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A novel, broadly applicable approach to isolation of fungi in diverse growth media

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ABSTRACT

Creatinine (CRN) is a vertebrate metabolic waste product normally found in blood and urine. Previous work demonstrated that the hydrochloride salt of creatinine (CRN-HCl) acted as a potent inhibitor of bacterial replication. Creatinine hydrochloride does not inhibit the growth of yeasts or molds (i.e. fungi), making it a potentially useful addition to growth media to facilitate isolation of environmental or clinically relevant fungal species. Sabouraud dextrose agar is the current medium of choice for detection and isolation of fungi although it does not offer optimal nutritional requirements for some fungi and can permit growth of bacteria which may subsequently inhibit fungal growth and/or obscure fungal isolation. We show that CRN-HCl effectively suppresses bacterial growth in either liquid or solid agar media while allowing outgrowth of slower growing fungi using either experimentally prepared samples or environmental samples.

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1. Introduction

There is a need for different and selective media for the propagation and isolation of fungi for identification in clinical settings and for the detection of fungal presence in the environment (Reddy et al., 2013; Sandven and Lassen, 1999; Scognamiglio et al., 2010; Tormo-Molina et al., 2012). The isolation of both yeasts and molds (i.e., fungi) from clinical and environmental samples can be complicated by various factors. Fungal growth in clinical or environmental samples may often be inhibited due to increased growth rates of bacteria as well as bacterial production of deleterious metabolites. These factors can result in failure to detect fungi in mixed cultures (Hockey et al., 1982; Kerr, 1994). Additionally, fungi often have diverse and specific nutritional requirements which may limit the extent and robustness of the growth of a given organism in the absence of a specific nutrient (Gao and Liu, 2010; Singh and Agrawal, 1982). Sabouraud dextrose agar (SDA), originally defined by Raymond Sabouraud in the late 1800s, remains the medium of choice for the isolation of fungi (Odds, 1991; Sandven and Lassen, 1999). Sabouraud's formula, a basic glucose and peptone formulation adjusted to a pH of 5.6, uses the lower pH as the inhibitory growth factor for bacteria. Selective media such as SDA are used when a need exists to isolate fungi from mixed cultures containing bacteria. However, SDA does not inhibit all bacterial outgrowth: one study reported that in mixed clinical cultures containing both bacteria and yeast, yeast were detected on SDA in only 77% of samples tested while others were obscured by bacterial growth (Sandven and Lassen, 1999). Further, selective media like SDA may not provide the necessary nutrients or be the most optimal media for isolation of all types of fungi from either clinical or environmental samples (Das et al., 2010; Gao and Liu, 2010; Samaranayake et al., 1987; Sandven and Lassen, 1999; Scognamiglio et al., 2010; Singh and Agrawal, 1982). For example, when comparing SDA to other media, one study demonstrated that, in general, fungi associated with fungal keratitis grew more rapidly, and were detected more frequently, on media such as blood agar and chocolate agar versus SDA (Das et al., 2010); this suggested that SDA did not provide optimal nutrients and growth requirements for these fungi. While this is not a universal finding (Reddy et al., 2013), sufficient concern exists to identify a more efficient selective media than SDA.

The efficiency with which SDA functions for the isolation of fungi has been compared to other fungus identification media that employ antibiotics to suppress bacterial outgrowth. For example, after inoculation with clinical mixed cultures, more fungal isolates were propagated on inhibitory mold agar, a complex media containing chloramphenicol, than on SDA, although some genera were propagated only on SDA (Scognamiglio et al., 2010). Compared to modified Pagano–Levin agar (MPL) which contains both gentamicin and triphenyltetrazolium chloride, MPL proved to be more effective than SDA at isolating different yeast species from oral samples (Samaranayake et al., 1987). It is important to note that the addition of antibiotics such as chloramphenicol to growth media may also adversely affect the growth of certain fungal species (Smith and Marchant, 1969; Touimi-Benjelloun et al., 1976).

Abbreviations: CRN, creatinine; CRN-HCl, protonated creatinine, creatinine hydrochloride; LBG, Luria broth supplemented with 1% D-glucose; LBG-CRN, LBG containing 100 mM creatinine hydrochloride; SDA, Sabouraud dextrose agar; SDA-CRN, SDA containing 100 mM creatinine hydrochloride.

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Other reports have explored chemically augmented SDA formulations to increase fungal isolation specificity. For example, the addition of gallium (III) nitrate to SDA effectively inhibit bacterial growth, although at concentrations greater than 16 mM, gallium (III) nitrate also inhibited the growth of fungi as well as bacteria (Moore et al., 2009). Although these approaches are useful, they involve modifying different media with diverse reagents which increase costs for laboratories (Scognamiglio et al., 2010) in addition to increasing the difficulty in standardization of testing systems in clinical settings. The various formulations of SDA can also limit the type of media on which specific or new fungi might best be propagated. The ideal reagent would be one that, if added to any growth medium, could function for the isolation of fungi while efficiently suppressing all bacterial outgrowths.

Creatinine is a natural vertebrate metabolic waste product of the breakdown of the high-energy compound creatine phosphate (White et al., 1968). This primarily non-enzymatic reaction leads to a steady production of CRN in the body which is subsequently voided in urine; CRN is considered to have no biological function (Harris and Crabb, 2005). However, our previous work demonstrated that the addition of the protonated form of creatinine [creatinine hydrochloride (CRN-HCl)] to growth media inhibits bacterial growth without affecting the outgrowth of fungi, even at concentrations greater than 200 mM (McDonald et al., 2012). The mechanism by which CRN-HCl selectively inhibits prokaryotes has not been elucidated, although it may overwhelm efflux pumps, causing an accretion of protons in bacterial cells, thereby lowering intracellular pH and disrupting metabolic pathways (McDonald et al., 2012). Although the anhydrous form of CRN does not inhibit bacterial growth, the protonated form in either a hydrochloride or other salt, is highly effective as an antibacterial agent (McDonald et al., 2012). Because of its demonstrated ability to inhibit bacterial replication and to kill bacteria, we tested the hypothesis that the addition of CRN-HCl to different solid and liquid media will facilitate isolation of fungi while completely inhibiting bacterial outgrowth.

2. Materials and methods

2.1. Microbial strains and culture conditions

Two bacteria and two yeast strains were used in this study: *Staphylococcus aureus* (ATCC 29213, Manassas, VA, USA), *Escherichia coli* strain DH5α, a strain commonly used for molecular cloning (Invitrogen; Life Technologies, USA), *Saccharomyces cerevisiae* (common baker's yeast), and *Rhodotorula mucilaginosa* (an environmental isolate). Genus and species of microorganisms were verified as described below (Section 2.2). All microorganisms were grown routinely for 18 h at 32 °C shaking at 300 RPM in 100 mL cultures of LBG [Luria broth (LB; Amresco, Solon, OH, USA) supplemented with 1% *w/v* D-glucose (Fisher Scientific; Fair Lawn, NJ)]. Cultures were maintained on LBG agar. Cultures were individually prepared by inoculation with a single colony of each respective microorganism. Organism concentrations were determined by serial dilution and plating on LBG agar to determine colony-forming units (cfu) per mL prior to subsequent experimentation.

2.2. Enzymatic amplification of rRNA sequences for organism identification

Genus and species were determined for microorganisms by amplifying the appropriate rRNA sequence from chromosomal DNA. DNA was isolated from 2 mL cultures inoculated originally with a single colony using the Zymo ZR Fungal/Bacterial DNA Miniprep Kit (Zymo Research; Irvine, CA, USA) per manufacturer's protocol. Primers specific to bacterial 16S rRNA and fungal 18S rRNA are shown in Table 1 ([a] (Gargas and DePriest, 1996), [b] (Dewhirst et al., 1999)). Twenty microliter (μL) reactions were assembled with GoTaq Green Master Mix (Promega; Madison, WI, USA) with primers included at a final concentration of 0.5 μM, then cycled in a TC-312 thermal cycler (Techne; Duxford, Cambridge, UK) with the following conditions: initial denaturation for 5 min at 94 °C followed by 20 cycles (16S primers) of 10 s at 94 °C, 20 s at 39 °C, and 2 min at 72 °C or 40 cycles (18S primers) of 10 s at 94 °C, 20 s at 43 °C and 2 min at 72 °C, finishing with 5 min at 72 °C. Ten microliters of the 20 μL PCR reactions was analyzed on 1% agarose gels to verify successful amplification and expected size of the amplimer (data not shown). The remainder of the reaction was ethanol precipitated, resuspended in water, and submitted for sequence analysis at the UNMC sequencing core facility. Sequences were analyzed with Nucleotide BLAST using the National Center for Biotechnology Information (NCBI) GenBank.

Table 1
Primers used for sequencing of bacterial and fungal specimens. [a] (Gargas and DePriest, 1996), [b] (Dewhirst et al., 1999).

Primer	Sequence
Fungal 18S sequencing primers	
NS1[a]	5′-GTA GTC ATA TGC TTG TCT C-3′
NS8[a]	5′-TCC GCA GGT TCA CCT ACG GA-3′
Bacterial 16S sequencing primers	
F24[b]	5′-GAG TTT GAT YMT GGC TCA G-3′
F25[b]	5′-AAG GAG GTG WTC CAR CC-3′

2.3. Culture media

Liquid LBG was prepared as described previously (McDonald et al., 2012) and as indicated above. Nutrient agar was prepared by adding 1.5% (*w/v*) bacto agar (BD; Sparks, MD) to liquid LBG prior to sterilization. Sabouraud dextrose agar (SDA) was prepared using Sabouraud dextrose broth (Sigma Aldrich; St. Louis, MO) per manufacturer's recommendations with 1.5% *w/v* bacto agar. Agar media containing 100 mM CRN-HCl was prepared by first cooling liquid agar medium in a water bath to 56 °C, to which CRN-HCl (F.W. 149.6; Sigma-Aldrich St. Louis, MO, USA) was then added. Adding CRN-HCl to agar media prior to sterilization prevents cross-linking and solidification; after mixing to solubilize CRN-HCl, plates were poured. LBG or SDA agar media containing antibiotics were prepared by addition of a combined penicillin and streptomycin solution (Invitrogen; Grand Island, NY) to a final concentration of 100 I.U./mL and 100 μg/mL respectively, and ampicillin solution (Fisher Bio Reagents; Fair Lawn, NJ) to a final concentration of 100 μg/mL with media below 56 °C and immediately prior to pouring.

2.4. Measuring effects of CRN-HCl concentration on microorganism growth at varying inocula

Single colonies were inoculated into 50 mL LBG cultures in 250 mL Erlenmeyer flasks and shaken overnight as described above. Absorbance at 600 nm was measured for each culture using a spectrophotometer and cfu/mL determined for each culture (based on linear regression data constructed by plotting absorbance at 600 nm against cfu/mL for each microorganism). A series of ten-fold dilutions were performed in LBG for final concentrations of ca. 10^3–10^7 cfu/mL of each microorganism. Cultures in sterile 16 × 125 mm glass culture tubes were prepared in duplicate containing 4.25 mL of LBG, 250 μL CRN-HCl [at a concentration of either 400 mM, 800 mM, 1.2 M, 1.6 M, or 2 M (water instead of CRN-HCl was used as the control)], and 500 μL of culture dilution (at either 10^7, 10^5, or 10^3 cfu/mL). Cultures contained final concentrations of 20 mM, 40 mM, 60 mM, 80 mM, 100 mM or no CRN-HCl respectively, and 10^6, 10^4, or 10^2 cfu per mL respectively in total volumes of 5 mL. Tubes were placed at a slant in a 37 °C/300 RPM shaking incubator for 18 h. Following incubation, absorbances at 600 nm were recorded, and a series of tenfold dilutions of each culture in sterile water were made and plated on LBG agar in duplicate.