DIAGNOSTIC MICROBIOLOGY

Evaluation of a new selective medium for the isolation of *Corynebacterium urealyticum*

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A new selective medium (CBU agar) was compared with blood agar (BA) medium for primary isolation of *Corynebacterium urealyticum* from urine and skin samples of hospitalised patients. Overall, the CBU agar detected *C. urealyticum* in 14 (4.6%) of 302 urine samples and the BA medium detected the organism in four (1.3%), but most cultures which were positive only on CBU agar had $<10^4$ cfu/ml. Six strains of *C. urealyticum* were isolated from 60 skin samples with CBU agar, whereas none was detected with BA. Although most skin samples had heavy inocula, the selective agar facilitated the recognition of low colony counts (≤ 10 cfu/plate) of *C. urealyticum* by reducing the growth of competing flora. Challenge of the selective medium with reference and clinical strains showed that CBU agar was inhibitory for gram-negative bacteria and reduced the gram-positive flora, allowing the growth of *C. urealyticum* strains. The new selective medium appears to be a useful epidemiological tool to study urinary and skin colonisation by *C. urealyticum*.

Introduction

In the last two decades, *Corynebacterium urealyticum*, a urea-splitting and slow-growing bacterium, has been documented as an opportunist human pathogen, mainly in urinary tract infections [1–7]. Its fastidious nature and slow growth explain the difficulties encountered in its isolation from 24-h cultures of urine samples or from mixed cultures with fast-growing organisms. Two selective or semi-selective media have been developed to enhance the isolation of *C. urealyticum* from urine samples [8, 9]. Although the selective media improved the isolation of multiresistant *C. urealyticum* strains usually involved in human infections [8–11], occasionally, some β -lactam-susceptible strains were isolated only with the non-selective media [8, 11]. The evidence for non-multiresistant isolates of *C. urealyticum* in clinical samples [7, 8, 11–13] and an interest in understanding the epidemiology of such bacteria, prompted the development of a new selective medium for the isolation of *C. urealyticum*. Although this medium has been used previously to isolate *C. urealyticum* from urine [14] and environmental samples [15], the selectivity, quantitative recovery and colonial morphology have not been documented. This study describes the growth, selectivity studies and the performance of the selective medium to isolate multi-

ple-antibiotic resistant and non-multiresistant strains of *C. urealyticum* from urine and skin samples.

Materials and methods

Culture media

The novel selective medium (CBU agar) has the following composition (/L): Heart Infusion Agar (Difco Laboratories, Detroit, MI, USA) 40 g; L-cysteine hydrochloride (Merck, Germany) 0.128 g; phenol red (Merck) 0.01 g; urea (Merck) 20 g; glucose (Scharlau, Barcelona, Spain) 10 g; polysorbate 80 (Tween 80; Difco) 10 ml; fosfomicin (Cepa SA, Madrid, Spain) 50 mg; polymyxin B (Sigma) 2500 IU; aztreonam (Squibb, Barcelona, Spain) 16 mg; and amphotericin B (Squibb) 2 mg.

A CBU agar medium without antibiotics (CBUw/a) was prepared as a control for growth and selectivity studies. A heart infusion agar (Difco) with sheep blood 5% (BA) was used as a control to culture the clinical samples. Inocula for growth and selectivity studies were performed in Brain Heart Infusion Broth (Difco).

Antimicrobial susceptibility tests

To challenge the antimicrobial agents included in the selective medium, the susceptibilities of one multi-

resistant reference strain (*C. urealyticum* ATCC 43042^T) and six non-multiresistant stock collection strains to ampicillin, aztreonam, fosfomycin and polymixin B were tested. A microdilution method was performed in cation-supplemented Mueller-Hinton Broth (Difco) containing lysed horse blood 3%, following the guidelines described by the NCCLS for streptococci [16].

The antimicrobial susceptibility of *C. urealyticum* strains from the urine and skin samples was studied by an agar-diffusion method, as reported previously [17, 18].

Growth studies and quantitative recovery of C. urealyticum in the selective medium

Overnight broth cultures of two reference strains and five non-multiresistant stock collection strains of *C. urealyticum* (Table 1) were adjusted to 0.5 MacFarland turbidity and eight serial (1 in 10) dilutions were made with a phosphate buffer. Samples of 0.1 ml were plated in duplicate on CBU agar as well as on to CBUw/a agar. The inocula were spread over the entire plate area to obtain isolated colonies. Plates were incubated aerobically at 35°C for 72 h. They were examined daily and the following data were recorded: length of time for growth to appear, number of colonies, size and morphology, colour changes in the medium and Tween hydrolysis. To test the quantitative recovery of *C. urealyticum* in the CBU agar, colony counts were made from the 10⁵ dilution.

Selectivity

Quantitative and qualitative tests were performed to check the selectivity of the CBU agar. Reference strains and clinical isolates of species usually involved in urinary tract infections or belonging to healthy skin flora and some environmental or animal strains of diphtheroids were selected.

Firstly, a quantitative analysis was performed by inoculating CBU agar and CBUw/a agar plates with a defined inoculum of $(0.5-1) \times 10^3$ cfu/ml. The challenged strains were *C. jeikeium* LA-89, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 10028, *Proteus mirabilis* CCI, *Pseudomonas aeruginosa* ATCC 27835, *S. epidermidis* ATCC 12222, Coryneform CDC group F CDC-F5863 and *Candida albicans* ATCC 90028. In addition, the colonial morphology of control strains on the CBU agar base without antibiotics was compared with those of *C. urealyticum* on CBU agar.

Secondly, to evaluate the selectivity of the medium with a higher inoculum and a large number of strains from different genera (Table 2), inocula of c. 10⁴ cfu/spot were plated on to a selective CBU agar and

non-selective blood agar medium with a Steers type device and were incubated aerobically up to 72 h at 35°C and examined daily for growth.

Clinical samples

A total of 302 urine samples from 282 patients, hospitalised in different wards of the hospital was cultured to assess the reliability of CBU agar in the detection of *C. urealyticum* bacteriuria. Mid-stream urine samples were plated with a calibrated loop (0.05 ml) on to a non-selective BA and on a selective CBU agar.

The performance of the selective agar in the detection of *C. urealyticum* from healthy skin was also studied. Twenty in-patients were sampled at admission or during the first 24 h of hospitalisation. Skin surfaces of c. 4 cm² in the axilla, groin and anterior abdominal wall were sampled following a swab-rinse sampling method, as described previously [19]. Sixty skin samples were cultured quantitatively by inoculating three serially diluted (1 in 10) and one undiluted sample (0.1 ml) on to plates of CBU agar and BA.

Both CBU agar and BA plates were incubated at 37°C for 72 h. Colonies with typical *C. urealyticum* morphology and Gram's stain appearance and positive catalase reaction were tested for urease activity, glucose acidification and nitrate reduction. Only isolates that were urease positive, glucose and nitrate negative, were further identified to species level by standard methods [17, 20].

Results

All seven *C. urealyticum* strains tested by the microdilution assay were resistant to fosfomycin (MICs > 128 µg/ml), aztreonam (MICs > 128 µg/ml) and polymixin B (MICs 2–8 µg/ml). The reference strain and one non-multiresistant strain (NMS-1) were also resistant to ampicillin, with the remainder being susceptible (MICs ≤ 0.5 µg/ml).

In the growth studies, all stock and reference strains of *C. urealyticum* grew on the selective and non-selective media (Table 1). The most typical morphology of *C. urealyticum* in CBU agar was seen after incubation for 48–72 h, when the colonies were pale yellow, circular, 0.5–1.5 mm in diameter, opaque, glistening, smooth, convex, with entire edges and then, the whole medium was usually red, especially with high inocula. Occasionally, *C. urealyticum* strains formed a white halo surrounding the colonies, which may be caused by Tween 80 hydrolysis.

In the quantitative selectivity test performed with stock and reference strains, *C. jeikeium* LA-89 and Coryneform group F CDC-F5863 grew after 48 h on

Table 1. Quantitative recovery of *C. urealyticum* on CBU agar

Strains*	Data at	CBU w/a agar		CBU agar		Percentage recovery†
		Colony size (mm)	Number of colonies	Colony size (mm)	Number of colonies	
ATCC 43042	24 h	0	0	0	0	0
	48 h	0.8–1	115	1	99	86
	72 h	1–2	115	1.5–2	99	86
ATCC 43043	24 h	0.1–0.3	50	0	0	0
	48 h	1–1.5	50	1–1.5	25	50
	72 h	1–1.5	50	1–1.5	25	50
NMS-1	24 h	0	0	0	0	0
	48 h	0.8–2	25	0.1–1.5	25	100
	72 h	0.8–2	25	0.1–1.5	25	100
NMS-2	24 h	0.1–0.3	15	0	0	0
	48 h	1–1.5	55	0.1–1.5	50	90.9
	72 h	1.5–2	55	0.5–1.5	50	90.9
NMS-3	24 h	0.1	25	0	0	0
	48 h	0.1–1	25	0.5–1	25	100
	72 h	0.1–1	25	0.5–1	25	100
NMS-4	24 h	0	0	0	0	0
	48 h	0.8–1.2	50	0.3–1	50	100
	72 h	0.8–1.2	50	0.3–1.2	50	100
NMS-5	24 h	0.1–0.3	30	0	0	0
	48 h	1–1.5	30	1–1.2	50	166
	72 h	1–1.5	30	1–1.5	50	166

*NMS = Non-multiresistant stock collection strains of *C. urealyticum*.

†Percentage of quantitative recovery of *C. urealyticum* in CBU agar, compared with CBUw/a agar colony counts.

CBU agar and CBUw/a agar. The other reference strains grew on CBUw/a agar after incubation for 24 h, but no growth was detected on the CBU agar after incubation for 72 h. Staphylococci, enterococci, enterobacteria, *P. aeruginosa* and *C. albicans* had different colonial morphologies on CBUw/a agar and their morphotypes were easily distinguished from *C. urealyticum*. The results of the qualitative selectivity test are shown in Table 2.

C. urealyticum was detected in 14 (4.6%) of 302 urine cultures with CBU agar, but only four (1.3%) of those cultures were detected in both media. No *C. urealyticum* isolate was in pure culture and all except two had colony counts $< 10^5$ cfu/ml.

C. urealyticum and gram-negative bacilli were isolated on the BA plates from four urine samples. The rest of the samples harbouring *C. urealyticum* yielded mixed microbial flora (eight samples) or gram-negative bacilli (two samples) on the BA plates and *C. urealyticum* was missed. Only six samples yielded *C. urealyticum* in pure culture in CBU agar. Otherwise, eight mixed cultures with gram-positive cocci (two samples) or with other gram-positive bacilli (six samples) were obtained.

Seventy-one urine samples not harbouring *C. urealyticum* had positive cultures on the CBU agar plate. Gram-positive bacilli ($n = 55$), staphylococci ($n = 21$), yeasts ($n = 9$) and gram-negative bacilli ($n = 1$) were isolated. Thirteen *C. urealyticum* strains were multi-resistant and one strain was β -lactam-resistant.

Sixty skin swabs from three different sites of 20 in-patients were inoculated quantitatively on to BA and CBU agar. As most skin samples had heavy inocula, many undiluted samples had abundant growth on BA and CBU agar plates. Micrococci, staphylococci and coryneform bacteria were usually isolated from the skin on the BA plate, but on CBU agar, micrococci or staphylococci were scarcely and occasionally recovered, with coryneform bacteria being the predominant growth. Six strains of *C. urealyticum* were isolated in five samples from five patients with the CBU agar plates, but none was detected on BA. Although heavy growth was present on CBU agar, *C. urealyticum* had ≤ 10 colonies per plate in all but one of the positive cultures. Several antibiotypes were present among isolates: multiresistant, β -lactam-resistant and β -lactam-susceptible.

Discussion

C. urealyticum causes nosocomial infections, mainly urinary tract infections, in immunosuppressed patients with renal transplantation or urological diseases [1, 4]. The isolation of *C. urealyticum* from clinical samples is difficult because of its fastidious growth, especially in the presence of non-fastidious normal flora. Although prolonged incubation for 48 h or longer improved the isolation of *C. urealyticum* on non-selective media [11, 7, 21], the use of selective media enhanced the recovery of multiresistant *C. urealyticum* from urine samples [8–11]. Those selective media contain either β -lactam antibiotics [8] or clindamycin

Table 2. Qualitative selectivity test

Species	Number of strains tested/inhibited
Coryneform bacteria	
<i>Arcanobacterium haemolyticum</i>	7/7
<i>Actinomyces pyogenes</i>	4/4
' <i>C. aquaticum</i> '	4/2
' <i>C. bovis</i> '	2/0
<i>C. jeikeium</i>	
Multiresistant	36/0
Susceptible	3/0*
<i>C. minutissimum</i>	19/3
<i>C. pilosum/C. renale</i>	4/4
<i>C. pseudodiphtheriticum</i>	12/3*
<i>C. pseudotuberculosis</i>	4/0
<i>C. striatum</i>	11/1
<i>C. ulcerans</i>	6/0
<i>C. urealyticum</i>	
Multiresistant	37/0*
Susceptible	9/0*
<i>C. xerosis</i>	20/2
Coryneform CDC group A	8/3
Coryneform CDC group ANF	6/3
Coryneform CDC group F	15/5
<i>Erysipelothrix rhusiopathiae</i>	7/7
<i>Listeria monocytogenes</i>	16/1
<i>Oerskovia</i> spp.	4/0
<i>Rothia dentocariosa</i>	2/2
<i>Rhodococcus equi</i>	9/0
Subtotal	245/51 (20.8%)
Gram-negative bacilli	
<i>Escherichia coli</i>	53/53
<i>Acinetobacter</i> spp.	13/13
<i>Klebsiella pneumoniae</i>	15/15
<i>Citrobacter</i> spp.	6/6
<i>Enterobacter</i> spp.	14/13†
<i>Pseudomonas aeruginosa</i>	17/17
<i>Pseudomonas</i> spp.	4/2‡
<i>Serratia marcescens</i>	6/6
Subtotal	128/125 (97.6%)
Gram-positive cocci	
<i>Enterococcus</i> spp.	35/26
<i>S. aureus</i>	18/3
<i>Staphylococcus</i> spp. (coagulase negative)	6/2
Subtotal	59/31 (52.5%)

*Some strains grew poorly: *C. jeikeium* (1), *C. pseudodiphtheriticum* (5) and *C. urealyticum* (5).

†One multiresistant strain not inhibited.

‡Two multiresistant strains not inhibited.

plus nalidixic acid [9] to inhibit or reduce other organisms present in the sample. Occasionally, urine samples containing β -lactam-susceptible isolates of *C. urealyticum* were missed when plated on to β -lactam-containing media [8, 11] and several strains susceptible to β -lactam [12, 22] or clindamycin [22–24] have been reported. As these antibiotic supplements may represent a negative selection for fully susceptible strains, a different antibiotic supplement consisting of fosfomycin, aztreonam, polymyxin B and amphotericin B was selected. Fosfomycin has been used successfully in selective media for multiresistant corynebacteria [8, 25], so the 'in-vitro' inactivity against a multiresistant reference strain and six susceptible clinical stock collection strains was confirmed by a microdilution method. Aztreonam was selected because of its activity against gram-negative bacilli and all seven strains tested by microdilution were resistant (> 128 mg/L),

as previously reported for multiresistant strains [23]. Polymyxin B and amphotericin B were added at concentrations used in several selective media [9].

CBU agar is a lipid-enriched peptone-based agar like those previously described for the primary isolation of *C. jeikeium* [8, 25] and *C. urealyticum* [8, 9]. CBU agar is also made into a differential medium by the addition of urea, glucose and phenol red. *C. urealyticum* is a urea-splitting, asaccharolytic bacterium forming typical colonies surrounded by a red halo on CBU agar. Because of its strong urease activity, when > 10 colonies grew, the whole plate was pink and only the colonial morphology of *C. urealyticum* allowed its recognition. Other urease-producing bacteria isolated on the medium grew faster or were glucose fermenting and no clear red halo was seen. Although CBU agar allowed the isolation of antibiotic-susceptible and multiresistant strains of *C. urealyticum*, growth was slower on the selective medium as compared with the agar base without antibiotics. The final number, size and morphology of colonies was similar on the selective and non-selective media. The most typical colonial morphology was seen after incubation for 48–72 h, so we recommend incubation for at least 48 h for plates inoculated with clinical samples or 72 h for samples cultured for epidemiological purposes.

The selectivity of CBU agar was excellent against gram-negative bacilli, not only in the selectivity studies with reference or clinical strains, but also for the urine samples cultured. Other isolates that rarely grew on CBU agar included multiresistant enterobacteria or pseudomonas and were easily recognised by their colonial morphology or absence of urease. Although the selectivity of CBU agar to gram-positive cocci was good for reference strains, it was less than expected for clinical isolates tested by the qualitative method. Furthermore, there was poor selectivity against micrococci, staphylococci or enterococci when plated with the urine and skin samples. Again, it was possible to distinguish *C. urealyticum* isolates because their urease and colonial morphology differed. Several coryneform bacteria other than *C. urealyticum* grew on CBU agar, most were non-urease producing and formed colonies different to those of *C. urealyticum*.

The selective media enhanced the recovery of *C. urealyticum* [8–11] but their utility in the practical management of patients is controversial [9, 11, 14, 21]. Although the use of selective media for *C. urealyticum* to culture urine samples from patients at high risk has been sustained [9, 14], most isolates of *C. urealyticum* grown only from selective media had no clinical relevance [8, 10, 11, 21]. CBU agar also improved the primary isolation of *C. urealyticum* in urine samples from unselected in-patients, yielding a 3.5-fold increase (1.3–4.6%), as has been reported for other selective media [8, 10, 11, 14, 21]. Garcia-Bravo

et al. reported a two-fold increase in recovery of *C. urealyticum* from urine samples of renal transplant patients with this selective medium [14]. The clinical significance of the urine isolates in the present study was not analysed, but all *C. urealyticum* strains were in mixed culture and only two cultures had colony counts $\geq 10^5$ cfu/ml of *C. urealyticum* and *Pseudomonas aeruginosa*. Therefore, we do not recommend the selective medium for routine urine cultures, but just for epidemiological surveys.

Multiresistant strains of *C. urealyticum* have been isolated from the healthy skin of infected and colonised patients by plating the skin samples on to β -lactam-containing selective media [4, 8, 26]. As the presence of antibiotic-susceptible strains would be missed with such media, the reliability of CBU agar for the isolation of non-multiresistant skin strains was tested. The selective agar was plated with skin swabs from patients at their first day of hospitalisation. As has been shown with the growth studies and clinical samples, the selective medium had excellent sensitivity for the isolation of *C. urealyticum* (≤ 10 colonies), although a heavy growth was present on the plate. The CBU agar was effective in isolating multiresistant and antibiotic-susceptible strains of *C. urealyticum* from skin samples. Finally, CBU agar has been used successfully in a prospective study of environmental contamination by *C. urealyticum* in a hospital, showing its reliability in isolating the bacteria from air and surface samples [15].

In conclusion, CBU agar supports the growth of *C. urealyticum*, including those isolates which could be inhibited in other selective media, facilitates isolation by reducing or inhibiting the growth of competing flora, and produces typical colonies. This selective medium appears to be an excellent epidemiological tool for the isolation of *C. urealyticum* from human or environmental samples.

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