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Opinion of the Scientific Panel on Additives and Products or Substances used in Animal Feed on the updating of the criteria used in the assessment of bacteria for resistance to antibiotics of human or veterinary importance

(Question N° EFSA-Q-2004-079)

Adopted on 25 May 2005

SUMMARY

The emergence and the spread of resistance to antimicrobials in bacteria pose a threat to human and animal health and present a major financial cost. In an effort to decrease the development of resistance various actions have been taken at Community level, including the removal of all antibiotics used for growth promotion purposes from animal feed in 2006. With this objective, the Scientific Committee on Animal Nutrition (SCAN) adopted an opinion in July 2001 defining the criteria used to assess the presence or absence of resistance determinants to antibiotics in microbial feed additives.

As part of the self tasking activities, the FEEDAP Panel identified the need to focus on this area and has been requested to: i) revise the SCAN Opinion on the assessment of bacteria for resistance to antibiotics of human clinical or veterinary importance taking into consideration the data published after the adoption of the SCAN opinion. ii) define appropriate breakpoint values as indicative of the need for a more extensive assessment of the basis for the resistance and iii) consider whether the distinction between "intrinsic" and "acquired" resistance used as indicative of the probability of transfer of resistance is still valid for the safety assessment of microbial feed additives.

Resistance to a given antimicrobial can be inherent to a bacterial species or genus (intrinsic or natural resistance), or acquired through gain of exogenous DNA or by mutation of indigenous genes.

For the purpose of distinguishing strains harbouring acquired antimicrobial resistance from susceptible strains, the FEEDAP Panel defines new microbiological breakpoints for 13 antimicrobials, which were chosen to maximise the identification of resistance genotypes, to the most commonly used antimicrobials, by assessing the resistance phenotypes. The data used for the definition of microbiological breakpoints were derived from the body of research published and from national and European monitoring programmes.

The detection of the MIC (minimum inhibitory concentration) above the breakpoint levels, as identified by the FEEDAP Panel, for one or more antimicrobials requires further investigations to make the distinction between acquired and intrinsic resistance.

Where resistance has been acquired by a strain belonging to a taxonomic group naturally susceptible to an antimicrobial, then the degree of risk of transfer, generally is considered to be substantially greater than that associated with intrinsic resistance. The FEEDAP Panel considers that strains of bacteria carrying an acquired resistance to antimicrobial(s) should not be used as a feed additive, unless it can be demonstrated that it is a result of chromosomal mutation(s).

Key words: Antimicrobial resistance, acquired resistance, intrinsic resistance, genomic mutation, added genes, horizontal transfer, MIC, microbiological breakpoints, bacterial feed additives.

BACKGROUND

The emergence and spread of resistance to antimicrobials in bacteria posses a threat to human and animal health and presents a major financial and societal cost. Various actions have been taken at the Member State and Community level intended to encourage the prudent use of antimicrobial agents in primary health care and veterinary medicine (including the removal of all antibiotics used for growth promotion purposes from animal feed). These actions were taken in an effort to slow the development of resistance.

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 the safety of a number of Dossiers submitted for microbial products seeking Community approval as feed additives. As part of this assessment SCAN considered the presence of any determinants conferring resistance to antibiotics of human clinical or veterinary importance. Subsequently, in the interests of transparency, SCAN adopted an opinion[1] (July 2001) defining the criteria it used to assess the presence or absence of resistance determinants to antibiotics. In particular the microbiological breakpoints for 13 antibiotics, belonging to different groups of antibacterial compounds, were defined. These were not intended as definitive values or to be used to automatically disqualify an organism for use as a feed additive. Rather they defined a minimum inhibitory concentration (MIC) value, which if exceeded, triggered the need for a more extensive investigation to define the genetic basis of the observed resistance.

In the years following the first adoption of the SCAN Opinion, increased attention has been paid to the study of antibiotic resistance in bacterial species which are also used as additives in animal nutrition, such as lactic acid bacteria (Danielsen and Wind 2003; Florez *et al.* 2005; Gevers *et al.*, 2003; Gfeller *et al.*, 2003; Katla *et al.*, 2001) and enterococci (Coconcelli *et al.*, 2004; Ferber 2003; Franz *et al.*, 2004; Hayes *et al.*, 2004; Jensen *et al.*, 2003; Klare *et al.*, 2004; Leavis *et al., 2003;* Werner *et al.,* 2004,). In addition new genetic determinants coding for antimicrobial resistance have been described and new mobile genetic elements identified in these bacteria. Moreover, the availability of the data from genome or metagenome analysis for several bacterial species used for feed additive purposes opens new possibilities of investigation into the nature of intrinsic or acquired antimicrobial resistance. Due to the importance of this topic, an EU research project, (ACE-ART) was approved in the 6th Framework Programme. As a consequence of this research effort, in January 2003 SCAN formally had to revise some of the breakpoints included in its Opinion.

There is now a need for a further and more comprehensive revision of the document to take account of the more recent work in this area. Consequently the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) proposes to revise the original SCAN Opinion and to adopt it as an EFSA document defining the approach taken by the FEEDAP Panel to the assessment of bacterial products used as feed additives in relation to antimicrobial resistance.

TERMS OF REFERENCE

The FEEDAP Panel is requested to revise the Opinion of the former Scientific Committee on Animal Nutrition (SCAN) of the Directorate General on Health and Consumer Protection (DG SANCO) on the assessment of bacteria for resistance to antibiotics of human clinical or veterinary importance. This revision should take account of the body of research published since the original adoption of the SCAN Opinion.

[1] Opinion of the Scientific Committee on Animal Nutrition on the criteria for assessing the safety of micro-organisms resistant to antibiotics of human clinical and veterinary importance, adopted on 3 July 2001, revised on 24 January 2003. http://europa.eu.int/comm/food/fs/sc/scan/out108_en.pdf

The FEEDAP Panel is further requested to consider whether the breakpoints considered by SCAN as indicative of the need for a more extensive assessment of the basis for the resistance are still appropriate. If not, the FEEDAP Panel is asked to determine more appropriate values.

The FEEDAP Panel is also asked to consider whether the distinction between "intrinsic" and "acquired" resistance used as indicative of the probability of transfer of resistance is still valid.

The Panel is requested to reach agreement on the harmonized approach preferably before the end of 2004.

ASSESSMENT

1. Introduction

The rapid evolution of resistance is a response from bacteria to the dramatic change in their environment introduced by the extensive use of antimicrobials. Data on antimicrobial consumption in livestock in EU is scarce and has been reported in only a few countries (DANMAP, 2003; NORM/NORMVET 2002; SWEDRES/SVARM 2003; MARAN 2002; Mitema *et al.*, 2001). Programmes for monitoring of antimicrobial resistance in bacteria have been implemented in a number of the EU Member States. Examples of such monitoring programmes are DANMAP in Denmark, SVARM/SWEDRES in Sweden, NORM/NORM-VET in Norway, MARAN in The Netherlands and similar programmes exist in France (Sanders *et al.,* 2002), Spain (Moreno *et al.,* 2000) and the UK (Goodyear, 2002). These programmes include the major food animal species and food-borne zoonotic bacterial species such as *Salmonella* spp. and *Campylobacter* spp. Most monitoring programmes also include commensal bacteria such as *Escherichia coli* and *Enterococcus* spp. (indicator organisms) as well as animal pathogens. The data generated and reported from monitoring programmes in the EU have helped considerably to increase our knowledge of trends in patterns of antimicrobial resistance.

Directive 2003/99/EC on the monitoring of zoonoses and zoonotic agents[2], obliges Member States to monitor antimicrobial resistance in zoonotic agents and, if they present a threat to the public health, in other agents. The monitoring is mandatory for *Salmonella* and *Campylobacter* in bovines, pigs and poultry and products thereof. It should provide comparable data and supplement the monitoring conducted in human isolates. The Commission is in a process of preparing harmonised rules for *Salmonella*, *Campylobacter*, *E. coli* and enterococci. The results of the monitoring are to be reported to the Commission, who will forward them to EFSA. EFSA will analyse and summarise the results and include them in the annual Community Report on Zoonoses.

The extent to which antibiotic usage in farm animals contributes to the spread of antimicrobial resistance via the food chain and to resistance problems in humans is debated. Food is generally considered to be the most important vector for spread of resistance between man and animals. The greatest risk has been associated with food-borne zoonotic agents such as *Salmonella* and *Campylobacter* originating from livestock and the transfer of these microorganisms from animals to humans is well documented (Molbak, 2004; Threlfall *et al.,* 2000). Spread of resistance to the growth promoter streptothricine from porcine to human *E. coli* and later also to *Salmonella* and *Shigella* is a well documented example of gene transfer (Witte, 2000).

[2] Directive 2003/99/EC of the European Parliament and of the Council of 17 November 2003 on the monitoring of zoonoses and zoonotic agents, amending Council Decision 90/424/EEC and repealing Council Directive 92/117/EEC OJ L 325, 12.12.2003 p. 31.

The removal from the market of antibiotics used as growth promoters after January 1st 2006 (Regulation (EC) N°1831/2003)[3] emphasizes the need of reducing the spread of the genetic determinants for antimicrobial resistance in the food chain. Viable micro-organisms used as the active agent(s) in feed additives should not add to the pool of antimicrobial resistance genes already present in the gut bacterial population or otherwise increase the risk of transfer of drug resistance. The FEEDAP Panel maintains the safety assessment of bacteria intended for use as feed additives evaluating the risk related to antibiotic resistance and acknowledges the different potential for spread of intrinsic and acquired resistances to antimicrobials.

This document was submitted to public consultation, during two and a half months (December 2004 - February 2005). Contributions were received from Universities, National Institutes/Centres, National Authorities, Scientific Societies, European Institutions and the industry.

2. Intrinsic *vs* acquired resistance

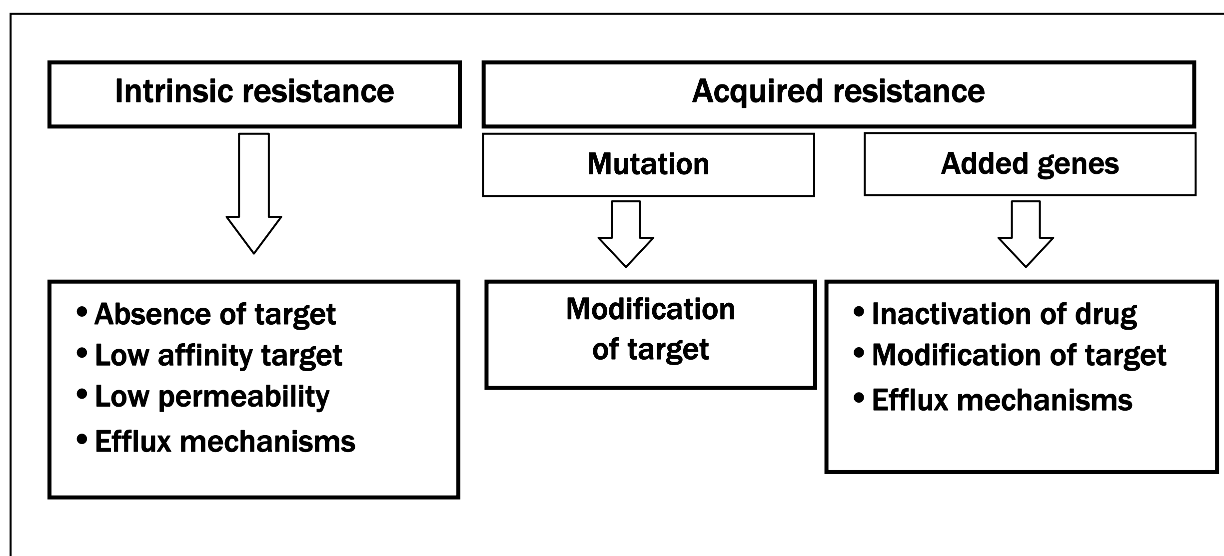
When resistance to an antimicrobial is inherent to a bacterial species it is generally referred to "intrinsic resistance" (sometimes called "natural resistance"), and it is typical of all strains of that species. In contrast, when a strain of a typically susceptible species is resistant to a given antimicrobial drug, it is considered to be an "acquired" resistance".

Three major mechanisms of antimicrobial resistance have been described: direct inactivation of the active molecule; loss of bacterial susceptibility to the antimicrobial by modification of the target of action; and reduction of the concentration of drug that reaches the target molecule without modification of the compound itself. The antibiotic defence mechanisms of intrinsic resistances are, in most of cases, related to the presence of low affinity targets, absence of targets, to decreased uptake, accumulation or efflux of drug (Figure 1).

The actual possibility of transfer of resistance to human or animal pathogenic bacteria which could result from the use of microbial products based on drug resistant strains is related to the genetic basis of resistance. Although it is reasonable to assume that gene transfer from viable micro-organisms will occur to other microorganisms in open environment such as the gastrointestinal tract, intrinsic resistance is presumed to present a minimal potential for horizontal spread, whereas acquired resistance mediated by added genes is considered as having a high potential for lateral spread.

[3] Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition. OJ L 268, 18.10.2003, p.29.

Figure 1. **Major mechanisms of intrinsic and acquired resistance**



3. Acquired resistance.

Acquired resistance can be due either to added genes (genes acquired by the bacteria via gain of exogenous DNA) or to the mutation of indigenous genes.

3.1 Genomic mutations

Chromosomal mutations can result in increased resistance to antimicrobials in different ways. The most frequent are mutations in genes coding for the target molecules of antimicrobials, usually altering the antibiotic-binding site. Examples are mutations in the penicillin binding protein gene (*e.g.,* Arbeloa *et al*., 2004), which results in high resistance to penicillin, mutations in 16S or 23S rRNA genes making *Helicobacter pylori* (Trieber *et al.,* 2002) and *Streptococcus pneumoniae* (Doktor *et al*., 2004) *resistant to* to tetracycline and erythromycin, respectively.

Mutations in regulators or regulatory regions can contribute to antimicrobial resistance by leading to the overproduction of either intrinsic resistance determinants, such as efflux pumps (Beinlich *et al*., 2001) or the target itself (Flensburg and Skold, 1984), which may overcome total inhibition by the drug.

Resistance by mutation of chromosomal genes presents a low risk of horizontal dissemination, and would generally be acceptable for the FEEDAP Panel. However, there could be unacceptable exceptions.

3.2 Added genes

Added genes are the result of gene exchange between bacteria. Although the resistance mechanisms associated with added genes are, in most cases, enzymatic modification leading to the direct inactivation of the active molecule, such as β-lactamases or acetyl transferases (Poole, 2002), added genes can code for mechanisms of decreased susceptibility to antimicrobials due to target modification or drug efflux.

The presence of added genes coding for antibiotic resistance, particularly when carried by mobile genetic elements, presents the greatest risk for horizontal dissemination of resistance.

3.2.1 Antimicrobial resistance gene exchange among bacteria

There are several known mechanisms for the horizontal transfer of resistance genes. One or several resistance genes could be located on a large plasmid, 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 (Grohmann *et al.,* 2003). The phenomenon is called conjugation. 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 (Burrus and Waldor, 2004). Transposons, which cannot replicate, but have to rely on the replication machinery of the chromosome or of a plasmid have been involved in spreading of antimicrobial resistance genes. Thus, they can move from plasmid to plasmid or from plasmid to chromosome and can carry several resistance genes, thereby substantially increasing the mobility of these genes. Integrons are genetic elements that can encode several different antibiotic resistance genes. They have been demonstrated to significantly contribute to the spread of antimicrobial resistance in gram-negative bacteria. 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 antimicrobial resistance genes. In multidrug-resistant clinical isolates integrons harbouring up to eight different antimicrobial resistance cassettes have been detected (Naas *et al*., 2001).

4. Criteria for identifying bacterial strains with acquired resistance to antimicrobials

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 antimicrobials of human or veterinary importance (Table 1). It is essential that such tests are made in a consistent manner using internationally recognised and standardised methods. As a basic requirement the MIC of the antimicrobial expressed as mg L^{-1} or µg mL^{-1} should be determined for each of the following substances: ampicillin, vancomycin, gentamicin, kanamycin, streptomycin, neomycin, erythromycin, clindamycin, quinupristin + dalfopristin (e.g., Synercid), tetracycline, chloramphenicol, trimethoprim and linezolid (Table 1). These antimicrobials were chosen to maximise the identification of resistance genotypes to the most commonly used antimicrobials by assessing the resistance phenotypes.

4.1. Microbiological breakpoints

For the purpose of distinguishing strains harbouring acquired antimicrobial resistances from susceptible strains, the FEEDAP Panel defines microbiological breakpoints. Microbiological breakpoints are set by studying the distribution of MICs of the chosen antimicrobials, in bacterial populations belonging to a single taxonomical unit (species or genus). The part of the population that clearly deviates from the normal susceptible populations is categorised as resistant. The data used for the definition of microbiological breakpoints, as reported in Table 1, were derived from the body of research published and from national and European monitoring programmes.

For the assessment of microorganisms used as feed additives, bacterial strains can be categorized as susceptible or resistant to antimicrobials:

- Susceptible (S): a microorganism is defined as susceptible when it is inhibited at breakpoint level of a specific antimicrobial in a defined phenotypic test system (S ≤ x mg L^{-1}).

- Resistant (R): a bacterial strain is defined as resistant when it is not inhibited at breakpoint level of a specific antimicrobial in a defined phenotypic test system (R > x mg L^{-1}).

The breakpoints identified should be seen as a pragmatic response intended to introduce consistency in the separation of strains with acquired resistance from susceptible strains. The breakpoint values are not intended for any purpose other than the assessment of microbial products for the possible presence of antimicrobial resistance. Identification of a MIC value above that shown in Table 1, would required a further investigation.

Table 1. **Microbiological breakpoints categorising bacteria as resistant (mg L^{-1}). Strains with MICs higher than the breakpoints below are considered as resistant.**

	Lactobacillus obligate homofermentative	*Lactobacillus* heterofermentative*	*Lactobacillus plantarum*	*Enterococcus*	*Pediococcus*	*Leuconostoc*	*Lactococcus lactis*	*Streptococcus thermophilus*	*Bacillus* spp	other Gram+
ampicillin	4	4	4	8	4	4	4	4	n.r.	2
vancomycin	4	n.r.	n.r.	8	n.r.	n.r.	4	4	4	4
gentamicin**	8	8	64	512	4	4	8	8	4	4
kanamycin **	16	16	64	1024	4	8	8	8	8	8
streptomycin**	16	16	64	1024	4	8	16	16	8	8
neomycin**	16	16	32	1024	8	8	8	8	8	8
erythromycin	4	4	4	4	4	4	4	4	4	4
clindamycin	4	4	4	4	4	4	4	4	4	4
quinupristin + dalfopristin	4	4	4	4	4	4	4	4	4	4
tetracycline	8	8	32	16	4	4	4	4	8	4
chloramphenicol	4	4	8	8	4	4	8	8	8	4
trimethoprim**	8	8	8	8	8	8	n.r.	n.r.	8	8
linezolid	4	4	4	4	4	4	4	4	4	4

n.r. not required.
*including *L. salivarius*
** possible interference of the growth medium

It is intended that the values in Table 1 will be reviewed on regular basis and modified when necessary, if new data becomes available. This may include data on Gram negative microorganisms, if they are introduced as feed additives.

4.2. Quantitative methods for the MIC determination

For the assessment of susceptibility of bacterial strains serial two-fold dilution procedures in agar or broth should be used and include relevant quality control strains. The tests should be performed according to standards such as the Clinical and Laboratory Standard Institute –CLSI- (formerly National Committee for Clinical Laboratory Standards – NCCLS) or similar. After

incubation, the MIC is defined as the lowest concentration of the antimicrobial that inhibits bacterial growth.

The existing body of scientific information related to that specific or related bacterial species must be considered when the procedure for MIC determination (dilution method, growth media and incubation conditions) is chosen, keeping in mind the possible interference of media and growth conditions. Media interference has been reported, for example, for lactic acid bacteria (Huys *et al*., 2002; Danielsen *et al*., 2004).

5. Defining the genetic basis of resistance

The detection of the MIC above the breakpoint levels identified by FEEDAP Panel, for one or more antimicrobials requires 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 at species level by means of molecular taxonomy methods.

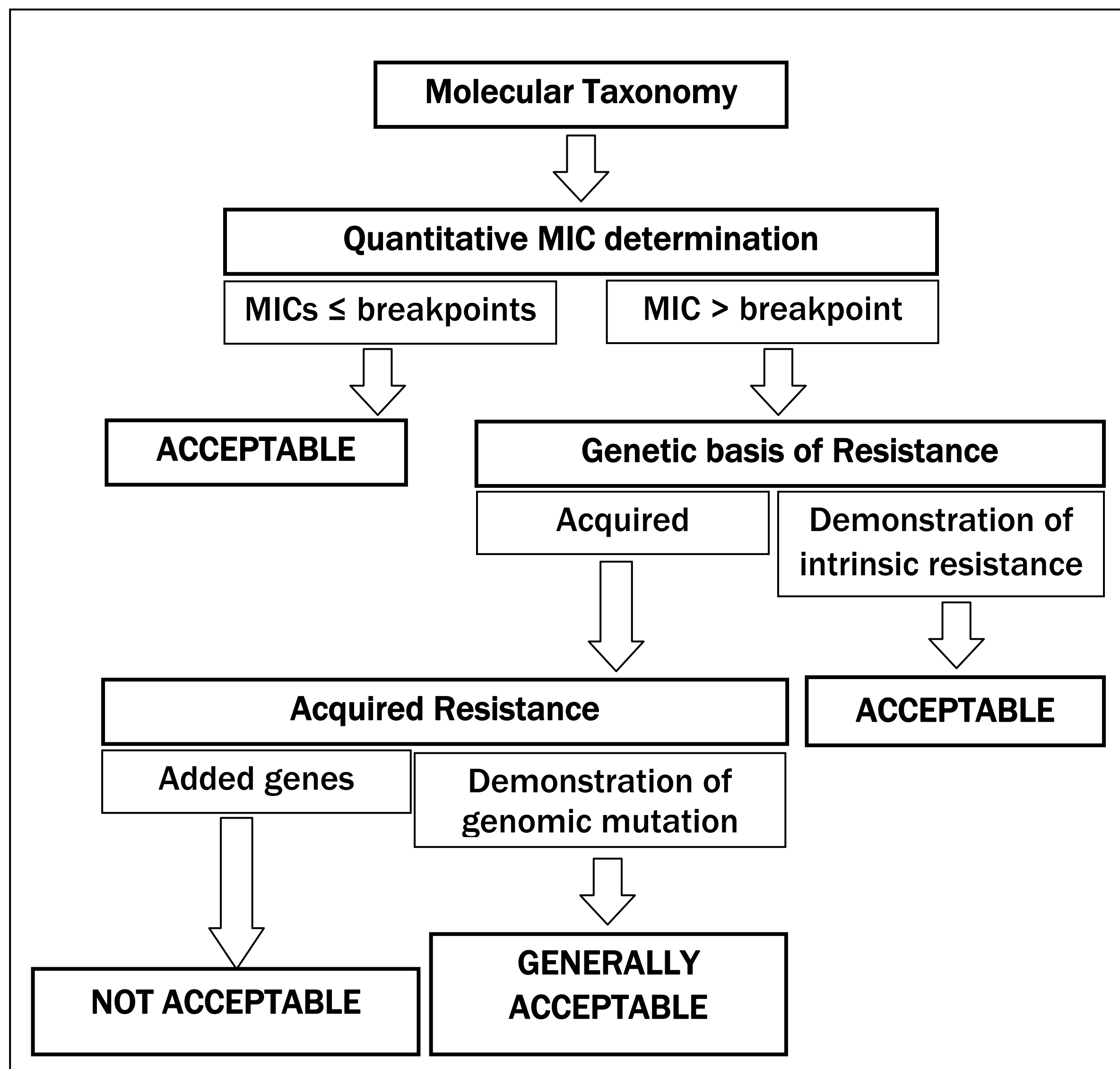
Where all strains within a given taxonomic group show phenotypic resistance to an antibiotic, that resistance can be intrinsic to the taxonomic group. If published data regarding the nature of intrinsic resistance of a specific bacterial group to a defined antimicrobial compound are not available, the structural nature and genetic basis of the resistance must be demonstrated analysing a representative selection of strains belonging to that taxonomical unit.

When a bacterial strain demonstrates higher resistance to specific antimicrobial compounds than the other strains of the same taxonomical unit, the presence of acquired resistance is indicated and additional information is needed on the genetic basis of the antimicrobial 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 antimicrobial resistance on mobile elements presents the highest risk for dissemination of resistance. The selection of micro-organisms for use as feed additives should be oriented towards the least resistant organism whenever possible.

The schema proposed by the FEEDAP Panel for the antimicrobial resistance assessment of a bacterial strain used as feed additive is shown in figure 2.

Figure 2. **Proposed scheme for the antimicrobial resistance assessment of a bacterial strain used as feed additive.**



6. Conclusions

From the assessment of the current scientific data, the FEEDAP Panel concludes that:

- Where all strains within a given taxonomic group show a common resistance to an antimicrobial, that resistance could be intrinsic to the taxonomic group. Provided that the gene (or genes) conferring resistance is (are) not associated with mobile genetic elements, the risk of transfer to other organisms can be considered as minimal.

- Where resistance has been acquired by a strain belonging to a taxonomic group naturally susceptible to an antibiotic, then the degree of risk of transfer generally is considered to be substantially greater than that associated with intrinsic resistance, unless it can be shown that the genetic basis of the acquired resistance is due to chromosomal mutation.

- Resistance by mutation of chromosomal genes presents a low risk of horizontal dissemination, and would generally be acceptable for the FEEDAP Panel.

- FEEDAP Panel considers that strains of bacteria carrying an acquired resistance to antimicrobial(s) should not be used as a feed additives, unless it can be demonstrated that it is a result of chromosomal mutation(s).

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