J. vet. Pharmacol. Therap. **28,** 3–20, 2005. REVIEW

Approaches in the safety evaluations of veterinary antimicrobial agents in food to determine the effects on the human intestinal microflora

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Cerniglia, C. E., Kotarski, S. Approaches in the safety evaluations of veterinary antimicrobial agents in food to determine the effects on the human intestinal microflora. *J. vet. Pharmacol. Therap.* **28,** 3–20.

The administration of antimicrobial agents to livestock creates potential for antibiotic residues to enter the food supply and be consumed by humans. Therefore, as a process of food animal drug registration, national regulatory agencies and international committees evaluate data regarding the chemical, microbiologic, pharmacokinetic, pharmacodynamic, pharmacologic, toxicologic, and antimicrobial properties of veterinary drugs to assess the safety of ingested antimicrobial residues to consumers. Currently, European, Australian and United States guidelines for veterinary drug registration require a safety assessment of microbiologic hazards from consumption of antimicrobial residues taking into account the potentially adverse effects on human intestinal microflora. The main concerns addressed are selection of resistant bacteria in the gastrointestinal tract and disruption of the colonization barrier of the resident intestinal microflora. Current requirements differ among national agencies. Efforts are ongoing internationally to review and harmonize approaches and test methods and protocols for application to these microbiologic safety evaluations of antimicrobial drug residues in food. This review describes the background to current regulatory approaches used in applying *in vitro* and *in vivo* methods to set a microbiologic acceptable daily intake for residues in food derived from animals treated with an antimicrobial agent. This paper also examines the current research needs to support these evaluations.

(Paper received 20 December 2003; accepted for publication 14 July 2004)

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INTRODUCTION

Antimicrobial agents are used in animal husbandry to treat disease, to control and prevent infections from spreading in herds and flocks, and for growth promotion. Countries worldwide rely on national regulatory agencies and international committees to evaluate the safety of all drugs used in food animals for potential human health risks, as an integral part of the drug registration process. These evaluations are based on all available and submitted data including chemical, microbiologic, pharmacologic, pharmacokinetic, pharmacodynamic, and toxicologic properties of veterinary drugs. There are two distinct safety evaluations unique to antimicrobial agents. One evaluation is the safety as use in animals relates to potential for development of antimicrobial-resistant bacteria and resistance determinants, which could spread via the food chain, or via zoonotic spread to humans. Guidance regarding the conduct of these evaluations has only recently been published by various national regulatory agencies, including the Australian Pesticides and Veterinary Medicines Authority (Australia National Registration Authority, 2000), the European Agency for the Evaluation of Medicinal Products Committee for Veterinary Medicinal Products (EMEA CVMP, 2002b) and the US Food and Drug Administration Center for Veterinary Medicine (US FDA CVM, 2003). A trilateral (EU-Japan-USA) programme entitled The International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH) was formed in 1996 to harmonize technical requirements for veterinary product registration. It also issued guidance regarding microbiologic safety of use (VICH, 2003). The second evaluation addresses the potential impacts to the human intestinal flora resulting from human ingestion of edible foodstuffs (meat, milk, eggs, and edible tissues) containing antimicrobial residue [parent drug or other compound(s)] formed from the metabolism of the drug used to treat the animal under label use. Historically, national regulatory authorities have used different approaches and changed regulatory approaches through the years to examine the safety of residue ingestion. The purpose of this review is to provide a

summary of the approaches and current status of the guidelines to evaluate the safe residue ingestion concentrations of antimicrobial agents in terms of their impact on human intestinal microflora.

IMPORTANCE OF THE HUMAN INTESTINAL MICROFLORA

The human intestinal flora is a balanced ecosystem that is very important in maintaining an individual's health. The microflora in the human gastrointestinal tract form an extremely complex, yet relatively stable, ecologic community, populated with over 10^{11} bacterial cells per gram of content and containing more than 400 bacterial species (Moore & Holdeman, 1974; Drasar & Duerden, 1991; Carman *et al.*, 1993). This high bacterial concentration accounts for about 30% of the fecal mass. Approximately 90% of the flora are obligate anaerobes, consisting of 30 different species. The predominant (cultivable) genera are *Bacteroides* spp., *Eubacterium* spp., *Bifidobacterium* spp., *Clostridium* spp., *Fusobacterium* spp., *Ruminococcus* spp., *Enterococcus* spp., *Peptococcus* spp., and *Peptostreptococcus* spp. The predominant bacteria are obligate anaerobes as the lower regions of the gastrointestinal tract form a highly reducing environment with a redox potential of −200 to −350 mv. Among the facultative anaerobic bacteria, the most commonly isolated species in feces is *Escherichia coli*, which can account for approximately 1% of fecal flora, although the concentrations can vary by orders of magnitude. Although there may be large individual variation in the proportions of the major species from person to person, the population sizes of different species from the same individual are stable (Moore & Holdeman, 1974; Moore & Moore, 1995). Intestinal microflora are an essential component of human physiology because they act as a barrier against colonization of the gastrointestinal tract by pathogenic bacteria (Vollaard & Clasener, 1994). They also play important roles in the digestion of dietary components and metabolism of drugs, xenobiotics, and nutrients and providing compounds such as short chain fatty acids and other essential nutrients that are later absorbed into the system (Chadwick *et al.*, 1992).

Although the microbial population in the gastrointestinal tract is generally stable, clinical studies have shown that therapeutic doses of antimicrobial agents may change the balance. Intestinal exposure to ingested antimicrobial agents that are poorly or incompletely absorbed, excreted in the bile, or reach the intestine through circulation and excretion from the intestinal mucosa can potentially alter the ecology of the intestinal microflora (Finegold *et al.*, 1983; Carman *et al.*, 1993; Edlund & Nord, 1999). The type or extent of change in the system will depend on the spectrum of action of the antimicrobial drug, its dose, the length of an individual's exposure to the drug, as well as the bioavailability, metabolism, distribution in the body and route of excretion. The lowest concentration of any antimicrobial drug that does not affect intestinal flora has not been examined to any great extent in the published literature, thus making the work by the agencies less than straightforward. However, studies using *in vitro* (continuous or semicontinuous flow culture systems) and *in vivo* human flora-associated (HFA) rodent test systems and in human volunteers have shown that therapeutic concentrations of antimicrobial drugs are capable of altering different parameters of the intestinal flora depending on the spectrum of action and concentration of a drug (Finegold *et al.*, 1983; Heimdahl *et al.*, 1985; Gorbach, 1993; Edlund & Nord, 1999). Thus, the question remains regarding safe ingestion concentrations. Furthermore, as individuals vary with respect to the composition of the flora, it is difficult, from either a scientific or regulatory standpoint, to define what magnitude of change in any one or more species is significant to the health and well-being of the individual.

The main concerns of adverse effects of antimicrobial drugs on human intestinal flora are selection of resistant bacteria and disruption of the colonization barrier (or barrier effect) of the resident intestinal flora. Colonization barrier or barrier effect is the 'limiting action' of normal flora on colonization of the bowel by exogenous or indigenous potentially pathogenic microorganisms (Vollaard & Clasener, 1994). Other effects, such as alteration of the metabolic activity of the flora may also be important. However, there is no documented evidence that antimicrobial agents cause human health effects (e.g. prolonged antimicrobial therapy, prolonged hospital stay, predisposition to infection, treatment failure) when present as residue concentrations already approved as safe by regulatory agencies.

METHODS FOR MEASURING THE EFFECTS OF ANTIMICROBIAL AGENTS ON THE HUMAN INTESTINAL MICROFLORA

Many *in vitro* and *in vivo* approaches can and have been used to examine impact of drugs on microbial flora in the gastrointestinal tract and have been the subject of review (Corpet, 1992, 1993a,b; EMEA CVMP, 1994; Woodward, 1998; Cerniglia & Kotarski, 1999; US FDA CVM, 2001, 2004; VICH, 2004). Each has intrinsic advantages and disadvantages in mimicking microflora interactions in the human large intestine to evaluate exposure of the antimicrobial agent to the intestinal microflora to determine effects on antimicrobial resistance selection and disruption of colonization resistance as reviewed by a number of experts (Table 1). While many of these experimental test systems and approaches can and have been used to assess the safety of veterinary drug residues for human consumption, none have been validated in accordance with the procedures proposed by the Inter-Agency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) (1997), wherein: (i) the observed end-points are validated to predict the biologic impact they intend to measure, and (ii) the test methods should provide repeatable results under standardized experimental procedures as confirmed by different laboratories.

More studies are needed to address the variability of protocols to test the effect of low concentrations of antimicrobial agents on human intestinal microflora and their relevance to human

Table 1. Methods for measuring the effects of antimicrobial compounds on the human intestinal microflora*

Method	Advantages	Disadvantages†	Recommended use in testing for
In vitro			
Short-term anaerobic incubation (1–4 h) of fecal suspensions	Rapid method to screen fecal specimens for immediate drug impacts on bacterial metabolism, bacterial numbers, resistant (insensitive) organisms Bacterial flora representative of original fecal inoculum Relatively inexpensive, simple to perform No ethical restrictions	Does not address long-term exposure effects on colonization barrier effects and resistance emergence Does not take into account host metabolism Models fecal flora, not necessarily intestinal flora The impact of variability of fecal inoculum on outcome has not been examined	Microbial metabolism of antimicrobials Fecal binding, chemical transformation and/or biotransformation to cause drug inactivation Relatively fast changes in bacterial- and drug-resistant populations, colonization barrier as a result of short-term exposure of the drug Impact of drug on bacterial functional end-points, including hydrolytic and reductive enzyme reactions, gas production, volatile and nonvolatile fatty acid formation
Pure culture determination of minimal inhibitory concentration (*MIC*)	Relatively simple to screen a number of cultures representative of human gastrointestinal tract Relatively inexpensive, simple to perform No ethical restrictions	Does not take into account drug interactions with fecal solids Does not fully model complexity of ecologic interactions in the intestinal tract Standardized *MIC* tests do not represent *in vivo* intestinal conditions (bacterial numbers, fecal solids, eH, pH, bacterial interactions, etc.) Does not provide data to address resistance emergence Models cultivable bacteria only Does not simulate microflora or account for host metabolism Does not address resistance emergence No consensus on how to summarize *MIC* output data to estimate a no effect concentration in humans	Spectrum of drug activity Direct effects of low concentrations of antimicrobial agents on a specific bacterial species
Continuous and semicontinuous culture systems	Samples are homogenous Bacterial complexity of fecal inoculum retained Models microflora interactions in the human large intestine Can examine long-term, continuous or pulsed drug exposures No ethical restrictions	Does not take into account host metabolism Intestinal (physiologic) conditions not represented Does not take into account drug interactions with solids found in fecal specimens Representative of resistant organisms or resistance determinants not established Models fecal flora not necessarily intestinal flora Impact of variability of fecal inoculum on outcome has not been examined Bacterial concentrations (10^9 cells/mL) are lower than in feces (10^{11} cells/g) Resource intensive, technically difficult, most expensive among the *in vitro* test systems Few Good Laboratory Practices laboratories available to conduct studies Studies needed to determine causes of variability in barrier effect studies before can be used for regulatory decisions	Determining no effect concentrations for functional end-points, including hydrolytic and reductive enzyme reactions, gas production, volatile and nonvolatile fatty acid formation, bacterial interactions, colonization barrier resistance emergence May be useful in determining potential for drug to be inactivated due to bacterial biotransformation

Table 1. Continued

Method	Advantages	Disadvantages†	Recommended use in testing for
Simulated gut models	Straightforward/simple model Models impact of food passage through stomach Relatively inexpensive and simple to perform Can screen a number of cultures representative of the human gastrointestinal tract Models host digestion processes that may impact drug activity No ethical restrictions	Microbiologic end-point is survival of tested bacterial isolates, which can be overly simplistic Does not fully model complexity of the gut system Does not account for host metabolism Intestinal conditions not represented (fecal solids, bacterial density, intestinal pH, bacterial interactions in the intestine) Models cultivable bacteria only Does not address resistance emergence Does not provide data to address resistance emergence Models cultivable bacteria only Does not simulate microflora No consensus how to summarize *MIC* output data to estimate a no effect concentration in humans	May be useful to examine the potential for the drug to be inactivated
Intestinal fed-batch culture	Bacterial complexity retained Can examine repeated, long-term or pulsed drug exposures to bacterial subcultures Relatively inexpensive, allowing experimental designs examining more than one source of fecal inoculation No ethical restrictions	May result in diminished complexity of flora with repeated transfers Bacterial populations are periodically destabilized on cyclic basis, which may impact any colonization barrier studies Does not take into account all aspects of host metabolism Intestinal (physiologic) conditions not represented Does not take into account drug interactions with solids found in fecal specimens No validation that resistance determinants in subcultures are representative of humans with repeated transfer The impact of variability of fecal inoculum on outcome has not been examined Models fecal flora not necessarily intestinal flora Bacterial concentrations (10^9 cells/mL) are lower than in feces (10^{11} cells/g)	Functional end-points, including hydrolytic and reductive enzyme reactions, gas production, volatile and nonvolatile fatty acid formation, bacterial interactions, colonization barrier effects, resistance emergence Potential for drug to be inactivated due to bacterial biotransformation
In vivo			
Conventional laboratory animals	Relatively simple to perform; can control wide variety of dietary and environmental parameters Ability to monitor flora in different gut regions as well as the feces Host metabolism occurs Bacterial complexity is high Least expensive among *in vivo* systems Limited ethical considerations	Intestinal microflora differ among animal species Human–host interactions not included High variability among individuals may occur Coprophagy in certain species does not mimic eating patterns of humans No validation that resistance determinants found in bacteria of GI tract of animals representative of those in humans Open system: bacterial contamination during trial	Determining resistance emergence, colonization resistance and functional end-points can be monitored May be useful in determining potential for drug to be inactivated due to metabolism or transformation in intestine

Table 1. Continued

Method	Advantages	Disadvantages†	Recommended use in testing for
Human flora associated rodents	Relevant to humans, since the inoculant is fecal flora from humans Can control variety of dietary and environmental parameters Ability of monitoring the flora in different gut regions as well as the feces Bacterial complexity is high Host metabolism occurs Limited ethical consideration	Expensive, germ-free animal facilities required Human–host interactions not included Labor intensive Coprophagy in certain species does not model human-eating patterns Introduction of a gut flora which is different from the conventional one, might affect the physiology of the human flora associated animals Validation needed that resistance determinants in flora are representative of those in humans Impact of fecal inoculum on outcome has not been examined Gut physiology (secretions, pH, peristalsis) of the germ-free animal may not be identical to that of humans Models fecal flora, not necessarily intestinal flora	Determining resistance emergence, colonization barrier effects, and functional end-points can be monitored May be useful in determining potential for drug to be inactivated due to metabolism or transformation in intestine
Human volunteers	Direct relevance to humans for safety evaluations No interspecies extrapolation has to be prepared Fecal samples can be collected to determine microbial population changes and number of organisms resistant to antimicrobial	Ethical issue of using human subjects Statistical significance due to human variability and high number of volunteers required to conduct study Long monitoring periods Most expensive system to use Fecal flora may not be representative of colonic flora	Determining resistance emergence and colonization barrier effects. May be used for veterinary drugs that have approval for use in human medicine

*Adapted from Rollins *et al.* (1975), Corpet (1992), Rumney and Rowland (1992, 1995), Nouws *et al.* (1994), EMEA CVMP (1994, 2001), McConville *et al.* (1995), Silley and Watson (1998), and Cerniglia and Kotarski (1999).

†With the exception of *MIC* tests, none of these test systems have standardized protocols, or have been evaluated for test system variability.

exposure before an appropriate design can be used in test validation. Further, none of these methods have been evaluated for their prediction of human health impact.

OVERVIEW OF SAFETY EVALUATION OF DRUGS ADMINISTERED TO FOOD ANIMALS

National regulatory authorities and international committees have developed methods and adopted regulatory approaches to evaluate the safety of residues in edible foodstuffs (milk, meat, eggs, and edible tissues such as fat, kidney, and liver) derived from animals treated with a specific drug. Antimicrobial residues are the compounds present in or on edible tissues of the treated animal as a result of label drug use. These residues can be comprised of the parent drug compound itself and/or the compound(s) resulting from the metabolism of the drug. Ultimately, residue formation is a function of the animal species and its metabolism, the drug, formulation, dose, method of administration, and time after drug administration. While the regulatory approaches vary, the objectives of the evaluation encompasses three basic evaluations and decisions: (i) the safe ingestion concentration quantified in terms of an acceptable daily intake (ADI) of residue for the lifetime of an individual without deleterious health effects; (ii) the maximum residue level (MRL; termed 'tolerance' by the USA) allowable in edible foodstuffs derived from treated animals and to be consumed by humans and (iii) the withdrawal time needed after the drug is administered for the residues to fall below the MRL so animals may enter the food chain for safe consumption by humans.

The ADI is based on an array of toxicologic safety evaluations taking into account acute and long-term exposure by ingestion of drug residues and the potential impact on humans. These impacts may include systemic toxicity, carcinogenicity, genotoxicity/mutagenicity, reproductive toxicity, developmental toxicity, teratology, neurotoxicity, immunotoxicity, allergenicity, ocular toxicity, cardiac toxicity and, in the case of antimicrobial agents, the safety for gastrointestinal microflora. The studies required for the evaluation are conducted by the sponsor under verifiable laboratory procedures as appropriate to the research and testing. A range of doses are selected and tested to include an oral dose without effect [termed the 'no effect level' (NOEL) quantified in terms of mg/kg equivalent body weight (BW)] in animals. Almost invariably, the NOEL is divided by additional safety factors (often in increments of 10), as appropriate, that take into account uncertainty in extrapolating safety in animals to safety in people, as well as any limitation of the study (e.g. the number of animals used, variability in sensitive populations, etc.). The ADI is then determined as a conservative estimate of the safe ingestion for humans based on the lowest ADI among a battery of toxicologic safety studies and applicable safety factor(s).

The ADI provides the basis for determining the MRL of the drug in the edible foodstuffs derived from the treated animals. The regulatory approach used to assign MRLs to milk, eggs and edible tissues, is dependent on the regulatory agency and beyond the scope of this review. Basically, the approaches take into consideration how much drug residue in a foodstuff derived from the animal species may be consumed on a daily basis and keep consumption of drug less than the ADI. To enable this, the drug sponsor provides data regarding the comparative metabolism in the intended and other species (including human, if such data are available), and the metabolism, elimination and tissue depletion of the drug in the intended species. Typically the daily intake of foodstuffs is inflated compared with expected ingestion rates. For example, in the case of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and EMEA CVMP, the approach assumes that an average person consumes daily 300 g of muscle, 100 g liver, 50 g kidney, 50 g fat, and 1.5 L of milk, all from a treated animal. Using this assumption, the MRL for each foodstuff is set so that, if a person were to consume this entire 'food basket' of foodstuffs (each foodstuff having the respective MRL) from a treated animal, the total consumption of residue would be below the ADI. The total amount of foodstuffs consumed, the underlying assumptions, and the statistical methods used to calculate the total residue consumption vary across agencies and are beyond the scope of this review.

National agencies establish legal drug withdrawal times for each drug, to assure that animals intended for human food are slaughtered at or after the drug residues in the tissues are below the MRL for each foodstuff. The drug sponsor provides drug residue decline data and also validates analytical methods for recovery and quantitation of parent drug and metabolite residues for injection site tissues, edible tissues, milk, and eggs, as appropriate from the animal species. Residues of treated animals are analyzed for total and specific residues, including parent drug to evaluate the drug absorption, distribution, metabolism, and excretion. Frequently, radiolabeled drugs administered at the label dose to the target species are used in these studies. Based on drug disposition and depletion studies, the tissue in the target species that has the longest drug depletion rate is identified, and is used as a basis for determining the withdrawal times for the animal. As a conservative measure to ensure safety, the slaughter time is adjusted (increased) to take into account the variation in target animal populations leading to the longest drug depletion of the longest depleting residue. While methodologic, statistical, and regulatory approaches vary among agencies (for example, see Concordet & Toutain, 1997a,b; EMEA CVMP, 1995; Friedlander *et al.*, 1999; Fisch, 2000; Martinez *et al.*, 2000; US FDA CVM, 1994), the goal is to ensure that the withdrawal time set for the drug, administered at maximum label dose and duration, will ensure that the residue will deplete to less than the MRL in all edible tissues among those individual animals that have the longer depletion rates for the drug.

As noted above, the regulatory approaches, test systems used, methodologies applied, and appropriation of safety factors to assign NOELs, ADIs, MRLs and withdrawal times for a specific drug indication can vary with the regulatory agency or review organization. Moreover, the withdrawal times can vary with specific formulation, and label use of the drug. As such, the MRLs and attendant withdrawal times can be substantially different among countries, which in turn can have substantial impacts on

the movement and sale of meat and meat products among countries. Moreover, not all countries have the same lists of approved drugs for use in animals. The Codex Alimentarius Commission, founded by the United Nations, sets the international standards for ADIs and MRLs for veterinary drug residues to protect consumers and facilitate trade. Codex relies on the JECFA to recommend the standard for the ADIs and the MRLs for veterinary drugs as discussed below. Withdrawal times remain the responsibility of the national authority.

INTERNATIONAL AND REGULATORY APPROACHES IN ASSESSMENT OF THE EFFECTS OF ANTIMICROBIAL DRUG RESIDUES FROM FOOD OF ANIMAL ORIGIN ON THE HUMAN INTESTINAL FLORA

Each national regulatory agency, JECFA, and the VICH organization have scientific experts that provide advice on the safety of veterinary drug residues and appropriate studies to determine their safety. The scientific advisory groups make recommendations that will later become standards when approved by the organizations. The Codex Alimentarius Commission sets standards for veterinary drug residues based on recommendations made by the JECFA through the Codex Committee on Residues of Veterinary Drugs in Food. The EMEA sets standards based on recommendations from the CVMP. The CVM is the regulatory agency responsible for review of veterinary medicines within the US FDA. The VICH recommends data requirements and protocols for determining human food safety of veterinary drugs, based on recommendations from the Safety Working Group.

The requirement for drug sponsors to account for the potential impact of antimicrobial drug residue on ingestion on the human intestinal flora first began in 1986 as a component of the deliberations of the drug registration or re-registration process. The US FDA CVM (1996, 2004), FAO/WHO (1988, 1995, 2000), and the EMEA CVMP (1994, 2001, 2002a) have since issued and updated guidance's reflecting the changing status, testing experience and increasing emphasis these agencies have placed on this evaluation over the years. Table 2 provides a comparative summary of the most recent approaches used by international committees and regulatory authorities.

Joint Expert Committee on Food Additives

The JECFA is responsible for the safety assessment of veterinary drugs in foods and has been charged with advising and providing guidance to FAO and WHO member states and to the Codex Alimentarius Commission on four broad tasks: (i) to establish and further elaborate principles for evaluating the safety of residues of veterinary drugs in foods and for determining acceptable and safe concentrations of such residues when the drugs are administered to food producing animals in accordance with good practice in the use of veterinary drugs; (ii) to determine criteria for appropriate methods of analysis for detecting or quantitating residues of veterinary drugs in foods; (iii) to evaluate or re-evaluate the safety of residues of certain veterinary drugs; (iv) to discuss and provide advice on matters of interest arising from the reports of the sessions of the Codex Committee on residues of veterinary foods. The microbiologic ADIs established by the JECFA Committee are listed in Table 3.

The JECFA initially addressed the microbiologic safety of veterinary drug residues in foods in June of 1987. The Committee concluded that the antimicrobial properties of veterinary drug residues would become the determining factor in safety evaluation when the toxicity of the substance is so low that their residues could be tolerated without any withdrawal period. In such case, the safety of the residues would be based on their danger to human health because of their selective pressure on the intestinal microflora favoring growth of micro-organisms with natural or acquired resistance (FAO/WHO, 1988).

Later in 1990, the Committee concluded that the most important risk was to the stability of the microbial flora and its barrier effect. Recognizing that *in vivo* models (e.g. germ-free rodents implanted with human intestinal flora) for such safety evaluations were not yet developed the Committee decided that minimal inhibitory concentrations (*MIC*s) for relevant intestinal bacteria could be used on a temporary basis for safety evaluations (FAO/WHO, 1990). In 1991, for the first time, JECFA calculated the ADI for an antimicrobial drug (spiramycin) using *MIC* data from four species of the dominant anaerobic flora. A formula was developed using the modal *MIC* of the bacteria tested, safety factors to cover different variables, the daily fecal bolus, the fraction of oral dose available, and the weight of humans (FAO/WHO, 1991).

This 'JECFA Formula Approach' has been used for approximately 10 years with minor modifications of the equation, including changing the definition of the MIC_{50}, substitution of the mass of colonic contents [220 g, based on the data by Cummings *et al.* (1990)] for daily fecal bolus since the human fecal weight of 150 g underestimates the colonic volume of a 60-kg person and refinement of the *MIC* summary (FAO/WHO, 1998). The current formula is:

$$\text{JECFA formula to derive an ADI} = \frac{MIC_{50}(\mu\text{g/g}) \times \text{Mass of colonic contents (220 g)}}{\text{Fraction of oral dose bioavailable} \times \text{Safety factor} \times \text{Weight of human (60 kg)}}$$

The MIC_{50} is currently defined as the minimum concentration of an antimicrobial drug that completely inhibits the growth of 50% of the cultures of a particular micro-organism, as judged by the naked eye, after a given period of incubation. For the purpose of the evaluation, the MIC_{50} value is the mean MIC_{50} of the relevant species tested. Alternatively, the lowest MIC_{50} value for the most sensitive species can be used.

In 1995, JECFA (FAO/WHO, 1995) discussed the use of a 'decision tree' approach (Fig. 1), which was later adopted during in the 52nd meeting of the JECFA Committee. This approach was first applied to an assignment of an ADI for lincomycin in 2000, and has served as a basis for all ensuing evaluations of

Table 2. Conceptual approaches for determining a microbiologic ADI

Reference	JECFA WHO Technical Report Series 893	CVMP EMEA/CVMP/234/01-FINAL Guideline	FDA FDA/CVM Draft Guidance for Industry No. 52*	VICH VICH Draft Guideline GL-36
Microbiologic effects of concern	Colonization barrier disruption Emergence of resistance Enzymatic activity directly linked to an adverse effect on human health	Reduction or elimination of barrier effect of normal intestinal flora Development or increase pool of resistant strains through resistance transference from normal flora	Disrupt protective barrier effect provided by intestinal microflora colonization barrier Emergence of resistance Change in metabolic activity of microflora	Colonization barrier disruption Increase in the populations of resistant bacteria
Conceptual approach	'Comprehensive decision tree approach' to determine need for microbiologic ADI Determine if drug residues are active, enter, and remain in colon. If no, use ADI based on toxicologic data. If yes, may use comprehensive literature survey to determine if drug residue will affect microflora at proposed toxicologic ADI If microbiologic ADI must be determined, address most sensitive effect(s) If concern is colonization barrier disruption, formula approach based on *MIC* data or other *in vitro* test may be used If concern is barrier disruption, resistance emergence, and/or enzymatic activity, *in vitro* or *in vivo* testing may be used	A formula approach based on *MIC* data and correction factors are used to determine a microbiologic ADI. Correction factors are assigned to take into account: (i) differences between *in vitro* and *in vivo* situations, and (ii) the potential of drug substance to cause selection and induction of resistance development	'Pathway approach' to determine need for microbiologic ADI Determine if drug residues are active, enter the colon, and remain in the colon If no, derive an ADI based on toxicologic data. If yes, use available human data to determine adverse effect(s) of concern Use *in vitro* or *in vivo* testing to derive a microbiologic ADI when concern is colonization barrier disruption and/or development of resistant bacteria and/or changes in microflora metabolism	'Sequence of steps' to determine the need for a microbiologic ADI Determine if drug residues are active, enter the colon, and remain in the colon. If no, use ADI based on toxicologic data. If yes, determine if there is scientific justification to decide whether a microbiologic ADI must be determined. If yes, a formula approach based on *MIC* data may be used when the concern is colonization barrier disruption *In vitro* or *in vivo* testing may be used when concern is colonization barrier disruption and/or development of resistant bacteria
Relevant data taken into account	Microbiologic activity (*MIC* of relevant intestinal bacteria) Availability of drug in colon (metabolism, pharmacokinetics) Activity of residue in the colon (fecal binding, inactivation, etc.) Effect of drug on GI flora *in vitro* or *in vivo*, and assessment of toxicologic ADI (changes in bacterial populations, colonization barrier, resistant bacterial populations, etc.) End-point(s) of concern for class of drug if a microbiologic ADI is needed Human data with appropriate safety factor	Microbiologic activity (*MIC* of relevant intestinal bacteria) Availability of drug in colon (metabolism, pharmacokinetics) Activity of residues in the colon (fecal binding, inactivation, etc.) Data to determine NOEL obtained in HFA rodents when induction of resistance and reduction of barrier effect are studied	Microbiologic activity (*MIC* of representatives of intestinal bacteria) Availability of drug in colon (metabolism, pharmacokinetics) Activity of residues in colon (fecal binding, inactivation, etc.) End-point(s) of concern for the class of drug if a microbiologic ADI is needed (*in vitro* or *in vivo* preliminary or definitive studies)	Microbiologic activity (*MIC* against relevant intestinal bacterial) Availability of drug in colon (adsorption, distribution, metabolism) Activity of residues in the colon (fecal binding, inactivation, etc.) Scientific justification to eliminate need for testing one or both end-points of concern (literature, case studies, etc.) End-point(s) of concern for class of drug if microbiologic ADI is needed

Table 2. Continued

Reference	JECFA WHO Technical Report Series 893	CVMP EMEA/CVMP/234/01-FINAL Guideline	FDA FDA/CVM Draft Guidance for Industry No. 52*	VICH VICH Draft Guideline GL-36
Testing to establish an ADI for increase in population of resistant bacteria	*In vitro* tests (continuous culture of fecal inocula) or *in vivo* test (HFA rodents, mice, rats, pigs) to examine changes in colonization barrier or resistance Challenge models with resistant strains to determine drug concentrations that do not select for resistance or does not select for challenge bacteria	*MIC* tests based on recognized guidelines (e.g. NCCLS), using different inoculum concentrations	*In vitro* or *in vivo* studies in model systems (as those recommended for colonization barrier disruption)	*In vitro* test systems: continuous, semicontinuous, and fed-batch cultures useful to evaluate long-term exposure *In vivo* test systems: HFA rodents, conventional animals Further studies needed for validation of *in vitro* and *in vivo* test systems

*This draft guidance may be further revised at a later date in accordance with recommendations from the VICH guidance development, concerning proper tests, model systems, and protocols.
ADI, acceptable daily intake; JECFA, Joint FAO/WHO Expert Committee on Food Additives; GI, gastrointestine; CVMP, Committee for Veterinary Medicinal Products; FDA, Food and Drug Administration; VICH, Veterinary International Cooperation on Harmonization; MIC, minimal inhibitory concentration; HFA, human flora-associated; NCCLS, National Committee for Clinical Laboratory Standards.

microbiologic impacts to the human intestinal flora (FAO/WHO, 2000), including cefuroxime, neomycin, and pirilimycin. The first three questions of the decision tree are intended to determine whether microbiologically active drug residue will even enter the colon of an individual if the person were to ingest the ADI limit derived from other toxicologic testing. To address these questions, the JECFA Expert Group uses data provided by the sponsor and the literature to determine whether the drug is microbiologically active, and whether any microbiologically active drug residue would enter the colon, taking into consideration the drug's absorption and metabolism characteristics, as well as amount of drug ingested, if the ADI were based on other toxicologic studies. If the Committee can use the data to show that microbiologically active residue does not enter the colon, then the ADI is not based on microbiologic end-points and the ADI derived from other toxicologic studies is assumed to address the concern of impact on microbiologic residue (Fig. 1). However, if review of data applicable to the first three questions affords reason to believe that microbiologically active residue could enter the colon, then all published literature and data provided by the sponsor regarding the characteristics of the drug and related classes are used to determine whether the ADI derived from toxicologic data is sufficiently low to protect the intestinal microflora. If the ADI is not sufficient, then available information about the drug and the drug class are used to identify effects, which could occur in the gastrointestinal microflora. If no information is available, then specific studies using an *in vitro* or an *in vivo* test system are used to determine the most sensitive adverse effect(s) of the antimicrobial agent on human intestinal microflora. The adverse effects of human health concerns to be considered are disruption of the colonization barrier (barrier effect), the selection of resistant bacteria in the colon, and change in metabolic activity of intestinal microflora.

The barrier effect (or colonization resistance) is the property of the flora that prevents overgrowth of transient potentially pathogenic micro-organisms, the outgrowth of indigenous potentially pathogenic micro-organisms, and/or proliferation of antimicrobial-resistant strains (Vollaard & Clasener, 1994). The barrier effect may be disrupted by the action of any antimicrobial drug in the intestinal microflora. If disruption of the colonization barrier is the end-point of concern, then either *in vitro* (e.g. continuous or semicontinuous culture systems) or *in vivo* test systems (e.g. HFA rodent test systems) may be used to determine a NOEL for this end-point. While these complex models of the human intestinal flora better approximate the human intestinal flora, there is some recognition that standardized antimicrobial susceptibility testing of at least 100 strains of bacteria normally inhabiting the colon may be used as a conservative approach to derive an ADI. The ADI derived from *MIC* data is conservative because the inoculum density used for testing is orders of magnitude lower than the bacterial population of the colon. In addition, the growth conditions in *MIC* testing (growth medium, pH, lack of fecal solids, lack of microbial interactions, and drug metabolism, etc.) minimize the potential of drug inactivation (Cerniglia & Kotarski, 1999). Thus, if the antibiotic concentration is below the concentrations that inhibit cell growth of the

Table 3. EMEA CVMP and JECFA published evaluations of microbiologic ADIs of antimicrobial agents *

		Microbiologic ADI (μg/kg) body weight		Reference*	
	Drug class	CVMP	JECFA	EMEA CVMP	JECFA reference
Apramycin 2	Aminoglycoside	40		EMEA/MRL/526/98-FINAL	
Bacitracin	Polypeptide	3.9		EMEA/MRL/768/00-FINAL, January 2001	
Cefacetrile	Cephalosporin	3.5		EMEA/MRL/784/01-FINAL, May 2001	
Cefalonium	Cephalosporin	15.3		EMEA/MRL/839/02-FINAL, September 2002	
Cefalexin	Cephalosporin	54.4		EMEA/MRL/627/99-FINAL, July 1999	
Cefazolin	Cephalosporin	10		EMEA/MRL/126/96-FINAL, July 1996	
Cefoperazone	Cephalosporin	2.8		EMEA/MRL/748/00-FINAL, July 2000	
Cefquinome	Cephalosporin	3.8		EMEA/MRL/005/95, 1995	
Cefapirin	Cephalosporin	2.54		EMEA/MRL/745/00-FINAL, February 2001	
Ceftiofur	Cephalosporin	20	50	EMEA/MRL/498/98-FINAL, July 1999	FAO/WHO (1996) (Food Additive Series 36)
Cefuroxime	Cephalosporin		30		FAO/WHO (2002) (Food Additive Series 49)
Clavulanic acid	Penicillin analog	88.4		EMEA/MRL/776/01-FINAL, February 2001	
Colistin	Cyclopeptide	5		EMEA/MRL/815/02-FINAL, January 2002	
Danofloxacin	Fluoroquinolone	600	37	EMEA/MRL/254/97-FINAL, September 1997	FAO/WHO (1997) (Food Additive Series 39)
Difloxacin	Fluoroquinolone	1.8		EMEA/MRL/154/96-FINAL January 1997	
Dihydrostreptomycin and streptomycin	Aminoglycoside	80	80	EMEA/MRL/810/01-FINAL, February 2002	FAO/WHO (1995) (Food Additive Series 34)
			120		FAO/WHO (1997) (Food Additive Series 39)
Doxycycline hyclate	Tetracycline	3		EMEA/MRL/101/96-FINAL, June 1996	
Enrofloxacin	Fluoroquinolone	6.2	2	EMEA/MRL/388/98-FINAL, July 1998	FAO/WHO (1997) (Food Additive Series 39)

Table 3. Continued

		Microbiologic ADI (μg/kg) body weight		Reference*	
	Drug class	CVMP	JECFA	EMEA CVMP	JECFA reference
Erythromycin	Macrolide	5		EMEA/MRL/720/99-FINAL, January 2000	
Florfenicol	Chloramphenicol analog	3		EMEA no date/report no.	
Flumequine	Quinolone	8.25	37	EMEA/MRL/624/99-FINAL, July 1999	FAO/WHO (1997) (Food Additive Series 39)
Gentamicin	Aminoglycoside	4	20	EMEA/MRL/803/01-FINAL, November 2001	FAO/WHO (1998) (Food Additive Series 41)
Josamycin	Macrolide	2 (provisional)		EMEA/MRL/011/95-FINAL, 1995	
Kanamycin	Aminoglycoside	8		MRL/514/98-FINAL, January 1999	
Lincomycin	Lincosamide	10	30	EMEA/MRL/497/98-FINAL, September 1998	FAO/WHO (2000) (Food Additive Series 45)
Marbofloxacin	Fluoroquinolone	4.5		EMEA/MRL/693/99-FINAL, October 1999	
Mecillinam	Penicillin	23.8		EMEA/MRL/462/98-FINAL, July 1998	
Nafcillin	Penicillin	4.4		EMEA/MRL/750/00-FINAL, April 2001	
Neomycin	Aminoglycoside	160	160	EMEA/MRL/816/02-FINAL, January 2002	FAO/WHO (1995) (Food Additive Series 34)
Novobiocin	Coumarin	1.25		EMEA/MRL/610/99-FINAL, corrigendum June 1999*	
Oxytetraycline, tetraycline, or chlortetraycline	Tetracycline	3	30	EMEA/MRL/023/95- 1995	FAO/WHO (1998) (Food Additive Series 41)
Pirlimycin	Lincosamide	6	8	EMEA/MRL/719/99-FINAL, January 2000	
Sarafloxacin	Fluoroquinolone	0.4	0.3	EMEA/MRL/160/96-FINAL, January 1997	FAO/WHO (1998) (Food Additive Series 41)
Spectinomycin	Aminocyclitol	40	40	EMEA/MRL/726/00-FINAL, March 2000	FAO/WHO (1994) (Food Additive Series 33)
Spiramycin	Macrolide	50	50	EMEA No date or code	FAO/WHO (1997) (Food Additive Series 34)
Thiamphenicol	Chloramphenicol analog	2.5	5	EMEA/MRL/256/97-FINAL, October 1997	FAO/WHO (2000) (Food Additive Series 43)
Tiamulin	Diterpene	32		EMEA/MRL/578/99-FINAL, August 1999	

Table 3. Continued

		Microbiologic ADI (μg/kg) body weight		Reference*	
	Drug class	CVMP	JECFA	EMEA CVMP	JECFA reference
Tilmicosin	Macrolide	4	40	EMEA/MRL/318/97-FINAL, November 1997	FAO/WHO (1996) (Food Additive Series 38)
Tylosin	Macrolide	6.06		EMEA/MRL/205/97-FINAL, April 1997	

*Note that all microbiologic ADIs published by JECFA and CVMP may not be listed in this Table. The final ADI established for each molecule is based on the lowest determination between the microbiologic ADI and the toxicologic ADI of the drug. Final ADIs are not listed in this Table and are beyond the scope of this review.
†The reports summarizing data leading to the establishment of the ADIs by the EMEA CVMP are available at the EMEA CVMP website at (http://www.emea.eu.int/index/indexv1.htm). The JECFA reports are published as the WHO Food Additive Series of toxicologic monographs, which describe comprehensively the data used by the committee during their risk assessment, are published in the WHO Food Additive Series, most of which are available electronically via the INCHEM website, at http://www.inchem.org/pages/jecfa.html, sponsored by the International Programme on Chemical Safety, a collaborative venture of the WHO.
EMEA, European Agency for the Evaluation of Medicinal Products Committee; ADI, acceptable daily intake; JECFA, Joint FAO/WHO Expert Committee on Food Additives; GI, gastrointestine; CVMP, Committee for Veterinary Medicinal Products; FDA, Food and Drug Administration; VICH, Veterinary International Cooperation on Harmonization; MIC, minimal inhibitory concentration; HFA, human flora-associated; NCCLS, National Committee for Clinical Laboratory Standards.

most sensitive groups of organisms tested, it may be assumed that the bacteria responsible for the barrier effect would not be affected and the *MIC* can be used as a basis to derive an ADI. If *MIC* testing is used as an option to derive an ADI, MIC_{50} determined by standard methods such as those of the National Committee for Clinical Laboratory Standards (NCCLS) (2002, 2003) of the most appropriate (sensitive) genus can be used to determine an ADI. It is recommended that at least 10 isolates from each of the most representative genera be collected from healthy human volunteers.

If antimicrobial resistance emergence from ingestion of residues is the concern, then *in vitro* (continuous culture of fecal inocula) or *in vivo* (mouse, rat, HFA rodent, pig) data are needed to show that expected residue concentrations in the colon do not change antibiotic resistance of resident populations of bacteria such as *Esch. coli*, *Enterococcus*, *Bacteroides* or other cultivable bacteria appropriate for the drug class.

If there are changes in enzymatic activity that are specifically linked to an adverse consequence in humans, as, for example, those cited by Gorbach (1993), then this microbiologic end-point may be appropriate for evaluation of some drugs. However, the need for this evaluation has met with some concern regarding the breadth of end-points it may encompass. The intestinal microflora catalyze a number of reactions including hydrolysis, reduction, degradation, and synthesis (Chadwick *et al.*, 1992). The biotransformations of compounds may be beneficial or have adverse toxicologic consequences in the host. Indicators of the metabolic activity of the intestinal microflora include measurements of hydrolytic enzymes (β-glucosidases, β-glucuronidase, arylsulfatase) reductases (nitroreductase, azoreductase, nitrate reductase), metabolism of bile acids and cholesterol, production of short chain fatty acids, determination of cellular fatty acids and sulfate reduction. Thus, the use of microbiologic end-points that measure any change in metabolic activity of microflora should be reconsidered. Of the multiple genera in human intestinal microflora, each one will have slightly different metabolic pathways that enable them to occupy a particular niche. Differences in oxygenation, depth of niche occupation and competitiveness with other microflora are further factors that will affect metabolism. Additionally, differences in the diet of the host will alter metabolism in unknown ways. Scientific literature has not established a specific metabolic activity concentration, or a specific magnitude of change that is considered to be indicative of an adverse effect to human health. Therefore, research should be conducted in this area in order to determine whether alterations in microbial activity are important in establishing a microbiological ADI.

European Agency for the Evaluation of Medicinal Products Committee for Veterinary Medicinal Products

The European Council issued a Council Regulation (EEC) 2377/90 requiring that the microbiologic effects of residues on human gut flora should be taken into account in establishing MRLs for antimicrobial compounds used in food producing animals. Later, EMEA CVMP (1994) adopted a Guideline which included the use

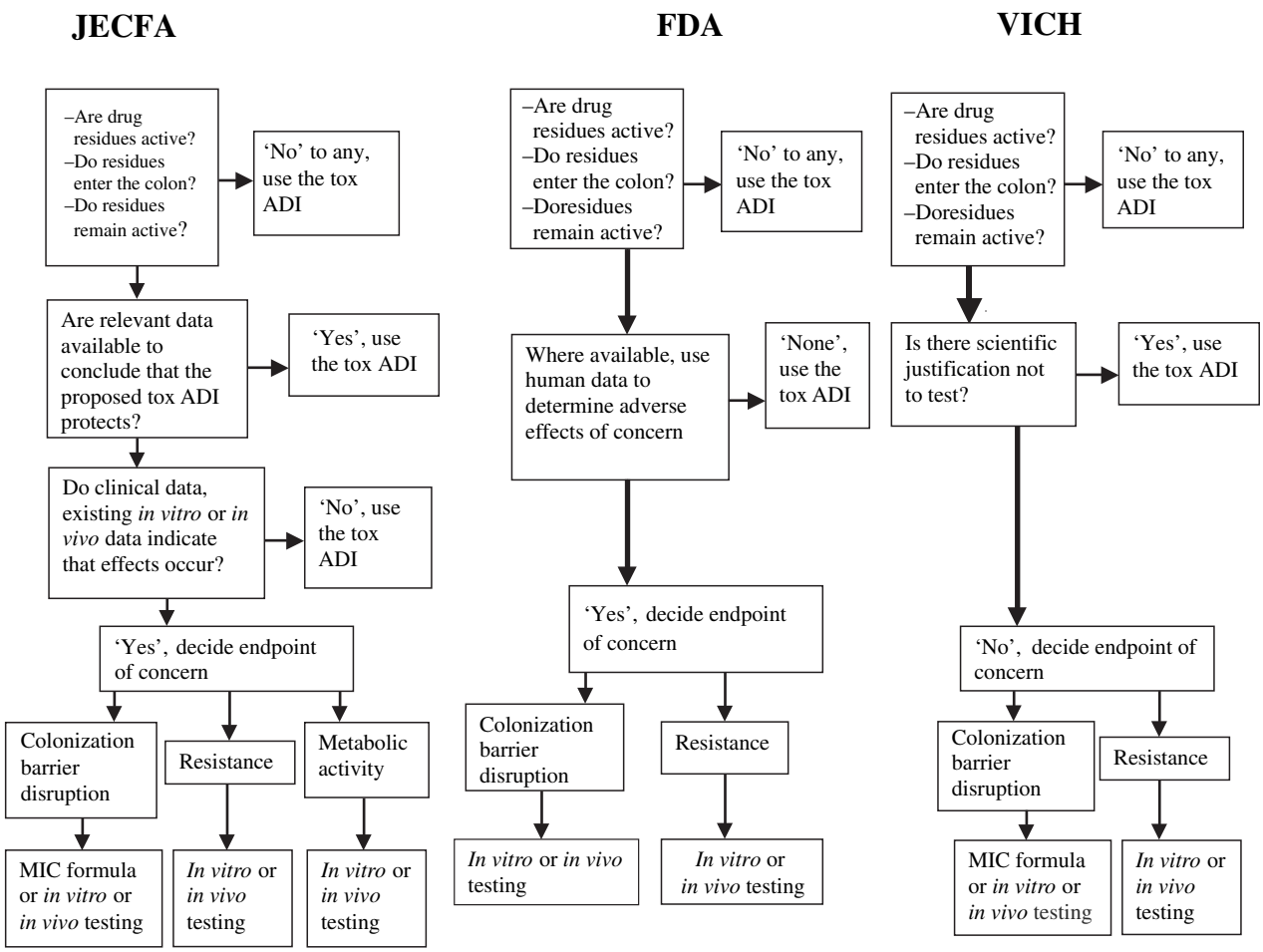

Fig. 1. Conceptual 'decision tree' approaches to derive a microbiological acceptable daily intake (ADI).

of a 'formula approach' to apply *MIC* data as a component part of an ADI determination for the evaluation of antimicrobial drug residues in food, which was used for the next 5 years until further review of the approach. Three types of data have been considered or applied by the EMEA CVMP in these evaluations: human data with an appropriate safety factor; data to demonstrate the NOEL determined in HFA rodents when the induction of resistance and reduction of the barrier effect are studied; or the calculation of a microbiologic ADI from *in vitro MIC* data, including *MIC* data determined under conditions similar to those in the colon. Typically, the *MIC* data have been used for most safety evaluations (see Table 3).

In April 2001, the EMEA CVMP published the latest guidance for consultation. The current revised guideline (EMEA CVMP, 2001, 2002a) states that the current CVMP approach is to be used as an interim measure until the adoption of a harmonized VICH guideline. The revised guideline states that the two endpoints of concern that should be addressed in the determination of a microbiologic ADI are reduction or elimination of the barrier effect of the normal flora and development of and/or increase in the pool of antibiotic-resistant strains of potentially pathogenic micro-organisms. The formula used in this Guideline is slightly different than the JECFA formula. The most recent version follows:

$$\text{CVMP formula to derive an ADI} = \frac{\frac{MIC_{50}\times \text{CF2}}{\text{CF1}} \times \text{Mass of colonic contents (220 g)}}{\text{Fraction of oral dose bioavailable for microorganisms} \times \text{Weight of human (60 kg)}}$$

MIC_{50} = in most circumstances, is the lower one-tailed 10% confidence limit of the mean MIC_{50} of all relevant susceptible genera; CF1 = correction factor to account for selection and induction of resistant organisms. Value varies from 1 to 5. Lack of resistance: a value of 3 would be used when there is evidence of nontransferable resistance and a value of 5 when transferable resistance is demonstrated. A value of 5 is also used if no data or inadequate data on resistance are available; CF2 = correction factor to account for differences in growth conditions between the *in vitro* and the *in vivo* situation. If no major differences (only limited effects on one single factor, e.g. changes only in bacterial densities) the value is 1. Values from 2 to 10 are used when different conditions are demonstrated.

The EMEA CVMP calculates and publishes both a toxicologic and a microbiologic ADI for antimicrobial drugs. The most relevant ADI (usually the lowest) is used to determine the ADI

(Freischem, 2000). A list of compounds and microbiologic ADIs determined by the EMEA CVMP is shown in Table 3.

US Food and Drug Administration Center for Veterinary Medicine approach

In the US FDA Federal Register of January 30, 1996 (US FDA CVM, 1996), the US FDA CVM published a Notice of Availability of Guidance Document No. 52 'Microbiological Testing of Antimicrobial Drug Residues in Food'. This document stated that the US FDA CVM considers antimicrobial activity as a valid end-point for establishing tolerances for antimicrobial drugs. The guidance also stated that antimicrobial drug residues present in food of animal origin should not cause any adverse effects on the ecology of the human intestinal microflora of consumers. The guidance identified antimicrobial drugs that would be exempt from additional microbiologic testing and those that would require testing. The reasons for exempting certain antimicrobial drugs from additional microbiologic testing included 'very low' residues present in the food, residues with limited antimicrobial activity, and drugs with no adverse effects on the human intestinal microflora at therapeutic doses.

Guidance No. 52 stated that 'very low' concentrations of antimicrobial drug residues present in food of animal origin would probably not disrupt the intestinal microflora or select for resistant micro-organisms and, therefore, would be 'safe' under Section 512 of the US Federal Food, Drug, and Cosmetic Act. Based on the best information available at that time, the CVM believed that a maximum ADI of 1.5 mg/person/day of microbiologically active antimicrobial drug residues present in the food qualified as 'very low' residues and should not produce adverse effects on the intestinal microflora. When establishing the maximum ADI of 1.5 mg/person/day, the US FDA CVM recognized that this threshold would need to be reevaluated when additional information was collected on the adequacy of this number for different classes of antimicrobial drugs.

The US FDA CVM has since published a final Guidance No. 52 'Microbiological Testing of Antimicrobial Drug Residues in Food' (US FDA CVM, 2004), based on information made available after 1995 concerning the effects of low doses of different classes of antimicrobial drugs on the human intestinal flora. The US FDA CVM is now recommending that sponsors use a 'pathway approach' (Fig. 1) to address the human food safety of antimicrobial drug residues which has been the basis for more recent drug evaluations for testing. This approach eliminates the use of any threshold, and provides a pathway by which antimicrobial agents are currently evaluated for a microbiologic ADI. This pathway represents a general approach. It is very similar to the decision tree used by JECFA and the draft VICH guideline (Table 2; Fig. 1).

Veterinary International Cooperation on Harmonization

The VICH was formed in 1996 to develop harmonized registration requirements for veterinary medicinal products between the USA, the EU and Japan. Representatives include delegates from the pharmaceutical industry and regulatory authorities of the USA, the EU, Japan, and observers from Australia/New Zealand and, more recently, Canada. These representatives work to review and recommend, as appropriate, harmonized approaches to testing and evaluating the safety of drugs used in food-producing animals [for full overview, see the review by Thompson (1999), and the VICH website at http://www.vich.eudra.org/htm/guidelines.htm]. In 1999, the VICH charged a Microbial Safety Task Force of experts to write recommendations regarding the test methods for impacts of residues on intestinal flora. The Task Force reports directly to the VICH Safety Working Group, which addresses more broadly the harmonization of toxicologic test methods to be used for safety evaluations of drug residues. The Task Force has completed its mandate and its recommendations have received international review. The approach recognizes the lack of standardization of current methodologies available and embodies decision tree and pathway concepts used in the JECFA and US FDA CVM approaches. In cases where microbiologically active residue will enter the colon, the use of an *MIC* calculation may be used to evaluate the potential for barrier effects the application of other test systems such as continuous culture and HFA animal models are also relevant (VICH, 2004).

ASSESSING THE RISK OF EXPOSURE TO ANTIMICROBIAL RESIDUES

Across all regulatory approaches, the 'microbiological' ADI is used to establish a safe residue ingestion concentration for humans that will guard against the risk that ingestion of microbiologically active drug residue will: (i) increase the concentrations of resistant bacteria, potentially comprising antibiotic therapies in humans; and (ii) adversely impact the colonization barrier formed by the intestinal bacteria, potentially comprising the natural defense mechanisms against opportunistic infection in the intestine. In addition to these potential hazards, regulatory authorities assign another 'toxicological' ADI based on potential hazards of carcinogenicity, genotoxicity, reproductive toxicity, developmental toxicity, and allergenicity. The toxicologic end-point or microbiologic end-point resulting in the lowest ADI ultimately drives the overall ADI, thus ensuring that the most sensitive effect across all aspects of potential toxicologic hazards is used to establish the appropriate MRLs for meat, milk, eggs, and edible tissues, and the withdrawal time.

The JECFA and the EU have had the longest history of applying relevant data to derive a microbiological ADI by their respective approaches. Thus, in some cases the ADIs derived can be different (Table 3). The most dramatic case is the two different microbiologic ADIs established for danofloxacin (600 vs. 37 μg/kg, established by the EMEA CVMP and JECFA, respectively). The difference is probably due to the fact that each review group interpreted and differently applied the *in vitro* data (i.e. *MIC* data and fecal-binding data) and *in vivo* data (drug bioavailability data) and safety factors for this molecule, as evidenced in the formulas used by the groups. However, the final ADI for this

drug was driven by the lower toxicologic ADI, which was 20 and 24 μg/kg for EMEA CVMP and JECFA, respectively, so ultimately there was little difference in the final ADI established by the JECFA and EMEA CVMP.

Our literature review did not reveal documentation that the current approved concentrations of residues in foods derived from animals treated with antibiotics are adversely impacting the intestinal microflora. Gathering evidence to determine whether or not currently approved safe antibiotic residue ingestion concentrations in food can truly modify colonization barrier, or the antimicrobial resistance profile of human gut microflora, and compromise antimicrobial therapies, is problematic for the following reasons. The assignments of ADIs, MRLs, and drug withdrawal times are intended to ensure safety of foodstuffs to the consumers and therefore incorporate a number of conservatisms and safety factors in their assignment and application. Food commodities in which residues are present might not be part of the daily diet of the consumer or might not be present in the edible portion of the commodity (Fitzpatrick, 1995). Given the safety evaluations and established ADIs, MRLs, and withdrawal periods already in place, antimicrobial residues in foods make up a small to negligible fraction of total antimicrobials to which humans are exposed to in terms of either frequency or dose. Therefore, it seems unlikely they contribute significantly to resistance development or colonization barrier disruption of intestinal microflora in humans. Not all food-producing animals will have a tissue residue concentration at the MRL. The MRLs and the withdrawal times are specifically derived to take into account the worst case scenario wherein the highest label dose for the longest label duration is administered to the animal subpopulation that has the longest depletion rates for the slowest depleting residue of the drug in question. Moreover, in practice, not all animals are treated with the drug. If they are treated, frequently the objective for treatment is so that they can be raised to market weight, often well after the drug approved withdrawal period has expired. Furthermore, people do not generally eat a full ADI on a regular basis, as they generally do not consume foodstuffs all from the same treated animal. If they do ingest food where residues are present, the degradation of residues associated with food processing and cooking may result in lower concentrations of microbiologically active residues in the prepared food. *In vitro* adsorption, chemical or bio-inactivation via metabolism and dilution of antimicrobial residues in the human gut may further lower the availability of any residue that is ingested. Therefore, dietary consumption of microbiologically active residue of veterinary antimicrobials is unlikely to play a role in the development of antimicrobial resistance or colonization barrier disruption.

Given the conservative nature of the assignment of ADIs, MRLs, withdrawal times, and since most animals enter the food chain after legally established withdrawal times, it is understandable that there have been no reported instances in which adverse reactions to humans have been documented. However, the failure to report an instance does not necessarily meant that no instances have occurred, and certainly does not negate the concern. Thus, regulatory agencies require microbiologic, toxicologic and chemical residue studies as part of the safety evaluation of veterinary drugs to set the ADIs, MRLs, and drug withdrawal times to limit any risk of unnecessary exposure to a person ingesting the food commodity. The resulting safety evaluation and procedures to set ADIs, MRLs, and drug withdrawal times for antimicrobials are not expected to cause toxic reactions in target species or in humans as long as they are used at the correct dosage and at the concentrations permitted.

The toxicologic end-point or microbiologic end-point resulting in the lowest ADI ultimately drives the overall ADI. This hazard analysis, coupled with exposure assessment based on a robust residue and depletion analysis, as well as conservative assumptions regarding potential ingestion rates by individuals, helps to minimize the risk of exposure to any toxicologic potential for the consumer. Most antimicrobial residues, if present in food, would be at concentrations too low for toxic effects.

When the drug is approved and used in compliance with the established dose and drug withdrawal times, the exposure of the drug is controlled to maximize the likelihood that residues will be below the MRL established for milk, eggs, or edible tissue and thereby minimize the risk of harmful effects to the individual.

CURRENT RESEARCH GAPS

Based on a review of the literature, as well as our own observations, we believe that there are certain data gaps that need to be addressed.

There appears to be consensus worldwide that if it can be shown that residues are readily inactivated before entering the colon, then the concern of their microbiologic impact is mitigated. However, it is less apparent what approaches and methodologies are best applied to address this. It is not possible to conduct such studies in humans because of ethical concerns in testing a drug destined for animal use, in humans. Thus, results of studies in animals are extrapolated or used directly in calculating the percentage of an ingested veterinary drug dose that is bioavailable to the gastrointestinal microflora. Similarly direct measurements of fecal active or inactive drug in animals, treated at therapeutic dose regimens, are used as a basis to determine bioavailability to the intestinal flora. While these are useful approximations, the extent of availability or inactivation of the drug residue may be dependent on the dose and thus information is lacking what microbiologically active drug concentrations enter the colon when residue concentrations are ingested by experimental animals. Similarly, in *in vitro* studies designed to examine inactivation of antimicrobial drugs because of binding or bacterial metabolism, it would seem approaches should be applied that examine this at drug concentrations representative of residue concentrations expected (proposed) in foodstuffs. Studies designed to take into account impacts of ingested residue concentrations, as opposed to ingested therapeutic concentrations, will support the initial portion of the 'decision tree' approaches to determine whether microbiologically active residues actually enter the colon. If no

microbiologic activity is detected, then the standard toxicologic ADI is used.

Various *in vitro* and *in vivo* models of human intestinal microflora have been used in basic research to examine the impact of various antimicrobial agents on the colon microbial ecosystem (Table 1). These test systems are still in the development phase, and as such have not been validated for their reproducibility or predictive value in determining a NOEL for residues of antimicrobial agents for their effects on the colonization barrier or resistance emergence in humans. Therefore, research is needed to validate and determine the predictive capabilities of *in vitro* or *in vivo* test systems in identifying adverse human health effects. The ecology of normal intestinal microflora of both animals and humans is incompletely understood. The extent of variation among resistant or nonresistant bacterial populations and their metabolic activities in an individual or among individuals has not been evaluated quantitatively. Therefore, research is needed to establish a database regarding the variability of the intestinal microflora normally among or within individuals to determine what magnitude of change in resistant populations or metabolic activities after exposure of antimicrobial drug residues in food to the consumer is relevant to human health. Model systems should be developed that are representative of the inherent variability within individuals and normal intestinal microflora.

Exposure of intestinal microflora to low concentrations of antimicrobial residues contained in food could cause an increase in resistant bacterial populations because of the acquisition of new genetic resistance determinants and/or because of mutation. Also the potential to increase the proportions of populations of existing bacteria in the gastrointestinal tract that are already resistant to the antimicrobial agent is possible. While quantifying an increase in resistance is experimentally achievable, it is not clear whether the bacteria enumerated, or the magnitude of detected change in the resistance of the enumerated bacteria has human health consequences, especially in light of the variability existent among individuals. Humans are colonized with resistant bacteria to varying extents, depending on the individual and the antibiotic resistance determinant. If the detected change is within variability normally encountered among humans, then it is debatable whether the detected increase is important. Test protocols and methodologies to define the magnitude of resistance increase in the intestinal microflora of humans that is of concern that can be extrapolated from a test system and thereby define the implications of a detected increase in resistance are needed. Currently, there are no consensus opinions on the magnitude of change in resistant populations that has human health implications, regardless of the test system used.

CONCLUSIONS

The ingestion of residues of antimicrobial compounds in food of animal origin has the potential risk to human health to compromise the colonization barrier, leading to pathogenic bacteria overgrowth or compromise antimicrobial therapy in humans by exerting a selective pressure on the intestinal microflora thus favoring the growth of micro-organisms with natural or acquired resistance. An extensive literature review did not reveal any evidence of such human health effects occurring as a result of antimicrobials present as residues in foods. However, the failure to find recorded adverse health effects in this regard does not negate the human health concern. To address this; regulatory agencies accordingly have put into place requirements for a safety assessment for this potential. These requirements continue to challenge scientists, given the complexity and variability of the gut flora, variation among and within individuals, and the difficulties in defining this variation and the magnitude of changes that have human health impact.

A harmonized approach is needed in evaluating the veterinary antimicrobial drug residues in food based on their effects on the human intestinal microflora. The EU, the USA, and international regulatory organizations have different approaches as outlined in this review. A VICH Safety Working Group Task Force has proposed a unified approach in evaluating data to determine the impact of veterinary antimicrobial drug residues in food and the human intestinal microflora. It is quite similar to the JECFA and US FDA decision tree and pathway approaches and is currently under international review. It is anticipated that this approach will be considered by national and international regulatory authorities and committees involved in the safety evaluation and risk assessment of chemicals in food derived from animals to ensure consistency and transparency in the determination of microbiologic ADIs.

ACKNOWLEDGMENTS

Authors thank members of the VICH Safety Working Group and Dr Richard Ellis, Center for Veterinary Medicine, FDA for providing advice concerning regulatory approaches, methodology, and JECFA microbiological ADIs; Dr Haydee Fernandeez, Center for Veterinary Medicine, for providing tabulated conceptual approaches to derive a microbiological ADI; and Sandra Malone and Diana Mathews for administrative assistance in the preparation of this manuscript.

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