Antibiotic Resistance Profile of Unknown Bacteria from Selective Agar Plates

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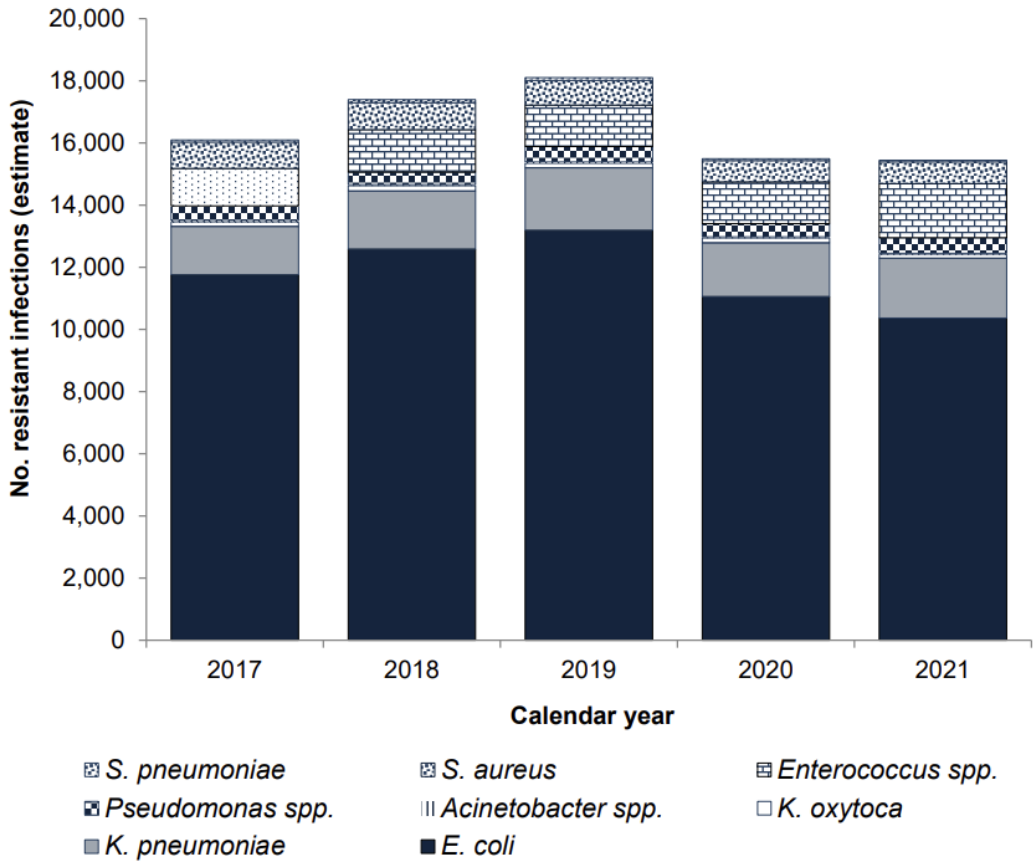
Introduction

Antibiotics are crucial for treating infections, especially in individuals with weakened immune systems. However, widespread availability and increased travel have led to the emergence of antibiotic-resistant bacteria, a global issue causing about 25,000 deaths annually in Europe. Resistance occurs when bacteria can survive in the presence of an antibiotic that typically inhibits them, rendering that antibiotic ineffective for treatment. (Sabtu et al., 2015).

Figure 1, from The UK Health Security Agency (2022), illustrates recent antibiotic-resistant rates in England. Solent NHS Trust (2023) implemented measures to combat Methicillin-resistant Staphylococcus aureus (MRSA) spread, reporting no MRSA bloodstream infections since 2013.

Figure 1: A graph reported by The UK Health Security Agency (2022) depicting the estimated number of antibiotic-resistant bloodstream infections in England between 2017 and 2021.



There are different mechanisms of action for antibiotics. Table 1 shows the methods of those used in this practical.

Table 1: A table to show the mechanism of action for the antibiotics used within the disk diffusion practical.

Antibiotic	Category of Antibiotic	Method of Action
Ampicillin	β-lactam	Inhibit Cell Wall Synthesis
Ofloxacin	Fluoroquinolone	Inhibit Nucleic Acid Synthesis
Rifampicin	Rifamycin	Inhibits Bacterial RNA Synthesis
Ciprofloxacin	Fluoroquinolone	Inhibit Nucleic Acid Synthesis
Tetracycline	Tetracycline	Inhibit Protein Synthesis
Streptomycin	Aminoglycoside	Inhibit Protein Synthesis

Resistance can be intrinsic or acquired through genetic mutations or genetic transfer (Sabtu et al., 2015). There are 4 mechanisms for antimicrobial resistance: limiting uptake of the drug, modifying a drug target, inactivating a drug, and active drug efflux. Gram negative bacteria, such as *Escherichia coli* identified in practical report 1, intrinsically limit the uptake of hydrophilic antibiotics such as glycopeptides due to the reduced permeability of their outer membrane (Reygaert, 2018).

Vancomycin-resistant *Staphylococcus aureus* (VRSA) exemplifies acquired resistance by altering a drug target. The *vanA* gene cluster, transferred from vancomycin-resistant *Enterococci* (VRE) to *Staphylococcus aureus*, changes peptidoglycan precursors crucial for vancomycin binding (Reygaert, 2018). Gene transfer methods include transformation, conjugation, and transduction (Burmeister, 2015). Given vancomycin's significance in treating MRSA, VRSA poses challenges in determining the optimal treatment (Cong et al., 2020).

Various methods, like epsilometer testing, MALDI-TOP MS, and PCR, are crucial for measuring antibiotic resistance, ensuring patients receive the appropriate treatment. This practical's goal is to evaluate antibiotic resistance in 4 known and 2 unknown samples using the disk diffusion method (Khan et al., 2019).

Method

Health and safety and personal protective equipment (PPE) measures were followed, including the use of gloves and a lab coat. Because of the infectious nature of the samples, precautions were taken when handling them. The COSHH evaluations for the substances use were also considered. Lowery (2023) provided the procedure for this practical.

The plates prepared in practical 1 for each sample were utilised in this practical. A sterile loop was used to transfer at least 4 colonies from each plate into separate tubes of Iso-Sensitest broth and mixed. The turbidity of the bacterial suspensions was then compared against a 0.5 McFarland standard to ensure that the concentration of the bacteria in the broth was acceptable. A micropipette was used to transfer 0.2ml of bacterial suspension onto an agar plate. A disposable tip was used and discarded after use. A plastic spreader was used to spread the bacterial suspension across the surface of the agar. Spreading was continued until the plate began to feel dry. The plastic spreader was then disposed of. This was repeated for all the samples. There were 6 samples in total- 4 known and 2 unknowns. A dispenser was used to plate 6 antibiotic disks onto each plate. Forceps were used to ensure the disks were correctly positioned. This includes ensuring they are not too close together or too close to the edges and that they are fully touching the agar surface. The plates were then incubated for 24 hours at 37°C. The zones of inhibition were then measured.

Results

Four control samples were used in this practical and the results were compared against ranges supplied by European Committee on Antimicrobial Susceptibility Testing (2023). The results compared with the ranges are shown in table 2.

A ruler was used to measure the zones of inhibition thrice and average was calculated. Table 3 shows the results of these samples compared with breakpoint ranges for each antibiotic disk used denoting if the sample is resistant (R) or susceptible (s). The ranges are supplied by European Committee on Antimicrobial Susceptibility Testing (2023). In practical 1, unknown sample A was identified as possibly *Streptococcus pyogenes* and sample B was identified as possible *Staphylococcus aureus*. This information was used to select the breakpoint ranges.

The European Committee on Antimicrobial Susceptibility Testing (EUCAST) do not provide control and breakpoint ranges for every antibiotic and organism. In these cases, the tables have been left blank, apart from *Bacillus subtilis* where EUCAST recommend using the ranges supplied for *Staphylococcus aureus*. The results for *Bacillus subtilis* indicate that this control did not work as expected as the zone of inhibition is not within the stated ranges. This could be due to the ranges being nonspecific, but it could also be down to error. It is also noted that there were two colonies growing within the zone of inhibition of the rifampicin disk. This could be due to not placing the disks properly or contamination. All other controls were within their given range.

Table 2: A table to show the zones of inhibition for each control sample used compared to the ranges supplied by European Committee on Antimicrobial Susceptibility Testing (2023)

	Ciprofloxacin CIP 5			Tetracycline TE 30			Streptomycin S 25			Ampicillin AMP 10			Ofloxacin OFX 5			Rifampicin RD 5		
Sample	Zone of Inhibition (mm)	Range (mm)	Control in range?	Zone of Inhibition (mm)	Range (mm)	Control in range?	Zone of Inhibition (mm)	Range (mm)	Control in range?	Zone of Inhibition (mm)	Range (mm)	Control in range?	Zone of Inhibition (mm)	Range (mm)	Control in range?	Zone of Inhibition (mm)	Range (mm)	Control in range?
Pseudomonas aeruginosa	31	25-33	Yes	6	n/a	n/a	22	n/a	n/a	16	n/a	n/a	29	n/a	n/a	0	n/a	n/a
Staphylococcus aureus	21	21-27	Yes	26	23-31	Yes	16	n/a	n/a	35	n/a	n/a	23	21-27	Yes	26	30-36	Yes
Bacillus subtilis *	31	21-27	No	19	23-31	No	26	n/a	n/a	26	15-21	no	27	21-27	Yes	13	30-36	No
Escherichia coli	33	29-37	Yes	17	n/a	n/a	19	n/a	n/a	16	15-22	Yes	29	29-33	Yes	10	n/a	n/a

Table 3: A table to compare the results from the disk diffusion test against breakpoint ranges supplied by European Committee on Antimicrobial Susceptibility Testing (2023) to determine if the bacteria is resistance or susceptible to the antibiotics tested.

	Ciprofloxacin CIP 5			Tetracycline TE 30			Streptomycin S 25			Ampicillin AMP 10			Ofloxacin OFX 5			Rifampicin RD 5		
Sample	Zone of Inhibition (mm)	Breakpoint Ranges (mm)	Result	Zone of Inhibition (mm)	Breakpoint Ranges (mm)	Result	Zone of Inhibition (mm)	Breakpoint Ranges (mm)	Result	Zone of Inhibition (mm)	Breakpoint Ranges (mm)	Result	Zone of Inhibition (mm)	Breakpoint Ranges (mm)	Result	Zone of Inhibition (mm)	Breakpoint Ranges (mm)	Result
Unknown A (Possible *Streptococcus pyogenes)*	23	n/a n/a	n/a	3	S ≥ 23 R < 23	R	14	n/a n/a	n/a	11	S ≥ 18 R < 18	R	22	n/a n/a	n/a	27	S ≥ 21 R < 21	S
Unknown B (Possible *Staphylococcus aureus)*	19	S ≥ 50 R < 21	R	22	S ≥ 22 R < 22	S	13	n/a n/a	n/a	28	n/a n/a	n/a	19	n/a n/a	n/a	2	S ≥ 26 R < 26	R

Discussion

Most controls in this experiment were as expected, aligning with the European Committee on Antimicrobial Susceptibility Testing (2023) ranges. This suggests a successful procedure, ensuring result quality. However, *Bacillus subtilis* results contradict this, with many zones of inhibition outside the specified range. Presence of two colonies in one disk's inhibition zone, as per Schwalbe et al. (2007), may result from mixed populations during bacterial isolation, influencing control results. To validate, re-isolation and repetition of the experiment are needed, highlighting a drawback in the manual aspect of this test.

In the previous gram stain practical, sample B suggested *Staphylococcus*, a gram-positive cocci bacterium. For such infections, fluoroquinolones, especially ofloxacin, are not the preferred initial antibiotic, as per the European Committee on Antimicrobial Susceptibility Testing (2023). Ofloxacin breakpoints for *Staphylococcus* are removed due to inferiority compared to other fluoroquinolones. Rising Staphylococcus resistance, particularly to ciprofloxacin and ofloxacin, makes them less suitable as first-line choices (Sharma et al., 2007). Fluoroquinolones are generally more effective against gram-negative than gram-positive bacteria (Cruciani & Bassetti, 1994).

Unknown sample A was identified as a possible *Streptococcus* infection. The European Committee on Antimicrobial Susceptibility Testing (2023) have reported the breakpoints for ciprofloxacin and ofloxacin as a dash (-). This means that these antibiotics are unsuitable to treat this infection. Due to this, the disk diffusion test could be improved in future by opting to test infections against antibiotics that would be relevant to the patient’s treatment.

Another way this test could be improved is by testing pairs of antibiotics that can be beneficial when resulted together. For example, if *Staphylococcus aureus* results as susceptible to benzylpenicillin and cefoxitin, it can be inferred that it is susceptible to

all penicillin-type antibiotics. If resistant to benzylpenicillin but susceptible to cefoxitin, it can be inferred that it is susceptible to β-lactam β-lactamase inhibitor combinations, isoxazolylpenicillins, and nafcillin. Therefore, testing these two antibiotics together could give more information. One strength of this test is that it can be easily manipulated to include the most relevant antibiotics for the bacteria.

Psirides (2020), in the Wellington ICU Drug Manual, provides a chart displaying antibiotic innate susceptibilities against various bacteria, revealing their spectra. Many penicillins exhibit broad spectra, effective against both gram-positive and negative bacteria. For instance, amoxicillin, closely related to ampicillin, demonstrates broad-spectrum activity. The chart also highlights fluoroquinolones' wide spectrum, however second-generation types, such as ciprofloxacin and ofloxacin, are better suited for gram-negative bacteria. Rifampicin is noted for its efficacy against gram-negative bacteria. These antibiotics might not have been best suited in this disk diffusion test since the unknown samples were identified as gram-positive.

Other methods are also available for antibiotic susceptibility testing. This includes the etest. The etest can test a wider range of antibiotics compared to disk diffusion and supplies a quantitative result of susceptibility which solves the issue of human error in interpretation in disk diffusion. However, it can be more expensive (Mayrhofer et al., 2008). Both these tests have a long incubation period. Tests have been conducted to attempt to improve this such as work done by Webber et al. (2022) which has shown incubation for 6 hours in a disk diffusion test, as opposed to 24 hours, may be possible.

Reference

- Burmeister, A.R. (2015) 'Horizontal gene transfer: Figure 1.', *Evolution, Medicine, and Public Health*, 2015(1), pp. 193–194. doi:10.1093/emph/eov018.
- Cong, Y., Yang, S. and Rao, X. (2020) 'Vancomycin resistant Staphylococcus aureus infections: A review of case updating and clinical features', *Journal of Advanced Research*, 21, pp. 169–176. doi:10.1016/j.jare.2019.10.005.
- European Committee on Antimicrobial Susceptibility Testing (2023a) *Breakpoint tables for interpretation of MICs and zone diameters*. Available at: https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_13.1_Breakpoint_Tables.pdf (Accessed: 14 November 2023).
- European Committee on Antimicrobial Susceptibility Testing (2023) *Routine and extended internal quality control for MIC determination and disk diffusion as recommended by EUCAST*. Available at: https://www.eucast.org/ast_of_bacteria/quality_control (Accessed: 14 November 2023).
- Khan, Z.A., Siddiqui, M.F. and Park, S. (2019) 'Current and emerging methods of antibiotic susceptibility testing', *Diagnostics*, 9(2), p. 49. doi:10.3390/diagnostics9020049.
- Lowery, C. (2023) 'Practical Manual'.
- Mayrhofer, S. *et al.* (2008) 'Comparison of broth microdilution, Etest, and agar disk diffusion methods for antimicrobial susceptibility testing of *lactobacillus acidophilus* group members', *Applied and Environmental Microbiology*, 74(12), pp. 3745–3748. doi:10.1128/aem.02849-07.
- Psirides, A. (2020) *Wellington ICU Drug Manual* [Preprint]. Available at: https://drug.wellingtonicu.com/PDF/WellingtonICUDrugManual.pdf (Accessed: 16 November 2023).
- Reygaert, W.C. (2018) 'An overview of the antimicrobial resistance mechanisms of bacteria', *AIMS Microbiology*, 4(3), pp. 482–501. doi:10.3934/microbiol.2018.3.482.
- Sabtu, N., Enoch, D.A. and Brown, N.M. (2015) 'Antibiotic resistance: What, why, where, when and how?', *British Medical Bulletin*, p. ldv041. doi:10.1093/bmb/ldv041.

- Schwalbe, R., Steele-Moore, L. and Goodwin, A.C. (2007) *Antimicrobial susceptibility testing protocols*. Boca Raton: CRC Press. Available at: https://books.google.co.uk/books?hl=en&lr=&id=FukzUi3sLvEC&oi=fnd&pg=PA53&dq=disk+diffusion+errors+and+troubleshooting&ots=VspW6J9afC&sig=XAVtmxP0D7Ava9GBQHPElw2fV2E&redir_esc=y#v=snippet&q=troubleshooting&f=false (Accessed: 16 November 2023).
- Sharma, S. *et al.* (2007) 'Activity of newer fluoroquinolones against gram-positive and gram-negative bacteria isolated from ocular infections: An in vitro comparison', *Indian Journal of Ophthalmology*, 55(1), p. 15. doi:10.4103/0301-4738.29489.
- Solent NHS Trust (2023) *Annual Reports and Accounts Incorporating the Quality Account 2022/23*. Available at: https://www.solent.nhs.uk/media/4409/annual-report-final-accounts-22-23-combined-final-230705.pdf (Accessed: 14 November 2023).
- UK Health Security Agency (2022) *English surveillance programme for antimicrobial utilisation and resistance (ESPAUR) Report 2021 to 2022*. Available at: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/1118310/ESPAUR-report-2021-to-2022.pdf (Accessed: 14 November 2023).
- Weber, D.M., Wallace, M.A. and Burnham, C.-A.D. (2022) 'Stop waiting for tomorrow: Disk Diffusion performed on early growth is an accurate method for antimicrobial susceptibility testing with reduced turnaround time', *Journal of Clinical Microbiology*, 60(5). doi:10.1128/jcm.03007-20.