Journal of Microbiological Methods 127 (2016) 164–171

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Evaluation of the in vitro growth of urinary tract infection-causing gram-negative and gram-positive bacteria in a proposed synthetic human urine (SHU) medium

Deepak S. Ipe, Glen C. Ulett *

School of Medical Science, and Menzies Health Institute Queensland, Griffith University, Gold Coast, QLD, 4222, Australia

ARTICLE INFO

Article history:
Received 13 May 2016
Received in revised form 10 June 2016
Accepted 12 June 2016
Available online 14 June 2016

Keywords:
Asymptomatic bacteriuria
Urinary tract infection
Urine
Synthetic human urine
Artificial urine
In vitro growth
ABU
Bacteriuria

ABSTRACT

Bacteriuria is a hallmark of urinary tract infection (UTI) and asymptomatic bacteriuria (ABU), which are among the most frequent infections in humans. A variety of gram-negative and gram-positive bacteria are associated with these infections but *Escherichia coli* contributes up to 80% of cases. Multiple bacterial species including *E. coli* can grow in human urine as a means to maintain colonization during infections. In vitro bacteriuria studies aimed at modeling microbial growth in urine have utilized various compositions of synthetic human urine (SHU) and a Composite SHU formulation was recently proposed. In this study, we sought to validate the recently proposed Composite SHU as a medium that supports the growth of several bacterial species that are known to grow in normal human urine and/or artificial urine. Comparative growth assays of gram-negative and gram-positive bacteria *E. coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Streptococcus agalactiae*, *Staphylococcus saprophyticus* and *Enterococcus faecalis* were undertaken using viable bacterial count and optical density measurements over a 48 h culture period. Three different SHU formulations were tested in various culture vessels, shaking conditions and volumes and showed that Composite SHU can support the robust growth of gram-negative bacteria but requires supplementation with 0.2% yeast extract to support the growth of gram-positive bacteria. Experiments are also presented that show an unexpected but major influence of *P. mirabilis* towards the ability to measure bacterial growth in generally accepted multiwell assays using absorbance readings, predicted to have a basis in the release of volatile organic compound(s) from *P. mirabilis* during growth in Composite SHU medium. This study represents an essential methodological validation of a more chemically defined type of synthetic urine that can be applied to study mechanisms of bacteriuria and we conclude will offer a useful in vitro model to investigate the basis of some of the most common infections of humans.

1. Introduction

Urinary tract infections (UTIs) are among the most common infectious diseases of humans and are associated with bladder (cystitis), kidney (pyelonephritis) and blood (urosepsis) infection as well as asymptomatic colonization. Asymptomatic bacteriuria (ABU) defines the isolation of a specified semi-quantitative count of bacteria from a person without signs or symptoms related to UTI (Nicolle et al., 2005; Rubin et al., 1992). Bacteriuria can persist in an individual for months or years, as reviews focused on the etiology of ABU have discussed elsewhere (Ipe et al., 2013; Schneeberger et al., 2014). Bacteruric potential refers to the ability of microbes to grow in urine, and endure local host defense mechanisms in the urinary tract (O'Grady and Cattell, 1966a, 1966b) that aim to eradicate bacteria from this unique host niche (Bates et al., 2004; Hand et al., 1971; Lomberg et al., 1983). A recent analysis of the bacterial traits that are known to support growth in urine revealed the diversity of potential mechanisms that contribute to bacteruric potential and bacteriuria (Ipe et al., 2016). For example, in ABU *Escherichia coli* 83972 (Klemm et al., 2006; Roos et al., 2006a, 2006b) *guaA* and *argC* are critical for growth in urine, as are guanine (or guanine-derived products) (Russo et al., 1996) and arginine metabolism (de Evgrafov et al., 2012). Other bacteria, including *Enterococcus faecalis* (La Rosa et al., 2012; Vebo et al., 2010), *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Klebsiella pneumoniae* (Storer et al., 2011), *Staphylococcus saprophyticus* (Sakinc et al., 2009) and *Streptococcus agalactiae* (Ipe et al., 2015) can also grow in normal human urine.

Normal human urine is highly variable in chemical constituency (Bouatra et al., 2013). Urine from a healthy adult contains glucose (0.2–0.6 mM) (Shaykhutdinov et al., 2009), creatine (0.38–55.6 mM) (Barr et al., 2005; Shaykhutdinov et al., 2009), and glycine with low levels of amino acids such as histidine, glutamine, methionine, proline, glutamate, arginine, cysteine and leucine (Bouatra et al., 2013; Guo

* Corresponding author at: School of Medical Science, and Menzies Health Institute Queensland, Griffith University, Gold Coast, QLD 4222, Australia.
E-mail address: g.ulett@griffith.edu.au (G.C. Ulett).

http://dx.doi.org/10.1016/j.mimet.2016.06.013
0167-7012/

Table 1
Constituents and their concentrations in proposed composite SHU media recipe for studies of infection.

Constituent[a]	Basal SHU (mM)	Amount per 1 l (g)
NaCl	100 mM	5.8440
Na_2SO_4	17.0 mM	2.4147
Urea	280 mM	16.8168
KCl	38.0 mM	2.8329
$CaCl_2$	4.0 mM	0.4439
Creatinine	9.0 mM	1.0181
$Na_3C_6H_5O_7$	3.4 mM	1.9999
NH_4Cl	20.0 mM	1.0698
$MgSO_4$	3.2 mM	0.3852
$Na_2C_2O_4$	0.18 mM	0.0241
NaH_2PO_4	3.6 mM	0.5616
Na_2HPO_4	6.5 mM	0.9227
KH_2PO_4	16.0 mM	2.1774
$C_5H_4N_4O_3$	0.6 mM	0.1009
$NaHCO_3$	13.5 mM	1.1341
$MgCl_2 \cdot 6H_2O$	3.2 mM	0.6506
$C_3H_6O_3$	1.1 mM	0.0991
$FeSO_4 \cdot 7H_2O$	0.005 mM	0.0014

[a] Constituents are described with historical references including the concentration ranges used in prior studies of artificial urine to develop the proposed SHU formulation in (Ipe et al., 2016). To the Basal SHU medium (described in the Table) are added 0.1% (v/v) casamino acids to generate Composite SHU Medium, and 0.2% (v/v) yeast extract to support the growth of gram-positive bacteria.

and Li, 2009; Vebo et al., 2010). It contains trace fatty acids, citrate (1–2 mM) (Wishart et al., 2009), sucrose (70–200 μM) (Tasevska et al., 2005), and manganese (nM range) (Jarvisalo et al., 1992). Variations in the levels of some urinary constituents such as glucose can influence microbial growth; glucosuria, for example, enhances the growth of *E. coli* in urine (Geerlings et al., 1999). Urine is naturally antiseptic due to its chemical makeup; nitrite in acidified urine inhibits the growth of gram-negative bacteria (Carlsson et al., 2001), and urinary proteins such as Tamm–Horsfall protein are antimicrobial (Raffi et al., 2005; Saemann et al., 2005). The low pH of urine combined with high concentrations of urea and hypertonicity inhibits the growth of most urogenital tract commensals (Chambers and Lever, 1996; Kucheria et al., 2005; Sobel, 1985). However, some bacteria can counteract hypertonicity and resist dehydration (Chambers and Lever, 1996; Deutch et al., 2006), as reviewed elsewhere (Ipe et al., 2016). The complexity of normal human urine and its highly variable chemical constituency represents a challenge for standardizing research studies.

A key methodological approach that microbiological studies have relied on to understand the growth of bacteria in human urine is the use of synthetic human urine (SHU) (also termed artificial urine). Many studies have used SHU as a model of normal human urine to measure and compare the in vitro growth dynamics of different microbes. The advantages of SHU instead of normal human urine include more consistent chemical constituency compared to normal human urine that is usually collected from different individuals; known concentration combinations of the major constituents that comprise normal human urine and no requirement for ethical approval or limits on volume requirements. A recent review and comprehensive analysis of microbiological studies using SHU in the context of bacteriuria studies proposed a standardized SHU medium for modeling bacteriuria in vitro (Ipe et al., 2016). In the current study, we evaluated the recently proposed Composite SHU medium using various bacteria associated with UTI and ABU to validate the medium as one that can support the growth of diverse organisms that are known to grow in normal human urine.

2. Materials and methods

2.1. Bacteria and growth conditions

Several bacteria, representing six species associated with ABU and UTI, were used in this study to define the degree that SHU is able to support bacterial growth of organisms known to grow in normal human urine; ABU *E. coli* strain 83972, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Streptococcus agalactiae* strain ABSA 834, coagulase-negative *Staphylococcus saprophyticus*, and *Enterococcus faecalis*. Each clinical isolate was cultured from urine analyzed as part of diagnosis of ABU or UTI, and were sourced from culture collections at Griffith University as previously described (Ipe et al., 2015; Tan et al., 2012; Ulett et al., 2009). Challenge inocula were prepared by culturing gram-negative bacteria in lysogeny broth (LB), and gram-positive bacteria in Todd–Hewitt broth (THB), overnight in shaking (200 rpm) 1 ml cultures at 37 °C. The cultures were centrifuged at 8000 ×*g* for 5 min at room temperature, and pellets were washed (1×) in 1 ml phosphate-buffered saline (PBS) and resuspended in 1 ml Basal SHU. To avoid carryover of LB or THB into the experimental cultures, a separate tube containing 990 μl of fresh Basal SHU was inoculated with 10 μl of the washed and resuspended overnight culture mentioned above to generate a 1/100 dilution. This bacterial suspension was inoculated into culture plates or tubes using a further 1/100 dilution ratio into respective growth medium (final dilution of 1/10,000 from overnight culture). The starting culture densities for each species at the beginning of each experiment, which were determined retrospectively using colony counts on LB or THB agar, ranged between 4 and 5×10^4 CFU ml^{-1}.

2.2. Preparation of synthetic human urine (SHU) medium

The recipe for Composite SHU medium used in this study was described previously (Ipe et al., 2016) and is described in Table 1. In this study, Composite SHU medium, that contains 0.1% casamino acids

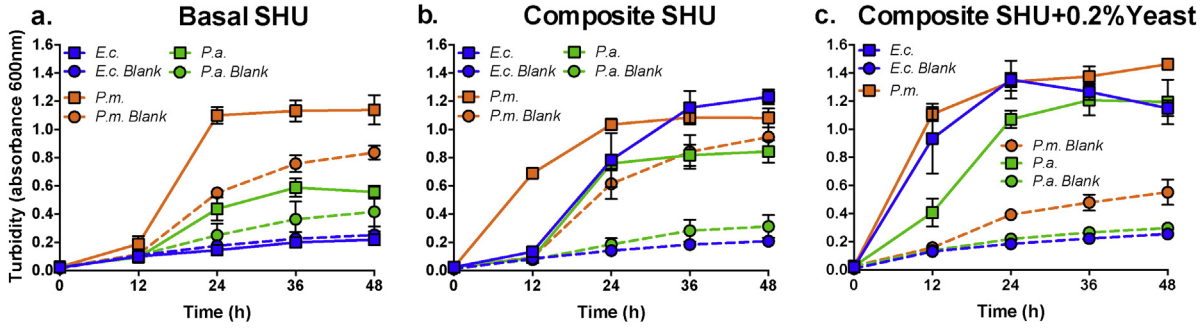

Fig. 1. Comparative growth of diverse gram-negative bacterial species associated with urinary tract infection in SHU medium according to culture turbidity ($OD600_{nm}$) measures. The data were derived using 200 μl cultures of *E. coli* (*E.c.*), *P. mirabilis* (*P.m.*), and *P. aeruginosa* (*P.a.*) in 96-well plates in duplicate, incorporating only a single bacterial species per plate in each experiment with negative control blank wells that were not inoculated with bacteria in each plate (Blank). The results show comparisons of (a) Basal SHU medium, (b) Composite SHU medium (contains 0.1% Casamino Acids), and (c) the later supplemented with 0.2% yeast extract. The data at each time point represent means ± SEM, calculated using pooled data from three independent assays. Statistical comparisons of growth between the groups were performed using AUC analysis followed by student's *t*-test, or one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons tests with a P value < 0.05 considered significant.