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[Editorial Note]

The Managing Editor of The Journal of Antibiotics has received information from the Japanese Society of Antimicrobials for Animals on their establishment of "Standards of the *In Vitro* Mutation Frequency Study and the Antimicrobial Activity Study in Gut" with an explanation as follows;

Background of Establishment of Test Standards for Veterinary Antimicrobial Drugs

In view of the recent rapid increase in incidence of infection with antimicrobial resistant bacteria in human medicine, there is international controversy as to the medical risk that is created by transfer of antimicrobial resistant bacteria and antimicrobial resistant genes, which may be produced through the processes of administration of antimicrobials to food-producing animals, via the food chain. According to the current international understanding, the possibility has not been validated sufficiently from the scientific aspect that antimicrobial resistant bacteria are selected in the process of administration of antimicrobials, as veterinary medicinal drugs or feed additives, to food-producing animals and directly affect human medicine. It is an undeniable fact that use of antimicrobials leads to selection of antimicrobial resistant bacteria. Therefore maximum control of antimicrobial resistant bacteria derived from food-producing animals should be indispensable.

Administration of antimicrobials to food-producing animals has contributed to improvement of productivity which can be achieved by treatment of infections and promotion of growth and has enabled stabilization of supply of inexpensive safe animal food products. In view of the importance of maintenance of the husbandry in Japan, no one actually agrees to the proposal of going back to the era before the development of antimicrobials.

In this situation, although there is no compelling reason to support urgent prohibition of administration of antimicrobials to food-producing animals, application of the conventional examinational guidance for registration of new veterinary medicinal drugs for food-producing animals to the substances of significance in human medicine containing substances of similar structure, such as fluoroquinolones and new cephem antibiotics, seemed to be difficult. Accordingly, International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Medicinal Products (VICH) provides the comprehensive guidelines for characterizing selection of antimicrobial resistance by bacteria which may adversely affect human health in order to establish the system of registry of antimicrobial drugs to be administered to food-producing animals. Regarding antimicrobial drugs of significance in human medicine as well as in veterinary medicine, continuous efforts have been made to control antimicrobial resistant bacteria by demanding more rigid test data in compliance with VICH guidelines in Japan. As for therapeutic antimicrobial drugs which are mixed in drinking water or feed, the possibilities of frequent selection of antimicrobial resistant bacteria have been pointed out and submission of the dossier of most rigid degree is requested.

Currently, Japanese Society of Antimicrobials for Animals recognizes the lack of technical test standards in compliance with VICH guidelines in the world and has established the test standards for "*in vitro* mutation frequency studies" to evaluate the appearance frequency and resistant level of resistant bacteria and those for studies on the "antimicrobial activity in gut" to estimate the effects on intestinal flora from professional aspects. Necessity to achieve international harmonization of the dossier to be submitted for approval of veterinary medicinal drugs has been widely insisted on in the world. Considering this situation, we concluded to issue Japanese test standards in the world. In the future, those who make the new veterinary antimicrobial drug application will be requested to submit the dossier in compliance with these test standards and the official approval will be given after thoroughly reviewing them.

Standards of the *In Vitro* Mutation Frequency Study and the Antimicrobial Activity Study in Gut

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In view of the recent rapid increase in incidence of infection with antimicrobial resistant bacteria in human medicine, there is international controversy as to the medical risk that is created by transfer of antimicrobial resistant bacteria and antimicrobial resistant genes, which may be produced through the processes of administration of antimicrobials to food-producing animals, via the food chain. Accordingly, International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Medicinal Products (VICH) provides the comprehensive guidelines for characterizing selection of antimicrobial resistance by bacteria which may adversely affect human health in order to establish the system of registry of antimicrobial drugs to be administered to food- producing animals.

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1. Introduction

Regulatory authorities of various countries such as FDA (Food and Drug Administration) and international organizations and groups such as VICH (International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Medicinal Products) and OIE (Office International Des Épizooties) are establishing the guidelines for the issue of resistance of veterinary antimicrobial drugs, and a part of the draft guideline has already been released[1,2]. This standard of studies was established in order that the sponsor (applicant for approval) conducts the study following the "Guidance on pre-approval information for registration of new veterinary medicinal products for food-producing animals with respect to antimicrobial resistance" (hereinafter referred to as the VICH antimicrobial resistance guideline) prepared by the Expert working group on antimicrobial resistance of VICH.

The VICH antimicrobial resistance guideline is a guidance for registration of therapeutic antimicrobial drugs intended for the use in food-producing animals in EU, Japan and USA, which provides the unified technical guidelines with respect to characterization of the selection of bacterial resistance concerned about human health. The information described in the VICH antimicrobial resistance guideline is classified into basic information and optional information. That is, the basic information is the antimicrobial class, the mechanism and type of action, antimicrobial spectrum of activity, resistance mechanisms and genetics, occurrence and rate of transfer of resistance genes, occurrence of cross-resistance, and pharmacokinetic data. On the other hand, the optional information is the *in vitro* mutation frequency studies, occurrence of co-resistance, antimicrobial drug activity in gut, other animal studies and historical information. Sponsors can choose to include some or all of this optional information at their own discretion.

The objective of this standard of studies is to clarify the study methods to obtain the following two information of different characteristics among this optional information. That is, the one is the "*in vitro* mutation frequency" study which is conducted to clarify the frequency of occurrence of mutants. The other is the study on the "antimicrobial activity in gut" to examine the degree of exposure of the test drug to intestinal flora.

The information obtained these two types of studies is not the one to evaluate the matters with respect to resistant mutants individually but used as a part of information to consider the effect of resistant mutants on humans comprehensively. For example, the discussion of the level of antimicrobial activity in gut should be used to evaluate synthetically the problem of resistant mutants together with the data of the MICs for foodborne pathogens and commensal organisms. Thus, the studies to be conducted according to this standard of studies provide a part of information required for characterizing the possibility of occurrence of resistance which may occur after administration of veterinary antimicrobial drugs to the animals subjected under the proposed conditions of use.

When these studies are conducted following this standard

of studies, the appropriate methods coming up to its objective according to the properties of the test drugs and the characteristics of test animals should be selected as appropriate. If animals are used, it is desirable to increase the precision of studies, to make effort to reduce the number of animals and to consider animal welfare. If the newly developed method is scientifically valid, it can be used.

1.1. Relationship to other guidelines and guidance

This document shows the study standard to conduct the *in vitro* mutation frequency studies and the study on the "antimicrobial activity in gut". This study standard is prepared in the form incorporating the related study methods from the already released guidelines and guidance[3,4,5,6,7] on veterinary medicinal products and ethical drugs as much as possible.

2. Test drugs and formulations

The bulk drugs and preparations of therapeutic antimicrobial drugs intended for the use in food-producing animals are referred to as the test drugs and formulations. The study using the final preparation from the early stage of development is rarely conducted, but it is necessary to use the final preparation in the study on the "antimicrobial activity in gut". In this study standard, cattle, pig and poultry are considered the main food-producing animals.

3. Study methods

3.1. *In vitro* mutation frequency studies

The VICH antimicrobial resistance guideline introduces the methods shown in the "Antimicrobials in Laboratory Medicine, 4th edition"[8], that is, the concentration gradient plate method (slanted plate method) and the nitrosoguanidine method (mutation induction method) as the *in vitro* mutation frequency studies. The objective of the concentration gradient plate method is limited to qualitative examination on whether or not resistant mutants to a certain drug easily occur. Additionally, the nitrosoguanidine method is a special method to isolate mutants occurring at the frequency below the detectable spontaneous mutation frequency using nitrosoguanidine of strong mutagenicity. In Japan, on the other hand, the resistance acquisition study by subculture at the increased dose of the test drug according to the study methods to evaluate the feed additives[9,10] or the study on appearance frequencies of spontaneous resistant mutants[11,12,13] have widely been used as the *in vitro* study methods for preparation of the attached data for application for approval of veterinary antimicrobial drugs. This document describes the concrete methods of the study on appearance frequencies of spontaneous resistant mutants, from which quantitative data can be obtained according to the meaning of the VICH antimicrobial resistance guideline to examine the appearance frequencies of spontaneous resistant mutants.

3.1.1. *In vitro* appearance frequencies of spontaneous resistant mutants

The appearance frequencies of resistant mutant to the test drug and the level of resistance of appeared resistant mutant were determined by the following test methods:

3.1.1.1. Test bacterial species

According to the characteristics of the test drug, *Enterococcus faecalis*, *E. faecium* or *Escherichia coli* shall be used as the test bacterial species. Multiple strains (at least three strains) per one of those bacterial species isolated from the test animals shall be used. As the control bacterial species, additionally, *E. faecalis* JCM7783 (=ATCC29212) strain or *E. faecium* JCM5804 (=ATCC19434) strain or *E. coli* JCM5491 (=ATCC25922) strain shall be used.

3.1.1.2. Test drugs

The test drug and the known drug of the same class as the control drug shall be used.

3.1.1.3. Culture media for enrichment and determination

For enrichment, a semisynthetic liquid medium based on the Mueller-Hinton medium, and for determination, a semisynthetic agar medium based on Mueller-Hinton medium shall be used. Irrespective of the product of any manufacturer, it is desirable to use the product of the same manufacturer for a series of studies, and the name of manufacturer shall be described clearly.

3.1.1.4. Concentrations of antimicrobial drug

The medium containing the drug at the concentration 4- and 8-times higher than that of the MIC determined in advance and, as the control, the medium not containing the drug used for determination of the count of inoculated bacteria shall be prepared. Additionally, the determination of MIC shall be conducted by the validated and standardized method as described in item 3.1.1.7.

3.1.1.5. Preparation of the bacterial solution for inoculation and the method of inoculation of bacteria

The test bacteria shall be incubated in a medium for enrichment at 37°C for 18 to 20 hours, diluted with a medium for enrichment, if necessary, or concentrated by centrifugation to prepare the inoculating bacterial solution of 10^9 CFU/mL. This inoculating bacterial solution shall be inoculated to 10 agar plates containing the drug at 0.1 mL per plate. Simultaneously, in order to determine the count of inoculated bacteria, a series of dilutions of inoculating bacterial solution shall be inoculated onto the medium not containing the drug.

3.1.1.6. Counting of colonies and calculation of appearance frequencies of resistant mutants

The colonies (resistant mutants) appearing on the drug-containing medium after incubation at 37°C for 18 to 20 hours shall be counted. From this number of colonies and the simultaneously determined count of inoculated bacteria, the appearance frequency of resistant mutants shall be calculated.

3.1.1.7. Determination of MIC of resistant mutants

At each added concentration of drug, multiple colonies (at least 5 colonies) shall randomly be taken from all colonies growing on the drug-containing medium to determine the MIC of the test drug. The determination of MIC shall follow the method of Japanese Society of Antimicrobials for Animals[14], Japan Society for Chemotherapy[15], or NCCLS[16,17].

3.2. Antimicrobial activity in gut

3.2.1. Method of quantification

It is necessary that, for the assay method used for determination of the concentrations of the test drug and metabolites contained in the collected sample, its sensitivity, precision and reproducibility shall be validated.

The assay method shall appropriately be selected according to the objectives of study, and the biological assay method by which the test drug and the metabolites with antimicrobial activity are determined without fractionation is one of the assay methods suitable for the objectives of this study. A certain method strongly correlated with the biological assay method and of better sensitivity, precision and reproducibility can be substituted, if any. The biological assay method is used in the study on the residue of veterinary medicinal products[6], in which the sensitivity, precision and reproducibility are the limit of detection of 0.05 ppm or lower, the recovery rate of 70% or higher in the spiked recovery experiment at 1 or 2 ppm and the coefficient of variation of about 10%, respectively.

3.2.2. Animal species

The study shall be conducted using the animal species to be clinically applied (test animal species) with an apparent history of use of feed and veterinary medicinal products.

3.2.3. Number of animals

It is necessary to conduct the study in the number of animals suitable to clarify the changes in the concentrations of the test drug and metabolites in feces and the distribution in the intestinal content after considering the precision of the study.

3.2.4. Route of administration

The route of administration shall be selected according to clinical application. If there are multiple routes of clinical administration, an appropriate route of administration shall be selected considering the toxicity in test animals and the route of administration used in the efficacy pharmacology study. If oral administration is selected, forced oral administration by gavage can be used, but it is necessary to use the final preparation as the test formulation.

3.2.5. Dose and frequency of administration

The dose shall be selected considering the expected clinical dose. If several levels of clinical dose are expected, the highest expected clinical dose shall generally be considered the dose. In principle, administration shall be conducted once.

3.2.6. Standard test methods

In the studies on absorption in the test animals, that is, the studies to examine the time-course of blood concentration and the studies to determine the distribution in main organs and tissues, feces and the intestinal content shall be collected, respectively, and the concentrations of the test drug and metabolites in the sample shall be determined to evaluate the pharmacokinetics. For these two studies, that it, the study to examine the "time-course of fecal concentration" and the one to examine the "concentration in the intestinal content" described in the item below, the study methods suitable for the objectives shall be selected considering the form of feeding of test animals and the characteristics of excretion. Additionally, the metabolites in these studies are those with antimicrobial activity.

Not taken in this document in detail, it is also possible to

conduct discussion using the data on the amount of the test drug and the composition and amount of metabolites in feces determined to examine urinary and fecal excretions in the test animals.

Additionally, if there is a possibility that the test drug adsorbs to feces or the intestinal content to affect its antimicrobial activity, the extent of decrease in the concentration of the test drug due to adsorption to feces shall be clarified.

3.2.6.1. Time-course of the concentrations in feces

In the study to examine the changes in blood concentrations (whole blood concentration, plasma concentration, and serum concentration) of the test drug, feces shall be collected with time. That is, the change in the fecal concentrations of the test drug and metabolites in the test animals shall be examined by a single-dose study. Basically, oral administration shall be conducted after fasting. The time-point of collection required to clarify the prosperity and decay of the test drug and metabolites shall be established, and feces shall be collected so as not to be contaminated with urine.

3.2.6.2. Concentration in the intestinal content

In the study to examine the distribution of the test drug in main organs and tissues, not only main organs and tissues but also the colonic content shall be collected to determine the concentrations of the test drug and metabolites. Since the distribution study is conducted to examine the distribution of the test drug in organs and tissues, its time-course and accumulation, if necessary, it shall principally be conducted by single administration. In order to show the time-course of distribution, additionally, sampling shall generally be conducted near the time-point showing the highest blood concentration and in the elimination phase, if necessary.

3.2.6.3. Effect of fecal binding

The feces collected from the test animals shall be used as the test material. An appropriate amount of appropriate buffer solution to the collected feces shall be added to prepare a suspension, and the reaction mixture prepared by addition of the test drug solution to the suspension shall be incubated at a certain temperature (for example, near the body temperature of animals) for a certain period. The concentration of the test drug in the supernatant obtained by centrifugation of the reaction mixture shall be determined before and after incubation to clarify the degree of decrease in the test drug concentration due to fecal binding. The test drug concentration and incubation period of the reaction mixture shall be decided with reference to the results obtained from the study to examine the pharmacological effects, the time-course of the fecal concentration previously described and the study on the concentration in the intestinal content.

4. Glossary

VICH: A trilateral (Japan, USA and EU) program aimed at harmonizing technical requirements for veterinary product registration, in which the trilateral organization for examination of approval and pharmaceutical parties participates. Its official name is the "International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Medicinal Products", which was started in April in 1996.

Food-producing animals: Although there are some regions considering sheep, goats, ducks and rabbits as major food-producing animals, cattle, pig and poultry are considered as major food-producing animals in this study standard according to the VICH antimicrobial resistance guideline. Additionally, since, for fish, the production system, the related bacterial population and the animal-originated concern about public health are basically different from those for cattle, pig and poultry, fish is omitted from the subject of this study standard same as the VICH antimicrobial resistance guideline.

Foodborne pathogens: Pathogens of which animals can be carriers in the gut and which become causes of foodborne poisoning in humans. *Salmonella* and *Campyrobacter* are applicable.

Commensal bacteria: Bacteria living in or on animals but not showing pathogenicity in animals. *Enterococci* and non-pathogenic *E. coli* are applicable.

Concentration gradient plate method (slanted plate method): One of the *in vitro* tests to examine appearance of resistant mutants, which is a method to select resistant mutants by growing bacteria on an agar plate consecutively increasing the concentrations of antimicrobials. That is, an agar containing antibiotics is poured onto the slanted and solidified agar to prepare agar plates of consecutive concentration gradient of antimicrobials. Mutants are selected by inoculating and spreading the culture medium of the test bacteria onto this plate.

Validation: For example, to establish the sensitivity,

precision, reproducibility and specificity of analytical method at the time when the test drug and its metabolites in the biological sample are quantified.

Bioassay: An assay method using the physiological effects on organisms as indices, which is used for determination of antimicrobial activity of antimicrobial substances. In the standard method, a disk (or stainless cylinder) is placed on an agar plate containing the test bacteria, and the sample solution is added and incubated. If the sample contains the substance of antimicrobial activity, a circular transparent zone (inhibition zone) produced around the disk due to inhibition of growth of the standard bacteria is formed. Using the regression equation determined from the concentration of the standard solution of the test antimicrobial and the diameter of its inhibition zone, the concentration of antimicrobial drug in the sample solution is calculated from the inhibition zone of the sample solution.

Studies on absorption, *etc.*: Studies conducted to clarify the pharmacokinetics of the drug in the body, which examine the absorption, distribution, metabolism and excretion of the test drug. For veterinary medicinal products, the test drug or the test formulations are administered to the test animals or experimental animals to examine the time-courses of the blood concentrations, the amount to be excreted into urine and feces, the amount to be excreted into bile and the concentrations in various organs and tissues and the metabolites in the body.

5. List of related guidelines, guidance and publications

1) VICH: Guidance on pre-approval information for registration of new veterinary medicinal products for food-producing animals with respect to antimicrobial resistance; for consultation at step 4 (2001)
2) OIE: Antimicrobial resistance: reports prepared by the OIE Ad hoc Group of experts on antimicrobial resistance (2001)
3) Ministry of Health, Labour and Welfare: Guidelines for non-clinical pharmacokinetic studies (1998)
4) Ministry of Health, Labour and Welfare: Clinical pharmacokinetic studies of medicinal products (2002)
5) Ministry of Health, Labour and Welfare: Guidance for safety evaluation of residual veterinary medicinal products in livestock food and aquatic food, Japanese Association of Residues and Safety in Animal Products, Tokyo (1998)
6) Ministry of Agriculture, Forestry and Fisheries: Guidelines for toxicity studies of new animal drugs. (5) Guidelines for residue tests (2000)
7) Ministry of Agriculture, Forestry and Fisheries: Guidelines, Detailed regulations for various studies for application for manufacturing (importing) approval of veterinary medicinal products intended for the use in fishery animals (2000)
8) RICE LB, BONOMO RA: Antimicrobials in laboratory medicine, Lorian V, ed., 4th ed., 482~483, Williams and Wilkins, Baltimore (1996)
9) TAKAHASHI I: Efficacy test methods of antimicrobial drugs, Kohanawa T, ed. 1st ed., 155~192. Fuji-Technosystem. Tokyo (1977)
10) Ministry of Agriculture, Forestry and Fisheries: Standards for Evaluation of feed additives and Outline of Procedures for Major Studies, 2nd ed., 63~68, Japan Scientific Feeds Association, Tokyo (1992)
11) SATO K, INOUE M & MITSUHASHI S: Chemotherapy, 32, S-1, 1~12 (1984)
12) BARRY AL,·THORNSBERRY C & JONES RN: Antimicrob Agents Chemother, 29, 40~43 (1986)
13) KAMATA S, YOSHIDA T, MATSUNAGA T & UCHIDA K.: Jpn Bull Anim Hyg, 33, 1~3 (1991)
14) Japanese Society of Antimicrobials for Animals: Determination of the minimum inhibitory concentration (MIC) of drugs on animal-derived bacteria. Proceedings of The Japanese Society of Antimicrobials for Animals, 18, 40~41 (1997)
15) Japanese Society of Chemotherapy: Re-revision of the method of determination of the minimum inhibitory concentration (MIC), Chemotherapy, 29, 76~77 (1981)
16) NCCLS: Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; approved standard (1999)
17) NCCLS: Development of in vitro susceptibility testing criteria and quality control parameters for veterinary antimicrobial agents; approved guideline (1999)