

S4 Regulatory guidelines (microbiology) for veterinary antimicrobial products

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INTRODUCTION

There have been a plethora of new microbiology guidelines introduced by the European Medicines Agency (EMA) in recent years. These have primarily been driven by the concerns that the imprudent use of antimicrobials in veterinary medicine is prejudicing their use in human medicine. Whilst a clear link between the use of antimicrobials in veterinary medicine and resistance development in human medicine remains to be demonstrated it is appropriate that all users of antimicrobial compounds examine the ways in which society uses these valuable resources.

The EMA is a decentralised body of the European Union with headquarters in London. Its main responsibility is the protection and promotion of public and animal health, through the evaluation and supervision of medicines for human and veterinary use. The EMA coordinates the evaluation and supervision of medicinal products throughout the European Union. The Agency brings together the scientific resources of the 25 EU Member States in a network of 42 national competent authorities. It cooperates closely with international partners, reinforcing the EU contribution to global harmonisation. The

EMA began its activities in 1995, when the European system for authorising medicinal products was introduced, providing for a centralised and a mutual recognition procedure. The EMA has a role in both, but is primarily involved in the centralised procedure. Where the centralised procedure is used, companies submit one single marketing authorisation application to the EMA. For veterinary medicines a single evaluation is carried out through the Committee for Medicinal Products for Veterinary Use (CVMP). If the relevant Committee concludes that quality, safety and efficacy of the medicinal product is sufficiently proven, it adopts a positive opinion. This is sent to the Commission to be transformed into a single market authorisation valid for the whole of the European Union.

Whilst CVMP is responsible for publishing respective guidelines on the EMA website (<http://www.ema.eu.int>) a welcome development in recent years has been VICH, a trilateral (EU-Japan-USA) programme aimed at harmonising technical requirements for veterinary product registration. Its full title is the International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products. VICH was officially launched in April 1996. The objectives of the VICH are:

1. to provide a forum for a constructive dialogue between regulatory authorities and the veterinary medicinal products industry on the real and perceived differences in the technical requirements for product registration in the EU, Japan and the USA, with the expectation that such a process may serve as a catalyst for a wider international harmonisation;
2. to identify areas where modifications in technical requirements or greater mutual acceptance of research and development procedures could lead to a more economical use of human, animal and material resources, without compromising safety;
3. to make recommendations on practical ways to achieve harmonisation in technical requirements affecting registration of veterinary products and to implement these recommendations in the three regions.

Once adopted VICH recommendations replace corresponding regional requirements. Of the guidelines that will be discussed in this chapter two are harmonised VICH guidelines. This initiative has clear benefits to sponsors in that a single data package should now satisfy the regulatory authorities in

Europe, Japan and the USA. This chapter, whilst focused on the European regulatory microbiology requirements for an antimicrobial compound is also relevant for the USA and Japan with respect to the harmonised guidelines.

SCOPE

This chapter will address the four guidelines with significant microbiology content, two of which are harmonised guidelines and two that solely relate to Europe;

CVMP/VICH/467/03-FINAL (Harmonised VICH Guideline 36)

Studies to evaluate the safety of residues of veterinary drugs in human food: general approach to establish a microbiological acceptable daily intake (ADI)

CVMP/VICH/644/01-FINAL (Harmonised VICH Guideline 27)

Guidance on pre-approval information for registration of new veterinary medicinal products for food producing animals with respect to antimicrobial resistance

EMA/CVMP/627/01-FINAL

Guideline for the demonstration of efficacy for veterinary medicinal products containing antimicrobial substances

EMA/CVMP/276/99-FINAL

Note for guidance for the assessment of the effect of antimicrobial substances on dairy starter cultures

It is often not appreciated that the requirements for a sponsor of a veterinary antimicrobial drug intended for use in food-producing animals are more onerous than those for companion animals or indeed for man. This is primarily because of the issue of potential drug residues being ingested in the human diet. Indeed this is the rationale behind CVMP/VICH/467/03-FINAL (Harmonised VICH Guideline 36). The rationale of CVMP/VICH/644/01-FINAL (Harmonised VICH Guideline 27) is to ensure that the potential for transfer of resistant organisms or resistance determinants from food animals to man through the food chain are minimised.

All the above guidelines are clearly detailed in full on the EMA website (<http://www.ema.eu.int/index/indexv1.htm>).

CVMP/VICH/467/03-FINAL (HARMONISED VICH GUIDELINE 36)

Studies to evaluate the safety of residues of veterinary drugs in human food: general approach to establish a microbiological ADI

Introduction

A variety of toxicological evaluations are performed to establish the safety of veterinary drug residues in human food. For drugs used in food-producing animals it is necessary to establish what is referred to as the ADI (Acceptable Daily Intake). This is defined as an estimate of the amount of a substance, expressed on a body weight basis, that can be ingested daily over a lifetime without appreciable risk to human health. It is necessary to determine a toxicological, pharmacological and microbiological ADI.

In recent years there has been an increasing awareness of the potential impact of residues on the gastro-intestinal flora. This is clearly a complex issue and has been handled in different ways by the regulators in different parts of the world. As a consequence and in an attempt to harmonise the different approaches a Microbiological Task Force was set up by VICH to draft a harmonised guideline. The Task Force recognised that the intestinal flora plays an important role in maintaining and protecting the health of individuals. This flora provides important functions to the host such as metabolising endogenous and exogenous compounds and dietary components; producing compounds that are later absorbed; and protecting against the invasion and colonisation by pathogenic micro-organisms. It is also widely accepted that ingested antimicrobial drugs can potentially alter the ecology of the intestinal flora reaching the colon due to incomplete absorption or being absorbed, circulated and excreted via bile or secreted through the intestinal mucosa. Taking all these issues into account it was agreed that the microbiological endpoints of current public health concern that should be considered when establishing a microbiological ADI are the disruption of the colonisation barrier and a measure of the increase of the population(s) of resistant bacteria. For the purposes of the guideline, resistance is defined as the increase of the population(s) of bacteria in the intestinal tract that is (are) insensitive to the test drug or other antimicrobial drugs. This effect may be due either to

the acquisition of resistance by organisms which were previously sensitive or to a relative increase in the proportion of organisms that are already less sensitive to the drug.

The present guideline is described as an attempt to address the complexity of the human intestinal flora and reduce uncertainty when determining microbiological ADIs. The guideline outlines a process for determining the need for a microbiological ADI and discusses test systems that take into account the complexity of the human intestinal flora. These test systems could be used for addressing the effects of antimicrobial drug residues on human intestinal flora for regulatory purposes. The guideline makes clear that further research is needed to confirm the reliability and validity of all test systems and it does not recommend any one particular system for use in regulatory decision-making. Instead, it provides recommendations for a harmonised approach to establish a microbiological ADI and offers test options rather than specifying a testing regimen. For a review of the history of this subject the reader is referred to the excellent review of Cerniglia and Kotarski¹.

The guideline requires the determination of two distinct microbiological ADI values and was adopted by CVMP on June 16th, 2004 and came into effect May, 2005. The essence of the guideline is summarised in the five steps outlined below and taken from Section 2 of CVMP/VICH/467/03-FINAL;

Steps in determining the need for a microbiological ADI

When determining the need for a microbiological ADI, the following sequence of steps is recommended. The data may be obtained experimentally, from the published literature, or other sources.

Step 1. Are residues of the drug, and (or) its metabolites, micro-biologically active against representatives of the human intestinal flora?

- Recommended data:
 - MIC data from the following relevant genera of intestinal bacteria (*E. coli*, and species of *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Enterococcus*, *Eubacterium (Collinsella)*, *Fusobacterium*, *Lactobacillus*, *Peptostreptococcus/Peptococcus*).

- It is recognised that the understanding of the relative importance of these micro-organisms is incomplete and that the taxonomic status of these organisms can change. The selection of organisms should take into account current scientific knowledge.
- If no information is available, assume that the compound and (or) its metabolites are microbiologically active.

Step 2. Do residues enter the human colon?

- Recommended data:
 - Absorption, distribution, metabolism, excretion (ADME), bioavailability, or similar data may provide information on the percentage of the ingested residue that enters the colon.
- If no information is available in humans, use appropriate animal data. If there is no available information, assume that 100% of the ingested residue enters the colon.

Step 3. Do the residues entering the human colon remain microbiologically active?

- Recommended data:
 - Data demonstrating loss of microbiological activity from *in vitro* inactivation studies of the drug incubated with faeces or data from *in vivo* studies evaluating the drug's microbiological activity in faeces or colon content of animals.

If the answer to any of questions in steps 1, 2, or 3 is "no", then the ADI will not be based on microbiological endpoints and the remaining steps need not be addressed.

Step 4. Assess whether there is any scientific justification to eliminate the need for testing either one or both endpoints of concern. Take into account available information regarding colonisation barrier disruption and resistance emergence for the drug. If a decision cannot be made based on the available information, both endpoints need to be examined.

Step 5. Determine the NOAECs/NOAELs for the endpoint(s) of concern as established in step 4. (NOAEC refers to no observable

adverse effect concentration and NOAEL to a no observable adverse effect level.) The most appropriate NOAEC/ NOAEL is used to determine the microbiological ADI.

The studies referred to in the guideline are complex and it is crucial that a sponsor fully understands all the issues and potential pitfalls before embarking on a series of studies. One of the positive aspects of this guideline is that it does offer the sponsor alternative approaches to addressing microbiological ADI determinations, however, it is my opinion that to fully exploit these opportunities the sponsor and the regulatory authorities must sit down together to discuss the most appropriate study approach for the respective active ingredient. One size does not fit all. It is also important to point out that the approach to the microbiological ADI determinations for colonisation barrier effects and resistance development are fundamentally different. In the former it is possible to carry out simple MIC studies and/or alternative short term *in vitro* approaches whereas the latter requires complex long-term population studies which can be carried out in *in vitro* or *in vivo* test systems.

CVMP/VICH/644/01-FINAL (HARMONISED VICH GUIDELINE 27)

Guidance on pre-approval information for registration of new veterinary medicinal products for food producing animals with respect to antimicrobial resistance

Introduction

The guideline was adopted by CVMP on 14 January, 2004 and came into effect on 15 December, 2004. It is accepted that the use of antimicrobial agents is likely to lead to selection of resistance irrespective of whether such compounds are administered to humans, animals or plants. The Guideline makes the important point that zoonotic organisms can, by definition, be transferred to humans from animals and thus it stands to reason that resistant zoonotic organisms can also be transferred to humans. What remains contentious is the transfer of antimicrobial-resistant non-zoonotic bacteria or their genetic material from animals to humans via the food chain.

The objective of Guideline CVMP/VICH/644/01-FINAL (GL 644)

is to provide harmonised technical guidance in the EU, Japan and the US for registration of antimicrobial veterinary medicinal products intended for use in food-producing animals with regard to characterisation of the potential for a given antimicrobial agent to select for resistant bacteria of human health concern.

GL 644 outlines the types of studies and data that are recommended to characterise the potential resistance development as it might occur in the food-producing animal under the proposed conditions of use of the product. This includes information, which describes attributes of the drug substance, the drug product, the nature of the resistance and the potential exposure of the gut flora in the target animal species. It does not account for post-slaughter factors such as processing of food products or kitchen hygiene that affect the potential human health impact.

GL 644 differentiates data into basic and additional. It is expected that a drug sponsor will provide all the “basic” data. In practice a sponsor will only provide additional data if requested to do so or if following an initial risk analysis it is clear that such data will need to be provided to allow the regulatory authorities to address issues of public health concern. Much of the basic information is fundamental to understanding the antimicrobial activity of a test compound. The guideline is very clearly structured and the relevant sections are as detailed below.

Basic Information

Antimicrobial class

Mechanism and type of antimicrobial action

It is rare for a new antimicrobial class to be first introduced into veterinary medicine although there are classes that are not used in human medicine such as the pleuromutilins. The information in this section can be taken from the literature, patent information, or specific mechanism of action studies undertaken by the sponsor. It is important for the sponsor to detail within this section whether the compound is bacteriostatic or bactericidal.

Antimicrobial spectrum of activity

General data

Information on the antimicrobial agent should be provided by the

sponsor including data from MIC (minimum inhibitory concentration) tests against a wide variety of micro-organisms or from literature studies, in order to determine the overall spectrum of activity. Where MICs are determined by the sponsor, the source of the isolates may be from culture collections, diagnostic laboratories, or other repositories. Where possible, MIC values should be determined with a validated and controlled method and the Guideline specifically cites the Clinical Laboratory Standards Institute (CLSI) (formerly NCCLS) Standard, M31-A2.² It is important in this section to include data on those genera that are not susceptible to the active compound.

MICs of target animal pathogens

The target pathogens are those that are indicated on the label claim. These data will also be required for Guideline EMEA/CVMP/627/01-FINAL that addresses product efficacy. The same data can clearly be used with regard to both guidelines.

MICs of food-borne pathogens and commensal organisms

Data should be presented to show MICs of food-borne pathogens and commensal organisms. This information may be based on published data or on studies done by the sponsor. The required data will clearly depend on the spectrum of activity of the active compound. The sponsor will be expected to provide data for the food-borne pathogens, *Salmonella enterica* and *Campylobacter* spp. and for the food-borne commensal organisms, *Escherichia coli* and *Enterococcus* spp.

It is important that the sponsor selects appropriate strains. Guidance is given and states that when possible, the strains included should be selected according to the following recommendations:

- Strains of relevant bacterial species/serotypes should be isolated from the proposed target animal species. When the product is intended for a broad range of animal species, the strains should be from the main food-producing species (e.g. cattle, pigs and poultry).
- Preferably, the strain collection should include recent isolates.

Information on the tested strains should include:

- identification at least to the species level;
- origin, source and data of isolation.

Experience suggests that if these criteria are not met then the sponsor will be asked to go away and provide additional data.

Antimicrobial resistance mechanisms and genetics

The guideline asks for information on the resistance mechanism(s) and information on the molecular genetic basis of resistance to the antimicrobial agent. Again it is acceptable for this information to come from literature or from studies carried out by the sponsor. It is pertinent to point out that if a sponsor claims to have a common mechanism of action with another compound yet provides MIC data that differentiate the compound from the comparator the authorities are likely to ask for additional data that explain the difference in susceptibility data.

Occurrence and rate of transfer of antimicrobial resistance genes

This requirement is relatively new within the European regulatory system although once again the data can be from the literature or from studies carried out by the sponsor. Within this section the guideline suggests that the sponsor may consider including data on target animal pathogens, relevant food-borne pathogens, and relevant commensal organisms.

Occurrence of cross-resistance and co-resistance

Information on cross-resistance to the antimicrobial agent and co-resistance of the antimicrobial agent in question with other antimicrobial agents needs to be provided. It always used to be sufficient to provide only phenotypic data but now the guideline states that if available, a genotypic description of strains should be provided. Clearly the choice of antimicrobials for the co-resistance study will be dependent in part on the antimicrobial class of the test product.

Pharmacokinetic data

Within any antimicrobial dossier there will have been a need to determine pharmacokinetic data. These data are needed in order to predict the antimicrobial activity of the test compound within the

intestinal tract. It is crucial that levels of active compound within the intestinal tract are adequately determined such that their potential impact on zoonotic and commensal bacteria can be assessed.

Additional Information

A sponsor must decide whether the following data are likely to be useful in order for the authorities to assess any impact of the active compound on public health. *In vitro* mutation frequency studies, antimicrobial agent activity in intestinal tract, other animal studies conducted to help characterise the rate and extent of resistance development associated with the proposed use of the antimicrobial product. This may include data from clinical studies conducted in support of other sections of the dossier.

With regard to the animal studies the guideline makes the important point that, “the predictive value of the results of such studies is yet to be established with regards to resistance development. Therefore the results of such studies should be interpreted in the context of all other pre-approval information described in this document.”

When available and relevant, supporting information from literature or studies on previously approved uses of the drug product or related products may be provided.

When all these data have been assembled the sponsor, normally through an Expert Report, is expected to characterise the potential for the use of the product to select for antimicrobial-resistant bacteria of human health concern. In order to achieve this it is necessary to discuss the information provided in the previous sections in terms of the exposure of food-borne pathogens and commensal organisms to microbiologically active substance in the target animal after administration of the veterinary medicinal product under the proposed conditions of use. It is crucial that all the data are referred back to the intended use of the test compound in the target animal. Intuitively one can easily see that an antimicrobial compound administered orally in feed to a poultry flock is likely to present a greater challenge to public health than the same compound administered by injection to individual cattle.

EMA/CVMP/627/01-FINAL

Guideline for the demonstration of efficacy for veterinary medicinal products containing antimicrobial substances

Introduction

Guideline EMA/CVMP/627/01-FINAL for the demonstration of efficacy for veterinary medicinal products for use in all animals containing antimicrobial substances applies to antimicrobial substances for all routes of administration, except for intramammary administration. The guideline was adopted by CVMP, 11 December 2002 and came into effect 11 June 2003. It aims to ensure that the applicant can demonstrate therapeutic efficacy of an antimicrobial substance for given indications whilst using a therapeutic regimen that minimises the risk of selecting antimicrobial resistant bacteria.

Data Requirements

There is considerable cross-over with Guideline CVMP/VICH/644/01-FINAL, the following issues are common between the two; details on antimicrobial class, mode and mechanism of action, antimicrobial spectrum of activity, MIC data with respect to target animal pathogens, resistance mechanisms including addressing occurrence of cross and co-resistance. The guideline does state that the number of isolates for MIC determinations should be scientifically justified and representative of the EU area and details must be provided with respect to the origin of any tested isolates. This can clearly pose significant problems in some cases although it is true to say that the authorities are prepared to be pragmatic when strain availability is a significant issue.

In addition to that mentioned above there is a need to provide a range of other standard *in vitro* data.

Minimum Bactericidal Concentration (MBC)

The MBC is the lowest concentration of an antimicrobial substance which under defined *in vitro* conditions reduces bacterial counts by 99.9%. Clearly not all antimicrobial compounds are bactericidal but if a sponsor is claiming bactericidal activity they must provide supportive data against representative target pathogens. CLSI provide a detailed

guideline, M26-A, Methods for Determining Bactericidal Activity of Antimicrobial Agents, for carrying out such studies.³

Kinetics of Bacterial Killing

Whilst this appears to be a simple requirement and most microbiologists will be familiar with standard bacterial kill curves there is something of a sting in the tail. The guideline clearly states that, "Data on the kinetics of bacterial killing should be provided to enable the action of the antimicrobial against the target bacteria to be characterised". When unpacked this relates to characterising the nature of killing which is of course important to interpretation of the pharmacodynamic nature of the test compound. The resultant data thus need to address questions of bactericidal and bacteriostatic action as well as whether the active compound can be characterised as a time-dependent, concentration-dependent or co-dependent compound⁴. Well designed *in vitro* studies that take into consideration the pharmacokinetics of the test compound by the relevant route of administration can adequately address all these questions.

Post Antibiotic Effect (PAE)

PAE is defined as the period of persistent suppression of bacterial growth after short exposure to the active antimicrobial, after the concentration of antimicrobial substance has diminished below the MIC. PAE can be extremely important with regard to influencing the dosage interval. Standard *in vitro* protocols are perfectly adequate for addressing this important issue against target animal pathogens.

Other Information

Under this heading the guideline makes the comment that some environmental factors may influence the antimicrobial activity of a test drug. This may be especially important with respect to activity of the compound in specific infections. If indeed a claim is being sought for urinary tract infections a sponsor may consider it appropriate to look at *in vitro* activity in the presence of urine, or for a mastitis claim activity in milk would be appropriate. Similarly if a claim was being made for activity against abscesses it might be considered appropriate to look at the effect of incubation atmosphere on *in vitro* susceptibility.

Data Interpretation

The remainder of this guideline refers to how the data should be interpreted and addresses the very important issues of pharmacokinetic-pharmacodynamic (PK-PD) analysis, breakpoints and all the issues relating to clinical studies such as dose-determination, dose-confirmation, and field trials. Clearly the latter fall in the realm of the clinician whereas the former are the subject of the microbiological expert report.

It is not appropriate to discuss PK-PD analysis at length other than to say that it is directed towards obtaining the best correlation between clinical cure and antibacterial activity and can thus be used as one of the contributing factors to selecting an appropriate administration regimen which optimises dosing by maximising efficacy and restricting emergence of resistance. Guideline EMEA/CVMP/627/01-FINAL advises that when the product is aimed at more than one pathogen which is part of the same therapeutic indication, it may be useful, within the context of PK-PD analysis, to identify the bacterial species which is dose-limiting. As the pharmacokinetics of animal species are different it is necessary to address this on a case by case basis and within each animal species to consider the limiting bacterial species claim. It is also necessary to realise that PK-PD principles are antimicrobial class specific and need to be worked through for each “bug/drug” combination. It is also important to be mindful of the important issue which is highlighted in Guideline EMEA/CVMP/627/01-FINAL when it points out that it may be appropriate to use concentrations of the active substance in tissue or other biological fluid rather than those in serum or plasma in PK-PD studies.

A final comment needs to be made in relation to breakpoints. The guideline highlights the requirement to detail MIC distribution of recent representative isolates of target pathogens indicating the proportion of resistant isolates and the breakpoints used. CLSI uses microbiological, pharmacokinetic and clinical data to establish breakpoints⁵ and without such considerations it is not possible to consider what is truly clinically sensitive or resistant. The MIC distribution pattern for a large number of micro-organisms often enables identification of two or more populations of micro-organisms that can be differentiated by the presence or absence of resistance factors. “Susceptible” and “resistant” MIC breakpoints can thus be established to differentiate these

populations. Dudley and Ambrose⁶ highlighted the challenge of combining microbiological, pharmacokinetic and clinical data to produce a single susceptibility/resistance breakpoint and asked the question, “should a breakpoint detect resistance or predict the antimicrobial effects of a drug in a patient when the drug is administered as a normal dose?” These are two fundamentally different functions, it would appear that the considerable disagreement that often emerges in selecting breakpoints stems from a failure to recognise this difference and hence a desire to combine all information into a single function⁶. Dudley and Ambrose suggested that the dual objective for breakpoints to detect resistance/predict outcome of therapy will continue to fail in many circumstances, and continue to serve as a source of confusion among clinicians, clinical microbiologists, regulators and researchers. Moreover, in an age in which clinicians manage infections by empiric therapy of a patient syndrome, the need to consider the distribution of MICs as well as drug exposure when administering safe doses in target populations must be taken into account while establishing MIC breakpoints for susceptibility.

Indeed Kahlmeter *et al.*⁷ have recently published a review of the state of breakpoints within Europe in an attempt to harmonise this issue. These authors point out that the success or failure of antimicrobial therapy in bacterial infections is predicted ideally by antimicrobial susceptibility testing (AST), in which micro-organisms are divided into treatable and non-treatable categories on the basis of MIC breakpoints. In Europe, the categorisation was traditionally a clinical one and it was made irrespective of whether or not the organism harboured resistance mechanisms. MIC breakpoints generally divide bacteria into three categories of susceptibility: susceptible, intermediate or indeterminate, or resistant. These terms can be defined as susceptible (S – where the antimicrobial activity is associated with a likelihood of therapeutic success), intermediate or indeterminate (I - where the antimicrobial activity is associated with an indeterminate or uncertain therapeutic effect) and resistant (R – where the antimicrobial activity is associated with a higher than expected likelihood of therapeutic failure). MIC breakpoints are used either directly, as in MIC determination and ‘breakpoint’ susceptibility testing methods in broth or agar or indirectly when converted into inhibition zone diameters in disc diffusion techniques.

Kahlmeter *et al.*⁷ discussed that the lack of harmonised breakpoints

and methods in different countries, or even within the same country, often obviates meaningful comparison of resistance rates, monitoring of development of resistance in international surveillance systems and investigation of the effects of intervention strategies. Breakpoints have evolved to try to satisfy both the need to guide therapy and the need to detect biological resistance, often resulting in compromises that satisfy neither. It seems little more than chance that the different European breakpoint committees occasionally recommend the same breakpoints and use the same terminology and/or methods, one example is the quite remarkable differences in ciprofloxacin breakpoints for *Enterobacteriaceae*⁷.

MIC breakpoints are defined against a background of data, including therapeutic indications, clinical response data, dosing schedules, pharmacokinetics and pharmacodynamics, and other microbiological data. The process of determining breakpoints never was, and probably never will be, exact or strictly scientific⁷.

Mouton⁸ made a case to recognise that there is a difference between clinical and microbiological breakpoints. Whilst the microbiological breakpoint may be used to detect organisms that do not belong to the natural bacterial population, but somehow have acquired resistance and might be useful in recognising emergence of resistant subpopulations, the clinical breakpoint is of principal value to the clinician in that it results in a classification of susceptible, intermediate and resistant isolates. It is a parameter that is used in clinical practice and correlates with a measure of clinical efficacy. In discussing future developments, Mouton⁸ suggested that irrespective of approach any method should indicate the probability of successful eradication of the micro-organism and successful treatment.

My view is that there should be standardisation on the setting of breakpoints following the established CLSI procedure⁵.

EMEA/CVMP/276/99-FINAL

Note for guidance for the assessment of the effect of antimicrobial substances on dairy starter cultures

Introduction

The guideline was adopted by CVMP on 8 March 2000 and came into effect on 8 September, 2000. According to Volume VI of the Rules Governing Medicinal Products in the European Community the safety evaluation carried out in connection with the establishment of maximum residue limits for residues of veterinary medicinal products in food stuffs of animal origin under Council Regulation (EEC) No 2377/90 must include an assessment of the potential effects on micro-organisms used for industrial food processing, in particular as regards the manufacture of dairy products. Data to allow such assessment therefore must be provided for substances whose residues may appear in inhibitive concentrations in milk in connection with recommended treatment of the target species. When present in sufficient concentrations, antimicrobial substances may cause problems to the dairy industry by inhibiting the activity of bacterial starter cultures. The largest single problem caused by such compounds in fluid milk is slow or complete absence of acid production. Other problems may be encountered in the ripening of cheese, in polysaccharide formation in fermented milk products where deficient production gives rise to a decrease in viscosity, and in obtaining the correct flavour in various products.

Background

An understanding of the way starter cultures are used within the dairy industry is fundamental to considering the assessment of potential effects of antimicrobial residues on micro-organisms used for industrial food processing, as regards the manufacture of dairy products. Lactic acid bacteria may be used as single strains within the processing industry although more commonly they are used as a combination of different strains. It is in this area that there have been significant developments even in the last few years. The drive for a wider range of fermented milk products has led to an increased research and development input into this area of microbiology resulting in a better,

although still incomplete, understanding concerning the industrial use of starter cultures. The fermentation of the milk sugar, lactose, to lactic acid is the major function of starter cultures, however, there are important and significant differences between the use of starters for cheese and yoghurt manufacture. In yoghurt the metabolic activity of the starters has the purpose of lowering the pH although metabolites such as acetaldehyde contribute to the characteristic yoghurt flavour. In cheese manufacture there are differences, which in many cases reflect the variety of cheeses currently on the market and which are realised by use of different starters. A common rule is that mixtures of mesophilic strains (Mesophilic starter strains have an optimal growth temperature in the range 30–37°C) tend to be used for continental cheeses whilst thermophilic strains (Thermophilic strains have an optimal growth temperature in the range of 40–45°C) are used for Swiss type products. In all cases the metabolic activity of the starter culture is both to produce lactic acid during cheese manufacture and subsequently to contribute to the maturation of the cheese by release of proteolytic and lipolytic enzymes.

Choice of Test Cultures

Cheese, yoghurt and other fermented milk products are extremely complicated biological milieux and despite the extensive product history it is still accepted that our knowledge is far from complete. It has long been established that there is considerable diversity among the lactic acid bacteria that are used as starter cultures and that this itself has impeded the progress of microbiological research.

In addition to the development of new products as a driver for increased research effort in this area, ongoing developments in molecular microbiology have also provided new insights into the complexity of growth of starter cultures. Giraffa *et al.*⁹ stated that all the component micro-organisms in cheese starter cultures together make up a dynamic, complex mixed culture. Other workers have commented that many different strains are present in cheese starters with sometimes clearly different properties all of which are important in cheese manufacture, such as acidification and growth rate, phage resistance and proteolytic activity^{10,11}. Fortina *et al.*¹² have recently shown that even with the species *Lactobacillus helveticus* there are a number of biotypes that can differ significantly. Perhaps the most critical

finding in this work was that the biotype differences included the ability to ferment different sugars with a corresponding difference in acidifying activity and also amongst other things differences in antibiotic sensitivity patterns. The authors thus commented that this new understanding made way for the possibility of choosing *Lactobacillus helveticus* strains with specific biotechnological profiles which will influence the quality and variety of fermented dairy products.

It is for such reasons that the initial assessment of an antimicrobial compound should include a wide range of organisms in the initial MIC studies. The choice of cultures available to the dairy technologist is large as detailed in the guideline, which lists the *genera* of importance for the dairy industry as *Streptococcus*, *Enterococcus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Lactobacillus* and *Bifidobacterium*. The guideline acknowledges that the sensitivity of bacteria in starter cultures to antimicrobial substances varies widely and therefore the test package must include an appropriate selection of bacteria currently used in the dairy industry. It further details the fact that in almost all cases mixed cultures rather than single strains are the method of choice for the dairy processor and consequently the presented data should include commercially available monocultures and mixed cultures.

Mesophilic aromatic cultures are normally used for both cheese and fermented milk products in particular lactic butter and semi-hard cheese varieties with eyes. These cultures contained selected strains of *Leuconostoc cremoris*, *Streptococcus diacetylactis*, *Streptococcus cremoris* and *Streptococcus lactis*. They traditionally consist of several different strains of the above species and possess high phage resistance. The production of diacetyl (2, 3-butanedione) and carbon dioxide is characteristic of these cultures.

Mesophilic homofermentative-cultures are primarily used for cheddar and feta type cheese production. These cultures are highly phage resistant and contain selected strains of *Streptococcus cremoris* and *Streptococcus lactis*, they do not produce carbon dioxide.

Thermophilic lactic cultures are used for yoghurt and other fermented milk products. They contain selected strains of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* and have been selected for taste and viscosity properties.

The *Streptococcus thermophilus* strains are used in yoghurt and Italian type cheeses and can be used alone or in combination with other

starter cultures, as can *Lactobacillus bulgaricus*, the latter being particularly important in flavour production.

Lactobacillus acidophilus cultures are relatively slow growing with some strains of human origin. They are applied in mixtures especially with *Bifidobacterium* spp. for the production of probiotic milk products.

Starter cultures are also made up of *Lactobacillus acidophilus* and *Bifidobacterium* spp. and are generally used for the production of sweet or fermented probiotic milk products.

The other notable group is the *Lactobacillus helveticus* cultures that are normally applied alone or in mixtures with other lactic acid bacteria. They are characterised by strong acid formation and proteolysis.

It is pertinent to divide starter cultures into three product groupings, fermented milk products, cultured dairy products and cheese products.

Fermented milk products are covered by FAO/WHO Standards A-11(a) and A-11(b) and can be further divided into subgroups characterised as described:

Yoghurt: Proto-symbiotic cultures of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*

Acidophilus Milk: *Lactobacillus acidophilus*

Kefir: Starter culture prepared from kefir grains, *Lactobacillus kefir*, species of the genera *Leuconostoc*, *Lactococcus* and *Acetobacter* growing in a strong specific relationship. Kefir grains constitute both lactose fermenting yeasts (*Kluyveromyces marxianus*) and non-lactose-fermenting yeasts (*Saccharomyces omnisporus*, *Saccharomyces cerevisiae* and *Saccharomyces exiguus*)

Kumys: *Lactobacillus delbrueckii* subsp. *bulgaricus* and species of *Kluyveromyces marxianus*

Cultured dairy products will of course include *Bifidobacterium* spp. Cheese products by their very nature are extremely complex and characterised by using a wide range of starter mixtures.

Studies

MIC Studies

The strategy is to screen a range of commercially relevant organisms to determine the MIC of the test compound. As has already been discussed the now-established practice of using mixed culture starters demands that for studies to be more meaningful mixed culture MIC studies ought to be considered. In both cases it is also relevant to determine the effect of liquid milk on the MIC values and so the respective studies are best carried out in the presence and absence of milk. MICs can be expected to increase when carried out in liquid milk as a result of active compound binding to milk proteins and consequently being unavailable for antimicrobial activity.

It is important at the outset to understand that MIC studies were developed as a tool in clinical microbiology to help determine appropriate therapy in cases of infectious disease. The data do, therefore, need to be interpreted with care when relating pure culture *in vitro* studies to the understanding of complex microbial ecosystem, as is the case with industrial starter cultures.

It also must be understood that MIC screening using CLSI methodology necessitates the use of inoculum levels which are much lower than those that will be used during manufacture of dairy products. In commercial practice the inoculum level will be upwards of 5×10^8 cells per ml. The inoculum used in MIC studies is thus much lower than would be used in commercial practice. In the commercial situation the high numbers of bacteria will act to protect the population against antibiotic whereas in the standard MIC protocol the numbers of bacteria are much reduced reflecting a very sensitive and unreal test environment.

In summary the value of the pure and mixed culture MIC determined at a relatively low inoculum level is that it provides for a measure of overall intrinsic activity of the test compound against which other data may be compared. It should not be seen as a figure on which to base determination of no effect levels for an industrial process but merely as an indicator of those strains, which should be used for further study. This is consistent with the rationale detailed in Section 5 of Guideline EMEA/CVMP/276/99-FINAL.

Acid Inhibition Studies

Introduction

The primary function of starter cultures is to bring about a reduction in pH of the culture and central to the CVMP Guideline EMEA/CVMP/276/99-FINAL is the requirement to determine the concentration of antibiotic which will impact upon production of acid by the requisite starter cultures. The CVMP Guideline EMEA/CVMP/276/99-FINAL is very clear that cultures used for such studies should be commercially relevant.

Activity of the tested antimicrobial will be demonstrated by disturbing the starter culture acidification profile in a number of ways. Changes can be observed in the time between initiation of incubation and most rapid period of acidification, the rate of acidification and the terminal pH value. Such effects could be observed alone, in combination or not at all. It is important to relate this finding to the way starter cultures perform in an industrial setting. The acidification performance of starter cultures depends upon a variety of factors including incubation temperature, dosage rate, substrate composition and the presence or absence of inhibitory compounds. As might be expected there is an element of biological variability in the test system and for this reason appropriate replication must be carried out. In an industrial setting inhibitory activity would only be considered when increases in pH of 0.3 units or more were measured. This has been adopted as the threshold value below which variations in pH are considered insignificant and is written into the CVMP Guideline EMEA/CVMP/276/99-FINAL.

Test Endpoints

The inhibitory effect of the antimicrobial is determined as the difference in acid production in inoculated UHT milk containing the antimicrobial and inoculated control UHT milk. Initially the pH-time curve for inoculated control UHT milk is determined under standardised conditions. Subsequently the pH profile is determined in inoculated control UHT milk and in inoculated samples containing increasing concentrations of test compound, following a test incubation period which ensures a fall in pH in the control UHT milk equivalent to

the expected total decrease in pH. The concentration without effect for the antimicrobial compound is defined as the highest concentration in the UHT milk containing the most sensitive starter culture giving rise to a pH not differing from the pH in the control UHT milk by more than 0.3 pH units.

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