Inoculum standardisation device for the EUCAST disk-diffusion method

Support for antimicrobial susceptibility methodology is changing, and laboratories face testing times in this important area of practice. Here, Andrew Ferguson and Rahila Chaudhry assess different approaches.

In the clinical microbiology laboratory, antibiotic susceptibility testing (AST) is most often carried out in order to determine which antibiotics can be used to treat specific bacterial infections. It helps to assure susceptibility to drugs of choice for known bacteria and can detect possible drug resistance development in commonly encountered pathogens.[1]

The laboratory AST procedure is extremely important for individual patients, but on a much wider scale it has become essential due to recent rapid developments of antimicrobial resistance. In terms of antibiotic stewardship, AST is an extremely important part of the treatment selection process.[2] According to the World Health Organization, new resistance mechanisms are constantly emerging and pose increasingly serious threats to global public health. Without effective antimicrobial therapies, it is believed that many routine medical interventions will fail or become extremely dangerous to perform in the future. Currently, 700,000 people die of antimicrobial-resistant infections every year, and by 2050 this is predicted to rise to 10 million (Fig 1). It is also estimated that US$100 trillion could be lost due to the rise in drug-resistant infections.[3] With huge potential public health and global financial pressures being threatened, the magnitude of the resistance problem is now beginning to be accepted and tackled.[3]

The most commonly used method in UK laboratories was the British Society for Antimicrobial Chemotherapy (BSAC) technique; however, the BSAC is ceasing active support of its method, and laboratories are being encouraged to move to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) disk-diffusion technique.

All differences between the two methods to aid transition can be found in the BSAC *Difference between BSAC-EUCAST methods document*,[4] as outlined in Table 1.

With the arrival of ISO standard 15189:2012 in UK laboratories, there has been an increased emphasis on standardisation and compliance within traditional microbiology methods. This has been particularly apparent in AST due to the subjective nature of manual zone reading, inconsistent use of density checks, and the perishable nature of agar plates. This causes some difficulty in measurement of uncertainty (ISO15189:2012 5.5.1.4) and traceability

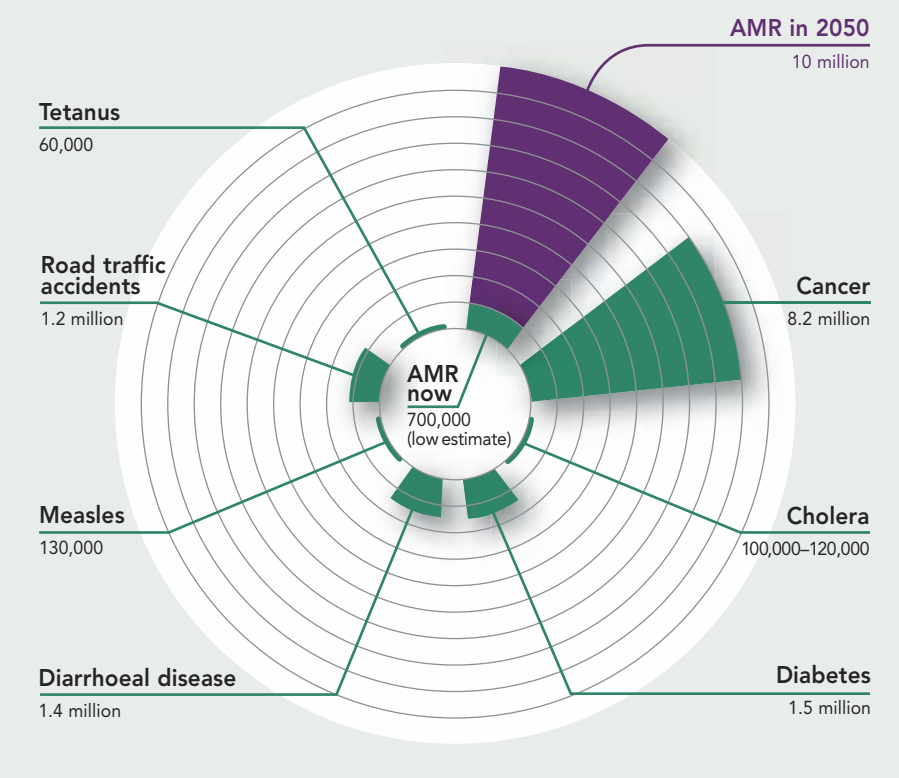

Fig 1. Current and predicted death rate from resistant infections every year.[3]

(ISO15189:2012 5.3.1.4). Devices such as Inoclic, which claim to ensure standardisation of inoculum, calibrated rulers and automated zone reading systems such as SIRscan can assist laboratories in achieving compliance.

At the Queen Elizabeth Hospital Birmingham (QEHB), the manual disk-diffusion method is utilised for fastidious organisms requiring CO_2 (eg *Neisseria gonorrhoeae*), when flexibility in the choice of antibiotics is required, or when there is a problem performing AST on the automated VITEK 2 system (bioMérieux). The main aim of this project was to internally verify, in accordance with ISO15189:2012, the EUCAST disk-diffusion technique by comparing AST categorisation results against those obtained for the BSAC disk-diffusion technique at QEHB. An additional aim for this study was to carry out an internal evaluation of the Inoclic device (Pro-Lab Diagnostics) by comparing results obtained using Inoclic with those obtained using the EUCAST disk-diffusion technique.

Standardisation

All fresh purity plates were obtained by subculturing from primary (mother) plates. All clinical isolates had their identification confirmed by matrix-assisted laser desorption/ionisation–time of flight (MALDI-TOF) mass spectrometry, the VITEK MS system (bioMérieux), prior to any AST being carried out.

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Test conditions were required to be standardised for all techniques to ensure the testing variable was limited to the BSAC, EUCAST or Inoclic disk-diffusion techniques. VITEK DensiCHEK plus (bioMérieux) was used throughout this study to check suspension densities.

Analysis

All non-concordant results were placed into one of four error categories:[5]

- **Very major** (false susceptible): categorised as sensitive, but resistant with reference method.
- **Major** (false resistant): categorised as resistant, but sensitive with reference method.
- **Minor**: categorised as resistant or sensitive, but intermediate with reference method.
- **Minor**: categorised as intermediate, but sensitive or resistant with reference method.

Although BSAC and EUCAST zone sizes would not be directly comparable due to specific differences in their methodologies, the categorisation of results produced should be directly concordant. Zone diameters were measured and interpreted as sensitive, intermediate or resistant according to relevant published clinical breakpoint tables. Where zone diameter reference data were not yet available – EUCAST have some in development – or when disk diffusion was not recommended, minimum inhibitory concentration (MIC) results were obtained using antibiotic gradient test strips; this allowed an interpretable result to be obtained. Each result categorisation obtained for EUCAST was compared to the reference result obtained for BSAC. Each result categorisation obtained for Inoclic was compared to the reference result obtained for EUCAST.

Results overview

Result categorisations were obtained for 11 quality control strains (Pro-Cult, Pro-Lab Diagnostics) and 100 different clinical isolates using BSAC, EUCAST and Inoclic techniques. Specified recommended quality control strains shown in Table 2 were used to include both susceptible and resistant strains.

Table 1. Summary of the key differences between BSAC and EUCAST.

Variable		Differences	
		BSAC	EUCAST
Media for non-fastidious organisms		IsoSensitest Agar (ISTA)	Muller Hinton Agar (MHE)
Media for fastidious organisms		IsoSensitest Agar + 5% defibrinated horse blood + 20 mg/L ß–NAD (Blood ISTA)	Muller Hinton Agar + 5% mechanically defibrinated horse blood + 20 mg/L ß–NAD (MHF)
General summary of different antibiotic disk strengths (µg)	Inoculum confluence	Semi-confluent	Confluent
	Inoculum preparation	Sterile water	Sterile saline
	Ampicillin	10	2
	Nitrofurantoin	200	100
	Erythromycin	5	15
	Tetracycline	10	30
	Gentamicin	200	30
	Rifampicin	2	5
	Cefuroxime	5	30
	Ciprofloxacin	1	5
	Pip/Tazobactam	85	36
	Ceftazidime	30	10
	Trimethoprim	2.5	5
	Cefoxitin	10	30
Incubations conditions	Non-fastidious organisms	36±1˚C in air for 19±1h	35±1˚C in air for 16–20h
	Fastidious organisms	36±1˚C in 4–6% CO_2 for 19±1h	35±1˚C in 5% CO_2 for 16–20h

Table 2. Control organisms tested.

Quality control organism (Pro-Cult, Pro-Lab)	Type strain	Product code
Pseudomonas aeruginosa	NCTC12903/ATCC27853	PLD10
Staphylococcus aureus (MRSA)	NCTC13373/ATCC43300	PLD91
Haemophilus influenzae	NCTC12699/ATCC49247	PLD81
Streptococcus pneumoniae	NCTC12977/ATCC49619	PLD95
Neisseria gonorrhoeae	NCTC12700/ATCC49226	PLD96
Haemophilus influenzae	NCTC12975/ATCC49766	PLD37
Staphylococcus aureus	NCTC12973/ATCC29213	PLD14
Streptococcus pyogenes	NCTC12696/ATCC19615	PLD20
Escherichia coli	NCTC12241/ATCC25922	PLD02
Enterobacter aerogenes	NCTC10006/ATCC13048	PLD26
Enterococcus faecalis	NCTC12697/ATCC29212	PLD18

These quality control strains were used to ensure that the methodology would detect resistance mediated by known resistance mechanisms.

A total of 2439 AST result categorisations were obtained for both BSAC and EUCAST techniques. For the Inoclic technique, 2442 AST result categorisations were obtained. There were slight differences in the amount of comparable result categorisations due to the fact that a few isolates lacked adequate growth.

Overall, out of 2439 comparable antibiotic results obtained, BSAC and EUCAST techniques produced 2362 (1664 QC results, 698 clinical sample results) concordant AST result categorisations (concordance level 96.84%). 77/2439 BSAC and EUCAST AST categorisation results did not show concordance (non-concordance level 3.16%). A comparison between EUCAST and Inoclic revealed that out of 2453 comparable antibiotic results, 2427 (1693 QC results, 734 clinical sample) were concordant results (concordance level 98.94%). 26/2453 EUCAST and Inoclic AST categorisation results did not show concordance (non-concordance level 1.06%).

Pseudomonas species

Of the 19 *Pseudomonas* spp. isolates that showed non-concordance, 17 were classed as minor category errors, while two were classed as major category errors. The major errors were both categorised as sensitive using the BSAC technique and resistant using the EUCAST technique. Using the Inoclic technique these same isolates were resistant, with colonies being present within a formed zone of inhibition. As the same MHE medium was used for the EUCAST and Inoclic techniques, this suggests increased expression of heteroresistance when using MHE agar.

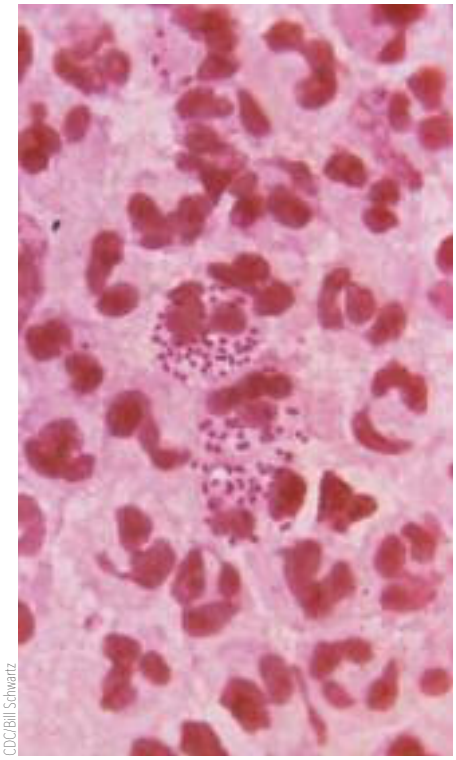
When comparing EUCAST with Inoclic, all 380 *Pseudomonas* spp. isolates showed results concordance.

Staphylococcus species

Two results that showed non-concordance (rifampicin categorised as intermediate using BSAC but sensitive using EUCAST) would both be classed as minor category errors and were very close to the S/I cut-off value. A comparison of result categorisations for *Staphylococcus* spp. using EUCAST and Inoclic techniques revealed 100% concordance.

Haemophilus influenzae

One antibiotic that showed non-concordance was co-amoxiclav, which was categorised as sensitive using the BSAC technique but resistant using the EUCAST technique. This result was classed as a major category error. However, the zones of inhibition measured were very close to the cut-off S/R clinical interpretation breakpoint, and, as there is no intermediate category for co-amoxiclav, a very minor difference in recorded zone size could result in a very major categorisation error. When comparing EUCAST with Inoclic, the six non-concordant results were due to no growth using the Inoclic technique.



CDC/Bill Schwartz

The fastidious organism *Neisseria gonorrhoeae* proved to be the most problematic during the study.

Streptococcus species

One antibiotic that showed non-concordance was tetracycline, which was categorised as sensitive using the BSAC technique but intermediate using the EUCAST technique. This discrepancy would be classed as a minor category error and once again the measured zones of inhibition were on the border of the S/I clinical breakpoint cut-off value. Comparing result categorisations for *Streptococcus* species using EUCAST and Inoclic revealed that the 220 control strain results showed 100% concordance. Of the 110 clinical isolates, 104 showed concordance (94.55%). Non-concordant results were due to one clinical isolate failing to grow.

Neisseria gonorrhoeae

Neisseria gonorrhoeae was the most problematic organism in this study. All 26 non-concordant result categorisations were classed as minor category errors. Of the 133 control strain result categorisations compared using the EUCAST and Inoclic techniques, 119 were concordant (89.47%). The 14 that showed non-concordance were all due to no growth using the EUCAST technique. If the 'no growth' results were removed from the study, there would have been 100% concordance between EUCAST

Table 3. Results data.

QC organisms	ATCC No	BSAC *vs.* EUCAST	Concordance (%)	EUCAST *vs.* Inoclic	Concordance (%)
P. aeruginosa	27853	200/200	100.00	200/200	100.00
S. aureus	43300	160/160	100.00	160/160	100.00
H. influenzae	49247	120/120	100.00	114/120	95.00
S. pneumoniae	49619	120/120	100.00	120/120	100.00
N. gonorrhoeae	49226	104/119	87.39	119/133	89.47
H. influenzae	49766	120/120	100.00	120/120	100.00
S. aureus	29213	160/160	100.00	160/160	100.00
S. pyogenes	19615	100/100	100.00	100/100	100.00
E. coli	25922	240/240	100.00	240/240	100.00
E. aerogenes	13048	240/240	100.00	240/240	100.00
E. faecalis	29212	100/120	83.33	120/120	100.00
QC concordance		1664/1699	97.94%	1693/1713	98.83%
Clinical samples		**BSAC *vs.* EUCAST**	**Concordance (%)**	**EUCAST *vs.* Inoclic**	**Concordance (%)**
Pseudomonas spp. (non-mucoid)		82/90	91.00	90/90	100.00
Pseudomonas spp. (mucoid)		79/90	87.78	90/90	100.00
S. pneumoniae		59/60	98.33	59/60	98.33
S. aureus (inc. MRSA)		70/70	100.00	70/70	100.00
Enterococcus spp.		55/60	91.70	60/60	100.00
Staphylococcus spp. (ex. *S. aureus*)		68/70	97.14	70/70	100.00
N. gonorrhoeae		59/70	84.29	70/70	100.00
Streptococcus spp. (ß-haemolytic)		50/50	100.00	45/50	90.00
Enterobacteriaceae (inc. *E. coli*)		117/120	97.50	120/120	100.00
H. influenzae		59/60	98.33	60/60	100.00
Clinical concordance		698/740	94.32%	734/740	99.19%
Overall total concordance		2362	96.84%	2427	98.94%

and Inoclic, with all 70 clinical samples being concordant.

Enterobacteriaceae

Three result categorisations that showed non-concordance were categorised as sensitive for BSAC but resistant for EUCAST. All would have been classed as major category errors. The result discrepancies once again produced colonies within a zone of inhibition; the colonies within the zones were checked and confirmed to be resistant. When comparing the EUCAST and Inoclic techniques, all 480 control strain results and all 120 clinical samples showed results concordance (100%).

***Enterococcus* species**

Of the non-concordant control strain results, all 20 were classed as minor category errors. Of the 60 results obtained for clinical samples, 55 were concordant (91.67%). Four of the five were classed as minor category errors, and one was classed as a major category error (sensitive using BSAC and resistant using EUCAST). All non-concordant results were observed with quinupristin-dalfopristin, which suggests a simple discrepancy between BSAC and EUCAST breakpoint tables. Comparing EUCAST with Inoclic, all 120 results obtained for the *Enterococcus faecalis* (ATCC 29212) control strain were concordant (100%). All 60 result categorisations obtained for clinical samples were concordant (100%).

In summary

The results obtained in this study allowed the EUCAST method to be verified against the currently used BSAC disk-diffusion technique. The data obtained (Table 3) also suggest that the Inoclic inoculum standardisation device is a robust and reliable method for routine laboratory use.

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