GIG CYMRU NHS WALES

Iechyd Cyhoeddus Cymru

Public Health Wales

Update on EUCAST susceptibility testing

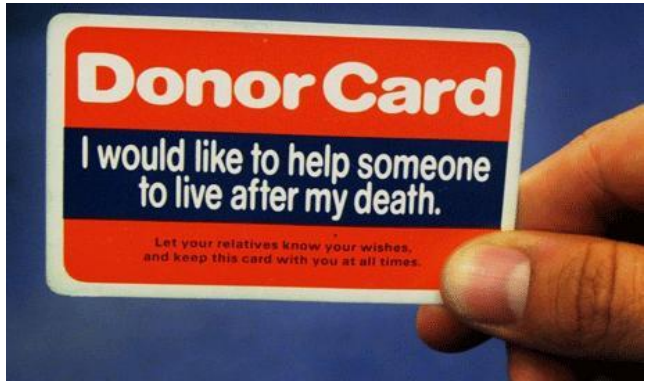
EUCAST EUROPEAN COMMITTEE ON ANTIMICROBIAL SUSCEPTIBILITY TESTING

European Society of Clinical Microbiology and Infectious Diseases

Dr Mandy Wootton

BSAC The British Society for Antimicrobial Chemotherapy

What antibiotics do for us!



The Problem with Infections

- 35,000 or 7% of all deaths in the UK are caused by infectious diseases.
- 66 different antibiotics prescribed - top 15 account for 98% in general practice and 88% in hospitals.
- 35 million courses of antibiotics are prescribed by GPs in England each year.
- Without antimicrobials, the rate of post-operative infection for clean surgery could be 0-50% and that about 30% of those with a serious infection will die.

So we have some resistant bacteria?

- **25,000** people per year in Europe die of sepsis caused by resistant bacteria

- **23,000** deaths per year from sepsis caused by resistant bacteria in United States
(conservative estimate)

- **1 child every 5 minutes** dies of infection caused by resistant bacteria in South East Asia

Increasing cost of AMR

Estimates of Burden of Antibacterial Resistance

European Union
population 500m

25,000 deaths per year

2.5m extra hospital days

Overall societal costs
(€ 900 million, hosp. days)
Approx. €1.5 billion per year

Source: ECDC 2007

Thailand
population 70m

>38,000 deaths

>3.2m hospital days

Overall societal costs
US$ 84.6–202.8 mill. direct
>US$1.3 billion indirect

Source: Pumart et al 2012

United States
population 300m

>23,000 deaths

>2.0m illnesses

Overall societal costs
Up to $20 billion direct
Up to $35 billion indirect

Source: US CDC 2013

From: World Health Organization: Global Report on Surveillance 2014.

Surveillance of resistance

Surveillance needs good quality, comparable susceptibility testing results

Harmonisation of breakpoints – we can now agree if something is resistant

Enterobactericeae 1975 - 2001

Committee	Amoxicillin	Cefotaxime	Piperacillin/ tazobactam
BSAC (UK)	8 / 16	2 / 2	16 / 16
CA-SFM (Fr)	4 / 16	4 / 32	8 / 64
CRG (NL)	2 / 16	4 / 8	0.25 / 4
DIN (Ger)	2 / 8	2 / 8	0.12 / 1
NWGA (Nor)	0.5 / 8	1 / 2	8 / 16
SRGA (Sw)	1 / 8	0.5 / 1	16 / 16
NCCLS (USA)	8 / 16	8 / 32	16 / 64

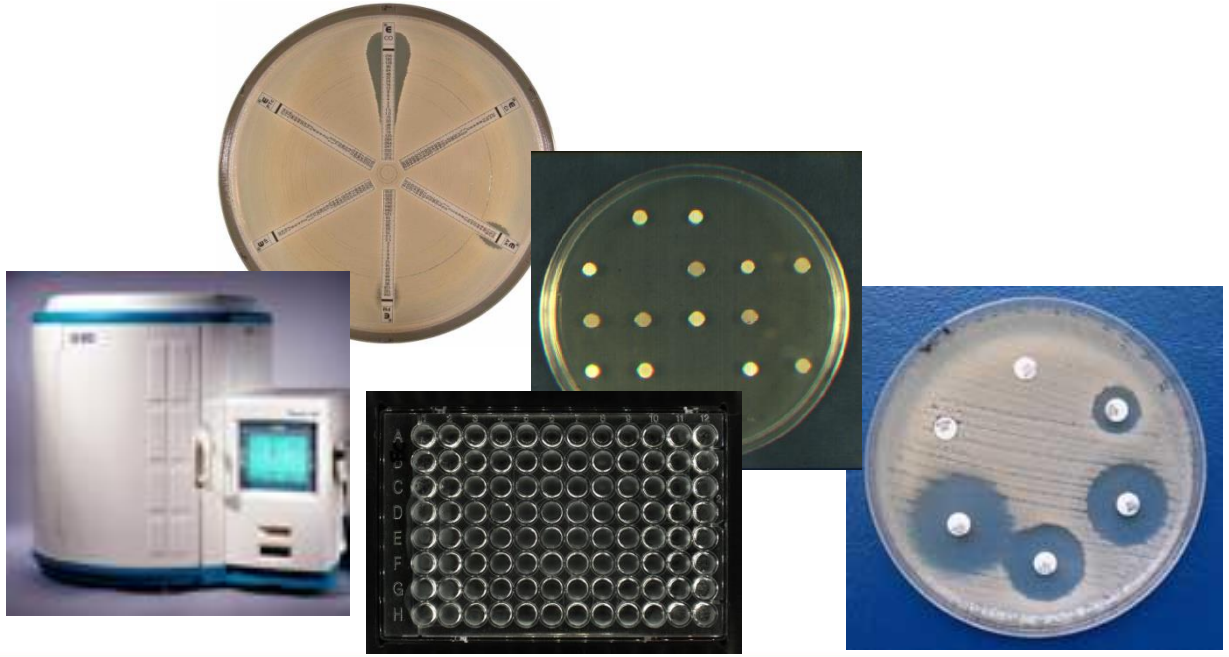
How to determine susceptibility/resistance

Minimum Inhibitory Concentration

- Macro broth dilution
- Micro broth dilution
- Agar dilution
- Gradient Strips
- Automated systems

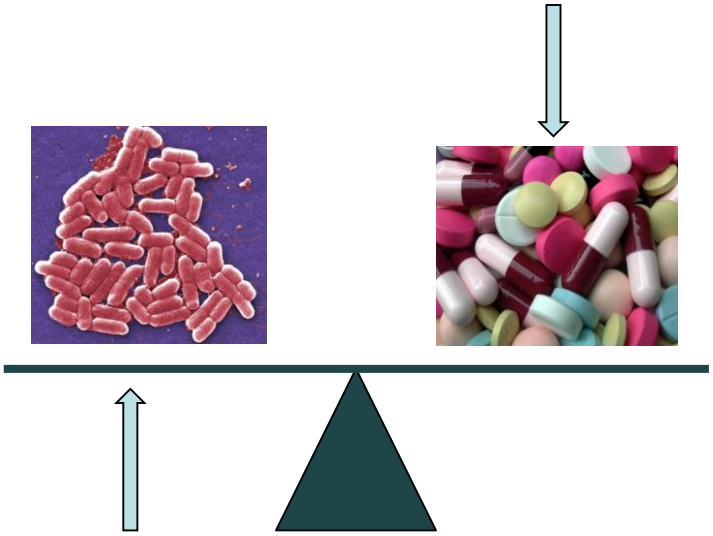
Category (S/I/R)

- Disc
- Breakpoint method



Standardisation

- Critical population of bacterial cells
  - Known inoculum
  - Defined media
- Defined media
  - Batch variation
  - pH
  - Gaseous environment

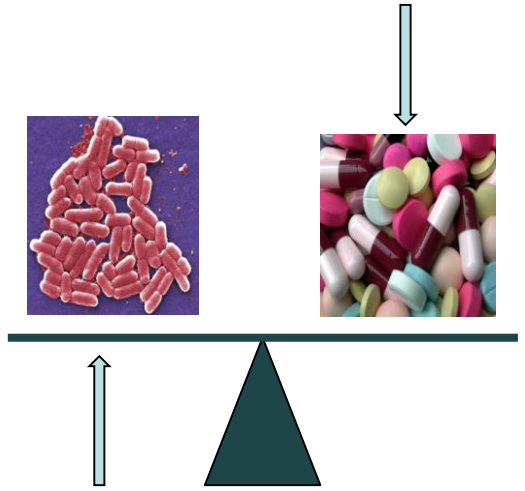


Drug	5.5	6.5	7.5	8.5
Streptomycin	+64	+16	1	-8
Gentamicin	+16	+4	1	-2
Tobramycin	+16	+8	1	-2
Amikacin	+32	+4	1	-2

Increased - Acidic	Increased - Alkaline	Decreased - Acidic
Amoxicillin	Amikacin	Azithromycin
Ampicillin	Clindamycin	Clindamycin
Cloxacillin	Metronidazole	Metronidazole
Doxycycline	Clarithromycin	
Minocycline	Erythromycin	
Nitrofurantoin	Nalidixic acid	
Piperacillin	Quinolones	
Tetracycline	Tobramycin	

Standardisation

- Defined media cont.
  - Cation concentration (Mg^{2+} & Ca^{2+})
  - Osmolarity (NaCl)
  - Thymine/thymidine
- Antimicrobial
  - Solvents with no antimicrobial activity
- Incubation temperature / time
  - Enterococci & glycopeptides
  - Staphylococci & cefoxitin



Trimethoprim and trimethoprim-sulfamethoxazole

- Follow the instructions for reading and read inner zones when double zones appear (see examples below).
- Ignore haze or faint growth up to the disk within a zone with otherwise clear zone edge.

Macrobroth dilution (tube dilution)

ON THE ANTIBACTERIAL ACTION OF CULTURES OF A PENICILLIUM, WITH SPECIAL REFERENCE TO THEIR USE IN THE ISOLATION OF *B. INFLUENZÆ*.

ALEXANDER FLEMING, F.R.C.S.

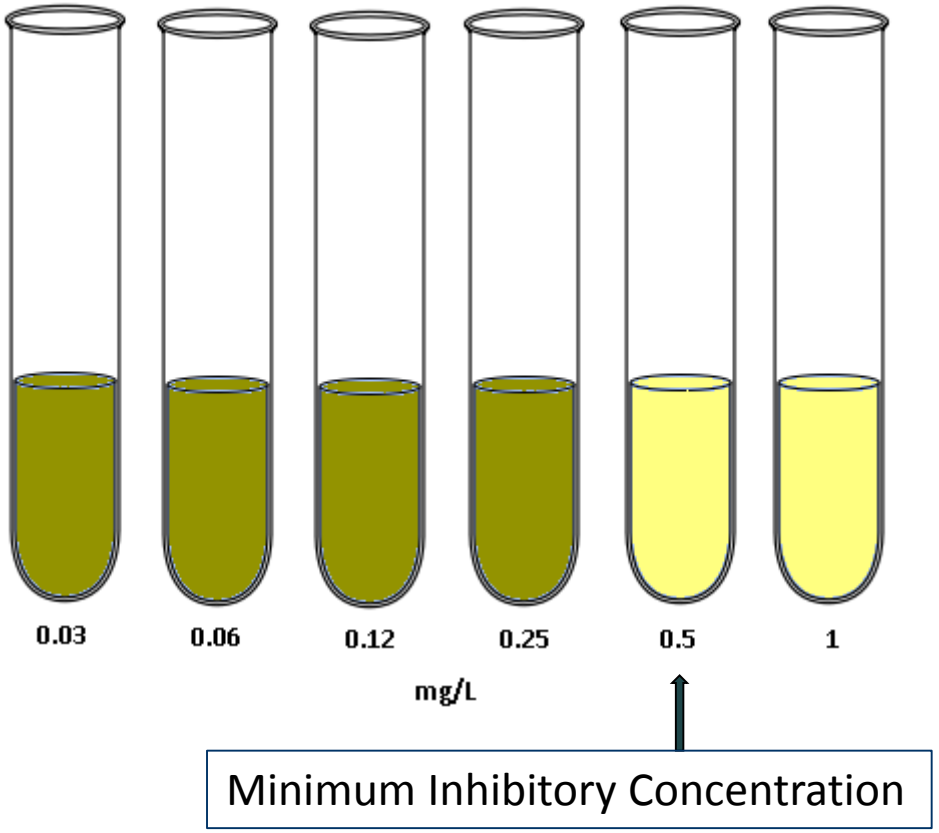
From the Laboratories of the Inoculation Department, St Mary's Hospital, London.

Received for publication May 10th, 1929.

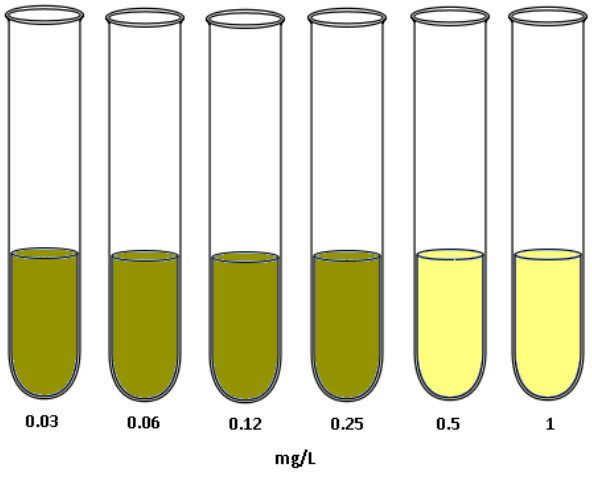
WHILE working with staphylococcus variants a number of culture-plates were set aside on the laboratory bench and examined from time to time. In the examinations these plates were necessarily exposed to the air and they became contaminated with various micro-organisms. It was noticed that around a large colony of a contaminating mould the staphylococcus colonies became transparent and were obviously undergoing lysis (see Fig. 1).

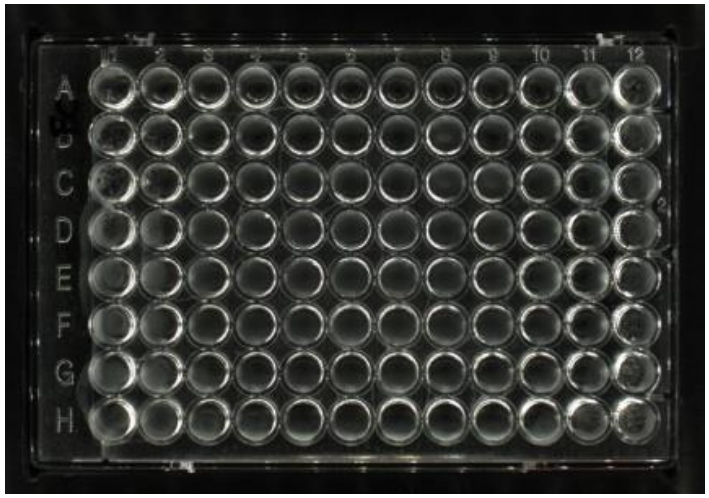
Macrobroth dilution (tube dilution)

- Broth media (1-2mL)
- Antimicrobial dilution: log2
  - Control tube
- Inoculum; direct or growth
  - touching 2 to 5 morphologically similar colonies
  - 0.5 McFarland (10^8); dilute to achieve 10^5
  - Control organisms
- Incubation
- Reading



Microbroth dilution





FINAL DRAFT

INTERNATIONAL STANDARD

ISO/FDIS 20776-1

ISO/TC **212**

Secretariat: **ANSI**

Voting begins on:
2006-07-13

Voting terminates on:
2006-09-13

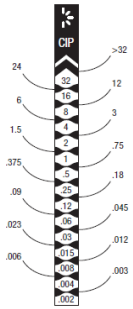
Clinical laboratory testing and *in vitro* diagnostic test systems — Susceptibility testing of infectious agents and evaluation of performance of antimicrobial susceptibility devices —

Part 1:
Reference methods for testing the *in vitro* activity of antimicrobial agents against bacteria involved in infectious diseases

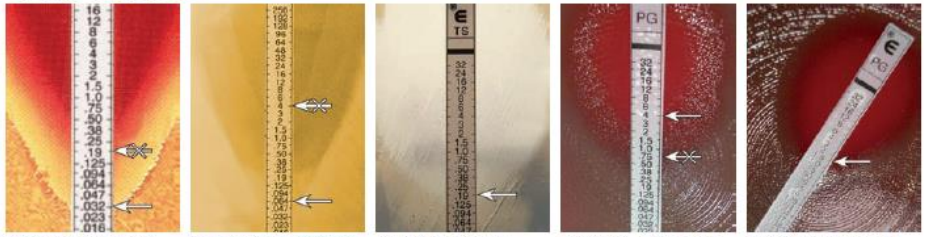
"Gold Standard"

Gradient strips

- Validated against MBD
- Easy to use
- Variety of manufacturers & antimicrobials
- QC
- Reading



ORGANISM EFFECTS



Ignore haemolysis (e.g. strep)
Read growth; **0.032** µg/mL

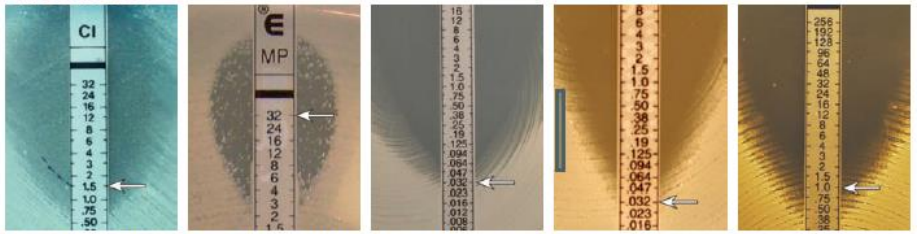
Ignore swarming (e.g. *Proteus* spp.)
Read growth edge; **0.064** µg/mL

S. maltophilia – trim/sulfa
Ignore haze in ellipse; **0.19** µg/mL

Pneumococci – ß-lactams, read all growth; **4** µg/mL

Pneumococci – ß-lactams, read haze/inner colonies; **1.5** µg/mL

DRUG EFFECTS



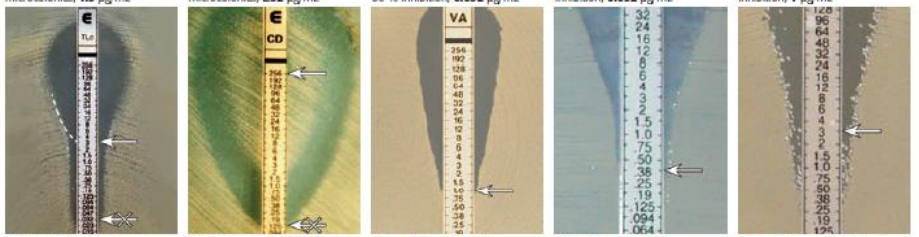
Bactericidal drugs – read hazes, microcolonies; **1.5** µg/mL

Bactericidal drugs – read macro/microcolonies; **≥32** µg/mL

Bacteriostatic drugs – read at 80 % inhibition; **0.032** µg/mL

Tigecycline – read at 80 % inhibition; **0.032** µg/mL

Linezolid – read at 90% inhibition; **1** µg/mL



Intrinsic activity, clavulanate
Extrapolate curve; **3** µg/mL

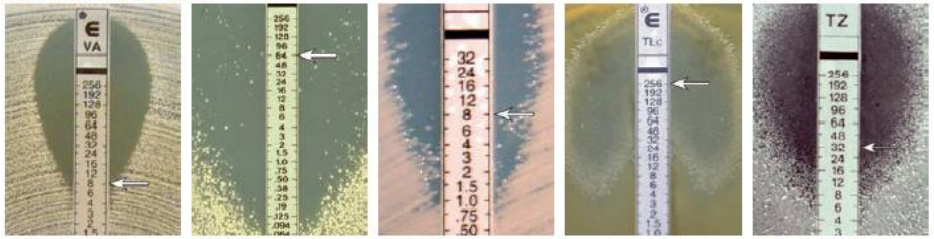
ß-lactams – paradoxical effect
Read all growth; **≥256** µg/mL

Glycopeptides – slim ellipse
Read end of dip; **1** µg/mL

Polypeptides – slim ellipse
Read bottom of dip; **0.38** µg/mL

Polypeptides – read colonies in the dip; **3** µg/mL

RESISTANCE EFFECTS



GISA/hGISA – vancomycin
Read all growth; **8** µg/mL

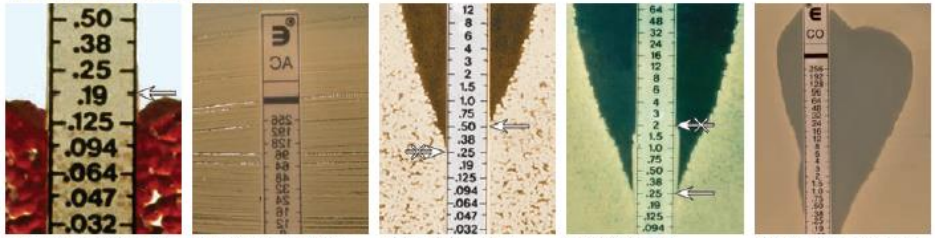
ORSA – oxacillin
Read all growth; **64** µg/mL

KPC – carbapenems
Read all colonies; **8** µg/mL

ß-lactamase induction by clavulanate; **≥256** µg/mL

Small colony variants – bactericidal drugs; **32** µg/mL

TECHNICAL AND HANDLING



Between markings – read upper value; **0.19** µg/mL

Etest strip placed upside down
Invalid; repeat the test

Uneven – read upper value; if >1 dilution, repeat the test

Ignore line of growth alongside strip; **0.25** µg/mL

Distorted ellipse – wet surface, invalid; repeat the test

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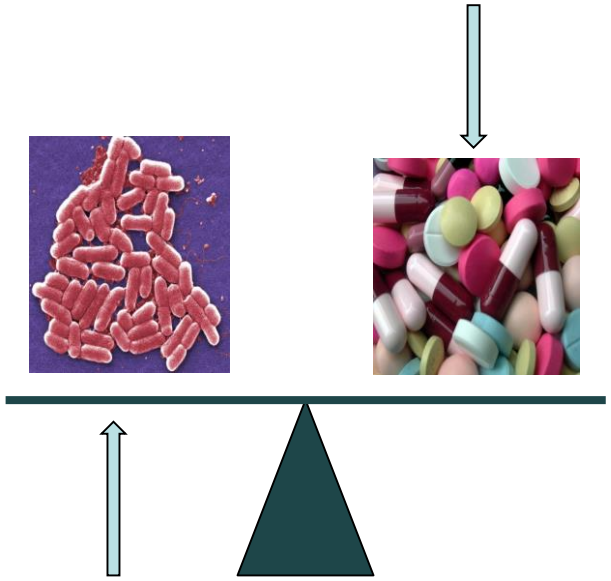
Automated AST systems

- More reliable/consistent results
  - Closer to standard
  - Reduced scope for error
  - Reproducible
- Easy to use
- Speed of results
  - 2-2.5 hrs for ID, 4-7hrs for AST
- Gram neg, Gram pos, Yeast panels
- Large range of antimicrobials

Disc diffusion method

- Most used method
- Cheap & easy to perform
- No MIC
- Correlated against MIC method
- Governed by three dynamics



Zone of inhibition formation:

Critical concentration: conc^{n} just capable of inhibiting growth & conc^{n} at zone edge at critical time

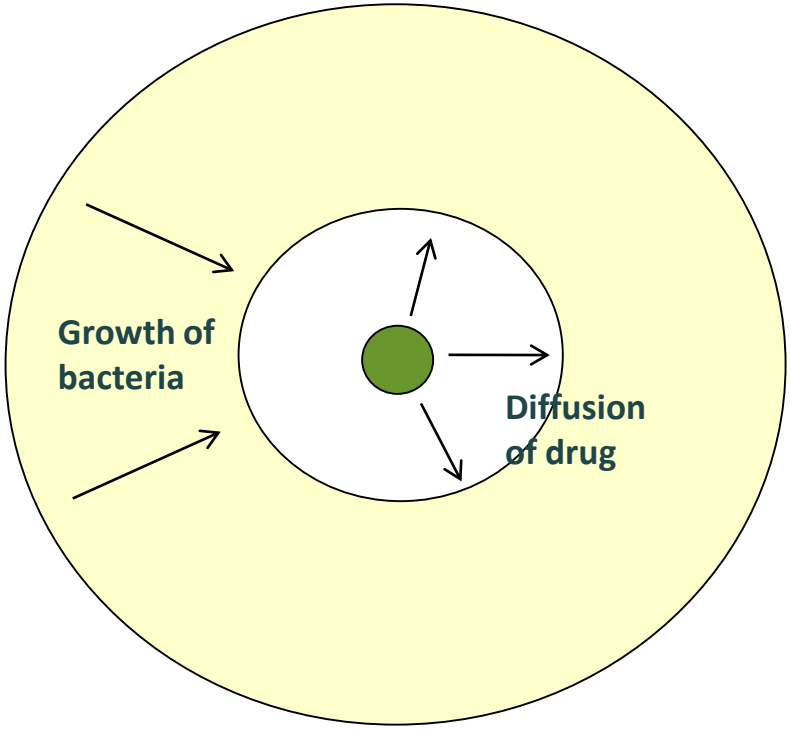
Critical time: time it takes for critical conc^{n} to be reached

Critical population: Number of bacterial cells found at the critical time at the ultimate zone edge

Disc diffusion method - Dynamics

Critical concentration

- Antimicrobial diffuses in a decreasing gradient
  - Diffusion rate
    - Depth of & osmolarity of agar
    - Initial concentration
    - Molecular size
    - Shape of drug
    - Charge of drug



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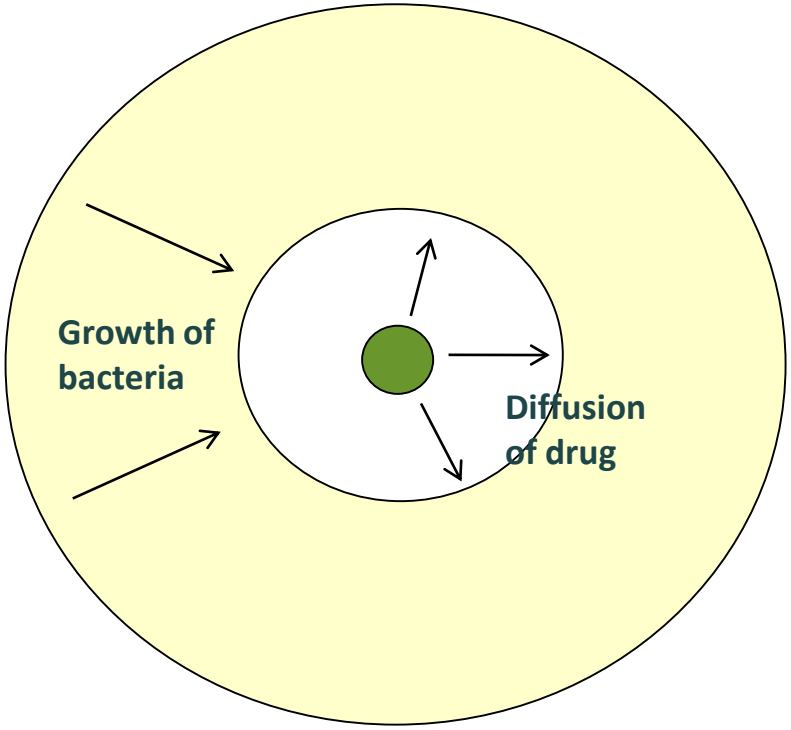
Disc diffusion method - Dynamics

Critical time

- 3 to 4 hours under standard conditions
- Standard: observable growth at 18hrs
- Longer for some bug:drug combinations

- Apply discs within 15-30mins after inoculation
- Incubate 15-30mins after application of discs



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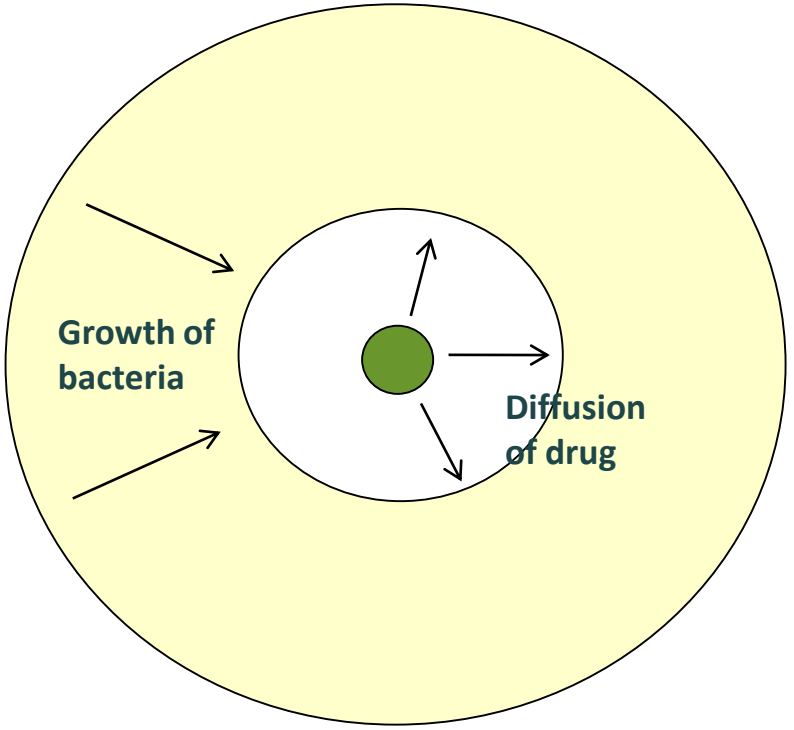
Disc diffusion method - Dynamics

Critical population

- Known inoculum
  - Too high: quicker to critical population, overwhelms antimicrobial so smaller zone (FALSE RESISTANCE)
  - Too low: slower to critical population, antimicrobial overwhelms bacteria (FALSE SUSCEPTIBILITY)
- Known lag phase & generation times
- Most antimicrobial work on dividing cells



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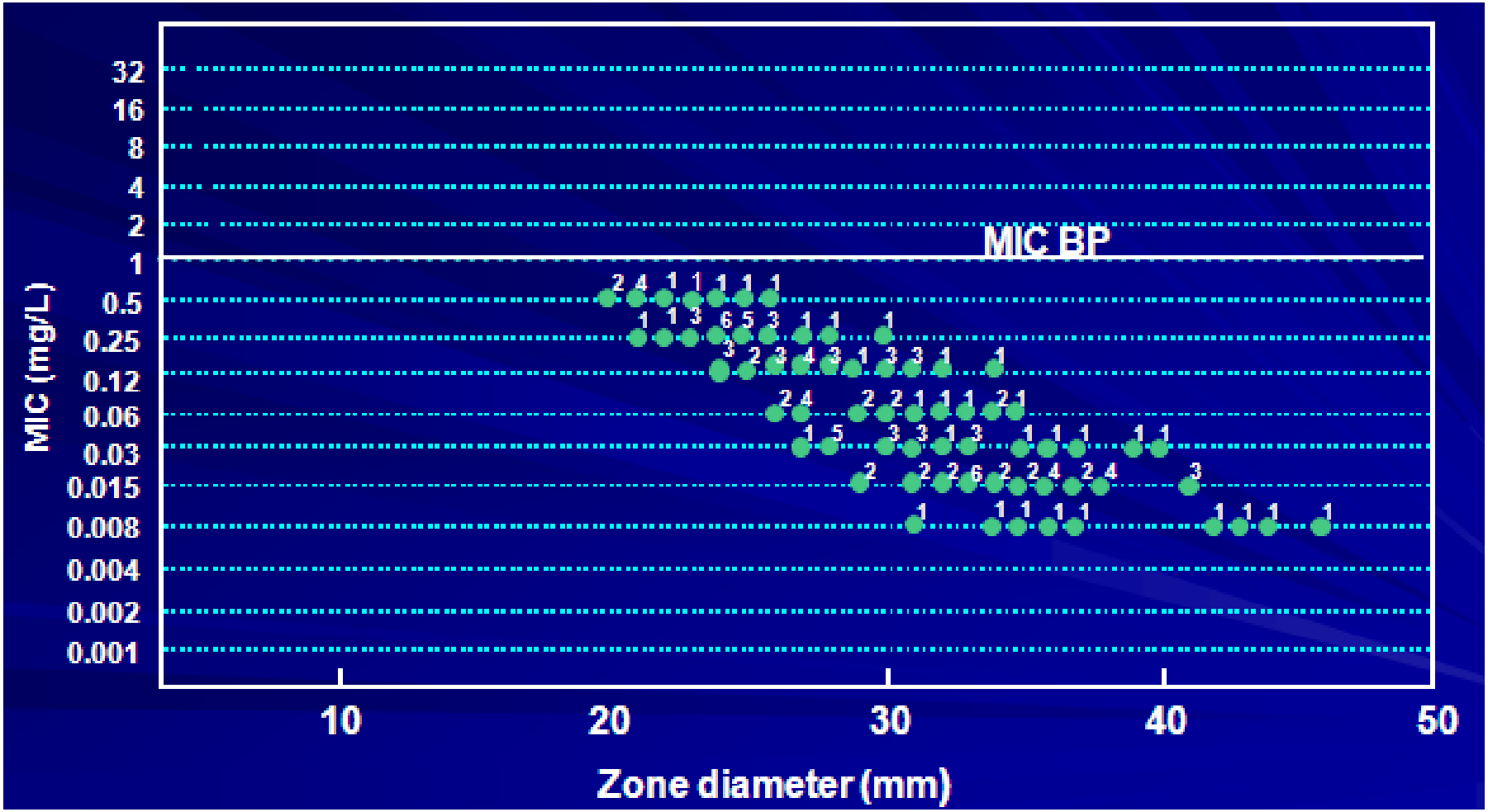
Disc diffusion method – correlation with MIC



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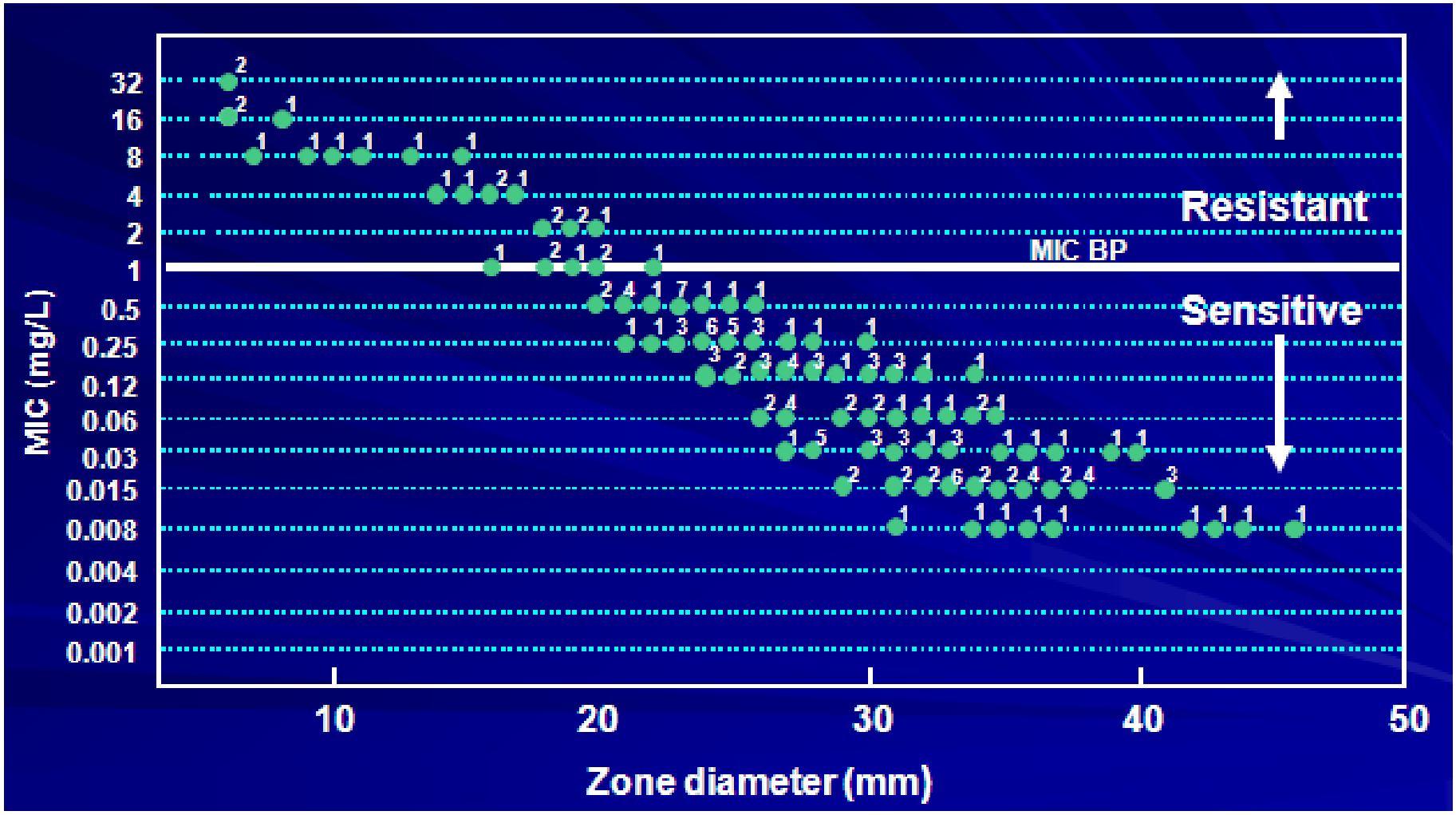

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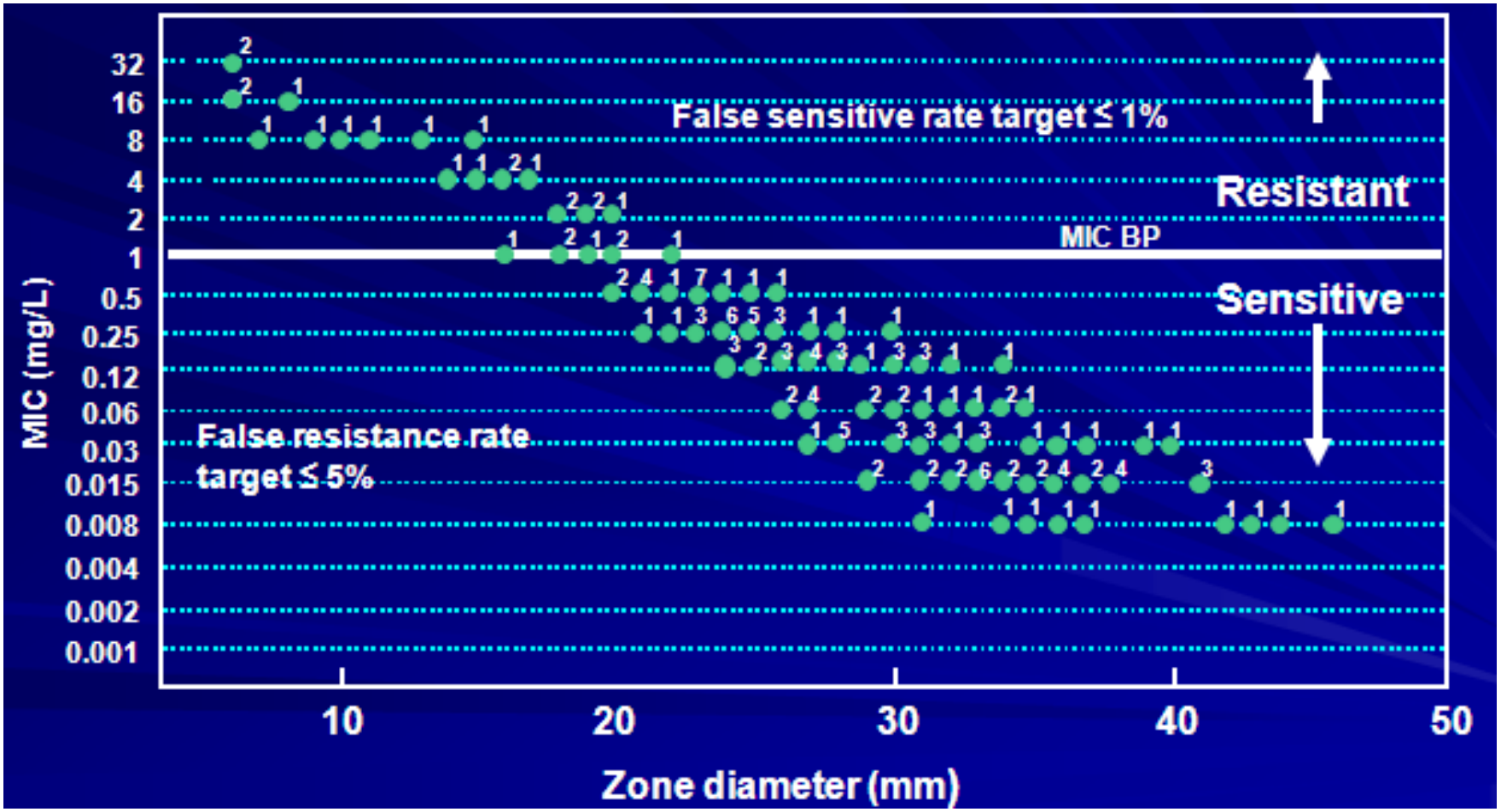
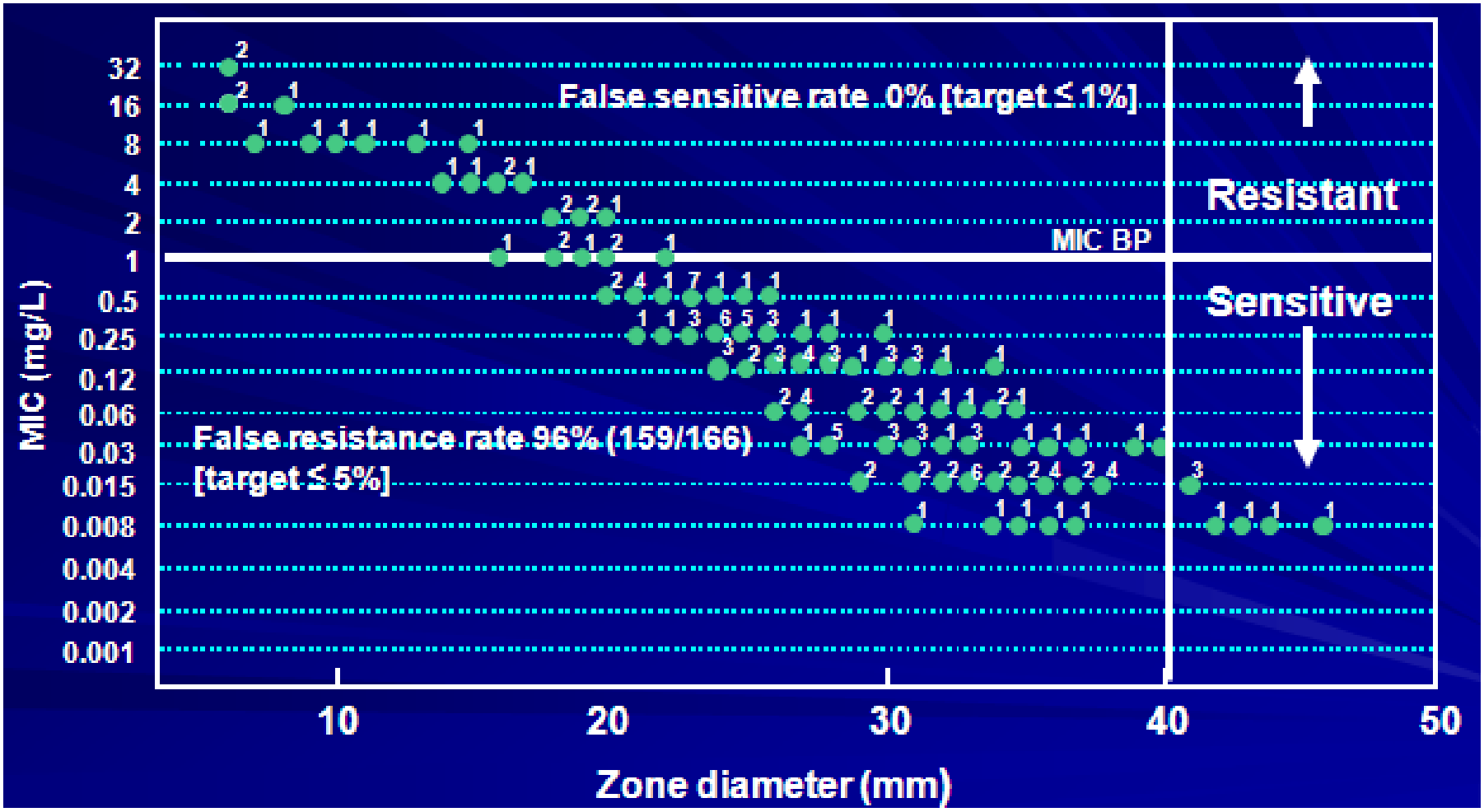
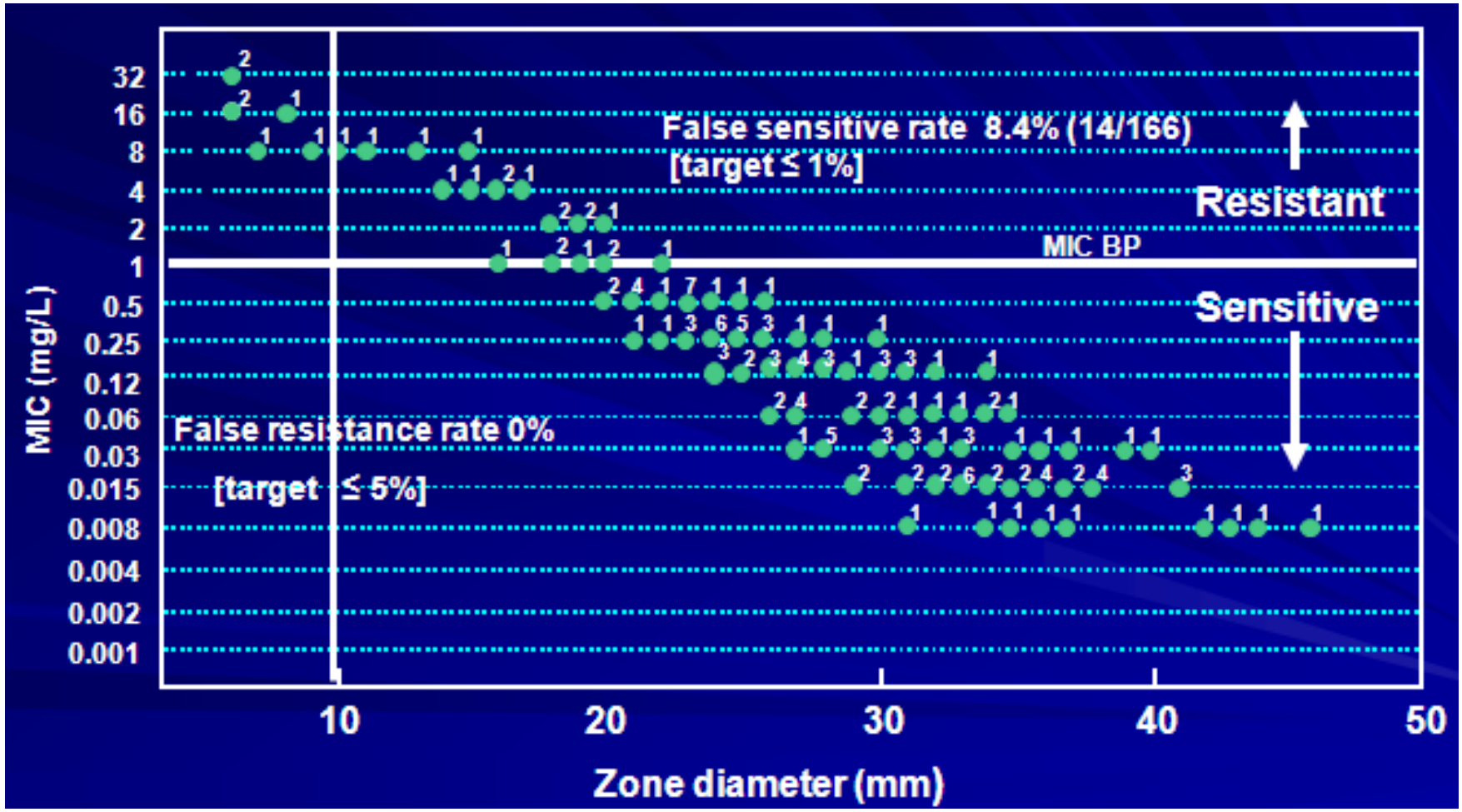


Disc diffusion method – correlation with MIC



Disc diffusion method – correlation with MIC



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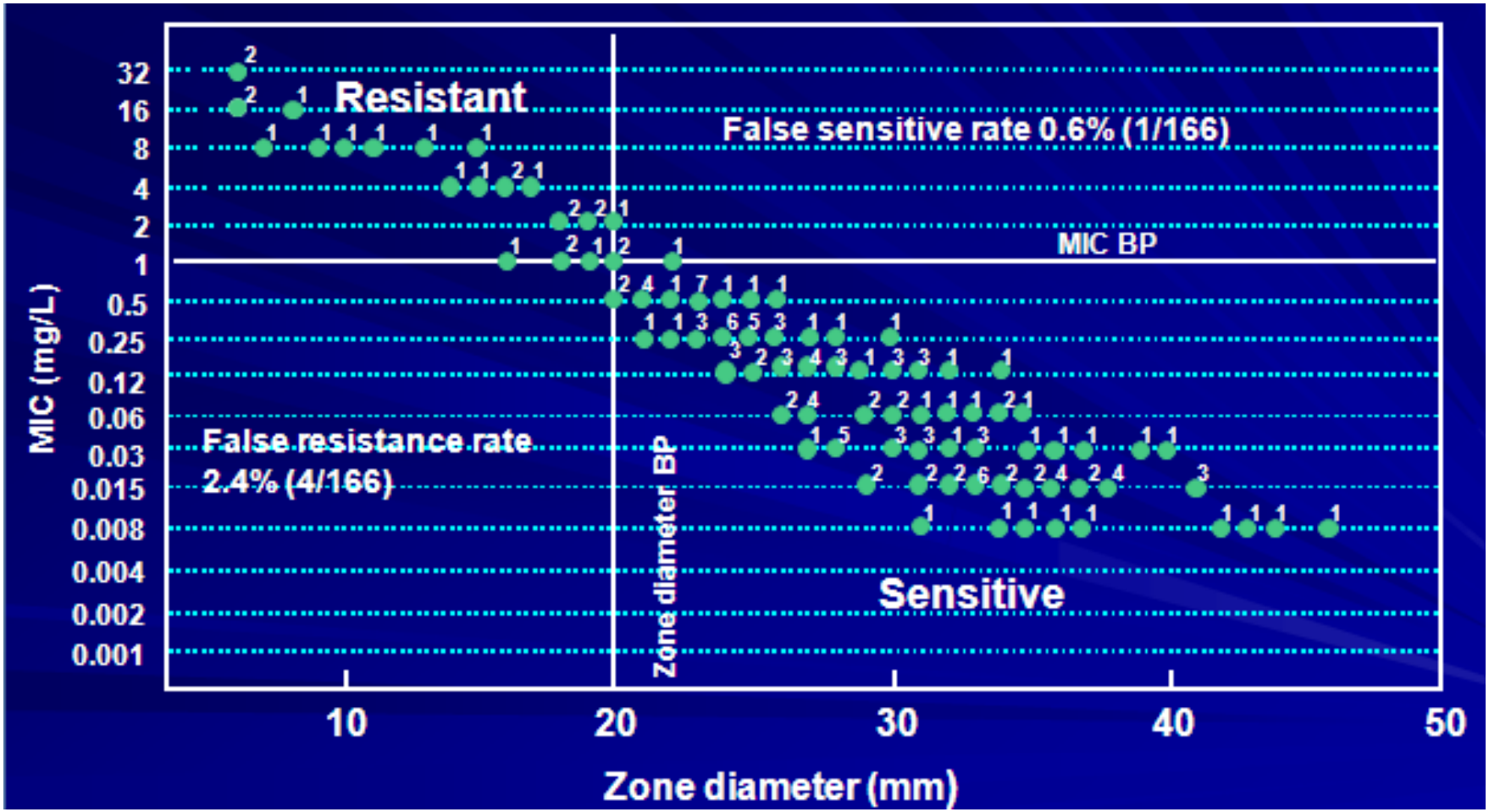


Disc diffusion method (BSAC)

- Antimicrobial in paper disc
- IsoSensitest agar (ISA) or ISA + 5% sheep blood + NAD
- Semi confluent inoculum by direct method
  - Touching 2-5 morphologically similar colonies
  - Dilute 0.5 McFarland

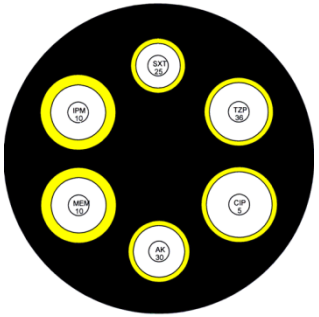
Dilute 1:100	Dilute 1:10	No dilution
β-Haemolytic streptococci	Staphylococci	*Neisseria gonorrhoeae*
Enterococci	*Serratia* spp.	*Campylobacter* spp.
Enterobacteriaceae	*Streptococcus pneumoniae*	
Pseudomonas spp.	*Neisseria meningitidis*	
Stenotrophomonas maltophilia	*Moraxella catarrhalis*	
Acinetobacter spp.	α-haemolytic streptococci	
Haemophilus spp.	*Clostridium perfringens*	
Pasteurella multocida	Coryneform organisms	
Bacteroides fragilis		
Bacteroides thetaiotaomicron		

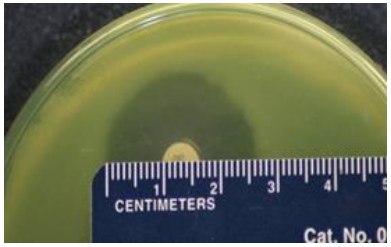
- Application of discs
- 15 minute rule

Disc diffusion method (BSAC)

- 18-20hr at 35-37˚ C in air
- VAN/TEIC resistance in enterococci: 24hr
- FOX resistance in Staphylococci: 35˚ C
- Campylobacter: 42˚ C microaerophilic for 24hr
- Fastidious orgs: 4-6% CO_2
- Anaerobes: 10% hydrogen/10%CO_2/80% Nitrogen

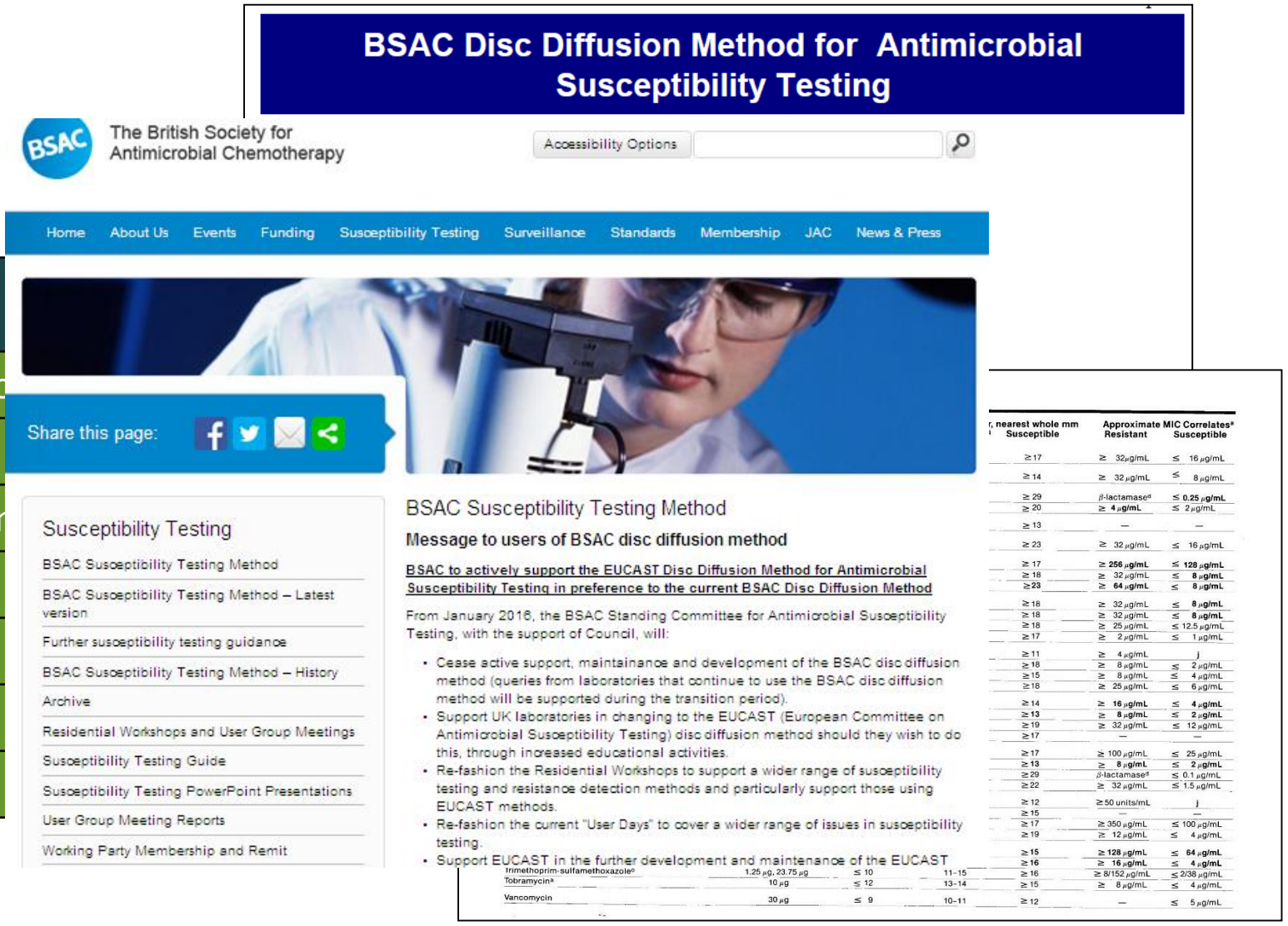
- Reading





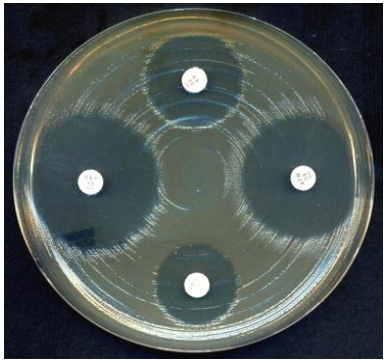
BSAC or EUCAST disc diffusion method

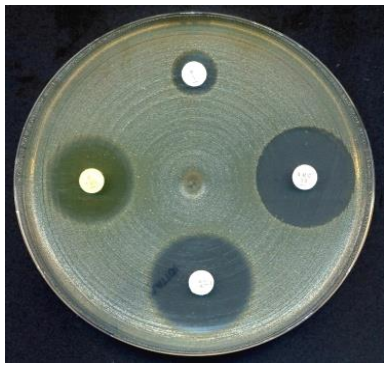
Committee	Country
BSAC	United Kingdo
CA-SFM	France
CRG	The Netherlar
DIN	Germany
NWGA	Norway
SRGA	Sweden
NCCLS (CLSI)	USA



EUCAST disk diffusion test

- Based on a well-known technique (Kirby-Bauer)
- Calibrated to EUCAST MIC breakpoints
- Methodology, breakpoints and QC criteria are freely available on the EUCAST website

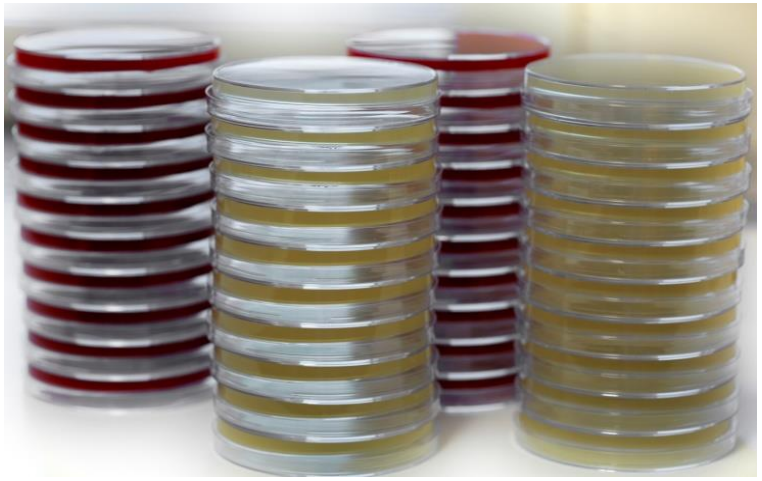





EUCAST susceptibility testing media

- **Mueller-Hinton agar (MH)**

  Enterobacteriaceae,
  Pseudomonas, staphylococci
  and enterococci

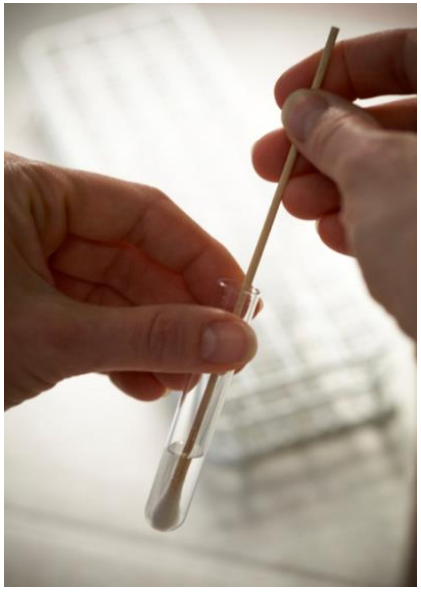


- **Mueller-Hinton agar with 5% defibrinated horse blood and 20 mg/L β-NAD (MH-F)**

  for fastidious organisms: *S. pneumoniae* and other streptococci, Haemophilus, Moraxella, Pasteurella, Listeria, Campylobacter, and Corynebacterium

Inoculum and incubation

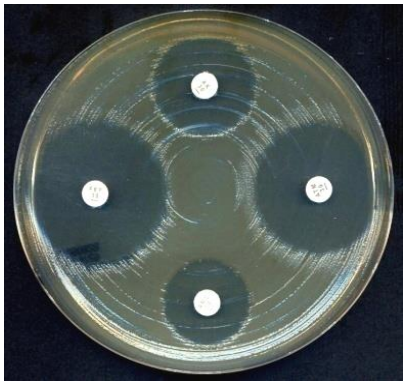
- The method requires an inoculum suspension equivalent to a 0.5 McFarland standard (0.85% saline)
- Inoculation should result in confluent growth without being too heavy
- Incubation for 16-20 h at 35±1ºC
  - MH plates in air
  - MH-F plates in 5% CO_2

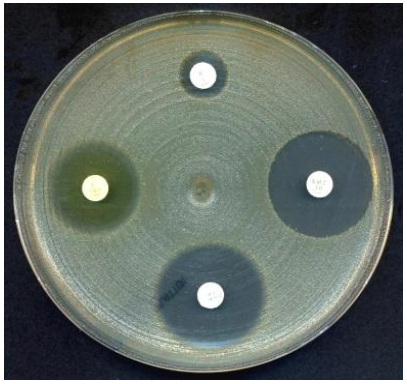


The 15-15-15 minute rule

- Use the inoculum within **15 minutes** of preparation – and always within 60 minutes
- Apply disks within **15 minutes** of inoculating plates
- Start incubation within **15 minutes** of application of disks

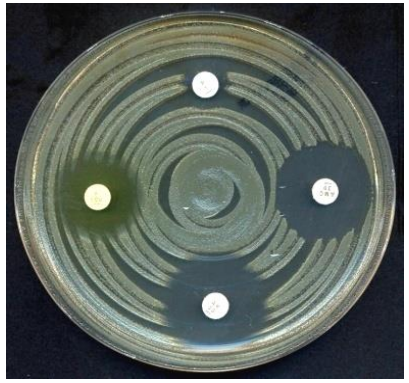
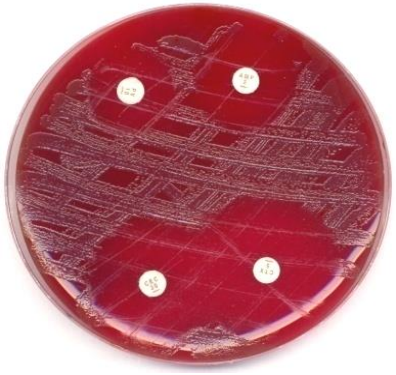
The growth should be confluent and evenly spread over the plate






Plates should look like this..



..and NOT like this!

Reading of zones

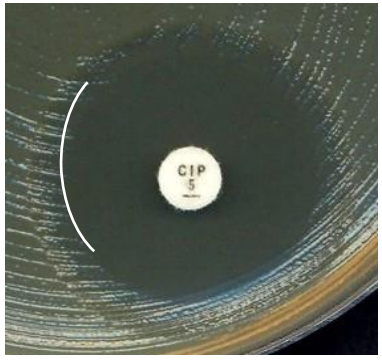
- MH plates
  Read zones from the back of the plate against a dark background and illuminated with reflected light

- MH-F plates
  Read zones from the front of the plate with the lid removed and illuminated with reflected light

Reading of zones

Read zone edges at the point where no obvious growth is detected by the unaided eye with the plate held about 30 cm from the eye.

Examples:



E. coli
Ciprofloxacin

S. aureus
Erythromycin

CoNS
Trimethoprim

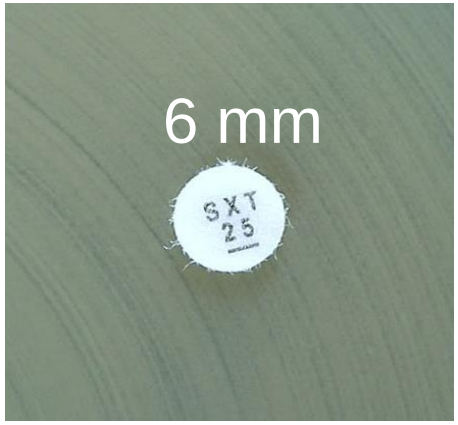
S. pneumoniae
Rifampicin

Reading guide available at www.eucast.org

Stenotrophomonas maltophilia and trimethoprim-sulfamethoxazole

- Ignore growth within the inhibition zone, which is common for *Stenotrophomonas maltophilia* and trimethoprim-sulfamethoxazole. The density of growth in the zone may vary from a fine haze to substantial growth.

Ignore growth and read an inhibition zone if any zone edge can be seen.
= Susceptible if zone diameter ≥ 16 mm



Growth up to the disk and no sign of inhibition zone = Resistant

Enterococci and vancomycin

- Examine with transmitted light (plate held up to light).
  - Fuzzy zone edges and colonies within zone indicate vancomycin resistance. If the zone diameter is ≥ 12 mm and the zone edge is fuzzy, investigate further.

E. faecalis
non-VRE

E. faecium
VRE

S. aureus and benzylpenicillin

- Examine with transmitted light (plate held up to light).
  - Disk diffusion is more reliable than MIC for detection of penicillinase producers, provided the zone diameter is measured AND the zone edge closely inspected.

sharp zone edge and
zone diameter ≥ 26 mm
= Resistant

fuzzy zone edge and
zone diameter ≥ 26 mm
= Susceptible

Developing breakpoints for new agents (with EMA)

- New glycopeptides
  - Dalbavancin (approved)
  - Oritavancin (approved)
- New oxazolidinone agent
  - Tedizolid (approved)
- β-lactam-inhibitor agents
  - Ceftolozane-tazobactam (in process)
  - Ceftazidime-avibactam (in process)
  - Carbapenem-inhibitor (in development)

Existing breakpoints under review

- Carbapenems
  - Imipenem, meropenem, ertapenem
  - Doripenem withdrawn in Europe
- Colistin
- Tigecycline for Enterobacteriaceae
- Fluoroquinolones

- Change in dosing or administration
- Change in indications
- Change in target organisms
- New clinical Pk/Pd data
- New resistance mechanisms
- New agents in class

Agents without EUCAST breakpoints and under consideration

- Temocillin
- Nitroxoline

} Expected 2016

Organisms with no breakpoints and EUCAST test

- Aerococcus spp.
- Kingella kingae

} On-going

- Nocardia spp.
- Aeromonas spp.
- Vibrio spp.
- Streptomyces spp.
- Leuconostoc spp.
- Lactobacillus spp.
- Pediococcus spp.
- Other HACEK organisms

} From 2016

<u>No disc method</u>

- Neisseria species
- Anaerobes

Aerococcus spp. and *Kingella kingae*
Disc diffusion

- EUCAST disk diffusion method for fastidious organisms
  - MH-F agar
  - McFarland 0.5
  - 5% CO_2
  - 16-20 h incubation
    - with possibility to prolong incubation to 40-44 h if growth is not-sufficient

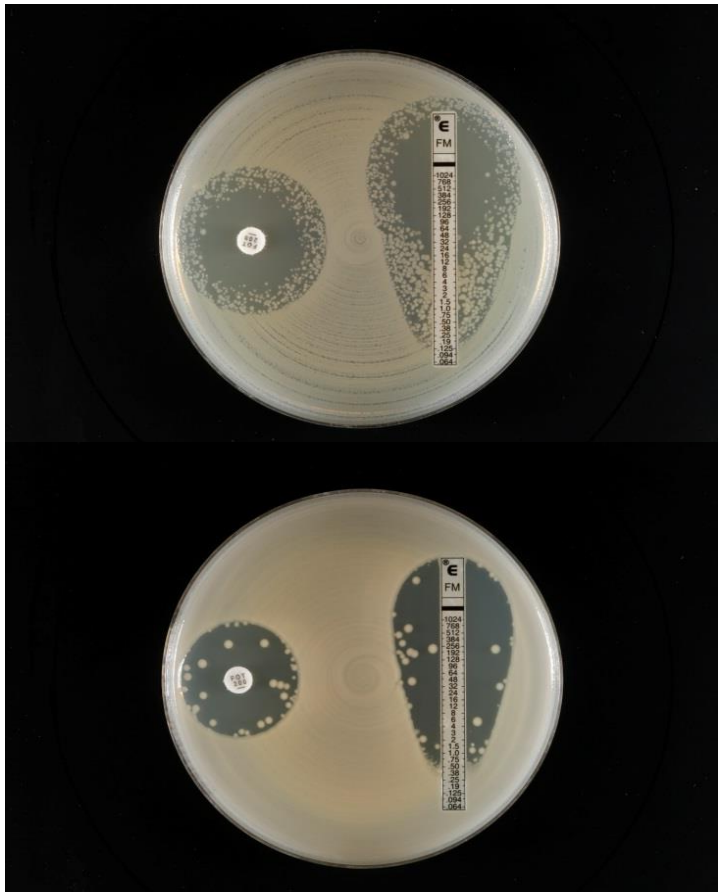
- Calibration vs. broth microdilution & agar dilution
  - MH-F broth / Mueller Hinton agar

Difficult tests: Fosfomycin

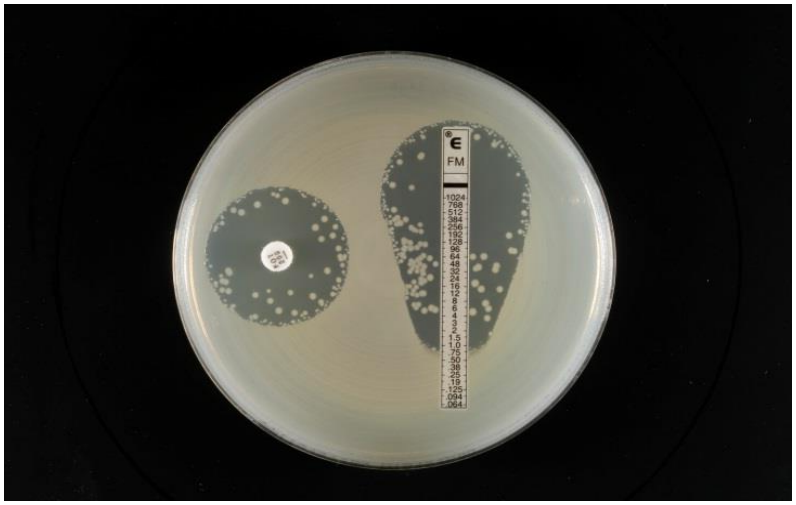
- Fosfomycin is difficult to test with all methods
  - Glucose-6-phosphate is added to improve results
- Agar dilution as reference
- Gradient tests
  - Etest: Disregard colonies if < 5
  - No specific reading instruction for MIC Test Strip
  - M.I.C.E not available
- No EUCAST disk test

AST results vs. WGS

”Resistance genes NOT likely, but results are uncertain”

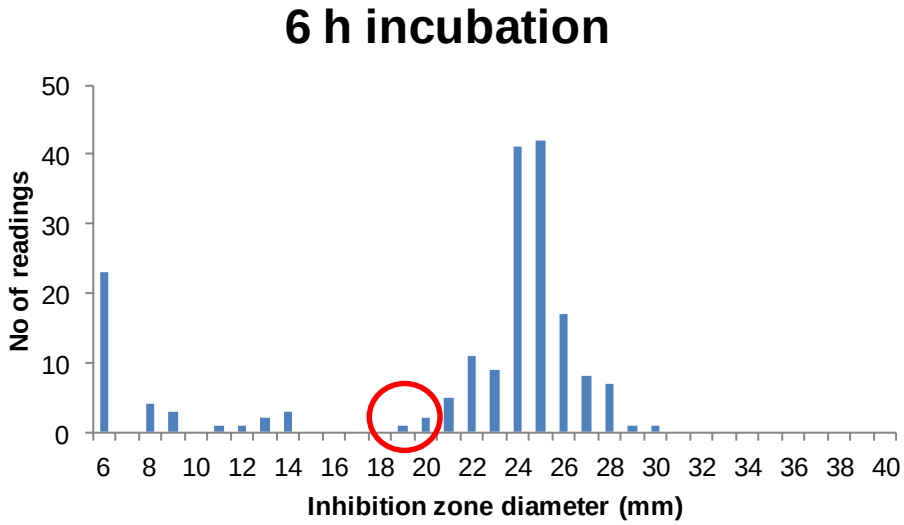


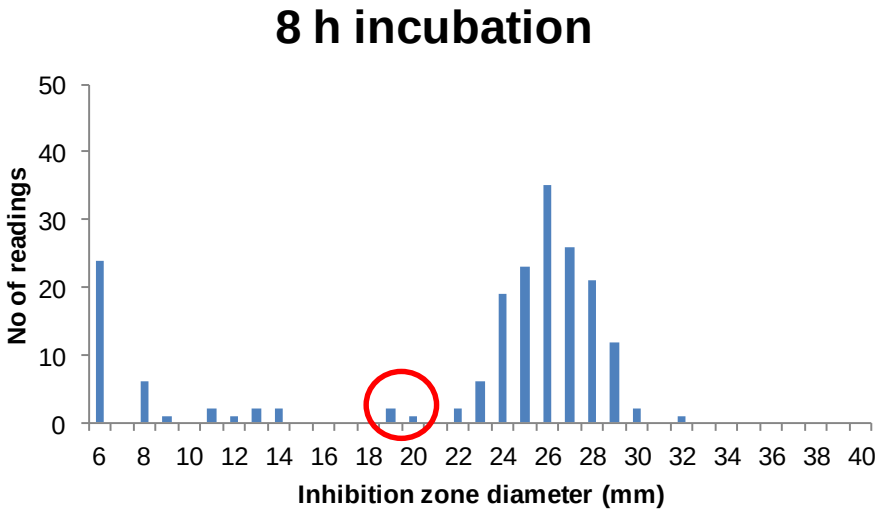
”Resistance genes likely, but results are uncertain”

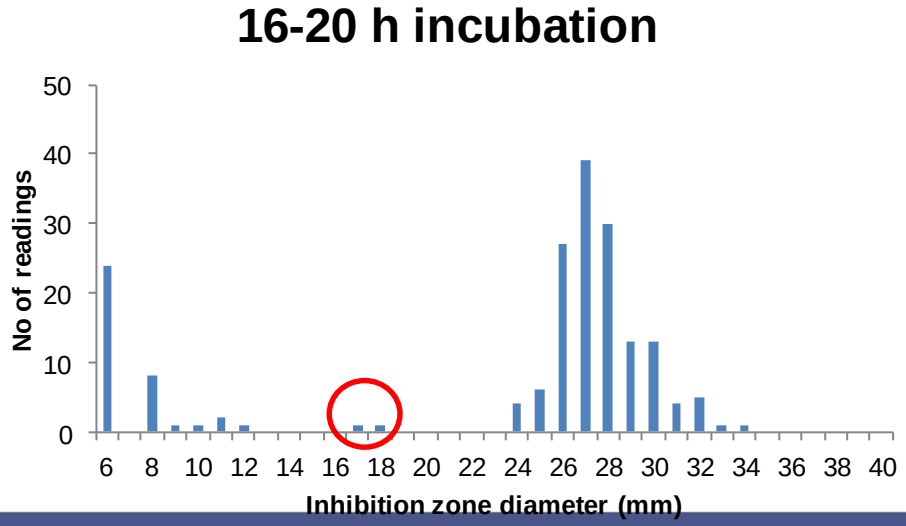


Early reading of disc diffusion tests

Examples with *E. coli* and cefotaxime 5 µg







- Poorer separation between wild type and non-wild type with short incubation time

- Testing of additional isolates with known resistance mechanisms is on-going

Other recent recommendations

- Improved screen for beta-lactam resistance in *S. pneumoniae*

- Screen for fluoroquinolone resistance in *Salmonella* spp. with the pefloxacin 5 µg disk
  - Skov et al. 2015 *Development of a Pefloxacin Disk Diffusion Method for Detection of Fluoroquinolone-resistant Salmonella Enterica*. J. Clin. Microbiol. (available online)

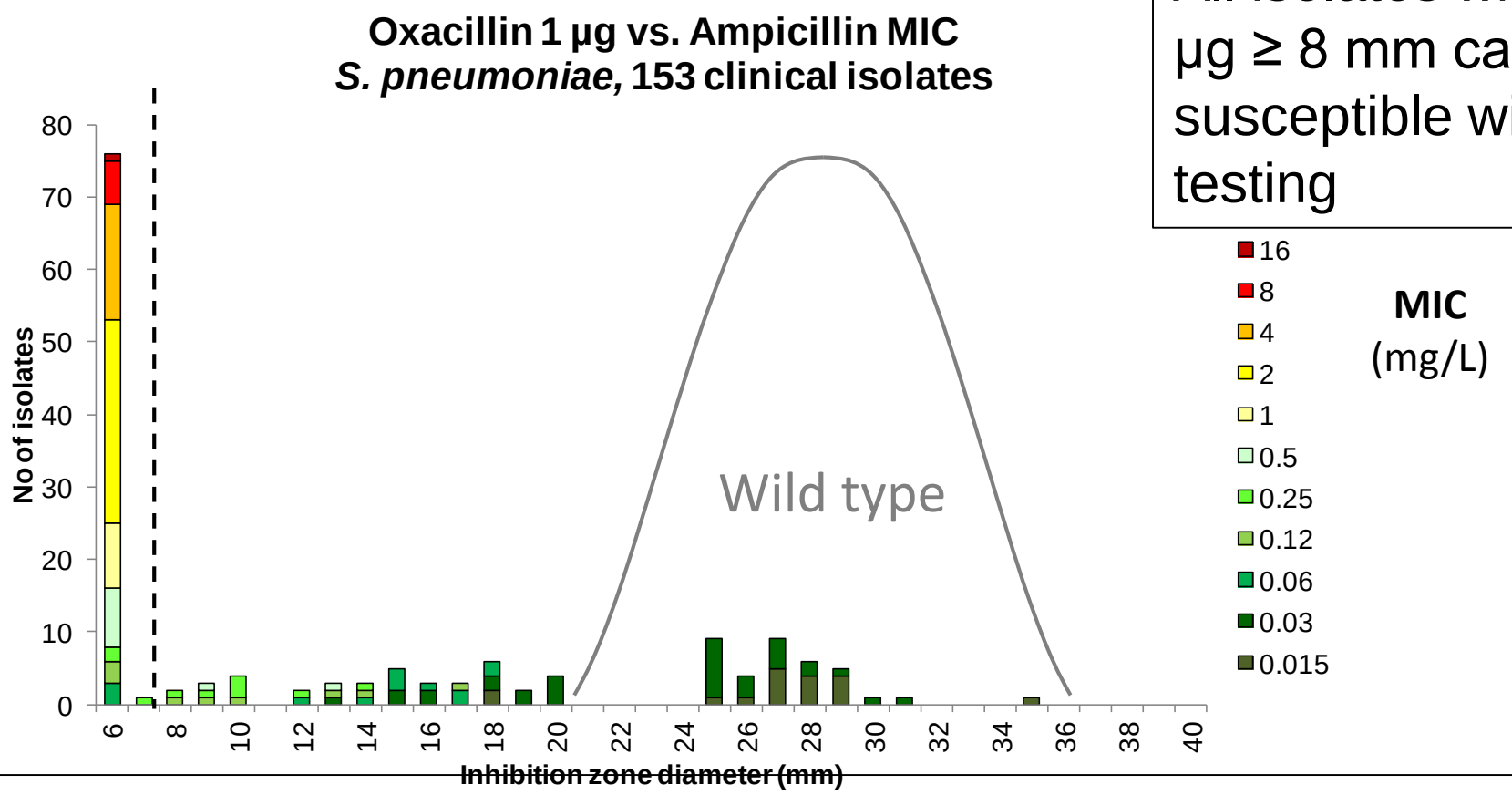
β-lactam resistance in *S. pneumoniae*

Oxacillin 1 µg	Agent	Further testing and/or interpretation
≥ 20 mm	All β-lactams	Report S to all agents with clinical breakpoints
	Benzylpenicillin	Meningitis: Report resistant Other infections: Determine the MIC
< 20 mm	Ampicillin, amoxicillin and piperacillin (± inhibitors) Cefepime, cefotaxime, ceftaroline, ceftobiprole and ceftriaxone	OXA ≥ 8 mm: Report S. In meningitis, consider determining the MIC. OXA < 8 mm: Determine the MIC
	Other β-lactams	Determine the MIC

EUCAST Breakpoint Table v 5.0

β-lactam resistance in *S. pneumoniae*

All isolates with oxacillin 1 µg ≥ 8 mm can be reported susceptible without further testing



Breakpoints

Ampicillin MIC	S≤0.5, R>2 mg/L
Oxacillin zone diameter (screen)	S≥8 mm

ECOFF

WT≤0.06 mg/L

Fluoroquinolone resistance in *Salmonella* spp.

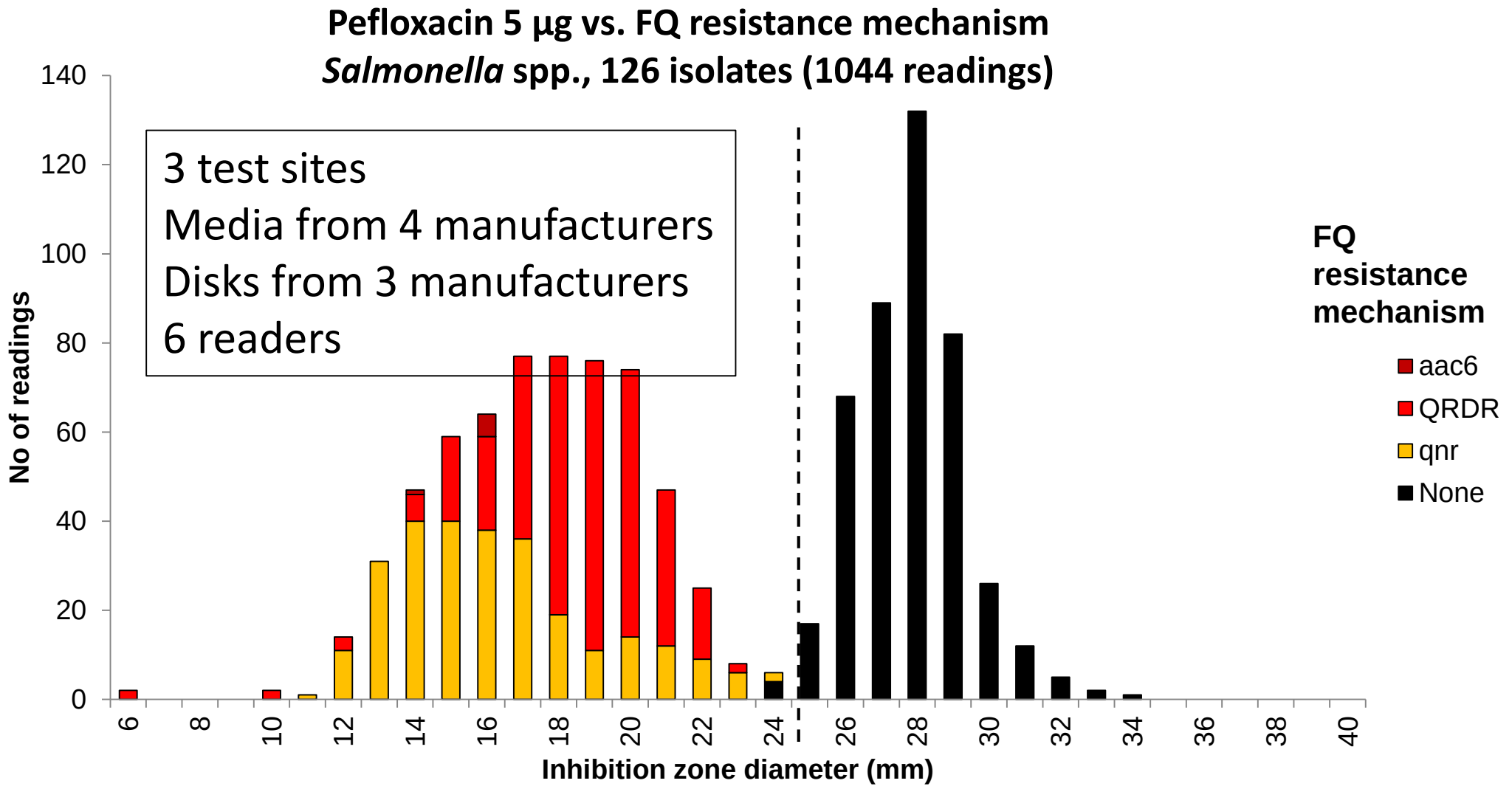
Enterobacteriaceae

Fluoroquinolones	MIC breakpoint (mg/L)		Disk content (µg)	Zone diameter breakpoint (mm)	
	S ≤	R >		S ≥	R <
Ciprofloxacin, *Salmonella* spp.	0.06	0.06		Note[A]	Note[A]
Pefloxacin (screen), *Salmonella* spp.	NA	NA	**5**	24	24

A. Susceptibility of *Salmonella* spp. to ciprofloxacin can be inferred from the pefloxacin disk diffusion susceptibility test result.

EUCAST Breakpoint Table v 5.0

Fluoroquinolone resistance in *Salmonella* spp.



Fluoroquinolone resistance in *Salmonella* spp.