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Review article

Molecular tools for the characterisation of antibiotic-resistant bacteria

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Abstract – This review will discuss a number of molecular tools which are currently used as well as some innovative approaches for the characterisation of antibiotic-resistant bacterial strains. Various methods involved in the detection and characterisation of genes and mutations associated with antibiotic resistance and that are used for strain typing as part of epidemiological studies, are described. Furthermore, a few examples are discussed in which the results of both gene and strain characterisation are combined to investigate the underlying mechanism of the spread of antibiotic resistance. Some of the available molecular techniques are heavily supported by the existence of databases on the Internet. These databases either contain a fast growing amount of sequence information or a large number of allelic or fingerprint profiles. The current progress in applied DNA technology and the ongoing projects on the elucidation of the whole genomic sequence of bacterial species have lead and will further lead to the development and application of sophisticated new strategies for the analysis of antibiotic resistant bacterial strains.

antibiotic resistance / molecular detection / molecular typing / horizontal gene transfer / new approaches

Résumé – Outils moléculaires pour la caractérisation de bactéries résistantes aux antibiotiques. Cette revue présente les outils moléculaires utilisés actuellement ainsi que des nouvelles approches pour la caractérisation de souches bactériennes résistantes aux antibiotiques. Différentes méthodes de détection et de caractérisation des gènes et des mutations impliqués dans la résistance aux antibiotiques, utilisées dans des études épidémiologiques, sont décrites. Quelques exemples sont présentés où sont associées les caractérisations du gène et de la souche bactérienne dans l'étude des mécanismes de diffusion de résistances aux antibiotiques. Certaines techniques moléculaires font

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largement appel aux banques de données disponibles sur Internet. Ces banques de données contiennent un nombre croissant d'informations comprenant des séquences ou des profils de typages moléculaires. Le progrès fait actuellement au niveau de technologies ADN appliquées et les projets en cours sur les séquençages de génomes entiers des espèces bactériennes ont conduit et conduiront progressivement au développement et à l'application de nouvelles stratégies sophistiquées pour l'analyse de souches bactériennes résistantes aux antibiotiques.

résistance aux antibiotiques / détection moléculaire / typage moléculaire / transfert horizontal de gènes / nouvelles approches

Table of contents

1. INTRODUCTION

Antibiotics are widely used to treat infectious diseases in both humans and animals, but the emergence of antibiotic resistance in previously susceptible bacterial populations is a very serious threat and now a major public health issue. The emergence and spread of antibiotic resistance must thus be fully understood in order to take the appropriate measures.

Resistance to antibiotics can be intrinsic where the micro-organism lacks the target site for the antibiotic drug, or antibiotic resistance can be acquired by means of changes in the genetic code of housekeeping genes or by uptake of foreign DNA containing antibiotic resistance genes. The emergence and rapid spread of resistance is not necessarily the result of selective pressure by antibiotics for resistant bacteria, but is more likely caused by the uptake of elements carrying resistance genes [24]. Antibiotic resistance

genes can reside on self-replicative extrachromosomal plasmids [19, 34] or can be part of non-self-replicative transposable genetic elements (transposons) [14]. Transposons are found within the bacterial genome or within the extrachromosomal plasmid. A third group of naturally occurring mobile genetic elements are integrons. Gene cassettes are part of these elements and contain one or more antibiotic resistance genes. Schwarz and Chaslus-Dancla discussed a number of aspects concerning bacterial resistance to antibiotics [58], among which resistance-mechanisms, resistance mediating elements and spread of antibiotic resistance genes.

Over the past few years, numerous molecular techniques have been set up for the investigation of an outbreak, in the epidemiological surveillance of antibiotic-resistant strains, in the identification of patterns of infections and to track the source of infection. Furthermore, molecular epidemiology of resistance allows to discriminate between clonal dissemination of a particular resistant strain and horizontal transfer of antibiotic resistance determinants between bacteria. In molecular epidemiology, a large number of different techniques have been used. The current progress in applied DNA technology and the fast increase in sequence information of the genome of a large number of microbial species [19] will indisputably lead to the development of more novel approaches to investigating antibiotic resistance.

This article will briefly review the available molecular techniques which are applied for the detection and identification of antibiotic resistance genes. Furthermore, molecular methods will be discussed for the genomic typing of strains as part of epidemiological investigations. Three examples will be described where both applications, gene characterisation and strain typing, are combined to investigate the underlying mechanism of the spread of resistance. Finally, novel molecular approaches and future developments, such as micro-array applications, will be discussed.

2. MOLECULAR TECHNIQUES FOR THE DETECTION AND CHARACTERISATION OF ANTIBIOTIC RESISTANCE GENES

2.1. Polymerase Chain Reaction (PCR)

The detection of antibiotic resistance genes requires a substantial level of specificity and sensitivity and in many cases speed can even be a requisite. The Polymerase Chain Reaction (PCR) is probably the technique of choice to meet all these requirements. PCR is based on repeated cycles in which the amount of a specific target DNA fragment is exponentially increased. This exponential amplification makes PCR very sensitive, since theoretically one DNA molecule can be detected. However, sensitivity also depends on sample preparation and the quality of the isolated DNA including the integrity of the isolated DNA and the presence of polymerase inhibitors. The required specificity is mainly determined by the choice of the primers in combination with the amplification conditions. The PCR is a rapid method and results can often be obtained in less than 24 hours, including the sample treatment to extract and purify the target DNA, PCR reaction itself, and separation and visualisation of PCR products on agarose gel. For these reasons, PCR is increasingly used to track resistance genes as well as to find specific gene mutations leading to resistance.

2.1.1. PCR-based detection of antibiotic resistance genes

The tracking of antibiotic resistance genes has been used to estimate their relative prevalence in bacterial populations. PCR has for instance been used for the analysis of 114 clinical erythromycin-resistant *Streptococcus pneumoniae* (ERSP) isolates, coming from an American multicentre, for the prevalence of two main mechanisms of macrolide resistance [59]. The primers used

were either specific for the macrolide efflux gene *mefE* or specific for the ribosomal methylase gene *ermAM*. Seventy (61%) ERSP isolates contained the macrolide efflux gene (*mefE*), whereas 36 isolates (32%) contained the biosomal methylase gene (*ermAM*). Even if other genes are probably involved in the resistance of the remaining isolates, these results indicate that the efflux mechanism is the predominant form of macrolide resistance in the United States. Interestingly, the latter resistance mechanism induces lower Minimum Inhibitory Concentration (MIC) values for clarithromycin than those determined by *ermAM* (4 $\mu g \cdot mL^{-1}$ vs. >128 $\mu g \cdot mL^{-1}$, respectively).

On the other hand, in a similar PCR study performed by Latini et al. [32] in Italian day-care centres, the results indicated that macrolide resistance of 75 ERSP was mainly due to the presence of the methylase gene *erm* in 73.6% of the strains while the efflux mechanism determined by *mefE* was only involved in the remaining 26.4%.

The PCR protocol has been adapted for various purposes. For instance, in order to improve gene identification, PCR is often followed by an additional hybridisation step with internal probes such as routinely performed in the Danish surveillance programme of clinical Vanomycin-resistant enterococci (VRE) isolates [51]. In other procedures the PCR protocol has been adapted to improve sensitivity and/or specificity in which the initial PCR is followed by a second PCR using a primer combination located within the amplified fragment. A variant of this "nested" PCR assay was used by Maeda et al. [36] for the analysis of *Helicobacter pylori* strains. In this case it was not actually meant to increase sensitivity but to simultaneously detect *Helicobacter pylori* and a mutation within the 23S rRNA gene associated with the threatening resistance to clarithromycin.

Several PCR protocols have been adapted for the detection of long DNA fragments that may contain several antibiotic resistance genes or for the simultaneous detection of several antibiotic genes at different chromosomal loci (multiplex PCR). Long PCR, for the amplification of fragments with a length between 5 and 11 kb, was used by Haaheim [23] prior to the structural analysis of the complete transposons Tn*1546* (VanA) and Tn*1547* in glycopeptide-resistant enterococci (GRE). For the simultaneous detection of the vancomycin resistance genes *vanA* and *vanB* by PCR, the annealing temperatures of the necessary primer pairs were chosen within the same temperature range to perform a so-called multiplex-PCR. In combination with microtitre plate hybridisation the multiplex PCR analysis of clinical isolates only took 8 hours instead of the required 24–48 hours when a culturing procedure is used [49]. By combining the results of three multiplex PCRs specific for genes coding for aminoglycoside acetyltransferases, nucleotidyltransferases and phosphotransferases, seven classes of aminoglycoside-resistant *Acinetobacter baumannii* strains responsible for nosocomial infections could be identified [44].

2.1.2. PCR-based detection of point mutations leading to antibiotic resistance

PCR followed by restriction analysis was used for the detection of mutations associated with resistance to antibiotics [25, 26], based on the fact that mutations leading to resistance can modify or create recognition sites for endonucleases [62]. Alternatively, oligonucleotidic primers have been designed for the specific detection of mutations related to resistance. A Mismatch Amplification Mutation Assay PCR (MAMA PCR) was successfully used for the detection of *gyrA* mutation in quinolone-resistant isolates of *Campylobacter jejuni* and *C. coli* [76, 77]. Similarly, an Allele-Specific PCR assay combined with Restriction Fragment Length Polymorphism (AS-PCR-RFLP) has been designed to simultaneously detect three common mutations related to quinolone

resistance at codons 81, 83 and 87 of the *gyrA* gene in *Salmonella* spp. [21]. Thus MAMA PCR and AS-PCR-RFLP could be proposed as rapid, simple, inexpensive alternatives to methods such as Single Strand Conformation Polymorphism (SSCP) or DNA sequencing for the detection of fluoroquinolone resistance mutation.

In conclusion, PCR is specific, rapid and sensitive. Its sensitivity can, however, be a drawback. On the one hand, false-positive reactions can easily be introduced through cross-contamination. On the other hand, PCR can be inhibited by a number of compounds present within the isolated DNA leading to false-negative results. Nevertheless, when the necessary precautions are implemented to prevent contamination and the correct controls are included, the number of both false-positive and negative reactions can be reduced to a minimum.

2.2. Nucleic acid analysis without amplification

For the detection of antibiotic resistance genes, PCR is far more favoured than procedures utilising hybridisation techniques. Hybridisations are, however, still required to confirm the lack of the targeted gene in the case of negative PCR results due to primer mismatching.

Hybridisation analyses are also still in use, especially for screening numerous samples. In this case, hybridisations are generally performed with bacterial colonies transferred onto filters. For instance, hybridisation with probes specific for nine antibiotic resistance genes was successfully used for the analysis of multi-resistant *E. coli* strains which were selected from 1200 strains isolated from the normal intestinal flora of healthy swine to prove that swine could be a considerable reservoir of antibiotic resistance genes that might be transferable to pathogens [61]. Considering developments in molecular technology, such as the use of miniaturised micro-arrays, hybridisation will probably become more important in future DNA applications as shown by recent reports on *Mycobacterium* species identification and rifampicin resistance typing obtained with high-density DNA probe arrays [65].

2.3. Bioinformatics and resources for nucleic acid analysis

Up to now a large number of antibiotic resistance genes have been discovered and described. For the development of tests for the detection and characterisation of antibiotic resistance genes, Internet services are available to retrieve the necessary genetic information and to explore the already sequenced bacterial genomes. Services are: database similarity searches such as Blast, sequence retrieval (e.g. Entrez), alignment interfaces, comparative genomics and a number of general tools. For sequence and service information see: http://gonow.to/genomes;www.ncbi.nlm.gov; www.genome.ad.jp/kegg/kegg.html; www.tigr.org; www.embl-heidelberg.de/services.

3. MOLECULAR TYPING METHODS USED FOR THE CHARACTERISATION OF ANTIBIOTIC-RESISTANT BACTERIA

Molecular typing methods are very essential to track the source and vehicle of transmission and to confirm and delineate the pattern of transmission during an outbreak but also to determine genome organisation and evolution. Molecular typing methods can contribute to the evaluation of control measures, they are essential in investigations to distinguish between clonal spread and horizontal gene transfer and are used to monitor the global distribution of antibiotic-resistant strains.

For the genomic characterisation of isolates a large number of different molecular

techniques are applied. The method with the highest resolution at the various taxonomic levels is sequencing, but this is not broadly applied as yet since this method has not been developed in a simple and automated routine in each laboratory and because of its relatively high costs. Molecular typing is therefore mostly performed with generic fingerprint methods, which require no or only little knowledge of sequences. Methods are either based on the analysis of the extra-chromosomal plasmids or are directed at specific loci on the chromosomal DNA or increasingly at various parts of the complete genome. Figure 1 summarises the application of a number of typing methods at the different taxonomic levels.

3.1. Plasmid profiling

As plasmids often carry antibiotic resistance determinants, plasmid fingerprinting can be used for epidemiological purposes. In epidemiological studies, the relatedness of isolated bacterial strains can be determined from the number and size of the plasmids and their restriction profiles. For example, *Aeromonas salmonicida* could be characterised by restriction enzyme analysis of the high-molecular-weight R-plasmids mediating oxytetracycline (OT) resistance [3]. Plasmid profiling was also used by Llanes et al. [34] in combination with hybridisation analysis with the virulence probe *spcCD-oriE* to characterise amoxillin resistance among *Salmonella* spp. In combination with genotypic analysis by pulsed field gel electrophoresis (PFGE) of *Xba*I macro-restricted chromosomal DNA, the study proved that the spread of these strains resulted both from gene transfer and clonal dissemination.

However, the weakness of the analysis of bacterial strains by plasmid profiling is inherent to the fact that plasmids are mobile extrachromosomal elements that can be lost

	family	genus	species	subspecies	strain
DNA sequencing	■	■	■	■	■
MLST	■	■	■	■	■
ARDRA		■	■	■	
tRNA-PCR		■	■	■	
ITS-PCR			■	■	
RFLP			■	■	■
PFGE			■	■	■
AFLP			■	■	■
AP-PCR			■	■	■
RAPD			■	■	■
REP-PCR			■	■	■

Figure 1. Applicability of various typing methods at the different taxonomic levels. Modified from [35, 57]. Sequencing techniques, like Multi Locus Sequence Typing (MLST), are capable to resolve bacteria at all taxonomic levels. Methods directed to ribosomal (ARDRA, Amplified Ribosomal DNA Restriction Analysis) or transfer (tRNA-PCR) RNA genes are able to resolve bacteria at the genus, species and sub-species level. The PCR technique (ITS-PCR, Internal Transcribed Spacer-PCR) amplifying the spacer sequence between the 16S and 23S RNA genes can only be used at the species and the subspecies level. The direct genomic methods, Restriction Fragment Length Polymorphism (RFLP) and Pulse Field Gel Electrophoresis (PFGE), are discriminative at species, subspecies and strain level. For the analysis of bacteria at these levels also the PCR based genomic fingerprint methods such as, Amplified Fragment Length Polymorphism (AFLP), Arbitrarily primed PCR (AP-PCR), Random Amplified DNA polymorphism and PCR fingerprint methods directed to repetitive elements, like REP-PCR, are applicable.

or acquired easily, thus it is well known that epidemiologically related strains can exhibit different plasmid profiles.

3.2. Locus-specific analysis of chromosomal DNA

Multiple regions of the bacterial genome can be used as targets for locus-specific analysis. One of the most frequently used regions is the multicopy essential operons encoding for ribosomal RNA because this locus is highly conserved throughout the whole life tree and thus allows the designation of conserved probes for hybridisation or PCR. Before the general use of PCR, the RFLP-Southern blotting technique in which probes are derived from the 16S and 23S rRNA genes, also known as the ribotyping technique, has been applied successfully in many studies for the differentiation of bacterial strains, e.g. [74]. Ribotyping results in profiles only consisting of a small number of bands which are easy to analyse. However, this feature also limits the ability of the technique to distinguish between closely related strains.

As already mentioned, the probes used in locus-specific Southern blotting studies can also be derived from other genetic sites such as an Insertion Sequence (IS). These are mobile DNA elements that are able to integrate within the bacterial genome. As their position and number can vary from strain to strain, this diversity has been used for typing purposes such as with IS*6110* typing of *Mycobacterium tuberculosis* [69] or IS*200* fingerprinting of *Salmonella enterica* [46, 47].

Presently, the cumbersome blotting techniques have been largely replaced by PCR-based locus-specific RFLP. PCR ribotyping strategy has been reported early and largely used with primers specific for conserved regions in the 16S and 23S rRNA genes [31, 42]. Two kinds of ribosomal PCR-RFLP with different resolutive powers were used. Analysis of the 16S rRNA gene (*rrs*) alone is used as an easy and cheap alternative to *rrs* sequencing [43]. Like the 16S sequencing, it allows bacterium identification at a taxonomic level corresponding to the species or a group of closely related species with identical *rrs* [18, 22]. This level of taxonomic resolution is often enough to differentiate between bacterial species in complex community, and a variety of methods that discriminate *rrs* based on their differential electrophoretic mobility has been set up such as the single strand conformation polymorphism of *rrs* [33]. As a result, *rrs* related polymorphism reflects phylogenetic relationship better than epidemiological connection [6].

Epidemiological studies require bacterial characterisation at infra-specific level which can be obtained by PCR-RFLP analysis of the intergenic region spanning between the 16S and 23S rRNA genes (*rrs-rrl*) [45]. A number of eubacterial ribosomal operons have been sequenced and it has been observed that the spacer sequences show extensive species-specific variation. On this basis, particular rRNA sequences that are species- or group-specific have been exploited in the construction of oligonucleotides that have been used as probes to identify bacteria and also for in situ detection of bacteria. Spacer-length polymorphism is used in the Ribosomal Intergenic Spacer Analysis (RISA) method to evaluate the diversity of bacterial communities [52].

The necessary ribosomal information for primer design and the selection of relevant frequent cutting restriction enzymes can be obtained through the Ribosomal Database Project (http://www.cme.msu.edu/RDP/) done for the phylogenetic interest of *rrs*. New technique developments for bacterial identification and genetic profiling of bacterial abundance still use *rrs* but with specific oligonucleotide arrays [4, 20, 56], and even high-density DNA probe arrays [65].

3.3. Whole genome DNA fingerprinting

3.3.1. Pulsed Field Gel Electrophoresis (PFGE) of chromosomal DNA

The best standardised and commonly used fingerprinting method based on the analysis of the bacterial genome is Pulsed Field Gel Electrophoresis (PFGE) (see http://www.cdc.gov/ncidod/dbmd/pulsenet/pulsenet.htm). This procedure requires the cutting of the bacterial DNA with very rare cutting restriction enzymes followed by agarose gel electrophoresis under specific electrophoretic conditions in order to separate the generated large fragments. Tenover et al. [63] formulated the criteria for interpreting PFGE patterns of macrorestriction genomic fragments. Four categories were identified: indistinguishable, closely related, possibly related and different from the outbreak pattern. Closely related patterns differed by a single genetic event, a point mutation or an indel (an insertion or a deletion) leading to a typical number of fragment differences between 1 and 3; possibly related strains differed by two independent genetic events, typically leading to 4 to 6 fragment differences; unrelated patterns exhibited 7 or more differences.

3.3.2. Multilocus sequence typing (MLST)

PFGE targets infrequent restriction enzyme sites in the genome which are chosen arbitrarily to give maximal variation within the population, and the observed variation thus corresponds to unknown regions varying very rapidly. A MultiLocus Sequence Typing (MLST) approach has been proposed that is based upon the sequences of short internal fragments of seven housekeeping genes in order to define sequence types corresponding to alleles at each of the seven loci [37]. MLST has been used with several pathogenic species, including *S. pneumoniae* [15], *Neisseria meningitidis* [75], *Campylobacter jejuni* [13], as well as to type uncultured bacteria directly from cerebrospinal fluid [17], or for the characterisation of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus* [16]. The overwhelming advantage of MLST is that sequence data are truly exchangeable between laboratories contrary to MultiLocus Enzyme Electrophoresis (MLEE). However, like the latter method, MLST targets coding regions and uses variation that accumulates very slowly in the population contrary to pulsefield-electrophoresis (PFGE) or arbitrarily primed PCR described above. As a result, MLST is used for long-term epidemiology and for the identification of lineages that have an increased propensity to cause disease [37]. Investigators can determine the allelic profile of their isolates by comparing the obtained sequence with the sequence available in the databases (http://mlst.zoo.ox.ac.uk). This web-site also provides the investigators the necessary primer sequences in order to amplify the gene fragments.

MLST becomes more broadly available as companies now offer sequence services at reasonable costs, but other methods of genome fingerprinting are still largely used in most laboratories.

3.3.3. Arbitrarily primed PCR multilocus fingerprinting methods

A considerable number of genomic molecular typing methods are based on PCR. In general, PCR-based fingerprinting methods are rapid, simple and easy to perform and allow the analysis of the entire genome. Single short primers with random sequences (10 bases in length) are used in Arbitrarily Primed-PCR (AP-PCR) or Random Amplification of Polymorphic DNA (RAPD) analysis to amplify multiple fragments of the bacterial DNA under low stringency conditions. Due to these low stringency PCR conditions, these methods are extremely sensitive to slight changes within the different PCR parameters such as type of

polymerase and thermocycler, DNA concentration and DNA isolation methods (for a review see [67]). Moreover, van Belkum et al. [68] concluded that "inter-institute standardisation will be very hard to achieve" and that arbitrarily PCR fingerprint methods are not suitable to serve as a reference method.

Versalovic et al. [71] described a fingerprinting method, which can be performed under higher stringency conditions, using primers derived from bacterial repetitive sequences present within bacterial genomes. Repetitive sequence based fingerprinting can either be directed against the 38 bp Repetitive Extragenic Palindromic elements (REP-PCR), which consist of 6 degenerate positions and a 5 bp variable loop in between a conserved palindromic stem, or to the Enterobacterial Repetitive Intergenic Consensus sequences such as in ERIC-PCR. ERIC sequences are 126 bp in length containing a highly conserved central inverted repeat and are located in extragenic regions of the bacterial genome. BOX sequences are other types of repetitive elements which have been used for DNA typing. BOX elements have no sequence relationship to either REP or ERIC sequences, and are located within intergenic regions. They are mosaic repetitive elements composed of various combinations of three subunit sequences referred to as boxA, boxB and boxC, with molecular lengths of 59, 45 and 50 nucleotides, respectively. Most of the fingerprinting techniques mentioned above were described by Louws et al. [35] and have been applied for the analysis of antibiotic resistance genes [8, 11, 40]. RAPD and ERIC-PCR are generally much more discriminative than PFGE, distinguishing different genotypes among isolates considered as belonging to the same clone by PFGE. The BOX-PCR has a lower resolution capacity and delineates taxa corresponding to genomic species.

A relative new and promising genomic fingerprint technique is AFLP. AFLP stands for Amplified Fragment Length Polymorphism and has been developed by Vos et al. in 1995 [72]. AFLP combines universal applicability, discriminative power and a high level of reproducibility [28]. This technique, initially developed for plant genetic mapping, has been widely used in microbial typing [1, 2, 5, 28, 57] as also in the typing of antibiotic-resistant strains [27]. Briefly, AFLP consists of three steps. During the first step the bacterial DNA is cut with restriction enzyme(s) followed by adapter ligation. The second step comprises the selective amplification of a subset of the adapter-restriction fragments with primers complementary to the adapter sequence and restriction enzyme recognition site. The primers are extended with (an) arbitrarily chosen nucleotide(s) at their 3' end to enable the selective amplification. The analysis of the generated amplicons on typical sequencing gels takes place during the last and third step. An example of the analysis of fluorescently labelled AFLP fragments on an automatic sequence device is presented in Figure 2. Complex AFLP patterns can be compared by using global banding differences determined by the product-moment correlation coefficient following densitogram analyses, but the results of AFLP analysis performed on an automatic DNA sequencer can more correctly be used to measure the overall genome base mispairing between strains as described by Mougel et al. [41]. For the former, a number of commercial or free software packages are available (GelCompar, BioNumerics, ImageMaster), while the 'DistAFLP' program developed to measure genomic distances with AFLP is available for free on the ADE-4 web server http://pbil.univ-lyon1.fr/ADE-4/microb. Another technique based on the same principles as AFLP is Infrequent Restriction Site (IRS) PCR. This technique uses a rare cutter endonuclease and is in this respect comparable to PGFE. IRS-PCR has been used to study the diversity of *Legionella pneumophila* [55]. This method has a higher resolution than PGFE, but is less discriminative than AFLP. IRS-PCR is

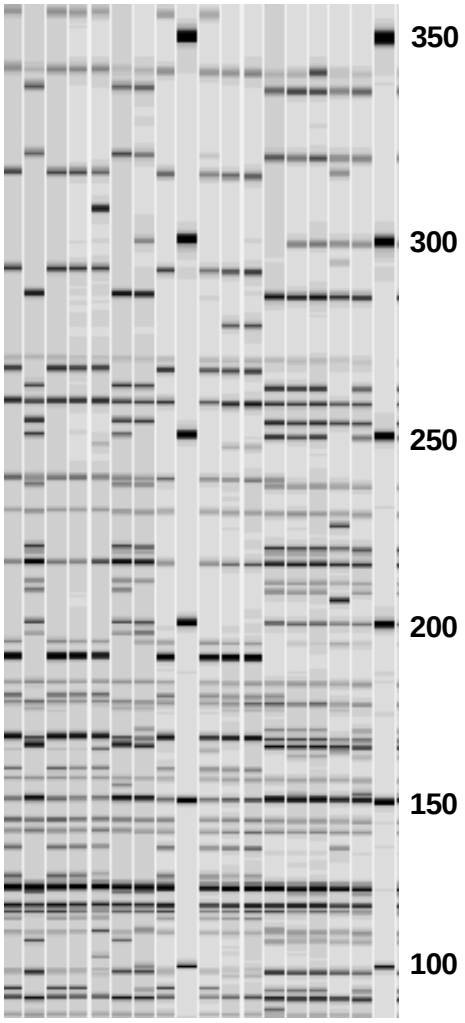

Figure 2. Digital electrophoresis image of AFLP fragments analysed on an ALF-Express automatic DNA sequencer. The sizes (in bp) of the marker bands are indicated.

accessible to most laboratories because fragments are separated in standard agarose gels instead of acrylamide gels such as those used in AFLP.

As discussed above, a large number of molecular bacterial typing methods have been developed. Most of them rely on the analysis of fingerprint patterns. Molecular methods in general surpass phenotypic methods in typability, reproducibility and discriminative power. The method of choice highly depends on its performance of the above-mentioned criteria along with robustness, epidemiological concordance, operational ease, standardisation and inter-laboratory comparison capacity [39, 60]. These methods have different benefits and should be chosen in view of the question addressed and the accuracy of the response which is expected. Some molecular methods such as PFGE or the easier-to-handle IRS-PCR are perfectly suited for outbreak investigation, to determine for instance the extent and mode of transmission of an epidemic clone. Other methods, such as the highly discriminative MLST method, are more suited for long-term epidemiology to examine the global spread of epidemic and endemic clones. The easy-to-handle REP-PCR or RAPD are perfectly relevant for identifying a known strain for instance in experimental studies to recognise an inoculated strain. However, these methods are not relevant to analyse the genetic structure of bacterial populations and to accurately describe the individual genotypes. AFLP would be more suited to do this. Supported by data banks accessible through the web this method allows the indirect comparison of any newly isolated strains with previously analysed strains (Mougel et al. unpublished result). Moreover, AFLP is a versatile method that can be adapted to the genomic constraints of any bacterial species [41], it allows the delineation of genomic species of bacteria and could soon become a serious alternative to DNA-DNA relatedness studies. Additionally, AFLP data banks could also facilitate species identification. Species identification could also be achieved by the easier to handle BOX-PCR, which is an interesting alternative in the first steps of etiological investigations, but the method

could hardly be used to construct public data banks.

4. HORIZONTAL GENE TRANSFER VERSUS CLONAL SPREAD

Molecular techniques are used both to characterise the antibiotic resistance gene(s) and to analyse the genome of the host bacterium. A combination of these two applications allow the discrimination between horizontal spread of antibiotic resistance genes and clonal dissemination of a particular strain. For a schematic representation see Figure 3.

An example of the horizontal spread of antibiotic resistance genes has been described by Keyes et al. [30]. They applied DNA-DNA hybridisation to assess the location of the florfenicol resistance gene, *flo*, on high-molecular weight plasmids of avian *E. coli* strains. In three out of the four investigated isolates the *flo* gene was found on different sized *Xba*I fragments originating from different sized plasmids. Furthermore, the florfenicol-resistant *E. coli* strains isolated from clinical samples of different poultry farms in Georgia and North Carolina exhibited different RAPD fingerprint patterns showing that they do not represent the dissemination of a clonal strain. Together, these findings suggested that the investigated avian *E. coli* strains had acquired the *flo* gene through independent events, which was more or less expected as the *flo* gene is located on extrachromosomal elements. For chromosomal-located antibiotic resistance genes it is sometimes more difficult to distinguish between horizontal gene transfer and clonal spread. Ridley and Threlfall [54] for instance investigated *Salmonella enterica* serovar Typhimurium phage-type DT104 isolates of human and animal origin from different European countries, from the USA, Trinidad and South Africa and concluded that the spread of resistance to ampicillin, chloramphenicol, streptomycin, sulphonamides, spectinomycin and tetracyclines (R-type ACSSuSpT) was of clonal origin. They found that all R-type ACSSuSpT strains contained the same two integron hotspots located on the same 10 kb chromosomal *Xba*I-fragment. One copy contained the *ant* (3")-*Ia* gene, responsible for resistance to streptomycin and spectinomycin, and the other copy the bla_{PSE-1} gene, coding for β-lactamase PSE-1 (CARB-2). The 10 kb fragment was not found in drug-sensitive isolates and isolates with another R-type. Thus irrespective of source and country of origin the investigated R-type ACSSuSpT strains contained the same inserted gene cassettes. In addition to these findings, other investigations [9, 10] however showed that the chromosomally located antibiotic resistance genes also occur in other phage-types rather than DT104 and more interestingly also in another *Salmonella* serovar, namely *Salmonella* Agona. This could be indicative of horizontal transfer of the antibiotic resistance gene cluster, perhaps phage-mediated. Therefore, in the case of DT104 probably both situations, clonal dissemination and horizontal gene transfer can occur.

Willems et al. [73] reported the detailed analysis of Tn*1546*-like elements in vancomycin-resistant enterococci from humans and animals and concluded that the observed genetic diversity within the elements could be exploited for tracing the routes of transmission of vancomycin resistance genes. Different molecular methods were used for these studies. PFGE electrophoresis was used to determine the genetic relationship between the different strains. RFLP was used to construct the physical map of the various Tn*1546*-types. PCR was used to amplify overlapping fragments and sequencing was applied to determine the sequence of these overlapping fragments and further analyse the mutations within all of the isolates. The observed genetic diversity among the Tn*1546*-like elements was successfully exploited to prove the presence of a common vancomycin resistance gene pool between human and farm animal isolates in

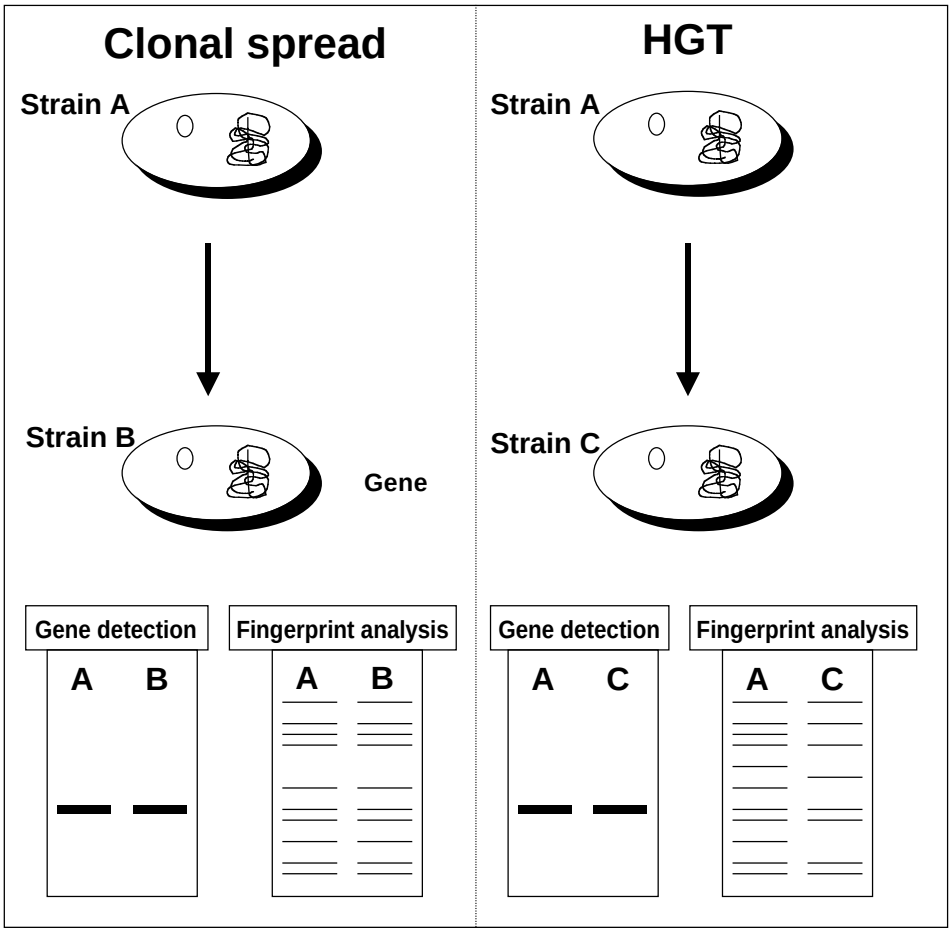

Figure 3. The molecular strategy to analyse different isolates to elucidate the underlying mechanism of the spread of resistance. HGT= Horizontal Gene Transfer.

the Netherlands. Furthermore, the observed diversity was exploited to trace the routes of transmission of vancomycin-resistant enterococci from hospitals in Oxford and Chicago. Identical *vanA* transposons were found in genetically different enterococci strains, which suggested that horizontal gene transfer could play a role in the transmission of vancomycin resistance.

5. NEW APPROACHES IN ANTIBIOTIC RESISTANCE GENE DETECTION AND IN MOLECULAR ANALYSIS OF ANTIBIOTIC-RESISTANT STRAINS

As described above, the PCR protocol is a commonly used procedure for the

detection of antibiotic resistance genes. The most important progress in PCR technology has been achieved on the level of the detection of the amplified product, in particular the use of fluorescent dyes [7]. Fluorescent dyes are used to circumvent the time-consuming gel-electrophoresis step and allow the detection of more amplicons within the same reaction tube. The most important advantage, however, is that these dyes allow a real-time detection of the amplicon for quantitative purposes. Quantification of target DNA is performed by measuring the increasing amount of fluorescence which is proportional to the increasing amount of DNA generated during the ongoing PCR process. Another advantage of using fluorescent dyes is that there is no need to open the reaction vial after amplification. This significantly reduces the risk of contamination.

DelVecchio et al. [12] applied real-time PCR to detect antibiotic resistance genes in *Enterococcus feacalis* and *Enterococcus faecium*. The PCR was carried out in the presence of SYBR Green I. However, for maximum product identification the hybridisation probe format, instead of the SYBR Green I format, is used in the LightCycler for DNA detection and quantification. This detection method needs two specially designed sequence specific oligonucleotides each labelled with a fluorescent dye. Upon hybridisation the two fluorescence dyes are positioned in close proximity to each other to allow the transfer of energy, referred to as Fluorescence Resonance Energy Transfer (FRET), between the two dyes. The first dye (fluorescein) is excited by the LightCycler light emitting diode and its energy is transferred to the second dye (LC Red 640 or 705). Subsequently, the second dye emits red fluorescent light at a longer wavelength (see Fig. 4). Reischl et al. [53] used this FRET technology in a duplex LightCycler assay for the rapid detection of methicillin-resistant *Staphylococcus aureus*. The real-time PCR was directed at the *mecA* gene and a recently described *Staphylococcus aureus* specific fragment [38]. The presence or absence of the *mecA* gene could be correctly identified within 70 minutes. Thus real-time PCR is rapid, allows the simultaneous detection of bacterium and gene of interest and allows quantification. The TaqMan system of PE Applied Biosystems is also based on a sequence-detecting real-time PCR assay that uses fluorogenic probes. In solution the reporter is suppressed by the quencher dye due to their spatial proximity. After binding to its target the probe is cleaved by the 5' nuclease activity of the Taq DNA polymerase resulting in an increase in fluorescence. Commercial kits are available to detect *Salmonella*, *Listeria* and *E. coli* O157:H7.

Other types of molecules which are very suited for real-time PCR are Molecular Beacons. Upon binding to their target these hairpin-shaped oligonucleotide probe molecules undergo a conformational reorganisation that restores the fluorescence of an internally quenched fluorophore [66]. Molecular Beacons were used by Piatek et al. [50] for the detection of *Mycobacterium tuberculosis*.

Due to the current progress in applied DNA technology, DNA-mediated typing procedures will become more and more accessible for surveillance and these new approaches will lead to more reliable interlaboratory studies. Kamerbeek et al. [29] developed a system for *Mycobacterium tubercolosis* and van Leeuwen et al. [70] for *Staphylococcus aureus* which are well suited for inter-laboratory comparison and also provide the required discriminatory power for use in surveillance programmes. These genotyping methods, referred to as spoligotyping, use reverse dot-blot or line-blot binary hybridisation patterns of crude DNA or amplified fragments with clone-specific probes.

The power of all the techniques discussed above will be highly increased by the use of the novel micro-array or DNA chip technology. And it is expected that the power

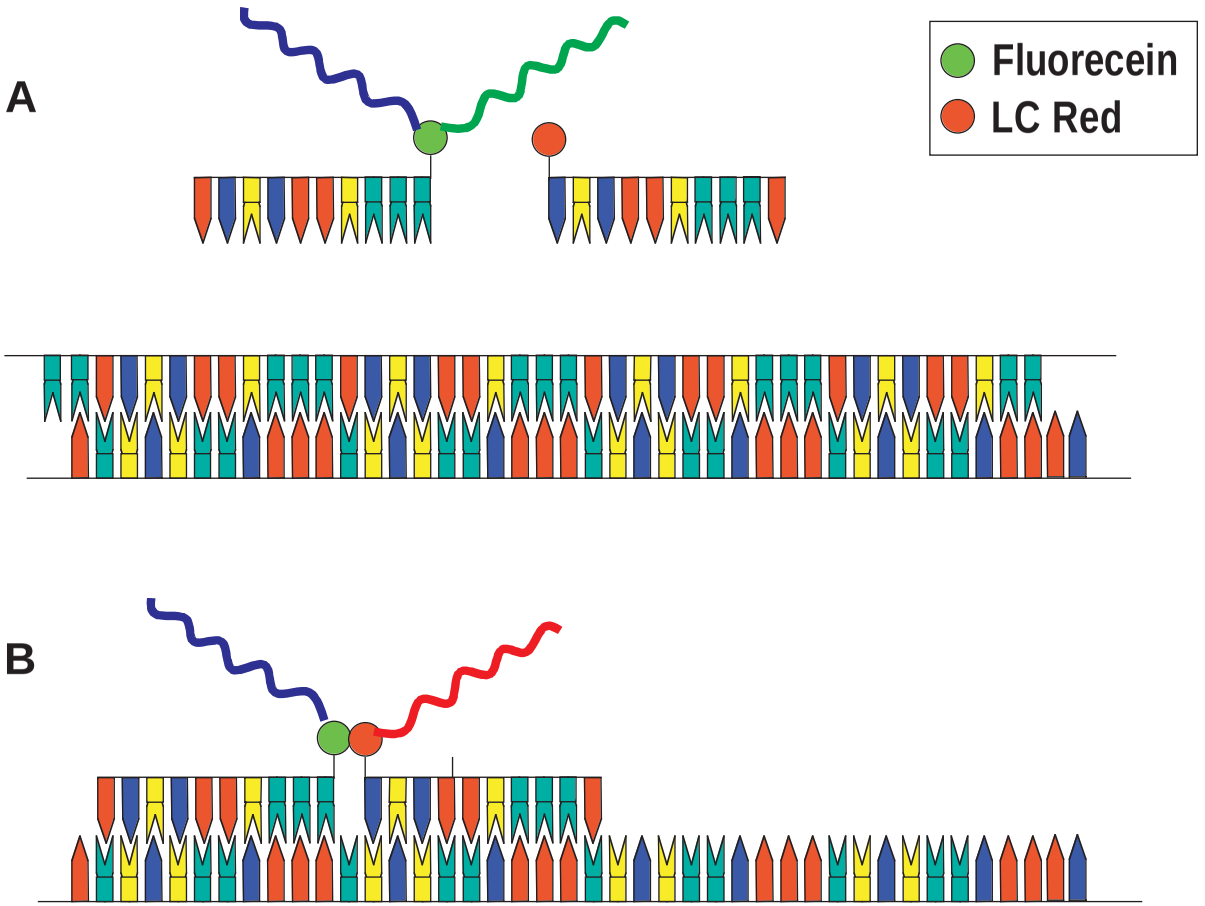

Figure 4. Schematic representation of Fluorescence Resonance Energy Transfer (FRET) as applied in the hybridisation probe format of the LightCycler. One probe is labelled with Fluorescein at its 3' end. The other probe is labelled with LightCycler-Red 640. (**A**) In solution, after exciting Fluorescein, no transfer of energy will take place between both dyes and no fluorescence will be detected at 640 nm. (**B**) Upon hybridisation, both probes hybridise in a head-to-tail arrangement, thereby bringing the two dyes into close proximity. The green light emitted by Fluorescein excites LC Red 640, which then emits red fluorescent light which is measured at the 640 nm channel.

of micro-array analysis will increase enormously by taking full advantage of the ongoing endeavour to elucidate the complete genomic information of a large variety of microbial species. Up to now the complete sequence information of more than 30 species is available and that of more than 100 is underway (see www.tigr.org) [19]. DNA micro-array is a novel technique and is comprised of different DNA molecules which are attached to a solid surface. The formerly used porous membranes have now been replaced by glass slides and substantial improvements in the techniques for spotting or synthesising DNA molecules on these glass slides have been made. Combined with advanced scanning devices, this has allowed the miniaturisation of the technique allowing to analyse a large number of DNA molecules simultaneously. In the field of antibiotic resistance micro-arrays could be applied in the near future to the screening of the bacterial genome for the presence of a large number of antibiotic resistance genes. Furthermore, within the same assay, the genome of the isolate could be typed. Micro-arrays are currently mainly used for comparative gene expression analysis and DNA resequencing. The latter application will certainly support MSLT and could also be applied for the screening of a large number of mutations associated with antibiotic resistance. Nevertheless, arrayed primer extension [64] or minisequencing [48] would be better suited for the analysis of mutations.

Molecular techniques have already proved their value in epidemiological studies. The fast increase in sequence information and progress in applied DNA technology will indisputably lead to a greater impact. In the near future, miniaturisation and high-throughput methods will enable the analysis of a large number of isolates simultaneously for the presence of a large number of genes or mutations associated with resistance combined with strain typing.

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