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Comparison of Smooth-Rough Form Variation and Antibiotic Susceptibility of *Escherichia coli* and *Clostridium perfringens* Isolates from Chickens, Pigs and Cattle

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닭, 돼지 및 소에서 분리한 대장균과 *Clostridium perfringens*의 S-R변이와 항생제의 감수성 비교

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국문요약

Acriflavine 시험법을 이용해서 수종의 화학물질을 첨가한 배지에 대장균과 *Clostridium perfringens*을 배양하여 변이를 유도한 후, S-R 변이와 변이 후의 항생제에 대한 감수성을 조사한 결과, 화학물질에 대한 S-R변이의 경우, 대장균은 mercuric chloride, cysteine, caffeine, glucose, nicotine 등의 순으로 나타났고, *C. perfringens*는 mercuric chloride, nicotine, caffeine, cysteine, glucose 등의 순으로 나타났으며 양자 공히 mercuric chloride가 가장 감수성이 높았다. 항생제에 대한 S-R 변이 후의 감수성은 대장균과, *C. perfingens*가 공히 S-R변이 후에는 감수성이 일반적으로 저하되는 경향이었다.

Key words: *Escherichia coli*, *Clostridium perfringens*, Smooth-Rough form variation, antibiotic susceptibility.

Ⅰ. Introduction

Acridine dyes, such as acriflavine and trypaflavin, are well known substances for the usage in differentiating from the smooth-form to rough-variant of bacteria (Ahn, 1960). White (1929) reported that the rough-forms of intestinal bacteria give rise to agglutination or sedimentation reaction when boiled with Millon' reagent which contains mercuric chloride compounds, while the S-forms do not.

Okamoto (1943) discovered later that the major role of the agglutination and sedimentation reaction is played by the Hg compounds contained in the Millon's reagent, and reported that not only the Millon's reagent but many heavy metal salts, such as $CuSO_4$, $AgNO_3$, $ZnCl_2$ etc., agglutinate the R-forms of almost all bacteria, and finally to this reaction gave the term "heavy metal ion reaction". Since then, the heavy metal ions have been

applied for the detection of R-forms of bacteria, though not so popular as the acridine dyes.

Although the strategy of ecological analysis was introduced in the social sciences over 40 years ago (Robinson, 1950), specialized epidemiologic discussions have become common only in the last decade (Greenland and Morgenstern, 1989; Greenland and Robins, 1994). These discussions may have been inspired by the recognition that, under special circumstances, ecologic studies can supply estimates of individual-level relative risks (Beral et al., 1979).

There is little information about the susceptibility of *Escherichia coli* and *Clostridium perfringens* to growth-enhancing antibiotics and the eventual development of resistance has not been adequately monitored (Jung, 1997). Dutta and Devriese (1980) studied on the susceptibility of strains isolated in 1979 from pigs, cattle, and poultry with growth -enhancing and therapeutical antibiotics available at that time. Antibiotic susceptibility of isolates pathogens from cases to almost antibiotics was decreased (Jung, 1999).

This study describes the S-R variation and antibiotic susceptibility tests of *E. coli* and *C. perfringens* isolates from mastitic cows, diarrheic pigs, diarrheic chickens, chickens with necrotic enteritis, piglets with enterotoxemia, and cattle with enterotoxemia using cysteine hydrochloride, glucose, caffeine sodium benzoate, mercuric chloride, and nicotine. This study hopes to help increase the productivity of animal farms and promote the nation's health by preventing human diseases due to *E. coli* and *C. perfringens*.

Ⅱ. Materials and Methods

1. Bacterial strains

Ninety-three isolates of *E. coli* were used in this study (Table 1). All were obtained from a single facility by Kwangju and Chonnam Animal Hygienic Center, Kwangju, Korea. These isolates had been submitted by local veterinarians for microbiologic examination: 37 of raw milk specimens from mastitic cows, 51 of fecal specimens from pigs with diarrhea, and 5 of fecal specimens from chickens with diarrhea. The control group of *E. coli* strains EC82 (O78:H$^-$) isolated from human beings was donated by Cho MJ from the Department of Microbiology, Kyungsang University, Medical College, Korea.

Table 1. Susceptible strains before form-variation of *Escherichia coli* isolates from cows with mastitis, pigs with diarrhea, and chickens with diarrhea

Antibiotics	Numbers of susceptible strains among 93 strains isolates from cases(%)
Cephalexin	86 (92.5)
Enrofloxacin	71 (76.3)
Gentamycin	86 (92.5)
Linsmycin	87 (93.5)
Naxcel	88 (94.6)
Neomycin	79 (84.9)
Norfloxacin	72 (77.4)

Also, twenty-four isolates of *C. perfringens* were used in this study (Table 3). All were obtained from a single facility by Veterinary Research Institute had been submitted by local veterinarians for microbiologic examination: 7 of small intestine and liver region specimens from chickens with necrotic enteritis, 14 of fecal specimens from piglets with enterotoxemia, and as well as 3 fecal specimens from cattle with enterotoxemia. The control group of *C. perfringens* NCTC8239 (Hobbs serotype 3) isolated from human beings was donated by Tsukamoto T, Osaka Prefectural Institute of Public Health, Japan.

2. Test for S-R Form Variation by Chemicals

In *E. coli*, tryptic soy broth (DIFCO) was prepared, consisting from 0.1 percent to 1.0 percent of cysteine hydrochloride (Sigma), dextrose (Junsei),

caffeine sodium benzoate (Sigma), mercuric chloride (Junsei), and nicotine (Fluka) respectively, then test strains were inoculated. Two hours incubation of the inoculated cells in the water-bath at 37℃ made it possible to induce the S-R variation but in some tubes it was not enough to discern the variated forms. Therefore, overnight setting at room temperature after the two hours incubation was required to produce a form-variation. Then, the slide agglutination test was performed by means of 0.2 % acriflavine solution (Sigma) for confirmation of test orgnism forms: non-agglutinated cells(−) was defined as S-form, strongly agglutinate one (++, +++) as R-form, and intermediate (±,+) as SR -form (Ahn, 1960). In *C. perfringens*, medium A (agar 20 g, beef extract 5 g, bactopeptone 10 g, NaCl 5 g, d.d.w. 1 L) and BHIA medium (brain-heart-in -fusion agar+1% thioglycollate, DIFCO) were prepared, and the other procedure for S-R form variation test was performed as same means of aforementioned *E. coli* (Ahn, 1960).

3. Antibiotic Susceptibility

Antibiotic susceptibility test was performed by means of a standardized susceptibility discs (FDA, 1976). Antibiotics were chosen based on their effectiveness against *E. coli* isolates from mastitic cows, diarrheic pigs, and diarrheic chickens: cephalexin (Dae Sung, Korea), enrofloxacin (DIFCO), gentamycin (BBL), linsmycin (A/S ROSCO, Denmark), neomycin (A/S ROSCO, Denmark), and norfloxacin (DIFCO). Also, Antibiotics were chosen based on their effectiveness against *C. perfringens* isolates from chickens with necrotic enteritis, piglets with enterotoxemia, and cattle with enterotoxemia: amikacin (BBL), cephalothin (BBL), erythromycin (BBL), gentamycin (BBL), kanamycin (BBL), nalidixic acid (BBL), penicillin (BBL), and tetracycline (BBL).

Ⅲ. Results

E. coli was observed to be most susceptible to naxcel (94.6%), linsmycin (93.5%), and both cephalexin (92.5%) and gentamycin (92.5%) as shown in Table 1. Varied R-form and intermediate SR -form of *E. coli* determined by means of 0.2% acriflavine test could be induced due to culturing in medium-containing of mercuric chloride, cysteine, caffeine, glucose, and nicotine in that order. Mercuric chloride ions were most effective in inducing form-variation. Isolated cells of *E. coli* were highly

Table 2. Antibiotics susceptibility of *Escherichia coli* after smooth-rough variants induced by chemicals (%)

Antibiotics	Cysteine (n=27)	Glucose (n=6)	Caffeine (n=9)	Mercuric chloride (n=49)	Nicotine (n=4)
Cephalexin	24(88.9)	6(100.0)	8(88.9)	45(91.8)	4(100.0)
Enrofloxacin	20(74.1)	4(66.7)	6(66.7)	37(75.5)	3(75.0)
Gentamycin	24(88.9)	5(83.3)	8(88.9)	44(90.0)	4(100.0)
Linsmycin	25(92.6)	6(100.0)	9(100.0)	46(93.9)	4(100.0)
Naxcel	26(96.3)	6(100.0)	9(100.0)	46(93.9)	4(100.0)
Neomycin	22(81.5)	5(83.3)	7(77.8)	41(83.7)	3(75.0)
Norfloxacin	21(77.8)	4(66.7)	6(66.7)	37(75.5)	3(75.0)
Control (EC 82)	±— ++	+ — ++	±— ++	++ — ++	- — ±

Remarks: n: numbers of isolates strains from cases after R form-variation (++ and +++) induced by chemicals consisting from 0.1 percent to 1.0 percent.

- — ++: degree of S-R form variation from - to ++ induced by chemicals of consisting from 0.1 percent to 1.0 percent of control group.

susceptible to some antibiotics, but variation from S-form to R-form seemed to modify the cell less susceptible to antibiotics as shown in Table 2.

C. perfringens was observed to be most susceptible to cephalothin (95.8%), and penicillin (95.8%), erythromycin (75.0%), and amikacin (71.0%) as shown in Table 3. R-form and SR-form variation of *C. perfringens* determined by means of 0.2% acriflavine test could be induced by culturing the cell in medium-containing of mercuric chloride, nicotine, caffeine, cysteine, and glucose in that order. Also, mercuric chloride ions were found most effective in inducing form-variation. The titers of this reaction were almost parallel with the degree of acriflavine reaction on slide in these results as shown in Table 2 and Table 4. Isolated cells of *C. perfringens* were highly susceptible to some antibiotics, but variation from S-form to R-form also seemed to modify the cell less susceptible to antibiotics as shown in Table 4.

Table 3. Susceptible strains before form-variation of *Clostridium perfringens* isolates from chickens with necrotic enteritis, piglets with enterotoxemia, and cattle with enterotoxemia

Antibiotics	Numbers of susceptible strains among 24 strains isolates from cases(%)
Amikacin	17 (71.0)
Cephalothin	23 (95.8)
Erythromycin	18 (75.0)
Gentamycin	1 (4.2)
Kanamycin	2 (8.3)
Nalidixin	13 (54.2)
Penicillin	23 (95.8)
Tetracyclin	2 (8.3)

Ⅳ. Discussion

E. coli and *C. perfringens* are considered to be a part of the normal microflora of the human and animal feces. Most strains of *E. coli* are harmless, but a few are pathogenic (Doyle and Cliver, 1990). *C. perfringens* is widespread in the soil and sewage, and is commonly found in the intestinal tract of human and various animals. It is an opportunistic organism and causes great economic losses by death due to chickens with necrotic

Table 4. Antibiotic susceptibility of *Clostridium perfringens* after smooth-rough variants induced by chemicals (%)

Antibiotics		Cysteine (n=10)	Glucose (n=8)	Caffeine (n=11)	Mercuric chloride (n=91)	Nicotine (n=12)
Amikacin		7(70.0)	6(75.0)	7(63.6)	62(68.1)	8(66.7)
Cephalothin		9(90.0)	8(100.0)	10(90.9)	86(94.5)	11(91.7)
Erythromycin		7(70.0)	5(62.5)	8(72.7)	68(74.7)	9(75.0)
Gentamycin		NT	NT	1(9.1)	3(3.3)	NT
Kanamycin		1(10.0)	1(12.5)	1(9.1)	6(6.6)	1(8.3)
Nalidixin		5(50.0)	5(62.5)	5(45.5)	48(52.7)	7(58.3)
Penicillin		9(90.0)	8(100.0)	10(90.9)	87(95.6)	11(91.7)
Tetracyclin		1(10.0)	NT	1(9.1)	7(7.7)	1(8.3)
Control	Medium A	- — +	- — -	- — -	++ — +++	- — -
(NCTC8239)	BHIA	± — +	- — +	- — +	+ — +++	- — +

Remarks: n: numbers of isolates strains from cases after R form-variation (++ and +++) induced by chemicals consisting from 0.1 percent to 1.0 percent.

NT: not detected.

- — +++: degree of S-R form variation from - to +++ induced by chemicals of consisting from 0.1 percent to 1.0 percent of control group.

enteritis, piglets with enterotoxemia, and cattle with enterotoxemia (Hobbs, 1965; Choudhary and Narayan, 1986).

Ahn (1960) reported that titers of acriflavine reaction in tubes were almost all parallel with the degree of acriflavine reaction on slides, and that Pb ions are most sensitive in differentiating the variation degree of *Shigella flexneri*. Clowes and Rowley (1955) suggested that this explanation might account for the isolation of many of these variants from toxic environments, and proposed that the inhibitor (antibiotics, metal ions etc.) permeates the variants at a lower rate, thus permitting their survival for a longer time than the corresponding, normally metabolizing organisms. They have further suggested that the general lowering of the metabolic rate might be due to a decrease in cell wall permeability. In general, R and SR variants by means of 0.2% acriflavine solution were induced by using mercuric chloride, cysteine, caffeine, glucose, and nicotine in order of effectiveness of *E. coli*. But, R and SR variants by means of 0.2% acriflavine solution were induced by using mercuric chloride, nicotine, caffeine, cysteine, and glucose of *C. perfringens* in that order. Mercuric chloride ions were most sensitive in differentiating the variation degree of *E. coli* and *C. perfringens*. The titers of this reaction were almost parallel with the degree of acriflavine reaction on slide in these results, which is in agreement with findings of Ahn (1960), Jung (1997a), and Jung (1997b).

E. coli was observed to be most susceptible to enrofloxacin (81.3%), gentamycin (68.8%), neomycin (62.5%), and kanamycin (59.4%) (Jung, 1995). Amikacin, amoxicillin/clavulanic acid, ceftiofur, colistin, and nitrofurantoin showed of 100% effectiveness against all isolates, and many of the isolates were also highly sensitive to cephalothin, gentamycin, neomycin, and norfloxacin. The bacteria showed a complete or substantial level of resistance to linsmycin, clindamycin, tiamulin, tylosin, erythromycin, chlortetracycline/tiamulin, tetracycline, triple sulfur, penicillin, and streptomycin (Kim, 1996). The effectiveness of antibiotics against *E. coli* were tested, and gentamycin was found to be the most effective (96.1%), whereas the susceptibilities to the other antibiotics were below 50% (Kim, 1994a). *E. coli* has been also observed to be highly susceptible to naxcel (94.6%), linsmycin (93.5%), and cephaloxin (92.5%), as well as gentamycin (92.5%). The same antibiotics, are studied with similar results of Jung (1994; 1995; 1997a). After S-R variation by use of cysteine, glucose, caffeine, mercuric chloride, and nicotine, these figures decrease somewhat.

The antibiotic susceptibility of 12 antibiotics against 30 isolated strains of *C. perfringens* that 27 or more of 30 strains (>90%) were susceptible to amoxicillin, ampicillin, and cephalothin (Kim, 1994 b). In antibiotic susceptibility tests on isolated strains of *C. perfringens*, all strains were found to be highly susceptible to cephalothin, penicillin, and chloramphenicol (Cho et al., 1990). 12 isolated strains of *C. perfringens* were highly susceptible to ampicillin, enrofloxacin, cephalothin, and penicillin (Park et al., 1994). *C. perfringens* was observed to be highly susceptible to cephalothin (95.8%) and penicillin (95.8%), as well as erythromycin (75.0%), and amikacin (71.0%). The same antibiotics, are studied with similar results of Jung (1997b), Kim (1994b), Cho et al. (1990), and Park et al. (1994). Also, after S-R variation by use of cysteine, glucose, caffeine, mercuric chloride, and nicotine, these figures decrease somewhat.

The antibiotic susceptibility was decreased current due to antibiotics misused and abused, generally. Although antibiotics and growth hormone can be misused when overfed to young animals to promote growth, the also to treat and prevent animal disease. Their misuse or abuse is thus an important issue for human health. This study is to

suggest that treat and prevent animal disease, to help reduce the occurrence of foodborne diseases and increase the productivity of animal farming.

V. Abstracts

Smooth form(S)-rough form(R) variation of *Escherichia coli* and *Clostridium perfringens*, while were determined by means of the acriflavine test, could be induced by culturing the bacteria in medium-containing of mercuric chloride, cysteine, caffeine, glucose, and nicotine, respectively. Varied R-form of *E. coli* determined by using 0.2% acriflavine could be induced of mercuric chloride , cysteine, caffeine, glucose, and nicotine in that order. But *C. perfringens* determined by using 0.2 % acriflavine could be induced of mercuric chloride, nicotine, caffeine, cysteine, and glucose in that order. In both *E. coli* and *C. perfringens*, mercuric chloride ions were most effective in inducing form -variation. Antibiotic susceptibility after variation from S-form to R-form seemed to have modified the cell less susceptible to antibiotics.

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