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ANTIBIOTIC RESISTANCE MECHANISM

Yeonhee Lee
Culture Collection of Antibiotic Resistant Microbes, College of Natural Science, Seoul Women's University, Seoul 139-774, Korea

Antibiotic resistance and the threat of new emerging infectious diseases are on the rise worldwide. Various data indicate that many important human pathogens now carry antibiotic resistance factors, sometimes to all or nearly all available drugs. The misuse of drugs, an expanded population of very old and very young people who are susceptible to infectious diseases, and a growing population group with AIDS, cancer, or other conditions that may lead to immunosuppression are among the factors that make these problems ever more serious. The relative utility of available antibiotics is eroding, tipping the balance in favor of multidrug-resistant pathogens. At the same time the search for new drugs or other novel agents to combat bacterial pathogens has lost much of its momentum because of high cost for developing and testing.

Veterinary diseases, particularly among animals used for large-scale food production, represent another arena in which the problems associated with antibiotic resistance are being played out. Antibiotics tend to be used prophylactically for entire flocks or herds at the first sign of illness among some of its members rather than waiting for the disease to be manifest in the entire group. The use of certain antibiotics and other growth promotants as low-level, nontherapeutic additives to animal feeds are performed widely. For instance, bacitracin is frequently added to chicken feed, highly effective in increasing flock productivity. Problems may arise when a class of such so-called family growth promotants includes representatives from a family of antibiotics that could be needed in human medicine. Drugs used in agricultural settings typically remain in the open environment and may flow out of growth ponds widely disseminated environmental contaminants. And data are accumulating that the resistant microbes in animal can infect human.

To solve this precarious problem of antibiotic resistance, a broad understanding what is going on in the hospital and environment situation including animal farms and the close cooperation among scientists, doctors, pharmaceutical industries, and government are necessary. In this talk, I will show you what we, scientistist can contribute to solve this world-wide problem and introduce the Culture Collection of Antibiotic Resistant Microbes which has been supported by a special grant for Research Materials Bank from KOSEF since 1999.

Antibiotic resistance mechanism

Antibiotic resistance mechanisms are like followings:

1. Change at the target site of each antibiotic. For example, quinolone resistance was due to the mutation in DNA gyrase and Topoisomerase IV and glycopeptide resistance was due to the mutation in rRNA sequence.

2. Reduced intracellular antibiotic concentration due to the decreased permeability and the existence or over-expression of efflux system. Most multi-drug resistant (MDR) bacteria have an efflux system with a broad specificity.

3. Degradation by enzymes. Many beta-lactam resistant bacteria produce the extracelluar beta-lactamase and degrade beta-lactam antibiotics before it enters bacterial cells .

4. Modification of antibiotics. Antibiotics are modified inside cells by acetylation or phosphorylation losing their inhibitory activities.

5. A new enzyme with similar function appears. MRSA produces a new cell wall cross-linking enzyme encoded by *mec* which has a low affinity to penicillin.

Methods to study the resistance mechanism

To find out the resistance mechanism following methods are being used.

1. MIC (Minimal inhibitory concentration): Antibiotics are serially diluted twofold in liquid media. Bacterial suspension adjusted to MacFarland Unit 0.5 is inoculated and their growth will be observed next day. MIC is the lowest concentration at which no growth is observed.

2. MBC (Minimal bactericidal concentration): Antibiotics are serially diluted twofold in solid media and bacterial suspension was inoculated onto it. After overnight incubation, the lowest concentration which produces no colony is determined as MBC.

3. Detection of degrading enzyme (beta-lactamase): Lay a disk containing cefinase onto a bacterial lawn and observe the color change or run IEF (Isoelectrofocusing gel electrophoresis) of cell extract and detect the beta-lactamase by adding nitrocefin.

4. Inhibitory activity on the target site: Isolate DNA gyrase and assay the IC50.

5. Permeability and efflux: Bacterial cells were incubated with antibiotics and the intracellular concentration of antibiotics is assayed with UV, fluorometer or HPLC. To detect the efflux system, add proton gradient inhibitor such as CCCP or reserpine to the reaction mixture.

6. Detection of modifying enzymes: Incubate the bacterial cell extract with antibiotics and add radioactive acetyl or phosphoryl group donor and detect the radioactively labeled protein on X-ray film.

7. Detect mutation in the target site: When the hot spot is already known and it consists a specific restriction site, the region, for example quinolone resistance determining region, is amplified with PCR and its RFLP is determined. In most cases, the gene encoding the target site

in the resistant strain is sequenced and its sequence is compared with that in the wild type strain.
8. Pulse-field gel electrophoresis: Many nosocomial bacteria are originated from one common strain and have the same resistant mechanism. To compare each strain, pulse field gel electrophoresis is widely used.
9. Rapid detection of the resistant strain using DNA chip.

Quinolone resistance mechanism

In the case of quinolone, genes responsible for quinolone resistance have been found only on the chromosome and genes for degrading and modifying quinolone have not been reported, yet. Until now, quinolone resistance is known to result from the target site mutation at *gyrA* of DNA gyrase and *parC* of topoisomerase IV, low permeability, and efflux. DNA gyrase is an enzyme which relaxes the supercoiled DNA by cutting and religating DNA. When a mutation near Tyr122 at the active site occurs, quinolone no long bind to the DNA-DNA gyrase complex and the cells become resistant to quinolone. Here are some research data we have gotten for the study of the quinolone resistanc machanism.

1. Target site mutation detected by PCR and RFLP.
Ser83 at *gyrA* is the hot spot and this site is *Hin*fI restriction site. When QRDR is amplified and cut with *Hif*I, a quinolone susceptible strain produces two fragments while a quinolone resistant strain produces only one fragment.
2. Inhibitory activity on the target site, DNA gyrase
When DNA gyrases were isolated from each resistant and susceptible strain, DNA gyrase in the resistant strain showed high IC50.
3. Target site sequencing
When *gyrA* and *parC* were amplified with PCR and sequenced, new mutations have been found.
4. Permeability.
Hydrophilic quinolones are imported through porins. So when porins are not expressed or under-expressed, quinolone permeability decreased resulting the low intracellular concentration and increasing the MICs. Hydrophobic quinolones are imported through lipid components and its permeability depends on the composition of the phospholipid bilayer.
5. Efflux.
Many multi-drug resistant (MDR) bacteria have an efflux system to quinolone, tetracycline, etc. To detect the efflux system, bacterial cells were incubated with quinolone in the presence of proton gradient uncoupler and the intracellular concentration was assayed.
6. SAR (Structure-Activity Relationship)

How to deal with the antibiotic resistance problem?

To solve the antibiotic resistance problem, each part-government, pharmaceutical industry, hospital, agricultural industry, and scientiests such as microbiologist and biochemist should cooperate. First, the government needs to prevent the occurrence of antibiotic resistance by making laws or at least set a guideline prohibiting the misuse and overuse of antibiotics. Second, the pharmaceutical industry should develop better drug delivery system (DDS), develop new antibiotics with a new target site, modify the existing drug with better activity, and develop an inhibitor of efflux system. Third, doctors should try to detect each pathogen rapidly, use narrow-spectrum antibiotics, avoid prescribing antibiotics for prophylactic purpose, follow up the resistance pattern, and report the occurrence of resistant strains. Fourth, the agricultural industry maybe the storage of resistant microbes. There is no law or guidelines for the antibiotic use in the agricultural industry in Korea. EU, Japan, and U.S.A. made guidelines to stop using antibiotics with the same resistance mechanism of antibiotics for human use. For example, avoparcin has the same resistance mechanism of vancomycin. More and more data have been reported that the animal bacteria can infect human. We need a rapid action to prohibit antibiotics for prophylactic use or growth promotant to curve the resistance problem due to the animal microbes. Finally, scientists should find out the new target site based on genomics and proteomics, perform SAR to develop new antibiotics, study the exact resistance mechanism, and develop new antibiotics to overcome the resistance mechanism. Each part should perform each responsibility to solve this world-wide problem.

Culture Collection of Antibiotic Resistant Microbes (http://www.swu.ac.kr/~ccarm)

Funtion

Characterize the resistance mechanism in different species for each antibiotic

Provide resistant microbes to scientists

Collection of Antibiotic Resistant Microbes

Aeromonas hydrophila, Aeromonas salmonicida, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus pneumoniae, Helicobacter pylori

MBC to each antibiotic, Mechanism (Permeability, efflux, target site mutation, modification, degrading enzymes)

Provide SAR data to the pharmaceutical industry, Develop the rapid detection kit, Service to farmers

REFERENCES

1. Chen CR, Malik M, Snyder M, Drlica K (1996) DNA gyrase and topoisomerase IV on the bacterial chromosome: quinolone-induced DNA cleavage. J Mol Biol 258: 627-37.
2. Heisig P (1996) Genetic evidence for a role of *parC* mutations in development of high-level fluoroquinolone resistance in *Escherichia coli*. AAC 40:879-85.
3. Hiasa H, Yousef DO, Marains KJ (1996) DNA strand cleavage is required for replication fork arrest by a frozen topoisomerase-quinolone-DNA ternary complex. J Biol Chem 271:26424-9.
4. Hooper DC (1995) Quinolone mode of action. Drugs 49(suppl.2):10-5.
5. Jalal S, Wretlind B (1998) Mechanisms of quinolone resistance in clinical strains of *Pseudomonas aeruginosa*. Microb Drug Resist 4:257-61.
6. Jorgensen JH, Weigel LM, Ferraro MJ, Swenson JM, Tenover FC (1999) Activities of newer fluoroquinolones against *Streptococcus pnemoniae* clinical isolates including those with mutations in the *gyrA, parC,* and *parE* loci. AAC 43:329-34.
7. Kohler T, Michea-Hamzehpour M, Pleisiat P, Kahr AL, Pechere JC (1997) Differential selectionof multidrug efflux systems by quinolones in *Pseudomonas aeruginosa*. AAC 41:2540-3.
8. Lee Y, Kim K, Pyun HE, Park W (1992) Characterization of ciprofloxacin, HK3140, norfloxacin, and ofloxacin resistant strains of *Escherichia coli*. Kor Biochem J 25:134-40.
9. Lee S, Lee Y (1998) Ofloxacin resistance mechanism in PA150 and PA300-clinical isolates of *Pseudomonas aeruginosa* in Korea. Arch Pharm Res 21:671-6.
10. Pan XS, Fisher LM (1998) DNA gyrase and topoisomerase IV are dual targets of clinafloxacin action in *Streptococcus pneumoniae*. AAC 42:2810-6.
11. Park S, Lee S, Lee Y (1996) Norfloxacin resistance mechanism of *Escherichia coli* 59-a clinical isolate in Korea. Mol Cells 4:469-472.
12. Yamano Y, Nishikawa T, Komatsu Y (1990) Outer membrane proteins responsible for the penetration of beta-lactams and quinolones in *Pseudomonas aeruginosa*. J Antimicrob Chemother 26:175-84.
13. Zhao X, Xu C, Domagala J, Drlica K (1997) DNA topoisomerse targets of the fluoroquinolones : a strategy for avoiding bacterial resistance. Proc Natl Acad Sci USA 94:13991-6.

Table 1. Target site mutations found in *Escherichia coli.*

DNA gyrase						Topoisomerase IV					
GyrA			GyrB			ParC			ParE		
Ala67	→	Ser	Ser426	→	Asn	Gly78	→	Asp	Leu445	→	His
Ser80	→	Arg	Lys447	→	Glu	**Gly78**	→	**Cys**			
Gly81	→	Asp				Ser80	→	Ile			
Gly81	→	Cys				Glu84	→	Gly			
Asp82	→	Gly				Glu84		Lsy			
Ser83	→	Ala									
Ser83	→	**Leu**									
Ser83	→	Trp									
Ala84	→	Pro									
Asp87	→	**Asn**									
Asp87	→	Gly									
Gln106	→	Arg									
Gln106	→	His									

Table 2. Target site mutations found in *Pseudomonas aeruginosa*

DNA gyrase				Topoisomerase IV	
GyrA			GyrB	ParC	ParE
Thr83	→	Ile			
Asp87	→	Asn			
Asp87	→	Gly			
Asp87	→	Tyr			
Asn116	→	**Tyr**			

Table 3. Ciprofloxacin and ofloxacin concentrations inside cells

CCCP	Quinolone	Susceptible strain	Resistant strain (PA150)	Resistant strain (PA300)

Figure 1. Intracellular concentration in the presence of CCCP.