et al., 2018).

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