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OPINION OF THE SCIENTIFIC COMMITTEE ON ANIMAL NUTRITION ON THE CRITERIA FOR ASSESSING THE SAFETY OF MICRO-ORGANISMS[1] RESISTANT TO ANTIBIOTICS OF HUMAN CLINICAL AND VETERINARY IMPORTANCE

(adopted on 3 July 2001, revised on 24 January 2003)

[1] **At present, resistance is not of concern in fungi and in this document, the term "micro-organisms" refers only to bacteria**

Acknowledgement

SCAN adopted this opinion on the basis of the preparatory work done by the SCAN ad'hoc Working Group, which was chaired by

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1. BACKGROUND

In 1993, the Council of the European Union requested the inclusion of micro-organisms in Council Directive 70/524/EEC. Micro-organisms used as additives in animal nutrition and authorised at national level by Member States had then to obtain a Community authorisation in accordance with the requirements of Council Directive 70/524/EEC.

In September 1996, the Scientific Committee on Animal Nutrition was requested by the Commission to assess for safety a number of dossiers submitted for micro-organisms seeking a Community approval as a feed additive. This led to the adoption on 26 September 1997 of the SCAN "Report on the use of certain micro-organisms as additives in feedingstuffs"[2]. The annex to this report lists the outcome of the evaluations and is regularly updated as the assessment of each dossier submitted to the Committee is concluded. Each assessment includes a consideration

[2] Report of the Scientific Committee on Animal Nutrition on the use of certain micro-organisms as additives in feedingstuffs of 26 September 1997 updated on 22 March 2001

of any identified resistance to antibiotics of human clinical and veterinary importance.

Because of serious concerns about the growing level of resistance to antibiotics in regular use in human medicine, the Scientific Steering Committee was asked by the European Commission to undertake a major review of the medical and non-medical use of antibiotics. Included in its Opinion of the 28th May 1999[3] was the recommendation that the use of antibiotics as growth promoters in animal nutrition should be phased out. This recommendation arose from the recognition that the presence of acquired resistance amongst the bacteria of the animal digestive tract, developed primarily as a result of exposure to antibiotics used as growth promoters, represents a large pool of resistance genes. Since ingestion of bacteria derived from animals is common, there is a consequent potential for resistance genes in these bacteria to be transferred to human bacteria, although the magnitude of this risk has yet to be established. The European Commission announced in the White Paper on Food Safety of 12 January 2000[4] its intention to pursue the prohibition or phasing-out of antibiotics used as growth promoters in the EU depending on their potential use in human and veterinary medicine as part of its broad strategy to control and contain antibiotic resistance.

The removal from the market of a number of antibiotics used as growth promoters and the stated intention to phase out those remaining in use, has changed the emphasis of the safety assessment of micro-organisms intended for use as feed additives. The earlier requirement that microbial additives should be compatible with antibiotic growth promoters is now both superfluous and undesirable. Viable micro-organisms used as the active agent(s) in feed additives should not add to the pool of transferable antibiotic resistance genes already present in the gut bacterial population or otherwise increase the likelihood of transfer of resistance.

This principle, if applied literally, would rule against most microbial products since micro-organisms susceptible to all groups of clinically important antibiotics are rarely encountered. However, the basis of resistance varies greatly as does the likelihood of intra- and inter-species resistance transfer. Any conclusion on the safety of microbial feed additives should be based on a current understanding of the mechanism of resistance and resistance transfer and should be proportionate to the risk identified while remaining consistent with Commission policy.

2. TERMS OF REFERENCE

In the light of the Scientific Steering Committee opinion on antimicrobial resistance of 28 May 1999, reinforced by its second opinion on antimicrobial resistance of 10-11 May 2001, and taking account of the current scientific knowledge, the Scientific Committee on Animal Nutrition is requested to review and document the criteria

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[3] Opinion of the Scientific Steering Committee on antimicrobial resistance of 28 May 1999, updated by the recent Second SSC Opinion on antimicrobial resistance of 10-11 May 2001

[4] COM (1999) 719 final

guiding its assessment of the safety of micro-organisms resistant to antibiotics of human clinical and veterinary importance intended for use as a feed additive.

3. INTRODUCTION: ANTIBIOTIC USE ON FARMS AND THE SPREAD OF RESISTANCE

The rapid evolution of resistance is a response from bacteria to the dramatic change in their environment introduced by the copious use of antibiotics. Globally some one to ten million tons of antibiotics have been distributed in the biosphere during an antibiotic era of only about sixty years duration. Of the 18,000 tons of antibiotics produced each year for medical and agricultural purposes in the United States, 12,600 tons are used for the non-therapeutic treatments of livestock in order to promote growth (Anon, 2001). In the EU and Switzerland, 1,600 tons of antibiotics, representing about 30 % of the total use of antibiotics in farm animals, were similarly used for growth promotion purposes in 1997 (FEDESA, 1998). These amounts of antibiotics have exerted a very strong selection pressure towards resistance among bacteria, which have adapted to this situation, mainly by a horizontal and promiscuous flow of resistance genes.

The need for action in relation to antibiotic use in farm animals is well illustrated by evidence that the rapid and efficient spread of resistance genes is not restricted only to mammalian systems but can be found in groundwater underlying farms (Chee-Sanford *et al.*, 2001). Tetracycline resistance genes were isolated from pig excreta from two farms in Illinois, U.S.A., where antibiotics were regularly used for disease control and growth promotion. The same resistance genes were also commonly found in taxonomically and ecologically diverse bacteria isolated in ground water downstream from farm manure deposits. This demonstrates, not only the effective horizontal spread of resistance genes among bacteria, but also that untreated ground water, used as drinking water in many rural areas, could be a way for antibiotic resistance to enter or re-enter the animal and human food chain. A farm with a selective pressure of antibiotics could thus be a point source of genetic contamination. This study also serves to illustrate the important role agriculture plays in the spread of resistance genes into the wider environment.

4. INTRINSIC AND ACQUIRED RESISTANCE

Resistance to a given antibiotic can be inherent to a bacterial species or genus (intrinsic or natural resistance). Intrinsic resistance helps to define the spectrum of activity of an antibiotic - the list of bacterial species usually susceptible to this antibiotic. By contrast, resistance may be acquired by some strains within a species usually susceptible to the antibiotic under consideration. Similar biochemical mechanisms are responsible for intrinsic and acquired resistances, *e.g.* enzymatic modification of the antibiotic, target modification or decreased uptake of the antibiotic. Table 1 gives examples of intrinsic resistances. In some cases, neither the biochemical mechanism nor the resistance gene responsible for intrinsic resistance have been identified.

The risk of transfer of resistance genes to human or animal pathogenic bacteria which could result from the use of microbial products is related in part to the genetic basis of resistance. Intrinsic resistance is presumed to present a minimal potential for spread (see below), whereas acquired resistances mediated by plasmids and

transposons are considered as having a high potential for spread. It should be stressed that no risk assessment study has been designed specifically to quantify the risk related to the use of resistant strains (intrinsic or acquired) as food or feed additives. The lack of risk assessment studies leads to use the term of hazard rather than the term of risk. However, the hazard can be qualified and discussed on the basis of current scientific knowledge.

Table 1. Examples of intrinsic resistance in Gram-positive and Gram-negative organisms.

Organism	Intrinsic resistance	Mechanism of resistance	Relevant gene
	Gram-positive organisms		
Enterococcus spp[*] and *Staphylococcus spp*[*]	nalidixic acid	low affinity gyrase	*gyrA*
Enterococcus faecalis	penicillin (low level)	low affinity PBPs	*pbp*
	aminoglycosides (low level)	decreased uptake	-
	lincomycin	Unknown	Unknown
	streptogramins A	Unknown	Unknown
Enterococcus faecium	amikacin, netilmicin, tobramycin	Acetylation	*aac(6')-I*
Enterococcus gallinarum	vancomycin	low affinity peptidoglycan precursors	*vanC1* (operon)
Enterococcus casseliflavus	vancomycin	low affinity peptidoglycan precursors	*vanC2* (operon)
Pediococcus acidilactici	vancomycin, teicoplanin	low affinity peptidoglycan precursors	ligase gene
Lactobacillus spp[*]	vancomycin, teicoplanin	low affinity peptidoglycan precursors	ligase gene
	Gram-negative organisms		
Enterobacteriaceae, *Pseudomonas*, *Haemophilus*	macrolides	outer membrane impermeability + efflux	*acrAB-tolC* (efflux)
Klebsiella	ampicillin	penicillinase	*shv-1*
Enterobacter	ampicillin-cephalothin	cephalosporinase	*ampC*

[*] Some but not all species

4.1. Spread of resistance related to intrinsic resistance

The notion of a stable and fixed bacterial chromosome distinct from a compartment of mobile genes borne on plasmids and transposons is obsolete and does not reflect the reality of the plasticity of the bacterial genomes. For instance, it is considered that only a portion of the chromosome of *Escherichia coli* is original and that the rest results from the acquisition of exogenous DNA. Exchange of DNA between bacteria via conjugation, transposition and transformation frequently occurs. In addition, bacterial DNA

has been shown able to transfer to eucaryotic cells. According to the International Human Genome Sequencing Consortium, 223 of the approximately 31 000 protein-coding genes (0.7%) are, judged by sequence homology, to be bacterial in origin (Relmann, 2001). The most significant was with a protein produced by *Mycobacterium tuberculosis*. Any gene responsible for intrinsic resistance can thus spread provided that it is flanked by insertion sequences. It can also be exported via a phage or from high frequency of recombination (Hfr) bacterial strains. Therefore, it is not surprising that several plasmid-borne genes of antibiotic resistance were found to originate from chromosomal genes. A well-known example is that of antibiotic-producing micro-organisms which avoid suicide by harbouring genes encoding resistance to the antibiotic they synthesize. These "intrinsic" resistance genes are considered as the source of most acquired resistances in pathogenic bacteria. The best evidence is provided by the predominant type of plasmid-mediated vancomycin resistance detected in *Enterococcus faecium,* the *vanA* resistance encoded by transposon Tn*1547*. Expression of *vanA* resistance requires the cooperation of seven genes clustered in a single operon. Homologous genes have been detected in the producer of vancomycin, *Amycolatopsis orientalis,* and in a soil bacterium intrinsically resistant to vancomycin, *Bacillus popilliae* (Rippere *et al*., 1998). The relatedness is not limited to the gene homology but extends to the organization of the entire operon which is identical in Tn*1547* and in the intrinsically resistant bacteria. Other examples in Gram-negative bacteria came from the characterization of plasmid-mediated beta-lactamases: plasmid-mediated penicillinase SHV-1 is identical to the chromosomal penicillinase of *Klebsiella pneumoniae*. Recently, several cephalosporinase genes have been detected on plasmids. The plasmid-mediated cephalosporinases DHA-1 and ACC-1 originate from the chromosomal (intrinsic) cephalosporinases of *Morganella morganii* and *Hafnia alvei*, respectively.

There are also several examples of the horizontal transfer of chromosomal fragments mediating resistance to antibiotics in *Neisseria*. Sulfonamide resistance in *Neisseria meningitidis* for example, has been shown to be caused by a complete or partial exchange of the gene for the target enzyme (dihydropteroate synthase) making the enzyme insensitive to sulfonamide, and the bacteria drug resistant. This horizontal transfer of genetic material has taken place by transformation and recombination, which is one general mechanism for transport of genetic material. In this mechanism, bacterial cells lyse releasing DNA, which is then taken up by living cells. This may lead to an incorporation of genetic material by homologous recombination. In the case of *Neisseria meningitidis* transformation can result in a chromosomal gene exchange or the formation of a mosaic gene, where one part is substituted to mediate resistance. The source of this resistance-mediating DNA is probably other *Neisseria* species, showing resistance to sulfonamide (Fermer *et al.*, 1995).

In studies by site-directed mutagenesis it has also been shown that differences in the amino acid sequence of the dihydropteroate synthase leading to sulfonamide resistance in *Neisseria meningitidis* are compensated for by other amino acid changes making the resistant enzyme as efficient as the susceptible enzyme. This means that resistance will not be selected against in the absence

of drug, but will remain as a consequence of the once heavy use of sulfonamide for the prophylaxis and treatment of bacterial meningitis. The phenomenon of horizontal transfer of chromosomal DNA mediating sulfonamide resistance has also been observed in *Streptococcus pyogenes* (Svedberg *et al.*, 1998). In this case, larger areas of chromosomal DNA, comprising several genes were found to have been transferred. Another well-known example is the development of resistance to beta-lactam antibiotics in pneumococci which is due to the development of altered forms of the high-molecular-weight penicillin-binding proteins (PBPs) that have decreased affinity for the antibiotics. Altered PBPs are encoded by mosaic genes that have emerged by recombinational events between the *pbp* genes of pneumococci and their homologues in closely related streptococcal species (Coffey *et al.*, 1995). These examples confirm that exchange of chromosomal DNA between bacteria exists in nature and is an efficient and necessary phenomenon important for the evolution of genomes.

The search for insertion sequences in the candidate organisms that carry intrinsic resistance that could be an indication for a higher potential for resistance spread does not appear feasible in practice because of the multiplicity of these elements. However, since, in theory at least, the antibiotic selection pressure might accelerate the export of chromosomal genes on mobile elements, the selection of micro-organisms for use as feed additive should be oriented towards the least resistant organism whenever possible.

4.2. Acquired resistance

Acquired resistance can be due either to acquired genes (genes acquired by the bacteria via gain of exogenous DNA) or to the mutation of indigenous genes. Clearly, the presence of antibiotic resistance on mobile elements presents the highest degree of danger for dissemination of resistance. By their nature, resistance by mutation of chromosomal genes presents the same low risk of dissemination as the intrinsic resistance detailed above. Tables 2 and 3 present examples of acquired resistance due to mobile elements and mutations.

Acquired resistance poses an immediate hazard, but the risk associated with the hazard is dependent on the nature of the acquired resistance. Characterization of acquired resistance genes thus is important for risk assessment in order to identify genes with known transferability, particularly in connection with insertion sequences, integrons or transposons**.**

Table 2. Examples of transferable acquired resistance in Gram-positive organisms.

Organism	Acquired resistance	Mechanism of resistance	Relevant gene
Staphylococcus spp.	Methicillin	low affinity PBP2A	*mecA*
	Gentamicin	modifying enzyme	*aac(6')-aph(2")*
	Kanamycin	modifying enzyme	*aph(3')-III*
	Kanamycin-tobramycin	modifying enzyme	*ant(4')-(4")*
	Macrolides	target modification efflux	*ermA, ermB, ermC, msrA*
	Tetracyclines	target modification efflux	*tetM, tetO, tetK, tetL*
Enterococcus spp	Gentamicin	modifying enzyme	*aac(6')-aph(2")*

Macrolides	target modification efflux	*ermA, ermB, ermC, mefA*
Tetracyclines	target modification efflux	*tetM, tetO, tetK, tetL*

Table 3. Examples of resistance due to mutation in Gram-positive organisms

Organism	Resistance	Mechanism	Mutation
E. faecium	Penicillin (high level)	target mutation	*pbp5*
S. pneumoniae	Erythromycin	target mutation	23S rRNA, L4, L22
Gram positive cocci	Quinolones	target mutation	*gyrA, parC, parE*
	Rifampin	target mutation	*rpoB*

4.3. Mechanisms of transfer of resistance

There are several known mechanisms for the horizontal transfer of resistance genes. One or several resistance genes could for example be located on a large plasmid (self-replicating, extra-chromosomal element), which by an internal genetic apparatus has the means of moving from bacterium to bacterium, often in a promiscuous way, and always leaving a copy behind. The phenomenon is called conjugation (see *section 6*). Small, non-conjugative plasmids carrying resistance genes can be mobilized between bacteria in the wake of large plasmid conjugation. Furthermore, there are several genetic mechanisms, located either on the chromosome or on a plasmid, which influence the likelihood of genetic transfer. Transposons, which cannot replicate, but have to rely on the replication machinery of the chromosome or of a plasmid present the least risk. They can, however, move from plasmid to plasmid or from plasmid to chromosome and can carry several resistance genes, thereby substantially increasing the mobility of these genes. The transposon can in turn carry a relatively recently discovered genetic element for the dissemination of resistance genes, the integron. The integron cannot move by itself, but carries a gene, the product of which (an integrase) can mobilize resistance genes, that are borne on the integron in the form of cassettes. The integrase can move these resistance cassettes in and out of the integron, thereby substantially increasing the horizontal mobility of antibiotic resistance genes. The described integron mechanism has only been seen in the context of moving resistance genes, but ancestors to it have recently been discovered in *Vibrio cholerae* and other bacteria (Rowe-Magnus *et al.*, 1999). Called superintegrons, they seem to have evolved as a mechanism to aid bacteria in adapting to environmental changes. This could serve as an illustrative example and explanation of the rapid development of bacterial mechanisms for the horizontal transfer of resistance genes. Under the heavy selection pressure of the ubiquitously distributed antibiotics, evolutionary old genetic mechanisms for gene transfer have adapted to the new antibiotic environment by recombination.

The spread of *dfr*9 provides a good example of efficient horizontal dissemination. This is a gene mediating trimethoprim resistance, originally found in isolates of *Escherichia coli* from pigs (Jansson *et al.*, 1992). It was found in *E. coli* to be situated on large conjugable plasmids and inserted in a disabled transposon, Tn*5393*, which in turn had been found earlier on a plasmid in the plant pathogen *Erwinia amylovora* (Chiou and Jones, 1993). Transposon Tn*5393* carries genes for streptomycin resistance, and apparently evolved in response to the spraying of apple orchards with streptomycin to

protect crops from fire blight caused by *Erwinia amylovora*. The trimethoprim resistance gene *dfr*9 codes for a resistant variation of dihydrofolate reductase, the target of trimethoprim. Its origin is unknown. The appearance of a new resistance gene under heavy selection pressure from trimethoprim used in pig rearing, and borne on a transposon earlier found in the very different context of a plant pathogen, illustrates the powerful ability of micro-organisms to effect a horizontal flow of genetic material mediating resistance.

5. CRITERIA FOR IDENTIFYING BACTERIAL STRAINS WITH ACQUIRED RESISTANCE TO ANTIBIOTICS

All bacterial products intended for use as feed additives must be examined to establish the susceptibility of the component strain(s) to a relevant range of antibiotics (see annex). It is important that such tests are made in a consistent manner using internationally recognised and standardised methods. As a basic requirement the minimum inhibitory concentration (MIC) of the antibiotic expressed as mg/l or µg/ml should be determined for each of the antibiotics listed in table 4.

Table 4. Microbiological breakpoints used by SCAN categorising bacterial species as resistant (mg/l). Strains with MICs equal to or higher than the breakpoints below are considered as resistant. R=Inherent resistance*.

Antibiotic	*Enterococcus faecium*	*Enterococcus faecalis*	*Pediococcus*	*Lactobacillus*	*Bacillus*
Ampicillin	8	8	2	2**	2**
Streptomycin	1024	1024	32	16	64
Kanamycin/ neomycin	1024	1024	32	32	64
Gentamicin	500	500	4	1	8
Chloramphenicol	16	16	16	16	16
Tetracycline	16	16	16	16	16
Erythromycin	4	4	4	4	4
Quinupristin/dalfopristin	4	R	4	4	4
Vancomycin	8	8	R	4**	4
Trimethoprim	8	8	16	32	8
cipro/enrofloxacin	4	2	16	4**	1
Linezolid	4	4	4	4	4
Rifampin	4	4	8	32	4

*MIC determination not necessary for species designated as inherently resistant to the antibiotic.
**Certain species are inherently resistant.

5.1. Dilution methods

For the assessment of antibiotic susceptibility of bacterial strains for use in microbial products agar or broth dilution procedures should be used and include relevant quality control strains. The tests should be performed according to national standards such as the NCCLS (National Committee for

Clinical Laboratory Standards) (NCCLS document M7-A3) or similar. When determining the MIC, serial dilutions of the antibiotic, usually two-step dilutions, are prepared in agar or broth. After incubation, usually over night, the MIC is defined as the lowest concentration of the antibiotic that inhibits bacterial growth.

5.2. Agar disk diffusion

In a clinical setting, other methods for determining the antibiotic susceptibility of bacteria, such as agar disk diffusion, have tended to replace the original dilution methods. The dilution methods have been regarded as too tedious, time consuming and also too expensive. However, the need for standardisation is even more important for disk diffusion methods than for other methods. Results obtained by disk diffusion are quantitative but the data in the form of zone diameters have to be correlated with data obtained with dilution methods (MICs) in order to be clinically useful. The inhibition zone diameters may be extrapolated to MICs and susceptibility categories through the calculation of regression lines (Ericsson and Sherris, 1971). The regression line for each antibiotic is mostly calculated for many bacterial species and the accuracy and usefulness of such results have been widely questioned over the years (Dickert *et al.*, 1981; Krasemann and Hildenbrand, 1980; Olsson-Liljequist *et al.*, 1997)**.** The results reported from disk diffusion tests are usually qualitative designating bacteria as susceptible, intermediate or resistant to the antibiotic in question. In view of SCAN, such methods are better avoided for the generation of MIC values.

5.3. Clinical breakpoints

In order to classify bacterial strains as susceptible or resistant to a certain antibiotic in the clinical situation, breakpoints are set. Their main function has been to guide clinicians in the choice of antibiotics. Usually breakpoints are used to categorise bacterial strains as susceptible (S), intermediate (I) or resistant (R). Such breakpoints are often referred to as clinical or pharmacological breakpoints and they may vary considerably between countries. The criteria mostly considered when categorising bacteria as S, I, or R for an antibiotic in the clinic are: pharmaco-kinetics (pharmaco-dynamics), the distribution of MICs in the bacterial population, toxicology, epidemiology of resistant strains and clinical experience. The intermediate category is used more or less as a buffer zone between the susceptible and resistant categories.

5.4. Microbiological breakpoints

For the more straightforward purpose of identifying bacterial strains with acquired and potentially transferable antibiotic resistance, microbiological breakpoints are more relevant than clinical breakpoints (Olsson-Liljequist *et al.*, 1997). Microbiological breakpoints are set by studying the distribution of MICs or inhibition zone diameters of a certain antibiotic for a bacterial population, for example in the form of histograms (Martel *et al.*, 1995; Olsson-Liljequist *et al.*, 1997). The part of the population that clearly deviates from the normal susceptible population of a bacterial species is categorised as resistant. Microbiological breakpoints have recently been introduced not only

in some of the antibiotic resistance monitoring programmes (DANMAP 1999), but also in the clinical situation (Olsson-Liljequist *et al.*, 1997). When using such breakpoints, most strains carrying transferable resistance genes associated with presently known transfer mechanisms will be clearly distinguished from susceptible strains. However, it is recognised that strains with non-transferable non-inherent antimicrobial resistance caused by chromosomal mutations are not always readily distinguished from susceptible strains by studying the distribution of MICs.

5.5. Breakpoints used by SCAN

To date only Gram-positive bacterial genera have been considered or used as additives in animal feed. These have included strains of *Enterococcus (*mostly *E. faecium), Pediococcus, Lactobacillus* (various species) and *Bacillus (B. subtilis, B. licheniformis* and *B. cereus*). A major obstacle for proposing breakpoints for these genera, with the exception of *Enterococcus* spp., is the general lack of relevant data. For some genera, such as *Lactobacillus,* there are no generally accepted standard procedures for MIC determination and information on MIC ranges in *Lactobacillus* spp. and in *Bacillus* spp. is limited (Barret and Jones, 1996, Weber *et al*., 1988, Zarazaga *et al*., 1999). MICs for *Enterococcus* spp. isolated from various sources (DANMAP 1999; Murray, 1990; Butaye *et al*., 1999; Butaye *et al*., 2001; Finch, 1996) and for *Pediococcus* spp. (Barrett and Jones, 1996; Swenson *et al*., 1990; Tancovic *et al*., 1999; Zarazaga *et al*., 1999) are better documented.

The breakpoints set in table 4 should be seen as a pragmatic response to introduce consistency in the separation of strains with acquired transferable resistance from susceptible strains. The MIC values, although as far as possible matching those in current use, are not intended for any purpose other than the assessment of microbial products for the possible presence of antibiotic resistance. In each case identification of an MIC value at or above that shown in table 4, in the view of SCAN, should require further investigation

6. CONJUGATIVE GENE TRANSFER

6.1. Background

Conjugation means genetic transfer from one bacterial strain to another through a direct cell to cell contact. The best studied example is the F-plasmid-mediated conjugative gene transfer in *Escherichia coli.* The F-plasmid contains the essential elements for conjugation, the origin of transfer (*oriT*) and approximately 40 different genes involved in the actual gene transfer process (*tra*-genes). Conjugation starts with the donor cell (harbouring the F-plasmid) and the recipient cell being brought together by the mediation of a specific structure called F-pilus. When a channel connecting the both bacterial cell surfaces has been formed the unidirectional DNA-transfer starts from the *oriT* site. The transfer is based on the rolling-cycle type of replication, where single-stranded DNA is generated as an intermediate product. This single-stranded DNA is transferred into the

recipient, where the complementary strand is synthesised (Lanka and Wilkins, 1995).

In addition to coding for its own transfer the F-plasmid can integrate itself into the bacterial chromosome. These Hfr strains can transfer long stretches of chromosomal DNA into the recipient cells and there recombine with the recipient chromosome.

Several conjugative plasmids both in Gram-negative and Gram-positive bacteria have been detected and described. While there are differences in the details, the overall conjugative processes appear to be similar. An important aspect of conjugation is mobilisation. This means that a conjugative plasmid can induce the transfer of a non-conjugative plasmid provided that the latter has an *oriT*-site of its own and also specific genes for mobilisation (*mob*-genes) (Lanka and Wilkins, 1995). In Gram-positive bacteria certain conjugative antibiotic resistance plasmids, such as pAMβ1 and pIP501, are capable of both mobilisation and also intergeneric conjugation (Tannock, 1987; Langella *et al.*, 1993). Conjugative elements are also associated with some transposons (Salyers *et al.*, 1998).

6.2. Detection of conjugation

Conjugation can be detected with bacterial mating experiments. The suspected donor with an antibiotic resistant phenotype is mixed with a recipient strain sensitive to the respective antibiotic, and the transfer of the resistance is subsequently checked. Also a positive control donor strain harbouring a known conjugative plasmid able to transfer into the recipient used, should be included in the test to ensure that the eventual negative results from the conjugation trials with the test donor are not a result of error in the experimental design. The conjugation frequencies are usually expressed as the number of transconjugants obtained per donor. This means that the detection limit is a conjugation frequency of approximately 10^{-9}. Frequencies of 10^{-6} to 10^{-5} of transconjugant cells are usually the highest experimentally obtainable.

In order to interprete the results of conjugation experiments correctly, it is important to differentiate the real transconjugants from spontaneous donor or recipient mutants resistant to selection antibiotics. For this purpose parallel experiments with separate donor and recipient cultures should be conducted in order to evaluate the respective mutation frequencies.

While the experiment is in principle simple, certain aspects should be taken into account in order to detect even low conjugation frequencies.

6.2.1. Choice of recipient

A recipient should be of the same species as the donor, be sensitive to the antibiotic to which the donor is resistant, but have a different resistance marker to allow for the counterselection against the donor. Where no strains with suitable counterselection markers are available, resistance mutants can usually be obtained by a variety of selection procedures. Resistance mutants against streptomycin, rifampicin or fusidic acid are usually readily obtained. Before the

actual conjugation experiments the MICs of the antibiotics for the recipient and donor strains, respectively, have to be determined.

6.2.2. Examples of the experimental procedure

It is essential to ensure maximum contact between the donor and recipient cells. There are two basic methods to achieve this, the plate mating and filter mating techniques (Tynkkynen *et al.*, 1998).

In plate mating the donor and recipient cells are first grown separately to late exponential phase in suitable liquid medium containing the antibiotic to which the respective strain is resistant. The cultures are then mixed in equal quantities, cells immediately harvested by centrifugation, washed and suspended in a small volume of antibiotic-free medium. This suspension is transferred as a drop on an agar plate (without antibiotics). After a suitable incubation time allowing visible growth on the plate the resulting thick bacterial mass is resuspended in a few ml of liquid medium. This suspension is plated on selection plates. These contain both the antibiotic, to which the donor is resistant but the recipient sensitive, and the other antibiotic allowing growth of the recipient but counter-selecting the donor. Thus, only cells having both the resistance traits as a result of conjugation are able to grow. In addition the mixtures are plated on media containing either of the selection antibiotics alone. This allows the enumeration of both the donor and recipient cells in the mixture.

The filter mating procedure is basically similar to the plate mating. The main difference is that the donor-recipient mixture is filtered through a filter, which is subsequently transferred to the agar plate to allow for bacterial growth and conjugation. The filter is then removed, the bacteria washed away and resuspended in fresh broth. The suspension is subsequently plated on selection and enumeration plates as described above.

7. THE REQUIREMENTS FOR DEMONSTRATION OF INTRINSIC RESISTANCE

The detection of resistance to one or more antibiotics tested under the conditions described above (*section 5*) and the absence of transferability of resistance (*section 6*) leads to further investigations to make the distinction between acquired and intrinsic resistance. Since intrinsic resistance is specific for a bacterial species or genus, an indispensable pre-requisite is the correct identification of the strain. Several recent studies have shown that the active agent in some commercialised products were not correctly identified (Green, 1999; Senesi, 2001; Hoa, 2000).

Some resistances are known to be intrinsic, for instance vancomycin resistance in *Pediococcus* spp. and in certain species of lactobacilli, and should not require further analysis. However, for clinically important antibiotics, the possibility of a plasmid-mediated resistance masked by the intrinsic resistance should be investigated. For instance a *vanA* or *vanB* plasmid-mediated resistance could be masked by the intrinsic vancomycin resistance in a *Pediococcus* strain and the presence of *van* genes should be checked by PCR or hybridization.

If the nature of resistance is not known, its probable intrinsic character can be established by confirming the resistance in a large number of strains (50-100) belonging to the same species. The presence of a resistance in all strains studied, however, is not definitive evidence that the resistance is intrinsic. Approximately 90% of *Staphylococcus aureus* strains are resistant to penicillin G by production of plasmid-mediated penicillinase. If the intrinsic character is assumed on the basis of the presence of the resistance trait in all studied strains, as much additional evidence as possible should be provided to confirm the assumption. Lack of detection by PCR or hybridization of a resistance gene known to be acquired (*e.g. tet* or *erm* genes) also is not definitive but is a strong indication of intrinsic resistance. In the field of Gram-positive bacteria, most studies on antibiotic resistance have focused on organisms pathogenic for man and animals. A considerable amount of knowledge has been accumulated through the years on the acquired resistance genes in these pathogens. By contrast, the knowledge gathered on organisms which are considered as weak pathogens or of minor medical importance is far more limited. A given mobile gene is generally preferentially distributed in a genus or species although this is not always the case. For instance, the *ermB* genes are widespread in streptococci and enterococci but are rare in human isolates of staphylococci which harbour preferentially *ermA* and *ermC* genes. Therefore, lack of detection of a gene known to be widely distributed in human or animal pathogens does not exclude the presence of a mobile gene not yet identified.

Lack of transfer of the resistance trait to a recipient strain does not necessarily mean that resistance is not transferable but rather that transfer was not detected. In addition, non-conjugative transposons or small plasmids are not self-transferable.

A summary of some proposed methods that can be used to provide evidence that resistance is intrinsic is shown in Table 5. The best evidence is based on genetic experiments, although this requires that the gene has been sequenced in part or in its entirety. Since it is not possible to propose a single definitive experiment, evidence of intrinsic resistance has to be based on the accumulation of data that is not definitive when taken individually, but convincing when taken together.

Table 5. Experiments that may be used to determine the intrinsic nature of a resistance gene.

1. Resistance detected in the totality of strains belonging to the same species (n=50-100).
2. Absence of *in vitro* transfer of the resistance
3. Lack of identification by PCR or hybridization of known resistance genes (*erm*, *tet*).
4. Isolation and determination of the sequence of the gene and its species specificity
5. Localization of the gene on the chromosome using a labeled probe specific for the gene. DNA of the bacteria can be digested with the *Iceu*I enzyme which cuts into the rRNA operons generally present in several copies on the chromosome and transferred to a nylon membrane. Co-hybridization of a fragment with a 16s rRNA probe and the probe specific for the gene provides a strong evidence for chromosomal location.
6. Determination of nucleotide sequences flanking the gene to show the presence, or otherwise, of house-keeping genes. It can also show the lack of insertion sequences.

8. CONCLUSION

- The concept of a stable and fixed bacterial chromosome distinct from a compartment of mobile genes borne on plasmids and transposons is outmoded and does not reflect the full plasticity of the bacterial genome. Consequently, all genes coding for antibiotic resistance in micro-organisms have to be considered to have the potential to be transferred to other microbial strains and thus to present a hazard.

- Although it is not yet possible to quantify the risk associated with the spread of resistance, differences in degree of risk can be identified depending on the genetic basis of the resistance trait.

- Where all, or virtually all, strains within a given taxonomic group show resistance to an antibiotic, that resistance can be considered inherent or intrinsic to the taxonomic group. Provided that the gene (or genes) conferring resistance is (are) not associated with a recognised mobile element, the risk of transfer to other organisms can be considered to be low.

- Where resistance has been be acquired by a strain belonging to a taxonomic group usually susceptible to an antibiotic, then the degree of risk of transfer generally is considered to be substantially greater than that associated with intrinsic resistance.

- If, however, the acquired resistance trait can be shown to be due to a gene mutation and that the mutated gene is boarded by other "house-keeping" genes, then the risk can be considered of similar magnitude to that of intrinsic resistance.

- SCAN considers that strains of bacteria carrying an acquired resistance to antibiotics used in human and veterinary medicine should not be used in microbial feed additives, unless it can be shown that the genetic basis of the resistance is due to gene mutation in a gene intrinsic to the bacterium.

9. MICRO-ORGANISMS IN HUMAN FOODS

Implementation of the conclusions above are consistent with the position adopted by the Commission on the need to take action to preserve the value of antibiotics in human and veterinary medicine. They should lead to the exclusion of any microbial feed additive containing one or more bacteria carrying resistance genes capable of being transferred to other bacteria. They are, however, far more stringent than those currently applied to live micro-organisms used in foods and consumed directly by humans. This seems to SCAN contrary to the stated desires of the Commission and iniquitous for producers of animal products. Accordingly SCAN recommends to the Commission that a consistency of approach should be adopted for all microbial products entering the food chain.

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ANNEX

Proposal of a test scheme

More detailed descriptions of MIC determination, transferability testing, PCR and other molecular methods to distinguish intrinsic from acquired resistance are given in chapters 5, 6 and 7.

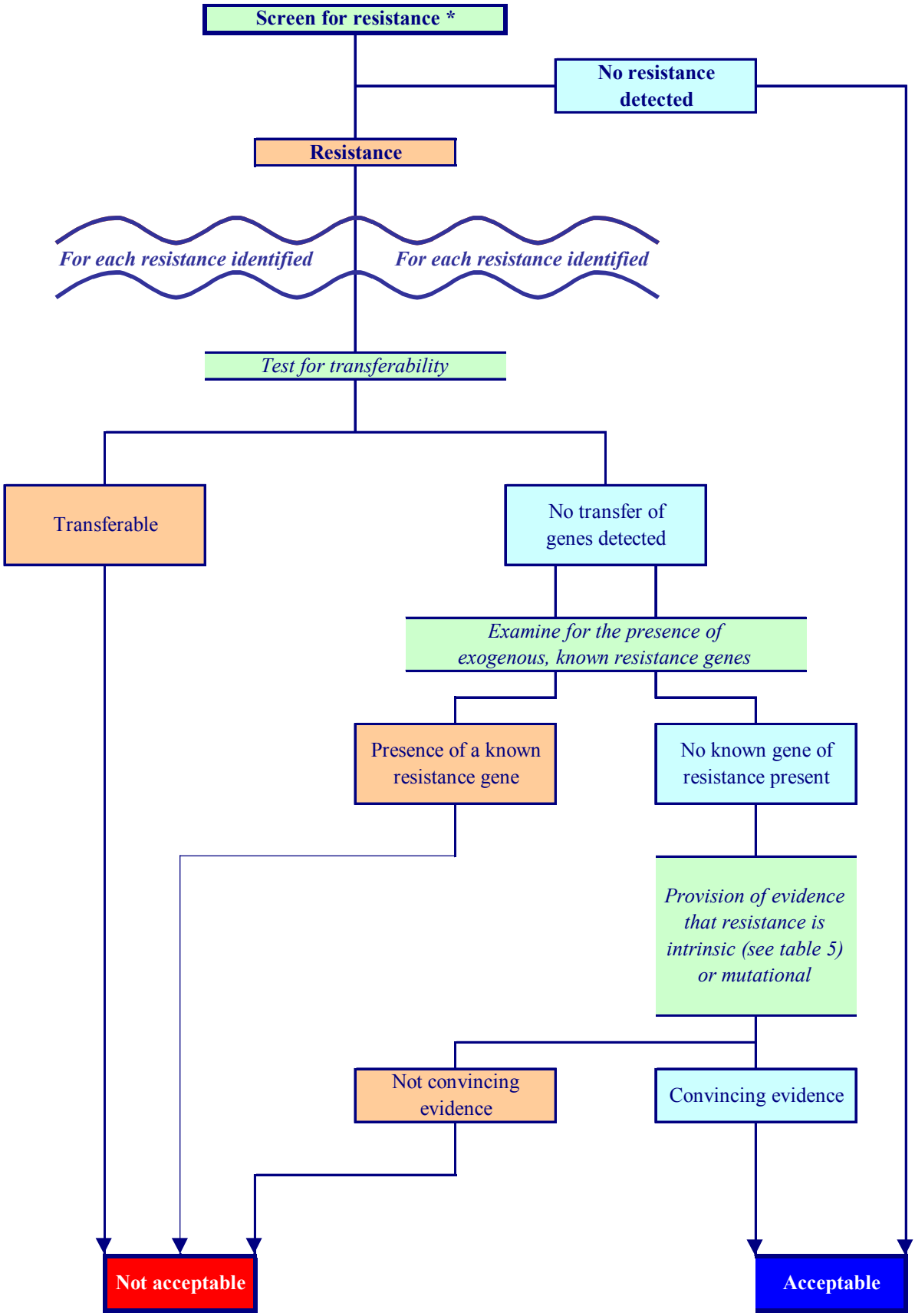
(1) Test for antibiotic susceptibility (MIC) – For those antibiotics to which the bacterial species is intrinsically resistant (shown as R in table 4) it is not necessary to determine an MIC.

(2) For species with known intrinsic resistance to an antibiotic such as vancomycin resistance in *Lactobacillus* and *Pediococcus,* absence of known resistance genes should be confirmed.

(3) Presence of plasmids should always be investigated.

(4) In case of suspected acquired resistance or intrinsic resistance, transferability tests are optional. If transferability of the resistance is proven, then the strain will not be considered for use in microbial products and further tests are superfluous.

(5) If the resistance trait(s) is not transferred in mating experiments, then it should be verified by molecular methods such as PCR and/or other methods that the resistance is caused by mutations in intrinsic genes (table 5, chapter 7).



*: For organisms intrinsically resistant to antibiotics, test of which is not required (see table 1), check the presence of exogenous and known genes, expression of which may be masked by intrinsic resistance