Lecture 4

Basics of Antimicrobial Resistance (AMR) Mechanisms and Antimicrobial Susceptibility Test (AST) Methods

動物分野AMRセンター
Veterinary AMR Center

WOAH Regional Hands-on Training for AMR National Laboratory Technicians

2026 Oct.

MAFF

Veterinary AMR Center, NVAL, JMAFF

National Veterinary Assay Laboratory
動物医薬品検査所

Today’s Topics

1. What Is an Antimicrobial?
2. What Is Antimicrobial Resistance?
3. How Is Antimicrobial Resistance Determined?

What Is an Antimicrobial?

- An **ANTIMICROBIAL** agent means a naturally occurring, semi-synthetic or synthetic substance that exhibits antimicrobial activity (kill or inhibit the growth of micro-organisms) at concentrations attainable *in vivo*.

- **ANTIBIOTICs** are a specific type of antimicrobial that are used against bacteria.

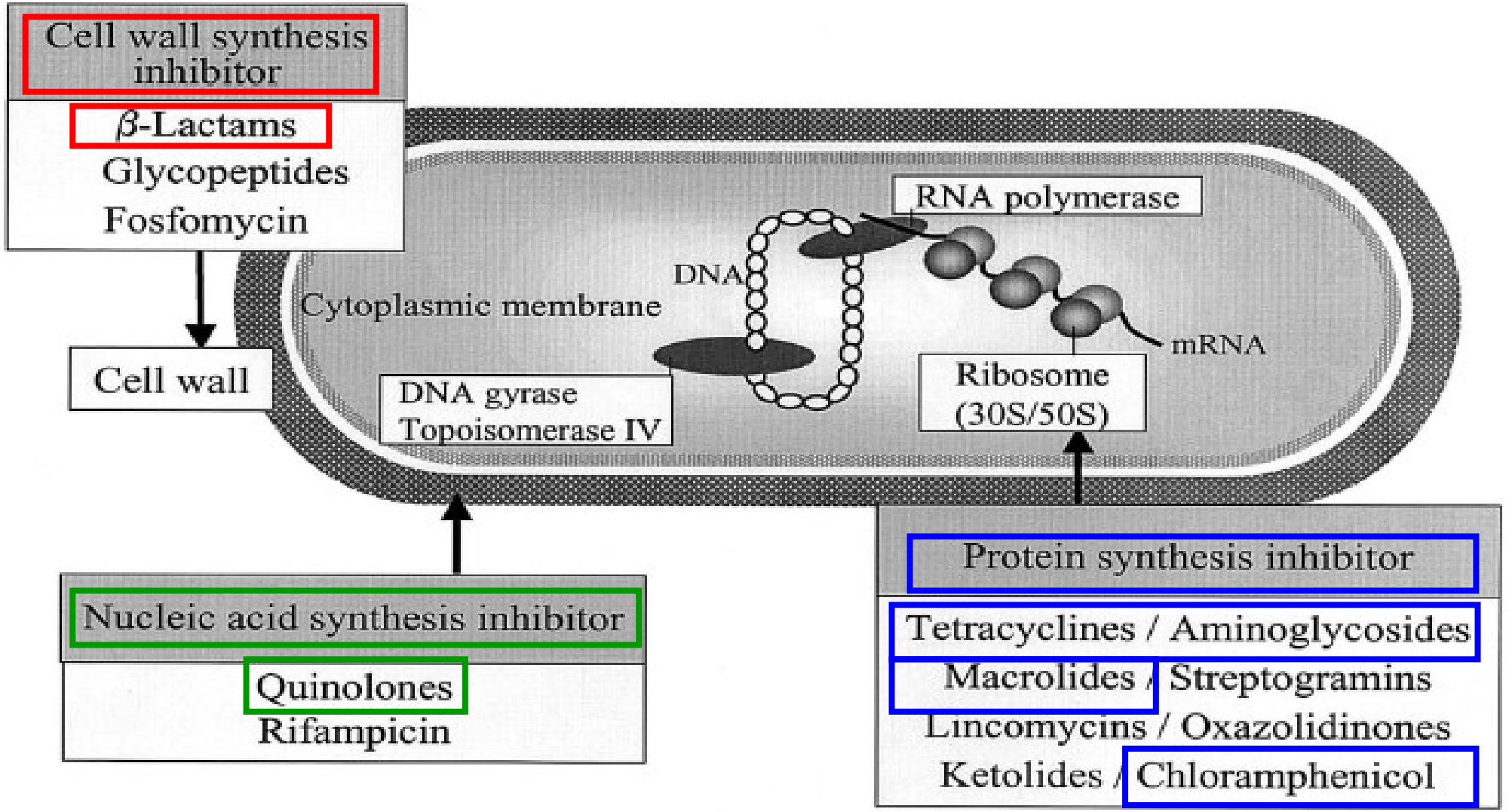
- All antibiotics are antimicrobials, but not all antimicrobials are antibiotics.

WOAH Definition
(https://www.woah.org/fileadmin/Home/eng/Health_standards/tahc/2018/en_glossaire.htm#terme_antibiotique)

Antimicrobial Classes

- Penicillins; Ampicillin (ABPC)
- Cephalosporins; (1st generation) Cefazolin (CEZ)
  (3rd generation) Ceftiofur (CTF)
- Aminoglycosides; Streptomycin (SM), Kanamycin (KM), Gentamicin (GM)
- Macrolides; Erythromycin (EM), Tylosin (TS)
- Tetracyclines; Tetracycline (TC), Oxytetracycline (OTC)
- (Am)Phenicols; Chloramphenicol (CP)
- Peptide (Polypeptide): Colistin (CL)
- Quinolones; Nalidixic acid (NA), (Fluoroquinolones) Enrofloxacin (ERFX)
- Sulfonamides; Sulfamethoxazole (SMX) (*+Trimethoprim (TMP))

How do antimicrobials act on bacteria?



Totsuka K.: Medical Practice 18(8): 1244, 2001

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What Are Antimicrobial Resistant Bacteria?

They are bacteria capable of growth in the presence of antimicrobials.

- Some bacteria are naturally resistant to certain types of antimicrobials (intrinsic resistance) .
- However, bacteria may also become resistant in two ways:

  1) by a genetic mutation (chromosomal mutation)

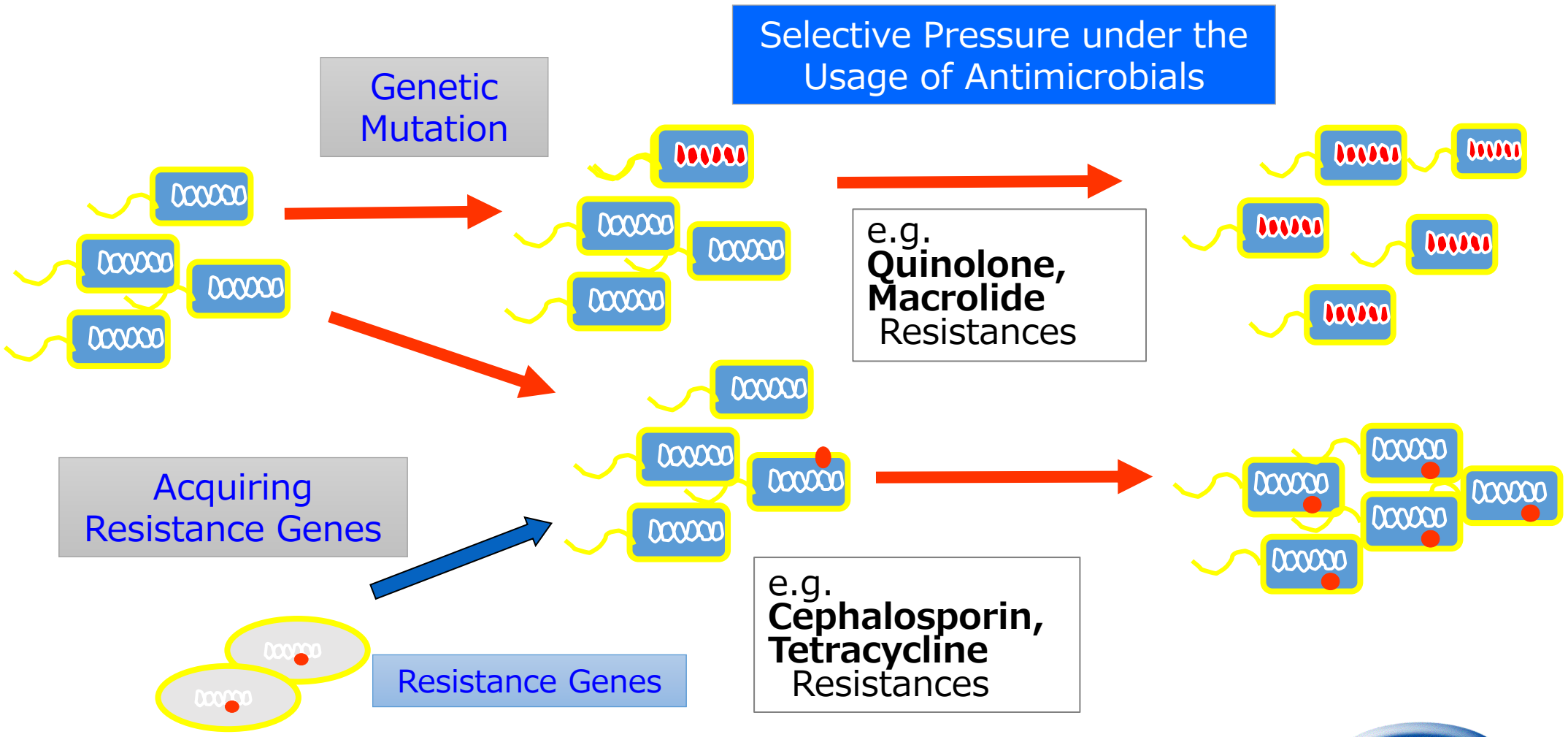
  2) by acquiring resistance genes from another bacterium (transmission of resistance).

Selection of Antimicrobial Resistant Bacteria

- The acquisition of resistant genes is occurring almost constantly irrespective of the presence of antimicrobials.

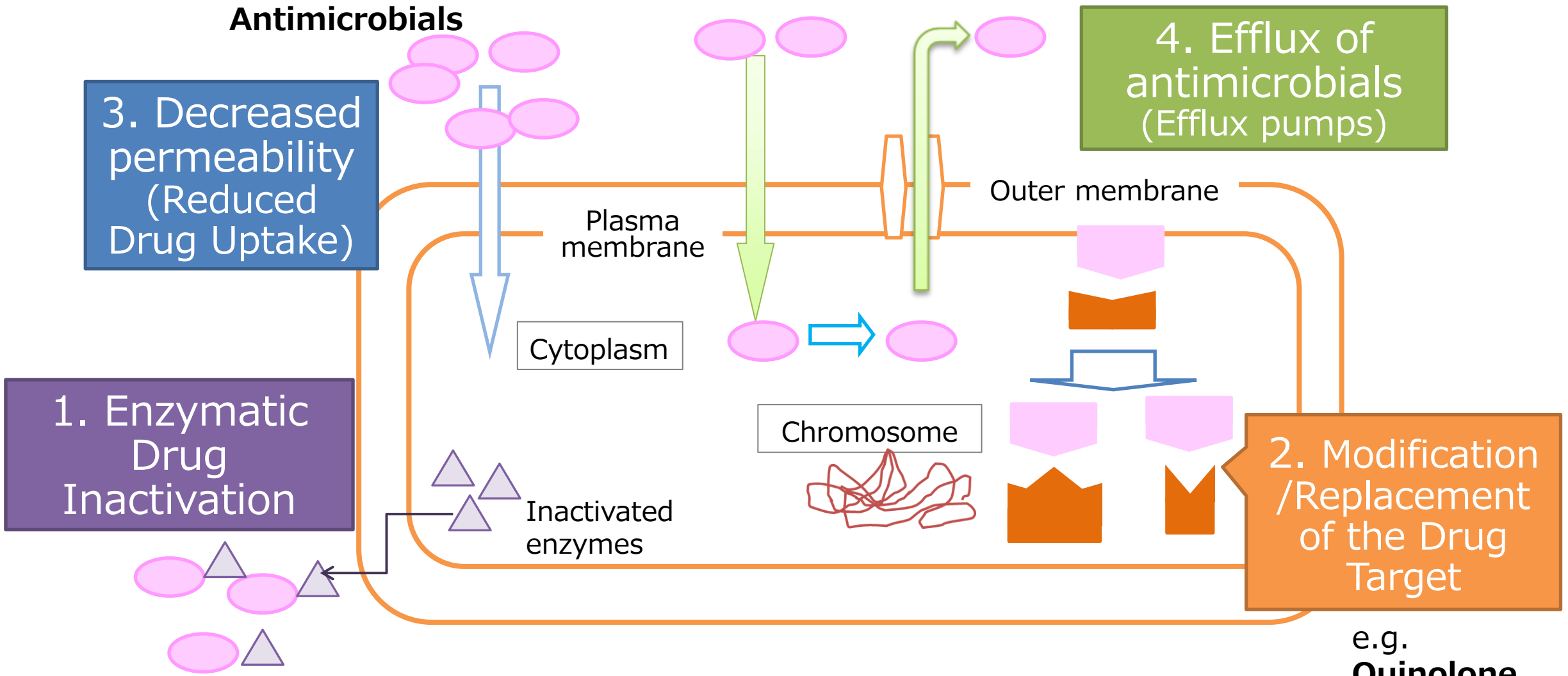
- However, antimicrobial-resistant bacteria can grow more advantageously than non-resistant bacteria in the presence of that specific antimicrobial (on-resistant bacteria antimicrobial-resistant bacteria survive and proliferate while non-resistant ones die away)
  →“selection of antimicrobial resistant bacteria”.

How does Antimicrobial Resistance Develop?



Antimicrobial-producing microorganisms,
Other Resistant bacteria

Mechanisms of Antimicrobial Resistance



1. Enzymatic Drug Inactivation

*Enzyme inactivation is the main mechanism of resistance to β-lactams, aminoglycosides, and phenicols.

*Drug-inactivating enzymes are generally associated with mobile genetic elements.

*The most widespread and clinically important enzymes are the β-lactamases and aminoglycoside-modifying enzymes.

*The β-lactamases hydrolyze the β-lactam ring of penicillins, cephalosporins, and/or carbapenems.

*The aminoglycoside-modifying enzymes catalyze transfer of an acetyl group (*N*-acetyltransferases) to amino groups or of a phosphoryl group (*O*-phosphotransferases) or a nucleotide (*O*-nucleotidyltransferases) to amino or hydroxyl groups in the aminoglycoside molecule.

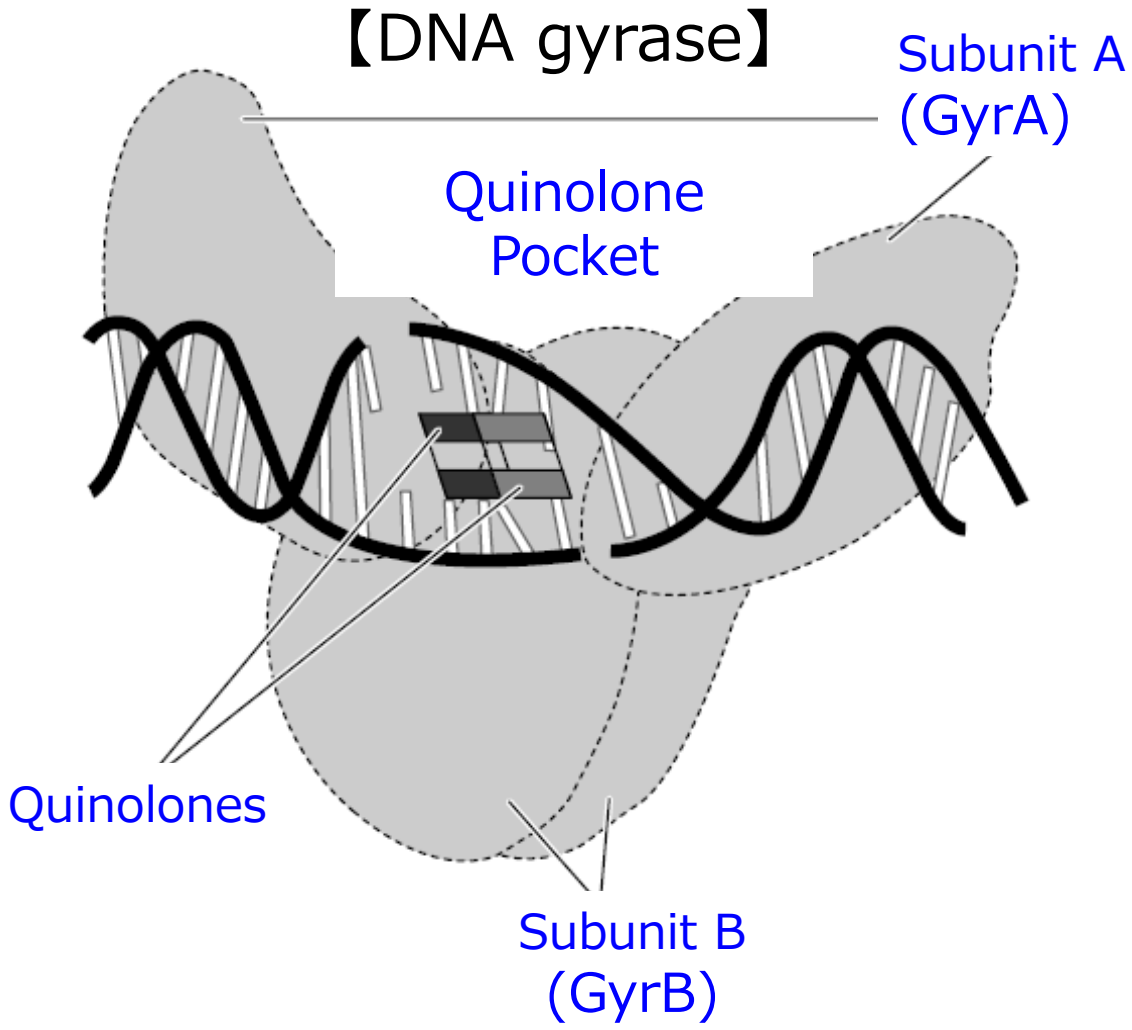
Guardabassi et al. *Antimicrobial resistance in Bacteria of animal origin* Chapter 1

2. Modification /Replacement of the Drug Target

【Quinolone Action】
Nucleic acid synthesis is a vital function for the bacterial cell.
Quinolones act on DNA synthesis, by target two enzymes that are essential for DNA unzipping: topoisomerase II (=DNA gyrase) and topoisomerase IV.

Mutations in gyrase and topoisomerase IV. (Point mutations of quinolone binding sites.)

【Topoisomerase IV】
Tetrameric structure, two A and two B subunit (encoded *parC* and *parE* (G-) or *grlA* and *grlB* (G+))



K. Hirai; *Jpn. J. Chemother*. 53 (6): 349-356, 2005

3. Decreased Permeability(Reduced Drug Uptake)

*In Gram-negative bacteria, hydrophilic drugs enter the bacterial cells through porins and hydrophobic drugs diffuse through the phospholipid layer.

*Mutation leading to loss, reduced size, or decreased expression of porins have been shown to confer acquired, generally lower level resistance to various antibacterial agents.

*For example, reduction in the expression of the OmpF porin has been shown to decrease the susceptibility of *E. coli* to quinolones, β-lactams, tetracyclines and chloramphenicol.

Guardabassi et al. *Antimicrobial resistance in Bacteria of animal origin* Chapter 1

4. Activation of Drug Efflux Pumps

*Active efflux is an energy-dependent mechanism used by bacteria as well as by eukaryotic cells and protozoa for extruding metabolites and foreign toxic compounds, including drugs.

*Some efflux pumps act on specific drugs (specific-drug-resistance (SDR) pumps), whereas others are active on multiple drug-resistance (MDR) pumps.

*SDR pumps are the most important mechanisms of resistance to TC, especially in Gram negative bacteria. (These pumps generally confer high-level resistance and are associated with mobile genetic elements.) MDR pumps generally confer low-level resistance and are frequently encoded by the chromosome.

Guardabassi et al. *Antimicrobial resistance in Bacteria of animal origin* Chapter 1

Cross-resistance and Co-resistance

- **Cross-resistance;** The situation in which resistance to one drug is associated with resistance to another drug and due to a single biochemical mechanism is defined as cross-resistance. Cross-resistance can occur between all members of a given antimicrobial class (e.g. sulfonamides), be limited to some of them (e.g. aminoglycosides), or involve antimicrobials belonging to different classes. Either target overlapping or unspecific drug efflux can cause the latter phenomenon.

- **Co-resistance**; which is due to the coexistence of genes or mutations in the same strain, each conferring resistance to a different class of drug. (e.g., gene cassette, genetic link on self-transferable plasmids.)

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Antimicrobial Susceptibility Testing(AST) Methods

Diffusion method

Disk method: Measures the diameters of inhibition zones by using disks containing a certain concentration of the antimicrobial.

Gradient MIC strips (E-test) : Measures the MIC by using a strip with stable gradient of antimicrobial concentrations.

Dilution method

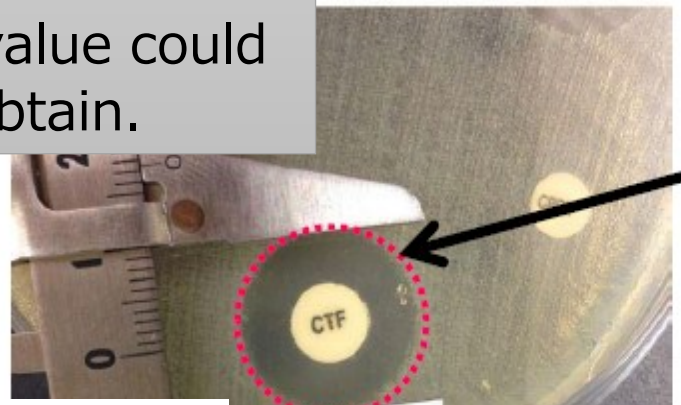
Agar dilution method: Measures the MIC by using agar plates with serial concentrations of the antimicrobial.

Broth microdilution method: Measures the MIC by using 96-well microplates containing liquid medium with serial concentrations of the antimicrobial.

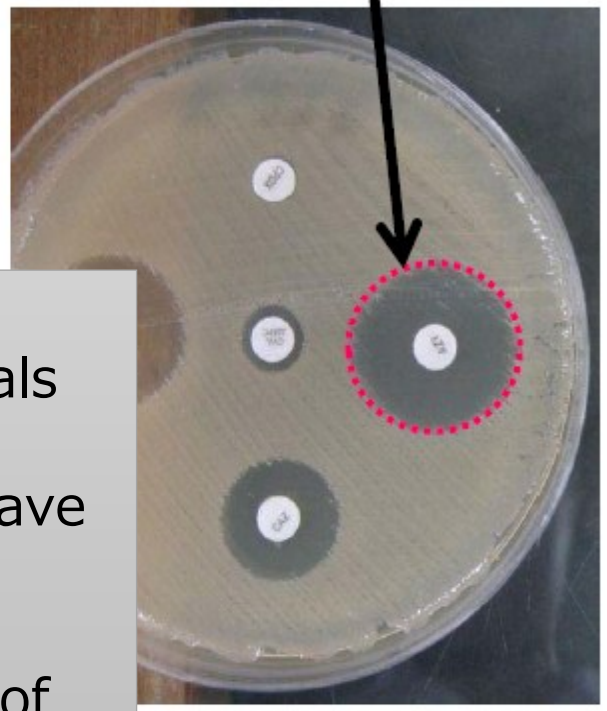
Whole Genome Sequence Analysis

Disk Diffusion Methods

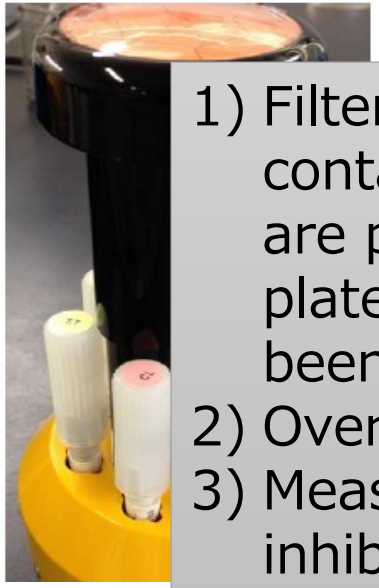
*Convenience, efficiency and the low cost.
*MIC value could not obtain.



Inhibition zone diameter



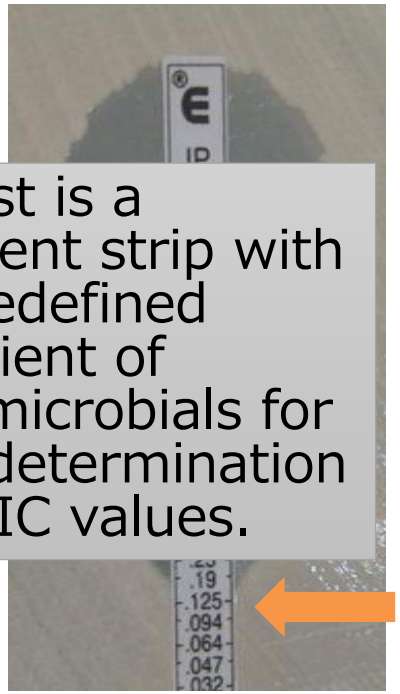
Dispenser



1) Filter-paper disks containing antimicrobials are placed on an agar plate where bacteria have been placed
2) Overnight incubation
3) Measure the diameter of inhibition zone.

Cf. Gradient MIC strips (E- test)

E-test is a reagent strip with a predefined gradient of antimicrobials for the determination of MIC values.

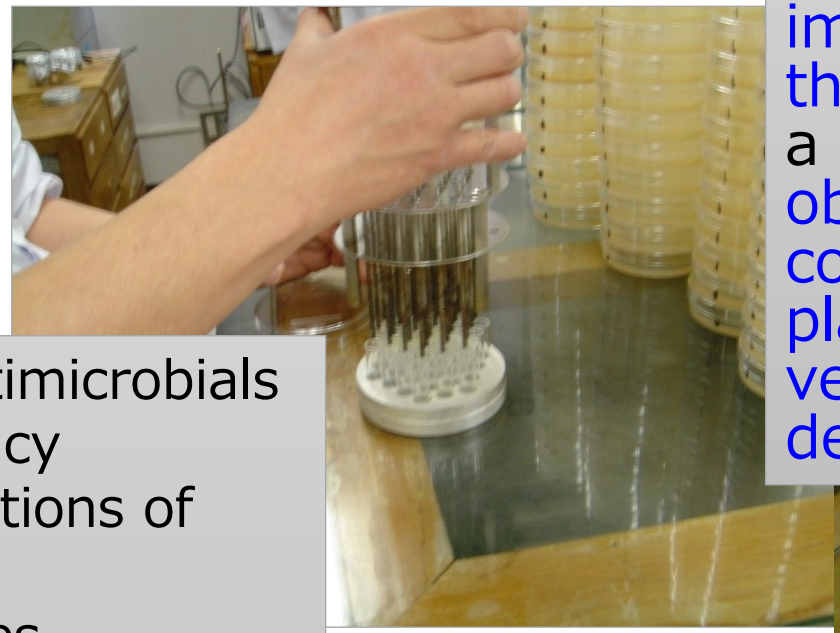


The MIC value is read from the scale in terms of μg/mL where the ellipse edge intersects the strip.

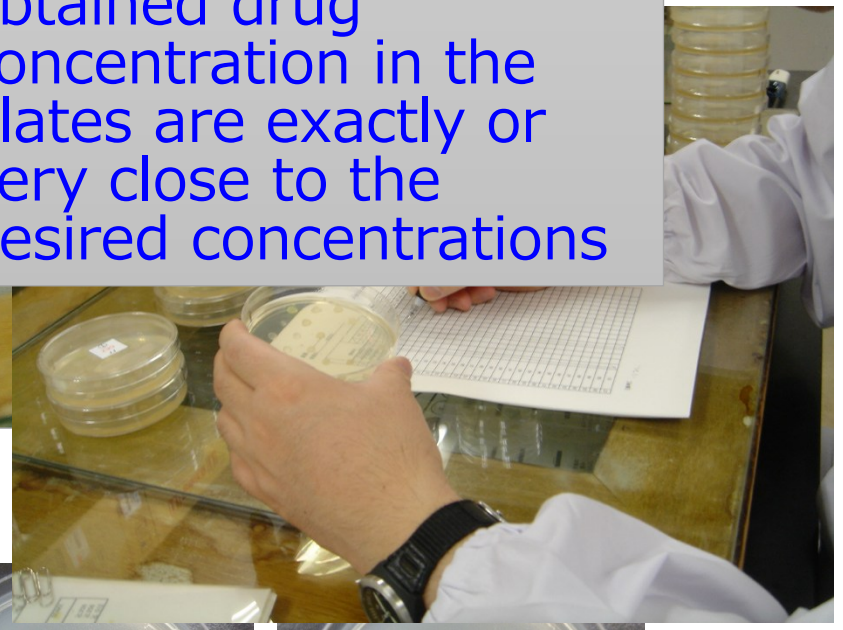
MIC
0.19 μg/ml

Agar Dilution Method



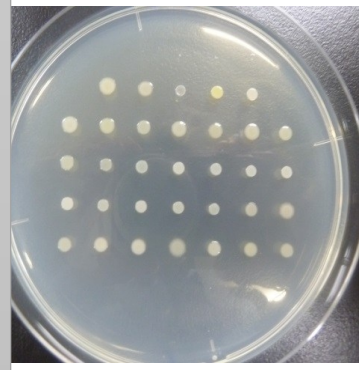


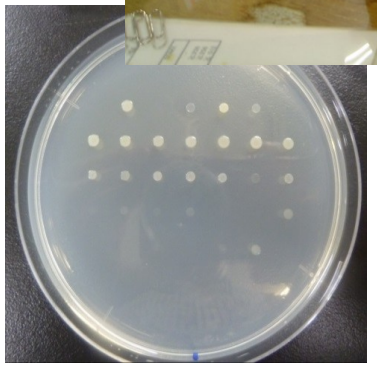
*It is extremely important to prepare the agar plates in such a way that the obtained drug concentration in the plates are exactly or very close to the desired concentrations

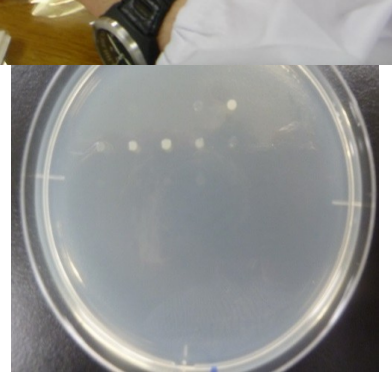


1) Prepare the antimicrobials of known potency
2) Make serial dilutions of them
3) Make agar plates containing serial-diluted antimicrobials
4) Inoculate the tested isolates on the plates with several standard bacterial strains
5) Incubate overnight
6) Measure the MIC.

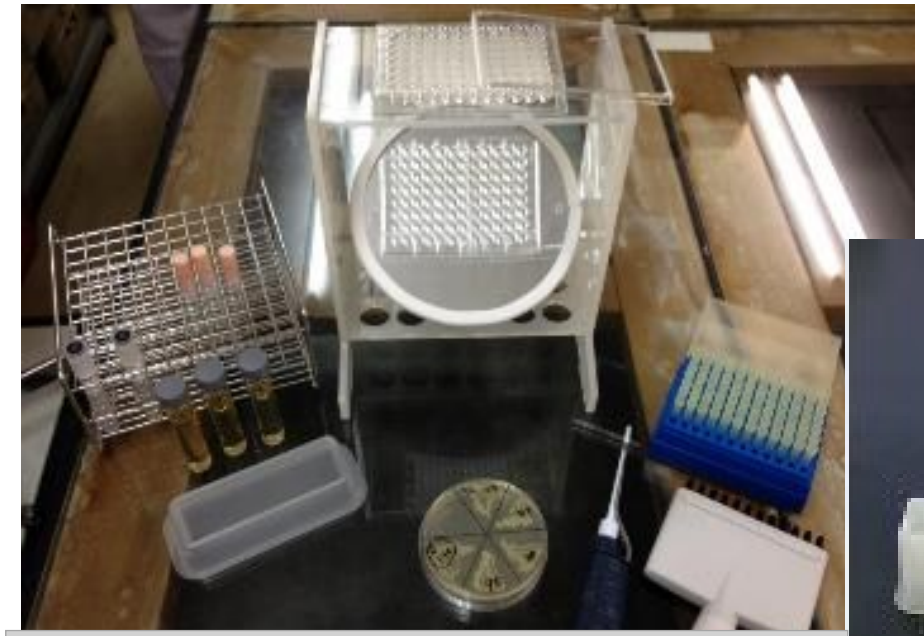








Broth Microdilution Method

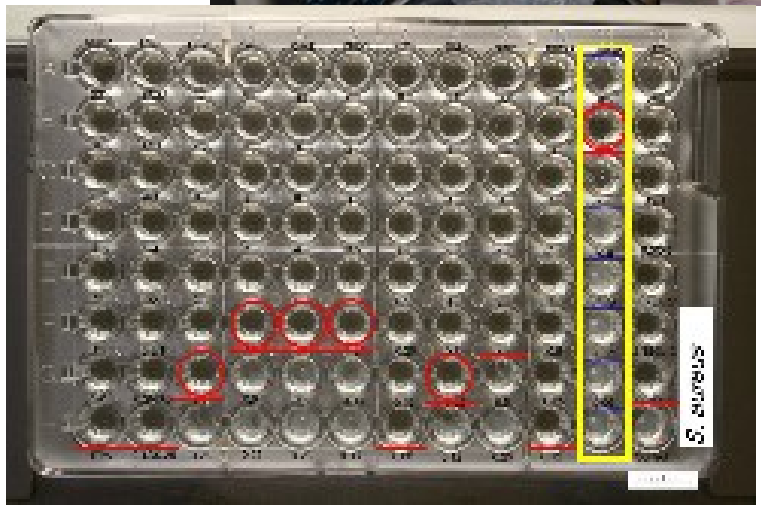




*We usually use commercialized microplates, which already contained a series of concentrations of antimicrobials.
*With these plate, MIC could be assayed easily
*But expensive ($10/plate)

Semi-automatic Inoculator
(Inoculatorβ Eiken)





Breakpoint/Cutoff

Epidemiological cutoff values: ECOFF, ECV

MIC（Minimum Inhibitory Concentration）or zone diameter value that separates microbial populations into those with and without acquired and/or mutational resistance based on their phenotypes (non-wild type or wild type), with the epidemiological cut off value defining the highest MIC or smallest zone diameter for the wild type population of isolates.

Clinical breakpoint: CBP

Clinical breakpoints are established using MIC distributions, pharmacokinetic/ pharmacodynamic data, clinical outcome data, when available.

CLSI VET01S 6th ed. p.174

Setting of Breakpoints

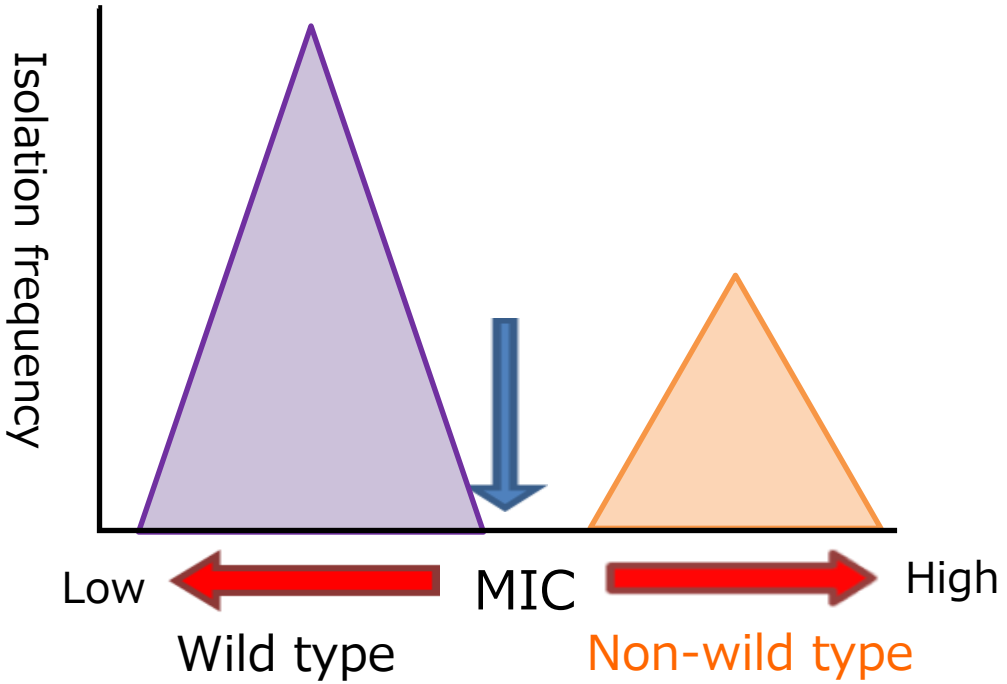
Epidemiological cutoff values (ECOFF)

ECOFFs (and TECOFFs) distinguish microorganisms without (wild type) and with phenotypically detectable acquired resistance mechanisms (non-wild type) to the agent in question.

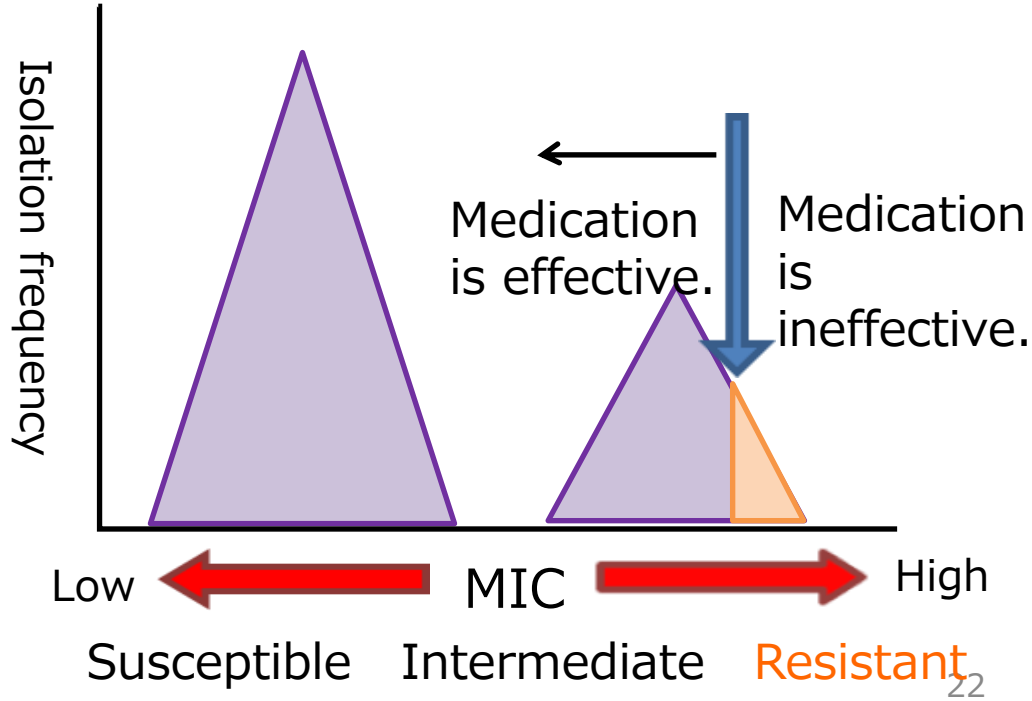
Clinical breakpoint (CBP)

Clinical breakpoints are defined based on pharmacokinetic/ pharmacodynamic data and clinical studies, as clinically susceptible (S), intermediate (I) or resistant (R).

(Example) **ECOFF**



(Example) **CBP**



S/I/R in Clinical Breakpoints

Clinical breakpoints typically are categorized into three groups

Susceptible: The organism should respond to therapy using recommended antimicrobial dosage for the given site of infection and species.

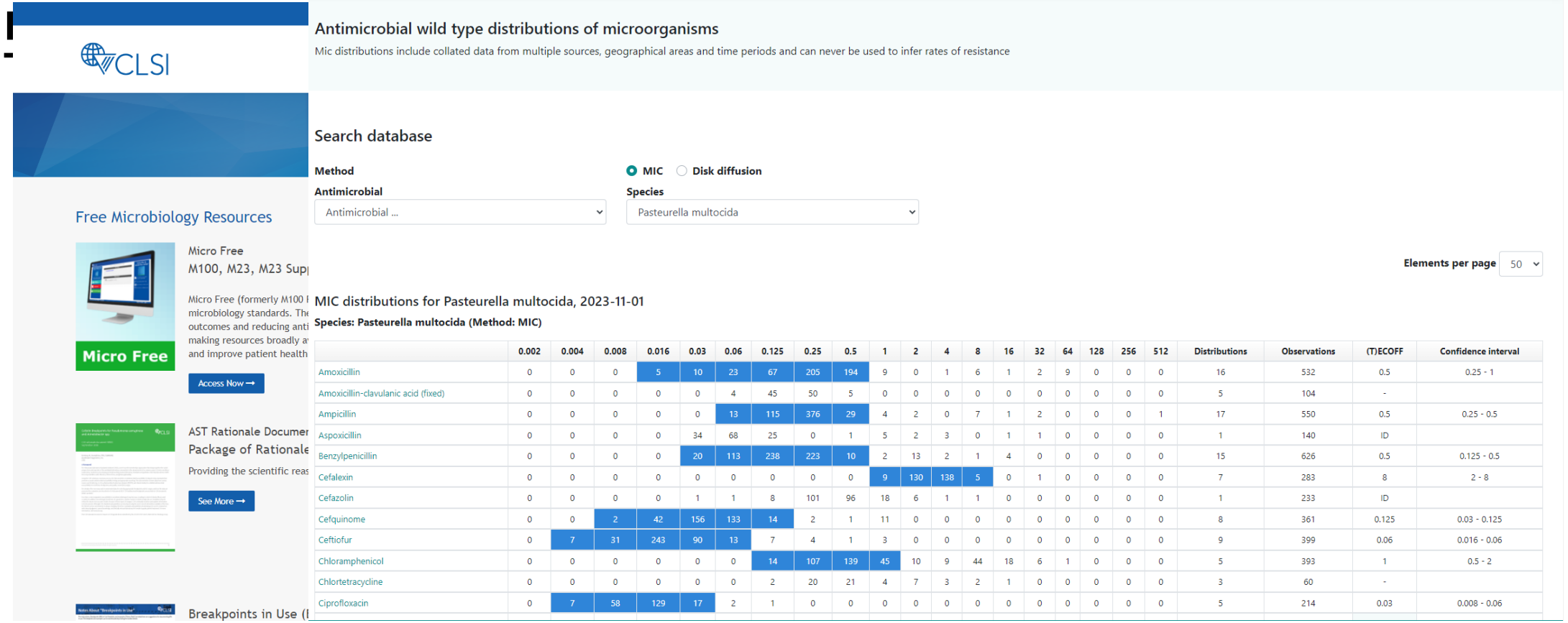
Intermediate: The organism's MIC approaches or exceeds the threshold for normal antimicrobial dosing, but clinical response is possible with higher doses or if the antimicrobial concentrates at the site of infection.

Resistant: The patient's organism should NOT be inhibited by concentrations achieved with normal dosing.

CLSI and EUCAST



- **CLSI (Clinical and Laboratory Standards Institute) : CBP**
  (https://clsi.org/all-free-resources/)
- **E**



	0.002	0.004	0.008	0.016	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	Distributions	Observations	(T)ECOFF	Confidence interval
Amoxicillin	0	0	0	5	10	23	67	205	194	9	0	1	6	1	2	9	0	0	0	16	532	0.5	0.25 - 1
Amoxicillin-clavulanic acid (fixed)	0	0	0	0	0	4	45	50	5	0	0	0	0	0	0	0	0	0	0	5	104	-	
Ampicillin	0	0	0	0	0	13	115	376	29	4	2	0	7	1	2	0	0	0	1	17	550	0.5	0.25 - 0.5
Aspoxicillin	0	0	0	0	34	68	25	0	1	5	2	3	0	1	1	0	0	0	0	1	140	ID	
Benzylpenicillin	0	0	0	0	20	113	238	223	10	2	13	2	1	4	0	0	0	0	0	15	626	0.5	0.125 - 0.5
Cefalexin	0	0	0	0	0	0	0	0	0	9	130	138	5	0	1	0	0	0	0	7	283	8	2 - 8
Cefazolin	0	0	0	0	1	1	8	101	96	18	6	1	1	0	0	0	0	0	0	1	233	ID	
Cefquinome	0	0	2	42	156	133	14	2	1	11	0	0	0	0	0	0	0	0	0	8	361	0.125	0.03 - 0.125
Ceftiofur	0	7	31	243	90	13	7	4	1	3	0	0	0	0	0	0	0	0	0	9	399	0.06	0.016 - 0.06
Chloramphenicol	0	0	0	0	0	0	14	107	139	45	10	9	44	18	6	1	0	0	0	5	393	1	0.5 - 2
Chlortetracycline	0	0	0	0	0	0	2	20	21	4	7	3	2	1	0	0	0	0	0	3	60	-	
Ciprofloxacin	0	7	58	129	17	2	1	0	0	0	0	0	0	0	0	0	0	0	0	5	214	0.03	0.008 - 0.06

CLSI and EUCAST

Caution!!

CLSI (Clinical and Laboratory Standards Institute) : CBP

Table 2A. Enterobacterales (Continued)

Test/ Report Group	Body Site	Antimicrobial Agent	Antimicrobial Agent Class or Subclass	Organism	Disk Content	Interpretive Categories and Zone Diameter Breakpoints, nearest whole mm			Interpretive Categories and MIC Breakpoints, µg/mL			Comments
						S	I	R	S	I	R	
Horses (Continued)												
A	Resp, SST	Enrofloxacin	Fluoroquinolones[c]	*E. coli*	-	-	-	-	≤ 0.12	0.25	≥ 0.5	
A	(adults)	Gentamicin	Aminoglycosides	Enterobacterales	10 µg	≥ 16	13-15	≤ 12	≤ 2	4	≥ 8	
A	Resp, SST	Minocycline	Tetracyclines	*E. coli*	-	-	-	-	≤ 0.12	0.25	≥ 0.5	See comment (10).
Cattle												
A	Metritis	Ampicillin	Penicillinase-labile penicillins	*E. coli*	-	-	-	-	≤ 0.03	0.06-0.12	≥ 0.25	
Swine												
A	Resp	Ceftiofur	Cephalosporins III	*Salmonella enterica* subsp. *Enterica* serovar Cholerasuis	30 µg	≥ 21	18-20	≤ 17	≤ 2	4	≥ 8	
A	Resp	Florfenicol	Phenicols	*S. enterica* subsp. *Enterica* serovar Choleraesuis	-	-	-	-	≤ 4	8	≥ 16	
Poultry												
B		Enrofloxacin	Fluoroquinolones[c]	*E. coli*	5 µg	≥ 23	17-22	≤ 16	≤ 0.25	0.5-1	≥ 2	**(11)** The US approval for poultry was withdrawn in September 2005.

If R column indicates ≥ 8, MIC =8 means Resistance

CLSI and EUCAST

Caution!!

EUCAST : ECOFF

MIC distributions for Pasteurella multocida, 2023-11-01

Species: Pasteurella multocida (Method: MIC)

	0.002	0.004	0.008	0.016	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	Distributions	Observations	(T)ECOFF	Confidence interval
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Benzylpenicillin	0	0	0	0	20	113	238	223	10	2	13	2	1	4							626	0.5	0.125 - 0.5
Cefalexin	0	0	0	0	0	0	0	0	0	9	130	138	5	0							283	8	2 - 8
Cefazolin	0	0	0	0	1	1	8	101	96	18	6	1	1	0							233	ID	
Cefquinome	0	0	2	42	156	133	14	2	1	11	0	0	0	0							361	0.125	0.03 - 0.125
Ceftiofur	0	7	31	243	90	13	7	4	1	3	0	0	0	0							399	0.06	0.016 - 0.06
Chloramphenicol	0	0	0	0	0	0	14	107	139	45	10	9	44	18							393	1	0.5 - 2
Chlortetracycline	0	0	0	0	0	0	2	20	21	4	7	3	2	1							60	-	
Ciprofloxacin	0	7	58	129	17	2	1	0	0	0	0	0	0	0							214	0.03	0.008 - 0.06
Clindamycin	0	0	0	0	0	0	0	1	1	12	32	61	42	18	5	0	0	0	0	1	172	ID	
Dihydrostreptomycin	0	0	0	0	0	0	0	0	1	6	6	5	42	41	8	1	4	26	0	1	140	-	0.25 - 1
Doxycycline	0	0	0	0	0	3	83	450	151	58	28	12	5	0	0	0	0	0	0	17	790	1	0.25 - 2

If (T)ECOFF indicates 8, MIC =8 is WT, MIC = 16 is non-WT.



Thank you !

MAFF

動物分野AMRセンター
Veterinary AMR Center

National Veterinary Assay Laboratory
動物医薬品検査所