A Biosensor Platform for Rapid Antimicrobial Susceptibility Testing Directly From Clinical Samples

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Abbreviations and Acronyms

AMP = ampicillin

AST = antimicrobial susceptibility testing

AXO = ceftriaxone

b-AST = biosensor based antimicrobial susceptibility test

cfu/ml = colony forming units/milliliter

CIP = ciprofloxacin

FEP = cefepime

GEN = gentamicin

ME = major error

MH = Mueller-Hinton

POS = no antibiotic control

R = antibiotic resistant

rRNA = ribosomal ribonucleic acid

S = antibiotic sensitive

SCI = spinal cord injury

SXT = trimethoprim-sulfamethoxazole

UTI = urinary tract infection

VAPAHCS = Veterans Affairs Palo Alto Health Care System

VME = very major error

Purpose: A significant barrier to efficient antibiotic management of infection is that the standard diagnostic methodologies do not provide results at the point of care. The delays between sample collection and bacterial culture and antibiotic susceptibility reporting have led to empirical use of antibiotics, contributing to the emergence of drug resistant pathogens. As a key step toward the development of a point of care device for determining the antibiotic susceptibility of urinary tract pathogens, we report on a biosensor based antimicrobial susceptibility test.

Materials and Methods: For assay development bacteria were cultured with or without antibiotics, and growth was quantitated by determining viable counts and electrochemical biosensor measurement of bacterial 16S rRNA. To determine antibiotic susceptibility directly from patient samples, urine was cultured on antibiotic plates for 2.5 hours and growth was determined by electrochemical measurement of bacterial 16S rRNA. For assay validation 252 urine samples were collected from patients at the Spinal Cord Injury Service at Veterans Affairs Palo Alto Health Care System. The biosensor based antimicrobial susceptibility test was completed for samples containing gram-negative organisms. Pathogen identification and antibiotic susceptibility results were compared between our assay and standard microbiological analysis.

Results: A direct biosensor quantitation of bacterial 16S rRNA can be used to monitor bacterial growth for a biosensor based antimicrobial susceptibility test. Clinical validation of a biosensor based antimicrobial susceptibility test with patient urine samples demonstrated that this test was 94% accurate in 368 pathogen-antibiotic tests compared to standard microbiological analysis.

Conclusions: This biosensor based antimicrobial susceptibility test, in concert with our previously described pathogen identification assay, can provide culture and susceptibility information directly from a urine sample within 3.5 hours.

Key Words: urinary tract infections, biosensing techniques, microbial sensitivity tests, point-of-care systems

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Emergence of drug resistant pathogens is an increasing problem worldwide, driven by the injudicious use of antibiotics and few new antibiotics. Standard microbiological diagnosis of bacterial infections such as UTI relies on culturing bacteria in a clinical microbiology laboratory. From sample collection 24 to 72 hours are typically required for culture and AST. Conventional methods of AST, including disk diffusion and microdilution, re-

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quire initial isolation of the pathogen from clinical samples, delaying the start of AST by at least 18 hours.[1–3] The increasing prevalence of drug resistant pathogens indicates a significant need for AST platforms that are capable of providing susceptibility data within hours rather than days.

Electrochemical biosensors are well suited for molecular diagnostics.[4] We previously described an electrochemical biosensor that uses sequence specific hybridization of bacterial 16S rRNA for the molecular identification of bacterial pathogens.[5,6] The hybridization of specific capture and detector probes to bacterial 16S rRNA at the sensor surface, followed by electrochemical signal amplification with an enzyme tag, transduces a molecular recognition event (DNA-RNA hybridization) into a quantitative electrical signal. Pathogen identification takes 1 hour and can be performed directly from urine without target purification or amplification. This assay was successfully validated using unknown urine samples from patients with a UTI, 1 of the most common bacterial infections.[7,8]

We report a rapid b-AST which combines the versatility of a phenotypic assay with genotypic specificity using molecular probes (fig. 1). Using 16S rRNA level to determine bacterial growth, b-AST measures bacterial phenotypic response to different antibiotics. With bacterial specific 16S rRNA probes, b-AST provides genotypic specificity and obviates the need for initial pathogen isolation. Finally, we demonstrated the clinical validation of b-AST in urine samples. We propose b-AST represents a significant advance in achieving point of care AST to impact clinical decision making.

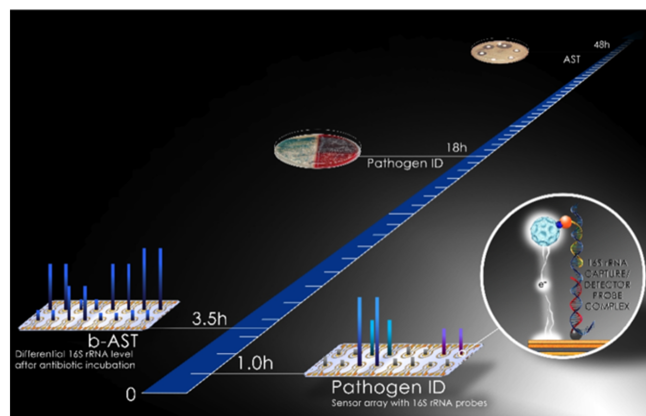


Figure 1. Comparison of conventional and biosensor based culture susceptibility analysis of urine. Urine sample is first tested for presence and identity of pathogens. For detection of pathogens sensors are functionalized with capture oligonucleotides targeting 16S rRNA of E. coli, P. mirabilis, P. aeruginosa, Enterococcus spp., Klebsiella-Enterobacter group, Enterobacteriaceae group, universal eubacterial and negative control. Electrochemical detection of pathogen 16S rRNA hybridization with capture and detector probes provides culture data within 1 hour of urine sample collection. If pathogens are identified, level of 16S rRNA from sample incubated without and with antibiotic is quantified on biosensor providing susceptibility data within 3.5 hours of urine sample collection. In contrast to b-AST, conventional culture and susceptibility can take up to 72 hours.

MATERIALS AND METHODS

Bacterial Strains

Escherichia coli 700928 (CFT073), Pseudomonas aeruginosa 10145 and Enterococcus faecalis 49532 were purchased from ATCC (Manassas, Virginia). Uropathogenic clinical isolates were obtained from clinical microbiology at VAPAHCS.

Biosensor Detection of Bacterial 16S rRNA

Electrochemical sensors were purchased from GeneFluidics (Monterey Park, California). Sensors were functionalized with oligonucleotides as previously described.[5] For pathogen identification from urine the cellular fraction was collected by centrifugation. Cultured bacteria and urine b-ASTs were assayed directly from culture using specific probes for E. coli, Klebsiella pneumonia, Proteus mirabilis, P. aeruginosa and E. faecalis, and Enterobacteriaceae or eubacterial for other species. Bacterial lysis and electrochemical biosensor detection of 16S rRNA were preformed as previously reported.[6,9]

Antibiotic Susceptibility Assay

Bacteria were inoculated into MH broth, grown to OD_{600} ~0.2, then diluted to OD_{600} ~0.02 in MH broth with or without antibiotic—AMP 32 μg/ml, CIP 4 μg/ml, SXT 4/76 μg/ml (Sigma, St. Louis, Missouri)—and incubated at 37C with shaking. Samples for OD_{600}, biosensor assay and/or determination of cfu/ml were taken at regular intervals during incubation. For antibiotic susceptibility from urine equal volumes of urine and MH were mixed, and 50 μl mixture was pipetted into the wells of a Sensititre® GN1F or GPN2F plate (containing dehydrated antibiotics and control wells without antibiotic. Antibiotics included AMP (8 to 64 μg/ml), CIP (0.5 to 4 μg/ml), SXT (0.5–9.5 to 4–76 μg/ml), AXO (4 to 64 μg/ml), GEN (2 to 16 μg/ml) and FEP (4 to 32 μg/ml). Plates were incubated at 37C with shaking for 2.5 hours. Biosensor assays were performed immediately after incubation or frozen at −80C for later assay because no significant difference was found in biosensor signal between fresh and frozen samples.[6]

Clinical Sample Collection

With approval from Stanford University institutional review board and informed patient consent, urine samples were prospectively collected from April 2008 to August 2009 from patients at the SCI Service at VAPAHCS. The decision to collect urine was made by the treating clinicians. For each subject 2 urine samples were collected by voiding, straight catheterization or directly from the indwelling catheter. One sample was sent for standard clinical microbiology culture and susceptibility, and the second was sent directly to our laboratory. All urines were cultured for b-AST and tested for the presence of bacteria by biosensor assay.

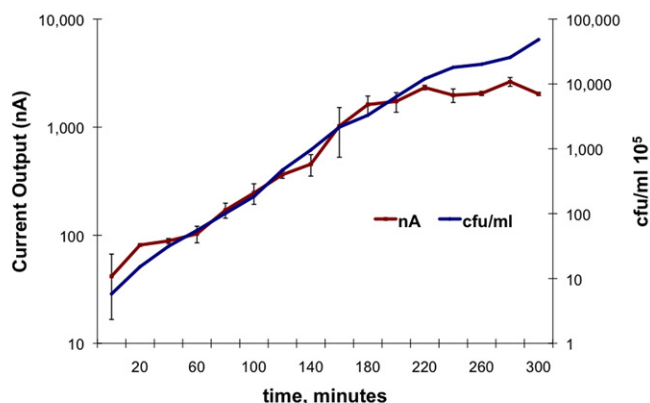


Figure 2. Biosensor detection 16S rRNA as bacterial growth marker. Growth of uropathogenic E. coli strain CFT073 measured over time by biosensor detection (nA) and quantitative plating data (cfu/ml). Result indicates that biosensor can measure bacterial growth over 4-log unit range, from 5×10^5 to 1×10^9 cfu/ml.

Statistical Analysis

To establish criteria for each antibiotic resistance in the b-AST the difference of the $\log_{10}$ of current output, nA, without antibiotic and with antibiotic, was calculated ($\log_{10}$(POS)-$\log_{10}$(ABX)). Using the clinical microbiology laboratory AST as the standard, the percent agreement of the biosensor was calculated for a range of threshold values of $\log_{10}$(POS)-$\log_{10}$(ABX). For each antibiotic the threshold yielding the highest agreement with the gold standard was chosen as the cutoff for resistance. Pathogen-antibiotic combinations identified as indeterminate by the clinical microbiology laboratory were excluded from analysis. AST is not routinely preformed on pathogens from samples containing more than 2 bacterial species by VAPAHCS clinical microbiology. Therefore, these samples were omitted from b-AST analysis due to the lack of comparison data.

RESULTS

Electrochemical Biosensor Measurement of Bacterial Growth

We examined whether electrochemical biosensor measurement of 16S rRNA could be used to monitor bacterial growth by comparing bacterial quantitation by viable counts (cfu/ml) with biosensor measurement of bacterial 16S rRNA. Uropathogenic E. coli was cultured, and samples taken at 20-minute intervals for quantitative plating and biosensor assay. Figure 2 shows the correlation between cfu/ml and biosensor measurement of 16S rRNA. Proportional increases in signal strength were observed over a 4 log unit range. A similar correlation between increased cell number and biosensor signal was observed with other common uropathogens including P. aeruginosa and E. faecalis (data not shown). These data demonstrate that the detection of 16S rRNA using our electrochemical biosensor can be used to measure bacterial growth.

Determination of Antibiotic Susceptibilities in Clinical Isolates

Next we examined the differential growth kinetics with and without antibiotics. Starting with common oral antibiotics for UTI treatment AMP, CIP and SXT, the growth of E. coli clinical isolates with different antibiotic susceptibility profiles was determined by b-AST (fig. 3). Isolates were cultured with or without the indicated antibiotics. Samples were taken at 15-minute intervals for biosensor assay. All strains grew well in MH broth without antibiotics. AMP, CIP and SXT inhibited growth of susceptible strains, while growth of resistant strains was comparable to the no antibiotic control. For example, the AMP resistant strain EC136 grew with and without AMP, and growth was effectively suppressed by CIP and SXT. The characterization of clinical isolates was expanded to 14 different clinical isolates including testing GEN and AXO with susceptible and resistant E. coli, and P. aeruginosa with CIP, GEN, AXO and SXT (data not shown). The clear differences in growth indicated that with less than 90 minutes of incubation measurement of 16S rRNA with the biosensor could be used for the determination of antibiotic susceptibility.

Rapid Determination of Antimicrobial Susceptibility in Urine

We tested whether b-AST could be done directly from urine. For this assay patient urine samples were mixed with media, then inoculated into each of the 96 wells of a commercial Sensititre plate[10] with

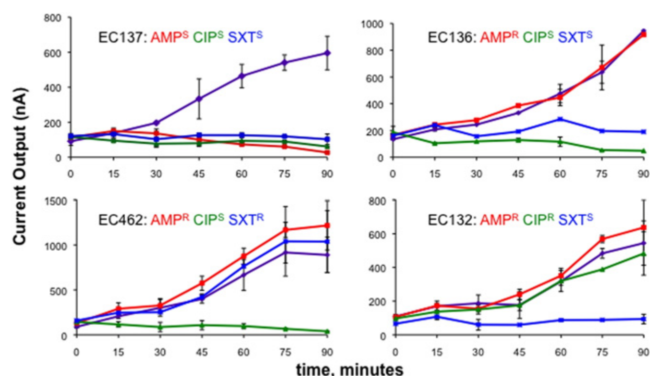


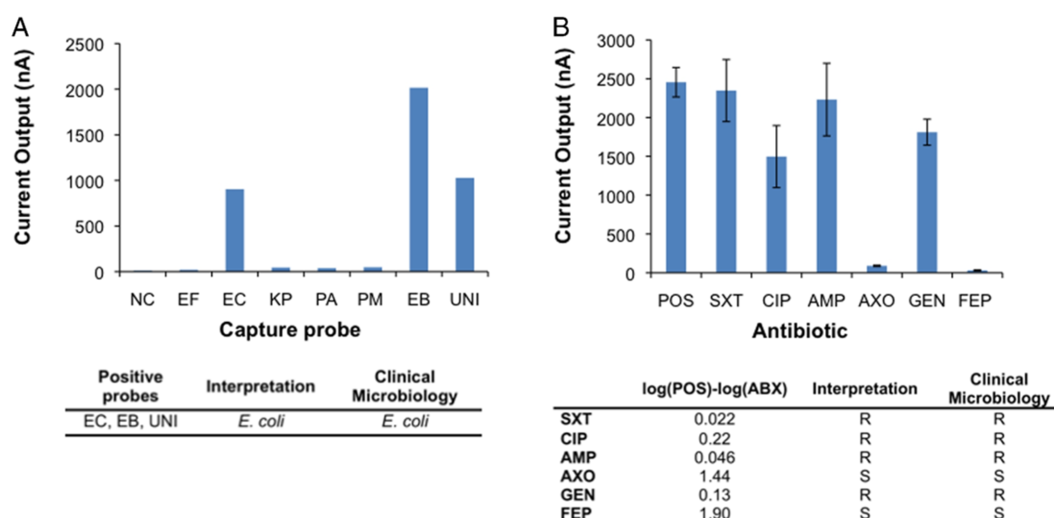
Figure 3. Antibiotic susceptibility determination of E. coli clinical isolates. E. coli isolated from patient urine samples with differing antibiotic resistance profiles was grown under 4 conditions of no antibiotic (purple line), AMP (red line), CIP (green line) and SXT (blue line). Time zero represents inoculation at early log phase growth. Biosensor measurements of 16S rRNA using E. coli probes were taken at 15-minute intervals. Antibiotic susceptibility profiles for clinical isolates as determined by biosensor assay were same as determined by conventional methods (VITEK® 2) at clinical microbiology laboratory.

a panel of antibiotics for gram-negative uropathogens. Antibiotic concentrations tested in the biosensor assay were the resistance breakpoints for each pathogen-antibiotic combination in accordance with Clinical and Laboratory Standards Institute guidelines.[11] After inoculation the plates were incubated for 2.5 hours. While with clinical isolates we saw growth differentiation between susceptibility and resistance in 90 minutes, longer incubation allowed pathogens at lower starting concentrations in the urine samples to grow to a concentration within the range of detection of the biosensor (fig. 3). In addition, a longer incubation time further differentiated antibiotic susceptibility and resistance. For biosensor analysis samples were lysed, species specific detector probes were added and then spotted onto a biosensor functionalized with species specific capture probes. The use of specific probes on the biosensor eliminated the need to separate the pathogen of interest from potential contaminants such as normal skin and genital flora, and allowed for the determination of the antibiotic susceptibility profiles of multiple pathogens in a single sample. The level of 16S rRNA measured from samples with each antibiotic was compared to the no antibiotic control. Figure 4 shows the results for the entire assay with a clinical urine sample. It was determined that the sample contained E. coli. Then b-AST comparison of different antibiotic conditions indicated that the E. coli identified was resistant to SXT, CIP, AMP and GEN, but sensitive to AXO and FEP (POS is no antibiotic control). Clinical microbiology analysis confirmed the results 3 days later, while the biosensor culture and susceptibility analysis was completed within 3.5 hours of collection.

Clinical Validation of b-AST

From April 2008 to August 2009, 222 patients were recruited from the SCI Service at VAPAHCS and 252 urine samples were collected. The majority of patients were male (97%) and required assistance for bladder emptying through intermittent catheterization (24%) or indwelling catheter (42%). Corresponding biosensor and clinical microbiology culture data were available for 215 of the samples. The clinical microbiology laboratory found that 157 (73%) of these samples contained bacteria. The pathogen identification assay reliably detected the appropriate pathogens with pathogen specific probes or in samples where pathogen specific probes were not available with the generic Enterobacteriaceae and/or universal eubacterial probes. Using the universal probe signal as the indicator for positive samples, the biosensor sensitivity was 92% and specificity was 97%, yielding a positive predictive value of 99% and a negative predictive value of 81%.

Because we previously found 84% of the culture positive samples from this patient population contained gram-negative pathogens,[6] we focused our efforts primarily on the antibiotics that target gram-negative organisms. Samples identified as containing Enterobacteriaceae or P. aeruginosa by biosensor were analyzed using b-AST. The most common pathogens analyzed by b-AST were E. coli (30) followed by K. pneumonia (17), P. mirabilis (8) and P. aeruginosa (4). Eleven other species of Enterobac-



Positive probes	Interpretation	Clinical Microbiology
EC, EB, UNI	*E. coli*	*E. coli*

	log(POS)-log(ABX)	Interpretation	Clinical Microbiology
SXT	0.022	R	R
CIP	0.22	R	R
AMP	0.046	R	R
AXO	1.44	S	S
GEN	0.13	R	R
FEP	1.90	S	S

Figure 4. Biosensor pathogen identification and antibiotic susceptibility directly from urine sample. *A*, biosensor signals more than 3 standard deviations over negative control (*NC*) for E. coli (*EC*). Enterobacteriaceae (*EB*) and universal (*UNI*) probes indicate urine sample contains E. coli. *EF*, E. faecalis. *KP*, K. pneumonia. *PA*, P. aeruginosa. *PM*, P. mirabilis. *B,* difference in growth between no antibiotic (*POS*), and AXO and FEP indicate that E. coli from sample is sensitive to AXO and FEP but resistant to other antibiotic tested. Interpretation of biosensors assay was confirmed by clinical microbiology culture and susceptibility analysis.

teriaceae made up the remainder of the samples. Once information was available from clinical microbiology, the AST profiles were compared. Comparison data were available for 72 pathogens, allowing for 368 observations between pathogen and antibiotic. A cutoff threshold for resistance was determined for each antibiotic that yielded the best agreement with the clinical microbiology laboratory AST. For example, for SXT a difference of 0.26 log units or less had the highest agreement (96%) with the lowest incidence of VME (1%) and ME (3%). Overall strains were identified as resistant if the log difference was 0.40 or less for AMP, 0.30 or less for CIP, 0.26 or less for SXT, 0.10 or less for AXO, 0.14 or less for GEN and 0.10 or less for FEP.

The overall agreement with the clinical microbiology AST was 94%, with only 1% VME (b-AST indicated a pathogen was susceptible to an antibiotic while the clinical microbiology AST found it resistant) and 4% ME (b-AST indicated a pathogen was resistant to an antibiotic while the clinical microbiology AST found it susceptible, see table). The accuracy of the b-AST was consistent for different antibiotics and pathogens with 90% or greater accuracy for each antibiotic, and 92% or greater accuracy for the most common pathogens.

Where b-AST differed with the clinical microbiology AST, the pathogen was isolated for additional testing. Retesting of isolates by b-AST found all but 1 observation in agreement with clinical microbiology AST. An E. coli isolate was reported as susceptible to CIP by clinical microbiology that b-AST from urine and with the isolate indicated was resistant. A subsequent disk diffusion assay revealed an intermediate zone of inhibition with this isolate (data not shown).

Clinical results of b-AST

	S	R	% Accuracy*	No. ME (%)†	No. VME (%)‡
Overall	267	101	94	16 (4)	4 (1)
AMP 32 μg/ml	17	41	93	3 (5)	1 (2)
CIP 4 μg/ml	49	22	90	7 (10)	0 (0)
SXT 4/76 μg/ml	45	26	96	2 (3)	1 (1)
AXO 64 μg/ml	56	5	98	0 (0)	1 (2)
GEN 16 μg/ml	65	7	94	3 (4)	1 (1)
FEP 32 μg/ml	35	0	97	1 (3)	0 (0)
E. coli	91	60	93	(5)	(2)
K. pneumonia	73	13	97	(3)	(0)
P. mirabilis	38	7	96	(4)	(0)
P. aeruginosa	4	8	92	(8)	(0)
Other Enterobacteriaceae	61	13	96	(3)	(1)

* Percent of b-AST results in agreement with clinical microbiology AST results.

† Pathogen was sensitive to antibiotic by clinical microbiology AST but was predicted to be resistant by b-AST.

‡ Pathogen was resistant to an antibiotic by clinical microbiology AST but predicted to be sensitive by b-AST.

While we primarily focused on a panel of antibiotics for gram-negative organisms, preliminary tests with gram-positive organisms were preformed. Four previously frozen urine samples containing Enterococcus or Staphylococcus aureus were inoculated onto plates with antibiotics for gram-positive organisms and assayed by b-AST (data not shown). This limited experience with gram-positive pathogens suggests b-AST can be optimized for gram-positive pathogens.

DISCUSSION

Building on our previous report of using the biosensor for multiplex identification of pathogens,[6] b-AST enables rapid determination of pathogen-antibiotic susceptibility directly from urine. While b-AST may be applicable to any body fluid, UTI diagnosis represents an ideal application given that urine is the most common sample sent to a clinical laboratory and UTI is among the most common bacterial infections.[7,8]

Similar to standard clinical microbiology AST, b-AST measures the phenotypic response of bacteria to antibiotics. Unlike standard ASTs, b-AST uses genetic detection of the bacterial growth with pathogen specific probes, thus eliminating the need for pathogen isolation. Others have described AST directly from urine samples containing a single pathogen using methods based on bioluminescent and optical detection.[12,13] In contrast, through the use of pathogen specific probes, b-AST can provide susceptibility analysis from urine containing multiple pathogens.

Other sensitive and accurate rapid ASTs in development use genotypic detection of antibiotic resistance genes.[14–18] However, a phenotypic approach may be superior as it is independent of specific genetic antibiotic resistance mechanisms. This feature is important because antibiotic resistance mechanisms are diverse and will continue to evolve.[19–21] Thus, our susceptibility assay will remain accurate and effective as new resistance mechanisms are elucidated.

Most discrepancies between b-AST and clinical microbiology AST were MEs in that b-AST demonstrated the strain was resistant while clinical microbiology found the strain to be susceptible. These errors, while significant, would not result in the selection of a potentially ineffective antibiotic. A potential cause of MEs is slower than expected pathogen growth. In samples with a low pathogen concentration delays in growth could result in final cultures near or below the level of detection of our sensor. In future studies the incubation time for the antibiotic susceptibility plates could be tailored to the starting concentration and bacterial species.

This may be essential when adding analysis of gram-positive organisms that generally have longer doubling times.

We measured growth with antibiotic at the resistance concentration recommended by the Clinical and Laboratory Standards Institute.[11] Thus, our assay provides a binary analysis— resistant or susceptible. This assay could be adapted for minimum inhibitory concentration determination by testing various antibiotic concentrations by b-AST. However, for a rapid test a simple susceptible or resistant end point for a panel of antibiotics may be sufficient in most clinical situations. When a more detailed analysis is required, samples could be sent to a clinical microbiology laboratory.

The biosensor pathogen identification and b-AST are currently manual bench top assays. However, all components of the assays are amenable to integration into an automated system to enable point of care application. As with other microdilution ASTs, samples can be grown in small volumes that can be integrated with microfluidic systems for sample preparation and delivery to the sensor. Efforts are currently under way to integrate the core technology into a microfluidic setup.[22,23]

CONCLUSIONS

We report a biosensor based AST that enables rapid determination of pathogen-antibiotic susceptibility directly from clinical samples. b-AST uses an electrochemical biosensor to detect differential 16S rRNA levels after short-term pathogen culture in the presence and absence of antibiotics. In a pilot clinical validation study using infected urine samples the overall agreement of b-AST with the standard clinical microbiology AST was 94%.

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