Quantitative colorimetric measurement of residual antimicrobials in the urine of patients with suspected urinary tract infection

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ABSTRACT

A simple microtitre plate assay is used to detect antimicrobial activity in clinical urine specimens and its potential as a screening tool is assessed. The assay is based on a colorimetric substrate, p-nitrophenyl-β-D-glucopyranoside, in combination with a *Bacillus subtilis* strain to detect antimicrobial residues. The assay identified antimicrobial activity in 31% of the 527 clinical urine samples tested. The majority of the samples (65%) came from the community, with the rest comprising hospital in-patients (19%) and out-patients (16%). The results demonstrated that there is an association between gender and the presence of inhibitory substances, as 40% of males and 27% of females tested positive. Just over two-fifths of hospital patients (46%) tested positive for inhibitory substances, compared to 26% of samples from community patients. Of the 306 samples that were culture-negative ($<10^4$ bacteria/mL), 42% were positive for inhibitory substances, compared with 17% among the remaining 221 samples. However, there was no evidence of an association between age and the presence of inhibitory substances. This study demonstrates that the bacteriostatic effect of the bacterial preservative boric acid is sufficient to upset the specificity of the assay. Furthermore, it has been suggested that antimicrobial activity can confuse the interpretation of culture results, as they have been found to play a major role in the occurrence of apparently sterile pyuria.

KEY WORDS: Antibiotic.
Boric acid.
Urinary tract infection.
Urine.

Introduction

Urinary tract infections (UTIs) are among the most prevalent bacterial infections affecting both sexes and all age groups. They also represent a significant proportion of the workload in most clinical microbiology laboratories. Confirmation of a urinary tract infection requires laboratory examination of a urinary sample, using a combination of microscopy and bacterial culture. Bacterial isolates then undergo antimicrobial sensitivity testing to provide the clinician with the appropriate choice of antibiotic treatment.[1]

The risk of suffering a UTI is dependent on age, underlying renal impairment and, most importantly, gender. Bacteriuria is significantly more prevalent in the female population and it increases with age in both sexes until the gender ratio of infection becomes nearly equal in the elderly.[1]

Escherichia coli causes 80-90% of acute uncomplicated bacterial UTIs in young women.[1] With nosocomial or hospital-acquired infections, the hospital environment is an important determinant of the nature of the infecting organism. *Proteus* sp., *Klebsiella* sp., *Enterobacter* sp., *Pseudomonas* sp., staphylococci and enterococci are more often isolated from in-patients, compared to the preponderance of *Escherichia coli* in an out-patient population.[2]

In elderly patients, the female:male UTI ratio becomes less significant. For patients of both sexes over the age of 65, the prevalence increases substantially, with at least 10% of men and 20% of women suffering from a UTI.[3]

It has long been suggested that the screening of urines for antimicrobial activity can be useful in the diagnosis of UTIs. It has also been suggested that the presence of antibiotics in a patient's urine prior to culture may have a negative effect on the accuracy of urinary culture, including the production of false-negative cultures.[4] A more recent study has suggested that bacterial growth inhibitors in urine are mainly due to the intake of antibiotics by patients and that this was found to play a major role in the occurrence of sterile pyuria and in reducing the numbers of specimens yielding significant bacterial growth.[5]

Several simple microbiological methods to identify antimicrobial agents in urine have been described using multisensitive bacterial strains, such as *E. coli*, *Micrococcus* sp. and *Bacillus stearothermophilus,* as indicator organisms.[6–7] It was also found that, in the presence of antimicrobials, isolates of *E. coli* were more resistant to gentamycin (75.3% *vs.* 48.7%), norfloxacin (85.2% *vs.* 64.6%) and co-trimoxazole (58.5% *vs.* 35.5%). It has been further suggested that screening urines for antimicrobial substances can be a useful tool when high levels of antimicrobial agents are used in the general community.[7]

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Table 1. Statistics of age groups.

Age	*n*	Range	Min	Max	Mean	SD
16 or less	63	15	1	16	5.92	5.240
17–49	169	32	17	49	33.28	9.209
50–64	95	14	50	64	57.39	4.401
65+	200	33	65	98	78.18	7.306

The present study aims to assess the ability of a simple microtitre plate assay to detect antimicrobial activity in clinical urine specimens. The microtitre plate assay undertaken was developed for use in screening for antimicrobial residues in porcine urine.[8] The urines to be tested will be selected from the samples arriving at the clinical microbiology laboratory from hospital in-patients and out-patients, and from the community.

This study will ascertain whether or not the microtitre plate assay is able to screen successfully for the presence of inhibitory substances in clinical urines and thereby determine the population of urines arriving at the laboratory that already contain antimicrobials. Statistical analysis is performed on the results to determine which factors (gender, age or source) affect the likelihood that a urine sample contains antimicrobial substances.

Materials and methods

Reagents

Ammonium sulphate, dipotassium hydrogen orthophosphate, potassium dihydrogen orthophosphate, sodium citrate and magnesium sulphate (BDH, Luttersworth, Leics. UK) were Analar grade. Yeast extract (Difco, East Moreley, Surrey, UK) was biological media grade. p-Nitrophenyl-β-D-glucopyranoside was obtained from Sigma (Poole, Dorset, UK).

B. subtilis (number 8054; National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland, UK) was used as the test strain throughout this study.

Bacillus growth medium (BM) was prepared by dissolving 0.2 g ammonium sulphate, 1.4 g dipotassium hydrogen orthophosphate, 0.6 g potassium dihydrogen orthophosphate, 0.1 g sodium citrate, 0.02 g magnesium sulphate and 0.01 g yeast extract in 100 mL deionised water. The medium was adjusted to pH 7.0 and sterilised by autoclaving at 121°C for 15 min. Double strength BM (DSBM) was also prepared using double the amounts of chemicals in 100 mL deionised water.

Cysteine lactose electrolyte-deficient (CLED) agar,[9] was prepared using 4 g peptone, 3 g Lab-Lemco powder, 4 g tryptone, 10 g lactose, 0.128 g L-cysteine, 0.02 g bromothymol blue and 15 g agar, dissolved in 1000 mL distilled water. The medium was then sterilised by autoclaving.

Columbia blood agar[10] was prepared using 23 g special peptone, 1 g starch, 5 g sodium chloride and 10 g agar, dissolved in 1000 mL distilled water. The medium was sterilised by autoclaving, then cooled to 50°C before adding 5% (v/v) sterile defibrinated blood.

Mueller-Hinton agar[11] was prepared using 300 g beef (dehydrated infusion), 17.5 g casein hydrolysate, 1.5 g starch and 17 g agar, dissolved in 1000 mL distilled water. The medium was sterilised by autoclaving.

A p-nitrophenyl-β-D-glucopyranoside stock solution was prepared by dissolving 1g in 100 mL deionised water to give a concentration of 10 mg/mL. This was filter sterilised using a 0.22 μm syringe filter (Whatman, Maidstone, Kent, UK).

Apparatus

Standard laboratory apparatus was used throughout for the preparation of media and chemical solutions. Clinical urine samples were selected for testing. The sample containers used where standard universal urine containers with and without boric acid (Sterilin UK).

Initial testing of the method was performed on a preliminary selection of both boric acid and non-boric acid urine samples. The main part of the study then focused on clinical samples supplied in non-boric acid containers. Urines were cultured using the filter-strip method[12] (Bacteruritest strip, Diamed Diagnostics). The primary culture medium used was CLED agar. Further examination of positive samples, including basic identification and antimicrobial sensitivities, was carried out using Columbia blood agar and Mueller-Hinton (with and without 5% [v/v] lysed horse blood) agar.

Basic identification of urinary pathogens from positive samples was carried out using basic grouping kits and specific identification reagents, including Staphaurex Plus (Remel Europe, Dartford, UK), colour catalase (ID-ASE, bioMerieux, France), 3% (w/v) KOH (K9860, BioConnections, UK), streptococcal grouping latex kit (Prolex, ProLab Diagnostics, Neston, UK), oxidase strips (CK3520, BioConnections UK) and rabbit coagulase plasma (ProLab Diagnostics).

The colorimetric assay was carried out using a standard Falcon 96-well microtitre plate (Becton Dickinson, France) incubated at 37°C in a Cultura mini-incubator (Almedica, Galmiz, Switzerland.) for 4.5 h. p-Nitrophenyl-β-D-glucopyranoside activity was measured in absorbance (*A*) units at 405 nm using an E-max precision microplate reader (Molecular Devices, USA).

Methodology

A total of 527 samples were included in the study, comprising 358 (68%) female samples and 169 (32%) male samples. For the purpose of this study, the subjects were divided into four cohort age groups. The largest percentage of the samples (38%) came from patients aged over 65. The second largest was the 17–49 group (32%), followed by the 50–64 group (18%) and ≤ 16 group (12%).

Table 2. Results of routine urine culture.

Culture result	Frequency	%
No significant growth	306	58
Doubtful/mixed growth	52	10
Coliforms isolated	127	24
Other pathogens isolated	42	8
Total	527	100

A high percentage of samples was received from the community. Almost two-thirds (65%) of the samples came from GPs and private nursing homes in the catchment area of the Belfast City Hospital. Other sources considered in this study included hospital in-patients (19%) and those attending out-patient clinics (16%). The majority of the samples included in the study came from the Belfast City Hospital Trust, with smaller numbers received from Lagan Valley, Downe and Muckamore Abbey hospitals.

Urine samples were cultured and incubated on CLED agar at 37°C for 18–24 h. The following day, samples with significant numbers of bacteria were subcultured for identification and sensitivity testing. All non-boric acid urines were then refrigerated prior to testing with the colorimetric assay.

A suspension of *B. subtilis* in the logarithmic phase was used in the assay to detect the presence of antimicrobials in the urine. Growth in the logarithmic phase was achieved by continued subculturing of an exponentially growing culture. An initial overnight culture was started by making a suspension of one colony of *B. subtilis* in 10 mL BM. The culture was propagated by inoculating 100 μL *B. subtilis* suspension to a fresh 10 mL BM.[8]

The reaction mixture for the assay was created by combining equal volumes of sterile DSBM and p-nitrophenyl-β-D-glucopyranoside stock solution. From this primary mixture, two 175-μL samples representing the negative control for the assay were added to the microtitre assay plate. To the remaining mixture, an amount of vortex-mixed *B. subtilis* suspension was added. The volume added was equivalent to a 1 in 100 dilution. Thus, for a 10-mL reaction mixture, 100 μL *B. subtilis* was added to create a final 'active' reaction mixture. After vortex-mixing, 175-μL amounts were added to the negative urine control wells and then to the remaining test wells.

The assay was initiated by adding 25 μL test urine to each test well, 25 μL negative urine to the negative urine control wells and 25 μL sterile water to the negative control wells. The microtitre plate was sealed with plate sealing film to prevent evaporation and incubated for 4.5 hrs at 37°C. The plate was removed and activity was read at 405 nm, measured in *A* units.

Lawn testing of antimicrobial activity was carried out using Columbia blood agar and a *B. subtilis* reference strain, with subsequent observation of a zone of inhibition produced after 24-h incubation. Urine samples that produced an increase in the colorimetric assay <0.1 *A* units were considered indicative of antimicrobial activity. To rule out the effects of other non-antimicrobial substances present in the urine that could affect the enzyme systems, it was necessary to visually demonstrate the presence of active antimicrobials in the urine by another method.

Table 3. Assay results by gender.

	Male		Female	
	Frequency	%	Frequency	%
Presence of inhibitory substances	67	40	97	27
Absence of inhibitory substances	102	60	261	73
Total	169	100	358	100

Antimicrobial activity was demonstrated by adding 20 μL test urine to the surface of a Columbia blood agar that was spread inoculated with a 0.1 McFarland density of a *B. subtilis* suspension. After 24-h incubation at 37°C, zone sizes were measured and recorded. As some antimicrobial substances, particularly the penicillins and related groups, are rapidly oxidised and degraded, every effort was made to reduce the time between arrival in the laboratory and testing.[6] As far as possible, the urines were refrigerated at all times between culture and antimicrobial testing.

Results

Table 1 shows descriptive statistics (range, minimum, maximum, mean and standard deviation [SD]) relating to the age groups studied. Table 2 shows the results of routine urine culture. Those of doubtful significance grew 10^4–10^5 bacteria/mL, and mixed growths were of three or more organisms. The culture-negative samples grew $<10^4$ bacteria/mL.

Visual demonstration of a sample's antimicrobial effect was performed on 31% (51/164) of the positive samples. Of these, 29.4% (15/51) were tested after seven days' storage, while the other 70.6% (36/51) were tested two or three days after arrival in the laboratory. Of the samples tested, 92.2% (47/51) displayed a zone of inhibition after 18+-h incubation at 37°C.

Of the samples that failed to produce a readable zone of inhibition, three were tested after seven days and one was tested after two to three days. Therefore, of the samples tested after two to three days of storage, 97.2% (35/36) displayed observable antimicrobial activity. An additional 20% (74/363) of samples that registered *A* increases >0.1 were also tested for antimicrobial activity, and all displayed no observable antimicrobial activity.

Based on the visual demonstration of antimicrobial effect, samples that showed an *A* increase <0.1 were considered to contain inhibitory substances. Samples that showed an increase >0.1 *A* units where considered to have allowed growth of the *B. subtilis* strain in the reaction mixture and were therefore considered negative. The results shown here indicate that 164 (31%) samples were considered positive for inhibitory substances, with the remaining 363 (69%) negative for the presence of inhibitory substances.

Table 3 shows the assay results by gender. χ^2 analysis showed significant evidence for an association between gender and assay result (χ^2= 8.43, P< 0.01). Table 4 shows assay results by age group. χ^2 analysis showed no significant association between age and assay result. (χ^2 = 6.963,

Table 4. Assay results by age group.

	Age group							
	16 or less		17–49		50–64		65+	
	Frequency	%	Frequency	%	Frequency	%	Frequency	%
Presence of inhibitory substances	20	32	41	24	29	31	74	37
Absence of inhibitory substances	43	68	128	76	66	69	126	63
Total	63	100	169	100	95	100	200	100

Table 5. Assay results by source.

	GP		Hospital OP		Hospital IP	
	Frequency	%	Frequency	%	Frequency	%
Presence of inhibitory substances	90	26	28	33	46	46
Absence of inhibitory substances	253	74	57	67	53	54
Total	343	100	85	100	99	100

Table 6. Assay results by culture result.

	No significant growth		Doubtful/mixed growth		Coliforms isolated		Other pathogens isolated	
	Frequency	%	Frequency	%	Frequency	%	Frequency	%
Presence of inhibitory substances	127	42	12	23	15	12	10	24
Absence of inhibitory substances	179	58	40	77	112	88	32	76
Total	306	100	52	100	127	100	42	100

$P = 0.07$). Table 5 shows assay results by sample source. The χ^2 test demonstrated evidence for a significant association between source and assay result ($\chi^2 = 14.818$, $P < 0.01$). Table 6 shows the assay results by culture result. Again, χ^2 analysis demonstrated an association between culture result and assay result ($\chi^2 = 40.097$, $P < 0.01$).

Discussion

The assay used here had previously been described and validated for the detection of five major antibiotics at concentrations below 1 μL/mL in porcine urine.[8] In the current study, visual determination of antimicrobial activity was possible in 97.5% of positive assay results after two to three days of storage. Samples that were tested after one week's storage displayed slightly less concordance, with 92.5% agreement. After storage at 4°C, little loss of antimicrobial activity was noted in some urine samples.[6]

Penicillins, in particular ampicillin, are known to oxidise rapidly and can break down spontaneously in urine or be broken down by β-lactamase-producing bacteria. Some antibiotics are also affected by the pH of urine; for example, erythromycin has been noted to produce reduced zone sizes in acidic urine.[6] Thus, deterioration of antibiotics in urine may explain why some samples displayed no observable antimicrobial activity after prolonged storage. It was therefore important to check zones of inhibition as soon as possible using fresh urine or samples stored for as short a time as possible.

Four of the samples indicated as positive by the criteria of the assay did not produce a readable zone of inhibition in the agar test. If antimicrobial deterioration is discounted, it is possible that the concentration of antimicrobial was insufficient to be detected on the agar screening test.

It has also been shown that urine from normal individuals can display an inherent antibacterial or bacteriostatic effect. Furthermore, antibacterial activity correlates with osmolarity, ammonia concentration and, in particular, urea concentration.[13] Thus, in some cases, it is possible that the bacteriostatic effect of urine may be sufficiently high as to cause a false-positive result.

Preliminary testing involved testing the effect, if any, of the bacteriostatic compound boric acid on the *B. subtilis* strain. Boric acid at a concentration of 20 g/L has been found to be largely bacteriostatic, with effects lasting up to 48 h.[14]

Initial tests showed that control urines tested in boric acid containers showed a 75% reduction in enzyme activity, compared to the same urine control in a non-boric acid container. This demonstrated that the bacteriostatic effect of boric acid is sufficiently large to upset the specificity of the assay.

Unfortunately, for the purposes of this study, the majority of clinical samples that arrived in the laboratory were in boric acid containers and had to be excluded from the study. This altered the initial estimate of the desired number of samples to be tested, as about 90% of clinical samples were

sent in boric acid containers. Therefore, the study took a subsample of clinical urine specimens (boric/non-boric acid), noting age, gender and source, and found that the non-boric acid subpopulation was representative of the whole population.

This study has shown that a significant number of samples arrive in the laboratory following antibiotic treatment. Ideally, where a UTI is suspected, a sample should be sent before antibiotic treatment is initiated. However, this will not always be possible as a patient may be receiving prophalytic treatment.

There is evidence to show that antibiotic treatment may lead to apparently sterile pyuria in some cases. In a study by Abu Shaqra,[5] an explanation was sought to explain a discrepancy between microscopic observations and culture diagnosis in urinary samples. Of 500 samples tested, 21% contained significant pyuria, yet only 14.4% yielded significant bacterial growth. Using *Staphylococcus aureus* (ATCC 29737), antimicrobial activity was detected in 19.2% specimens.

If antimicrobial activity confuses the interpretation of culture results in this study, some results from samples displaying antimicrobial activity could be adversely affected and not represent the true clinical picture. Further analysis of this group would be required to ascertain what effects antimicrobial activity has on bacterial culture.

The aim of clinical laboratory diagnostic procedures should be the detection of the abnormal presence of bacteria in the urinary tract, together with evidence of inflammation. In order to achieve accurate diagnosis, it is essential to adhere to the criteria for specimen collection and transport. Delays in transport can result in bacterial multiplication, leading to misdiagnosis.[15] When delay is unavoidable, refrigeration at 4°C is a satisfactory means of maintaining urinary bacterial preservation for culture.[16]

As refrigeration may not always be possible, use of urine containers containing the bacterial preservative boric acid is an alternative.[17] Boric acid at a concentration of 20 g/L is largely bacteriostatic, eliminates the false-positive results obtained with unpreserved specimens and lasts for up to 48 h.[14]

In 1957, Kass[18] helped to define the criteria for significant bacteriuria. He suggested that a count of 100,000 colony-forming units (cfu) per mL, or greater, of a single species of microorganism in a midstream specimen of urine (MSU) was almost invariably indicative of bladder bacteriuria. The semi-quantitative filter paper method[12] was used as the culturing technique for clinical urines. After overnight incubation, more than 25 colonies on the plate equated to $>10^5$ bacteria/mL urine.[19]

A screening method to detect the presence of antimicrobials in urine specimens may be a useful tool. In fact, screening is common in countries such as Taiwan, where antibiotics are commonly abused. In one particular study, 68% of hospital patients screened in three Taiwanese hospitals were positive for antimicrobial agents.[7]

One of the issues that has arisen is the incompatibility of boric acid with the current assay methodology. As the use of boric acid is recommended by the clinical laboratory, it would hamper any decision to introduce routine screening of urine samples by the assay under study. If routine screening were to be introduced, this would require a second urine sample to be sent in a standard non-boric acid container.

Other methods of antimicrobial screening, such as inoculating seeded agar,[6] usually require overnight culture before confirmation of the presence of antimicrobial substances. The advantage of the described test is that, with an incubation time of just over 4 h, samples that arrive in the morning could be screened before culturing in the afternoon. This would allow alterations to be made to the culture protocol, or, at the very least, a comment be added to the laboratory report to indicate the potentially deleterious effect of antimicrobial activity.

As the results of the assay showed, urine samples from hospital in-patients are more likely to contain antimicrobial substances. With this in mind, it could to possible to single out hospital patients for screening. Again, this would require provision of a second non-boric acid sample. In symptomatic, pyuric patients whose urine fails to yield significant growth, screening for antimicrobials could help to explain an apparently sterile bacteriuria.

If screening of samples for antimicrobials substances is to be considered, more work will be needed to refine the rationale for selection. The basis for screening could be identified by microscopic examination or the use of flow cytometry. Also, samples containing significant numbers of white blood cells or bacteria could be screened routinely for antimicrobials, which would give the laboratory additional information for use in interpretating culture results.

It has long been suggested that the widespread and continued use of particular antibiotics in the community drives the selective pressure for the development of antibiotic resistant organisms. An Icelandic study has shown that antimicrobial use, both individual and total antimicrobial consumption, in the community is statistically strongly associated with nasopharyngeal carriage of penicillin-resistant pneumococci in children.[20]

Societies in which antibiotics are used widely and often indiscriminately tend to show increased levels of multidrug-resistant organisms. The rise in bacterial resistance in community-acquired pathogens in Spain over the past 15 years continues to be a cause for concern, and perhaps reflects the excessive consumption of antimicrobials in the past and of the distribution of various therapeutic groups.[21] Elsewhere, high levels of antimicrobial usage among Taiwanese patients presenting at emergency departments, clinics and in the community may account for the extraordinarily high rates of antimicrobial drug resistance encountered in this population, and in the misdiagnosis of infectious disease.[22]

In recent years, multidrug resistance has become a widespread problem, particularly in long-term or acute healthcare facilities. In such cases, antimicrobial therapy must be balanced against the possibility that its use could promote further resistance.[23] This also highlights the increasing importance of culture and sensitivity testing prior to empirical antimicrobial therapy.

Increasing levels of antimicrobial resistance in hospitals are well documented; however, it is now apparent that resistance levels in the community are also on the increase. Several studies have presented a statistical link between increased consumption of antimicrobials and increasing community resistance rates. Two studies, one each from The Netherlands[24] and Slovenia,[25] have compiled resistance rates to fluoroquinolones in community-acquired UTIs.

Both indicate that increasing resistance of *E. coli* to fluoroquinolones is associated with an increase in consumption of these antibiotics in the community.

In conclusion, this study has shown that the colorimetric assay can successfully demonstrate the presence of antimicrobials in clinical urine samples. The test is quick, convenient and simple to perform. If carried out as part of the routine laboratory examination, this assay could help in the diagnosis of problematic urine cultures and also explain the discrepancies between symptoms, microscopy and culture results. □

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References

1 Jawetz E, Melnick JL, Adelberg EA, eds. *Medical microbiology* (20th edn). London: Prentice Hall International, 1995.
2 Turck M, Stamm WE. Nosocomial infection of the urinary tract. *Am J Med* 1981; **70**: 651–4.
3 Kaye D. Urinary tract infection in the elderly. *Bull NY Acad Med* 1980; **56**: 209–20.
4 Crotti D, Peratoner G. The presence of antimicrobial potential in urine samples and its interference in the evaluation of bacteriuria. *Quad Sclavo Diagn* 1982; **18**: 327–32.
5 Abu Shaqra QM. Antimicrobial activity in urine: effect on leukocyte count and bacterial culture results. *New Microbiol* 2001; **24**: 137–42.
6 Millar MR, Langdale P. Simple microbiological method for the identification of antimicrobial agents prescribed in general practice. *J Clin Microbiol* 1985; **21**: 741–4.
7 Wu JJ, Chien ML, Lee N. Microbiologic assay for detection of antimicrobial agents in urine. *J Formos Med Assoc* 1996; **95**: 464–8.
8 Buick RK, Greer NM, Elliot CT. A microtitre plate assay for the detection of antibiotics in porcine urine. *Analyst* 2000; **125**: 395–6.
9 Mackey JP, Sandys GH. Diagnosis of urinary tract infections. *BMJ* 1966; **1**: 1173–4.
10 Ellner PD, Stoessel CJ, Drakeford E, Vasi F. A new culture medium for medical bacteriology. *Am J Clin Path* 1966; **45**: 502–4.
11 Mueller JH, Hinton J. A protein-free medium for primary isolation of the Gonococcus and Meningococcus. *Proc Soc Exp Biol Med* 1941; **48**: 330–3.
12 Leigh DA, Williams JD. Method for the detection of significant bacteriuria in large groups of patients. *J Clin Path* 1964; **17**: 498–503.
13 Kaye D. Antibacterial activity of human urine. *J Clin Invest* 1968; **47**: 2374–90.
14 Lum KT, Meers PD. Boric acid converts urine into an effective bacteriostatic transport medium. *J Infect* 1989; **18**: 51–8.
15 Hindman R, Tronic B, Bartlett R. Effect of delay on culture of urine. *J Clin Microbiol* 1976; **4**: 102–3.
16 Lewis JF, Alexander JJ. Overnight refrigeration of urine specimens for culture. *South Med J* 1980; **73**: 351–2.
17 Brodie J, Porter IA. Boric-acid preservation of urine samples. *Lancet* 1971; **16**: 133.
18 Kass EH. Bacteriuria and the diagnosis of infections of the urinary tract; with observations on the use of methionine as a urinary antiseptic. *AMA Arch Intern Med* 1957; **100**: 709–14.
19 Hoeprich P. Culture of the urine. *J Lab Clin Med* 1960; **56**: 899.
20 Arason VA, Kristinsson KG, Sigurdsson JA *et al*. Do antimicrobials increase the carriage rate of penicillin-resistant pneumococci in children? Cross sectional prevalence study. *BMJ* 1996; **313**: 387–91.
21 Baquero F. Antibiotic resistance in Spain: what can be done? Task Force of the General Direction for Health Planning of the Spanish Ministry of Health. *Clin Infect Dis* 1996; **23**: 819–23.
22 Kunin CM, Liu YC. Excessive use of antibiotics in the community associated with delayed admission and masked diagnosis of infectious diseases. *J Microbiol Immunol Infect* 2002; **35**: 141–6.
23 Nicolle LE. Resistant pathogens in urinary tract infections. *J Am Getiatr Soc* 2002; **50** (Suppl l) 230–5.
24 Goettsch W, van Pelt W, Nagelkerke N *et al*. Increasing resistance to fluoroquinolones in *Escherichia coli* from urinary tract infections in The Netherlands. *J Antimicrob Chemother* 2000; **46**: 223–8.
25 Cizman M, Orazem A, Krizan-Hergouth V, Kolman J. Correlation between increased consumption of fluoroquinolones in outpatients and resistance of *Escherichia coli* from urinary tract infections. *J Antimicrob Chemother* 2001; **47**: 502.