DETECTION OF ANTIBIOTIC RESISTANCE GENES IN FOOD-ASSOCIATED BACTERIA

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SUMMARY

The increasing prevalence of acquired antibiotic resistance (AR) in bacteria constitutes a growing concern, especially in the last decades. Resistant pathogenic bacteria can lead to serious implications for the treatment of infectious diseases but the occurrence of AR determinants in commensal and environmental bacteria is important too, as these microorganisms represent a reservoir of AR genes that can be transferred to pathogens. A large number of studies has observed an ever increasing level of AR in food-related bacteria, such as enterococci, staphylococci and lactic acid bacteria. Consequently, food is considered an efficient vehicle for the diffusion of antibiotic resistances. Detection of AR in bacteria is required both to monitor the spread of resistant organisms and for determine the optimal antimicrobial therapy for clinical isolates. For these purposes, conventional phenotypic tests and/or more advanced nucleic acid-based methods can be applied. The technique to be used depends on the information that are required and on the targets under consideration.

KEY WORDS: antibiotic resistance genes, detection methods, food-associated bacteria.

RESUMEN

DETECCIÓN DE GENES DE RESISTENCIA A ANTIBIÓTICOS EN BACTERIAS ASOCIADAS A LOS ALIMENTOS

La creciente prevalencia de la resistencia adquirida a antibióticos (AR) en las bacterias constituye una preocupación creciente, especialmente en las últimas décadas. Las bacterias patógenas resistentes pueden conducir a complicaciones serias para el tratamiento de enfermedades infecciosas, pero la ocurrencia de determinantes de AR en bacterias comensalistas y del ambiente es también importante, dado que estos microorganismos representan un reservorio de genes AR que pueden ser transferidos a los patógenos. Un gran número de estudios han observado un nivel creciente de AR en bacterias relacionadas con los alimentos, tales como *Enterococcus*, *Staphylococcus* y bacterias ácido lácticas. Los alimentos son considerados un vehículo eficiente para la difusión de resistencias a antibióticos. La detección de AR en bacterias es necesaria para revelar la magnitud de organismos resistentes y para determinar la terapia antimicrobiana óptima para aislamientos clínicos. Para estos propósitos, pueden utilizarse las pruebas fenotípicas convencionales y/o métodos moleculares basados en ácidos nucleicos. La técnica utilizada depende de la información que sea requerida y los objetivos bajo consideración.

PALABRAS CLAVE: genes resistentes a antibióticos, métodos de detección, bacteria asociada a los alimentos.

Resistance to antimicrobials has existed since before they were introduced into human and veterinary medicine. Recent evidences however point to an increasing prevalence of antibiotic resistance (AR) in bacteria, thus constituting a growing concern, especially in the last decades. It is generally accepted that the main factor that is responsible for this phenomenon is the increased use of antibiotics both in human medicine and in animal husbandry as growth promoters. The extensive use of antibiotics creates a selective pressure that results in mutation of normal cellular genes, acquisition of pre-existing foreign resistance genes or a combination of these mechanisms. Resistance genes are however often imported and the same AR genes are found in bacteria belonging to different species and genera.

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The main mechanism of horizontal transfer in natural environments is conjugation. As AR genes are normally located on transmissible genetic elements like plasmids or transposons, their intra-, inter-specific and inter-generic transfer is possible (Sorum & L'Abée-Lund, 2002).

Antibiotic resistance, especially in pathogenic bacteria, has become an increasingly health problem which has serious implications for the treatment and prevention of infectious diseases in both humans and animals. On the other hand, the occurrence of AR determinants in commensal and environmental bacteria is important too, as these microorganisms constitute a reservoir of AR genes that can be transferred to pathogens.

In this context, foods containing resistant bacteria represent efficient vehicles for the diffusion of antibiotic resistances (Teuber & Perreten, 2000). A large number of studies has observed an ever increasing level of AR in food-related bacteria (Schlegelova *et al*., 2002; Hayes *et al*., 2003).

Additionally, concerns have been heightened over the increasing number of both pathogenic and commensal microorganisms that are resistant to multiple antibiotics.

Aim of this speak is to present the main methods that can be applied to detect antibiotic resistance in food-associated bacteria and to describe some recent studies showing the importance of food as a vehicle of AR genes to humans.

ANTIBIOTIC RESISTANCE

Bacteria may resist to an antimicrobial agent by means of intrinsic or acquired resistance mechanisms.

Intrinsic or natural resistance is a peculiar feature of a certain bacterial species that confers a resistance phenotype. For example, lactobacilli are naturally resistant to vancomycin.

In contrast, acquired resistance emerges when a bacterial strain, normally susceptible to an antibiotic, becomes resistant due to a genetic modification such as the acquisition of a new gene or gene cluster. This type of resistance is the matter of concern in the last decades for its diffusion in several bacterial species.

The main mechanisms that bacteria use against antibiotic action are: (i) presence of an enzyme that inactivates the molecule; (ii) production of an alternative enzyme that is not inhibited by the antimicrobial agent; (iii) mutation in the antibiotic's target with a reduced binding affinity; (iv) overproduction of the antibiotic's target; (v) reduced uptake of the antimicrobial molecule; (vi) active efflux of the antibiotic.

The major biochemical classes of antibiotics are aminoglycosides, beta-lactams, glycopeptides, macrolide-lincosamide-streptogramins (MLS) and tetracyclines. Resistance to a certain class of antibiotic can be achieved by one or more of the above described mechanisms and often different genes are known that confer such resistance phenotype.

Aminoglycoside resistance is mainly due to enzymatic inactivation of the antibiotic. Three classes of modifying enzymes are recognized: aminoglycoside nucleotidyl-transferase (ANT), aminoglycoside phosphotransferase (APH) and aminoglycoside acetyltransferase (AAC) and different genes are known that encode for these enzymes. Furthermore, the gene *aac(6')Ie-aph(2")Ia* encodes for a bifunctional enzyme that is able to transfer both an acetyl and a phosphorous group to the antibiotic.

Beta-lactam molecules can be hydrolysed by beta-lactamases (encoded by *blaZ* gene) or resistance may derive from the production of modified penicillin binding proteins (*mecA* gene) resulting in reduced affinity for beta-lactam.

The *van* gene clusters are responsible for the resistance to glycopeptides among which vancomycin and teicoplanin, antibiotics of clinical interest. The presence of the gene cluster *vanA* results in the production of a novel D-Ala-D-Ala ligase that drives the substitution of the peptidoglycan side chain with D-alanyl-D-lactate chain, which has less affinity to glycopeptides.

MLS resistance is mainly due to a posttranscriptional modification (a methylation) of the 23S rRNA which is the target of the antibiotic. Genes encoding these methylase are designated *erm* (erytromycin ribosome methylase) and several genes with this function are known (for example, *ermA*, *ermB*, *ermC*). Moreover, a number of macrolide resistance genes code for efflux proteins and belong to three different classes: *mef*, *mrs* ad *vga*.

Tetracycline resistance is encoded by several resistance genes: till now the number of known *tet* genes are 38 (Roberts, 2005). For example, *tet(K)* and *tet(L)* genes encode for tetracycline efflux protein; *tet(M)* and *tet(O)* are responsible for a ribosome protection mechanism; *tet(X)* codes for an enzyme that inactivates tetracyclines.

DETECTION METHODS

Conventional methods used to ascertain resistance to antibiotics are based on phenotypic tests performed on isolated bacteria. Currently, the most popular susceptibility testing methods are broth micro-dilution test, disk diffusion test and E-test.

The broth micro-dilution is the miniaturization of the tube-dilution method. Two-fold dilutions of antibiotics are added to individual wells in disposable plastic micro-dilution trays containing a liquid growth medium and then wells are inoculated with the bacterial suspension. Following incubation, the lowest concentration of antimicrobial which prevents visible growth represents the MIC, or minimum inhibitory concentration.

In the disk diffusion test (also known as Kirby-Bauer procedure) a standardized bacterial inoculum is applied onto the entire surface of an agar plate. Then, paper discs impregnated with different antibiotics are placed on the surface of the agar. The antibiotic agent diffuses into the agar, thereby preventing the growth of the bacteria in a zone around the disc. The width of the zone gives an indication of the sensitivity of the organism to the agents being tested.

E-test is a patented antimicrobial gradient technique. Essentially, dilutions of an antibiotic have been repackaged onto a plastic strip. The reagent strip is placed onto the surface of an inoculated agar plate and a continuous antibiotic gradient is formed under the strip. After incubation, the resulting inhibition ellipse intersects the scale at the MIC value.

As other phenotypic assays, all these methods are subjected to some disadvantages: only culturable bacteria can be tested and the results are strongly dependent to the physiological state of the cultures.

Antimicrobial susceptibility testing has two purposes. First, it is utilised clinically to predict the likely outcome of treating a patient's infection with a particular antimicrobial agent. Second, it can provide a quantitative measurement of susceptibility which can be used to monitor the emergence and prevalence of AR.

The use of nucleic acid-based methods is particularly useful for applications in basic research as well as in surveillance programs for antimicrobial resistance and food safety.

The main molecular techniques that can be employed for the detection of resistance determinants are hybridisation, polymerase chain reaction (PCR) and, recently, DNA arrays.

Hybridisation is one of the oldest molecular techniques and is based on the specific recognition and annealing of a DNA fragment to its complement sequence. The DNA in a sample is allowed to combine with a single-stranded probe marked with a fluorescent, chemiluminescent or radioactive molecule. This method has some disadvantages such as it needs large quantities of DNA and is time-consuming. However, hybridisation assays are routinely performed for the detection of AR genes in studies aimed to the plasmidic/genomic localization of such determinants (Foglia *et al*., 2003).

Indeed, an advance of the hybridisation method is the use of molecular beacons. In brief, beacons are hairpin-shaped oligonucleotide probes with attached a fluorophore and a "quencher" that quenches the fluorescence when it is next to the fluorophore. Hybridisation with the target sequence allows the fluorescence to be expressed because the fluorophore and the quencher are spatially separated. This application associated to PCR amplification makes possible the real-time monitoring technology.

PCR is the most frequently applied nucleic acid-based method. A number of primer pairs designed on specific target regions are now available for the amplification of nearly all known AR genes (some examples: Predari *et al*., 1991; Dutka-Malen *et al*., 1995; Sutcliffe *et al*., 1996; Kao *et al*., 2000). Thanks to its rapidity, sensitivity and reliability, this method is applied in several studies for clinical, epidemiological and food safety purposes.

DNA array is a powerful technology, developed in the last years, that is used in a number of different applications from large-scale gene expression to detection of specific genes. This technique is based on the principle of hybridisation and differs from the conventional assays because a collection of probes (up to some hundreds) is bound to a solid surface like glass or silicon. The length of the DNA probes is variable and depends on diverse factors such as the type of fragment (cDNA, PCR products, oligonucleotides) and the specificity of the assay. DNA array technology allows the screening of several sequences at the same time so it is useful to study the occurrence and/or the expression of many different genes.

The search of AR genes using DNA microarrays has first involved a low number of genes: the *mecA* gene, responsible for the meticillin resistance in staphylococci (Hamels *et al*., 2001) and, indirectly, the resistance against ciprofloxacin acquired by *Neisseria gonorrhoeae* due to a point mutation (Ng *et al*., 2002). Recently, a disposable microarray was developed for detection of up to 90 antibiotic resistance genes in Gram-positive bacteria (Perreten *et al*., 2005).

Finally, an application that need to be mentioned is DNA sequencing. It can be useful to confirm the results of molecular assays such as AR gene-specific PCRs and is applied to the study of gene variability (De Leener *et al*., 2005).

RESISTANT FOOD-ASSOCIATED BACTERIA

Resistant bacteria, belonging to the genera *Enterococcus* and *Staphylococcus* and the lactic acid bacteria (LAB) group, are ever more frequently being isolated from foods with a large consumption.

Enterococci are ubiquitous microorganisms as they belong to the endogenous flora of man and animals but also occur in a range of food products. Their role could be beneficial as they were retrieved in natural starter cultures used for the manufacture of a variety of cheeses and some strains are employed in adjunct starter cultures.

Staphylococci are widespread in nature, too. They are normal inhabitants of human and animal skin and mucous membranes. Besides, some coagulase-negative strains are used as starters being technologically important in the fermentation and ripening of fermented sausages (salami) and of some types of cheeses.

On the other hand (from the health point of view), the importance of these two genera is also connected to their implications in many nosocomial- and community-acquired infections. For example, *Staphylococcus aureus* and some coagulase-negative staphylococci are the most common causes of wound and blood infections, respectively; enterococci have been implicated in infective endocarditis and urinary tract infections.

Additionally, enterococci and staphylococci are two of the major microbial groups involved in resistance spread. In fact, Gram-positive cocci frequently carry intrinsic resistance to antimicrobials and are also able to rapidly acquire other resistances (Jeljaszewicz *et al*., 2000).

Resistance to tetracycline is frequently found among both enterococci and staphylococci and it is mainly due to the diffusion of plasmids and transposons carrying tetracycline-resistance (*tet*) genes. Genes coding for aminoglycoside-modifying enzymes are also diffused among both genera and the same AR genes (mainly aph3', ant4' and aac6'-aph2'') are present.

In the last years, special interest has been directed to vancomycin-resistant enterococci and methicillin-resistant staphylococci, especially *S. aureus*, because these resistances generate a serious therapeutic problem.

LAB, and in particular lactobacilli, represent an essential element of the commensals of human and animal body as well as of environmental microflora. Moreover, they are the main microorganisms employed as starter in the production of fermented foods of both animal and vegetable origin. Moreover, lactobacilli are often used as probiotics, so it is essential to verify that the single strains do not contain transferable AR genes.

The presence of AR genes in many LAB and their transfer by means of plasmids and conjugative transposons have been reported, as reviewed by Teuber *et al*. (1999). *erm*(B) and *tet*(M) were found in lactobacilli of dairy and human origin (Cataloluk and Gogebakan, 2004) as well as *tet*(M) and *tet*(S) were detected in lactobacilli and cocci isolated from raw meat (Gevers *et al*., 2003). Recently, *Streptococcus thermophilus* strains carrying *erm*(B) gene were isolated from milk and dairy products (Berruti *et al*., 2005; our unpublished data).

ANTIBIOTIC RESISTANCE IN FOOD AND IN FOOD CHAIN

As above mentioned, food has been recognised as an important vehicle for the spread of AR genes. Moreover, the number of studies regarding the presence of resistant bacteria in animals, foods of animal origin and humans has highlight their strong connection. Direct transmission of AR from animals to humans occurs when resistant bacteria are transferred. In a second, indirect way, resistant microorganisms transfer their AR genes horizontally to members of the human microflora.

Following, some recent studies showing examples of resistance spread in food products and AR genes transmission are depicted.

Donabedian *et al*. (2003) found that when gentamicin-resistant genes are present in resistant enterococci isolated from animals they are also present in the enterococci isolated from food products from the same animal species. They also observed undistinguishable enterococcal isolates with common resistant determinants in humans and food.

In order to study the prevalence and diversity of tetracycline-resistant (Tc^r) LAB, Gevers *et al*. (2003) analysed a number of different meat samples along the process line of fermented dry sausages. All raw meat components contained Tc^r LAB such as lactococci, lactobacilli, streptococci and enterococci. Characterisation of the resistance by PCR detection showed the presence of *tet(S), tet(M)* or both genes in most isolated strains. None of these isolates was found to contain *tet(K), tet(L)* or *tet(O)*.

A recent study of Huys *et al*. (2005) regards Tc^r *S. aureus* isolated from a poultry processing plant. Thirty-eight Tc^r isolates, originated from various processing stages, were characterised by different fingerprinting techniques (i.e. $(GTG)_5$-PCR, plasmid profiling and PFGE), and their resistance pattern (presence of some *tet* genes and resistance to most common antibiotics) was determined.

From this study it emerged that a Tc^r *S. aureus* clone carrying *tet(K)* gene was probably disseminated throughout the entire processing line and may thus end up in the final product. Such results support the importance of the clonal spread to the dissemination of resistant staphylococci also in food chains. Moreover, the authors observed that: (i) all *tet(K)*-positive strains shared a plasmid of 4.2-4.4 kb which could indicate the presence of a pT181-like plasmid; (ii) a strain carrying *tet(M)* gene was found to contain a transposon of the Tn*916*-Tn*1545* family. These findings confirm the role of plasmids and transposons in the diffusion of *tet(K)* and *tet(M)* genes, respectively.

The presence of antibiotic-resistant enterococci in different types of food products was extensively demonstrated. Until now, only one study (Rizzotti *et al*., 2005) has been carried out taking in consideration an entire production chain of food of animal origin, from animal farming (feedstuffs and feces) to commercialized food products (raw and processed meat, dry fermented sausages). The authors investigated the presence of 11 AR genes involved in resistance to aminoglycosides (*aac(6 ')Ie-aph(2 ")Ia* gene), beta-lactams (*blaZ, mecA*), glycopeptides (*vanA, vanB*), macrolide-lincosamide-streptogramins (*ermA, ermB, ermC*), and tetracyclines (*tet(M), tet(O), tet(K)*). PCR amplifications were performed both on DNA extracted directly from the samples and on 147 resistant enterococci isolated from these matrices. AR genes were detected in a high percentage of samples: the most frequently detected genes are *tet(K)* (80.5%), *ermB* and *tet(M)* (66.7%). The genes *tet(M)* and *ermB* were highly diffused in isolates, too, being present in 86.9 and 84.9%, respectively, of the resistant enterococci. Moreover, AFLP fingerprinting was applied to monitor the resistant enterococcal isolates along the production chain. Isolates of *E. faecalis* and *E. faecium* showing the same AFLP profile and AR gene pattern were detected in samples taken at different steps of the food chain suggesting three cases of bacterial clonal spread.

CONCLUSIONS

The technique to be used for antimicrobial resistance testing depends on the information required and on the targets under consideration.

Better understanding of the genetic basis of resistance has resulted in the development and application of molecular methods to detect the genetic changes or mutations present in resistant bacterial phenotypes. Nucleic acid-based detection systems offer rapid and sensitive methods to detect AR genes and are essential in order to elucidate resistance mechanisms.

On the other hand, the presence of an AR gene does not necessarily lead to phenotypic resistance, hence the results of molecular analysis should sometimes be associated with phenotypic assays. Moreover, the emergence of new resistance mechanisms or AR genes cannot be found using only specific molecular assays.

Regarding the prevalence and development of antimicrobial resistance four important areas of action are proposed (Scientific Steering Committee, 1999):

– prudent use of antimicrobials:
  (i) avoid their misuse in human and veterinary medi cine,
  (ii) regarding growth promoting agents, the use of agents from classes which are used in human or veterinary medicine should be phased out and ultimately abolished.
– prevention of infection and containment of resistant organisms: this action indirectly contributes to an overall reduction in antimicrobial usage.
– new modalities of prevention and treatment for infections: development of truly novel agents and of effective alternatives to antimicrobials as well as preventive therapies are necessary.
– monitoring the effects of interventions by research programs.

Concluding, the presence of AR genes in food-associated bacteria is a very complex and difficult problem that only with an interdisciplinary approach could be controlled. Fortunately, the advanced molecular methods, actually available, will help us in discovering protective strategies for human and animal well being.

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