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EPIZOOTIOLOGICAL AND MICROBIOLOGICAL METHODOLOGIES FOR MONITORING ANTI-MICROBIAL RESISTANCE AMONG ENTEROBACTERICEAE OF ANIMAL ORIGIN: A REVIEW

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SUMMARY

The emergence of anti-microbial resistant strains in the treatment of bacterial infections has demanded some critical revaluation of the continued use of these agents in agriculture. Current information on the spread of pathogenic and commensal bacteria of animal origin and their resistance genes highlight the existence of interactions between micro-ecological systems in different animal hosts and the environment. Protocols for appropriate epizootiological investigations of anti-microbial resistance in farming environments should therefore incorporate elements of such ecologic interactions. For example, to evaluate the risks of continued use of anti-microbials in animals and their impact on human health, veterinary monitoring of bacterial susceptibility should assume a broad structure that includes a large number of animal species, detection of resistance levels in bacteria resident in animals and animal-derived foods, and also incorporate deferent bacterial species especially veterinary pathogens, zoonotic bacteria and commensals. Similarly, application of molecular typing techniques alongside conventional phenotypic methods is preferred especially when testing for newly emerging as well as established resistance patterns.

KEY WORDS: Anti-microbial resistance, Enterobacteriaceae, Epizootiology, Molecular typing, Animal

INTRODUCTION

Antibiotics have been used in the therapy and prevention of animal and human infectious diseases since their discovery. In animal husbandry, the control and prevention of infectious diseases is of basic economic and public health importance. This has been dramatically improved by the introduction of antibiotics into the industry. For example, the idea of

adding small quantities of antibiotics to animal feeds to improve growth and the efficiency of utilization of the feed initiated by Moore *et al.* in 1946 and demonstrated with practical ration by Stokstad and Jukes in 1950 had such an economic impact that for years research on nutritional problems were devoted to the study of the effects of adding antibiotics to animal feeds and the mode of action of the antibiotics (Braude *et al.*, 1953; Maynard *et al.*, 1979).

The emergence of antimicrobial resistant strains in the treatment of bacterial infections has demanded some critical revaluation of the continued use of these agents in agriculture and has recently become the subject of debate (Apley *et al.*, 1998; Witte, 1998; Davis *et al.*, 1999). The idea that the use of antimicrobial agents is a powerful selective force that promotes the emergence of resistant strains is well supported (Okoli *et al.*, 2002; Lipsitch and Samore, 2002). However, observed persistence of bacteria resistance to antibiotics long after their withdrawal from farmyards or even before their introduction into veterinary practice remains disturbing may be linked to interspecies and environmental dissemination of multi-drug-resistant clones (Davis *et al.*, 1999).

In a farming environment, bacterial resistance to antimicrobial agents is frequently caused by the acquisition of new genes by means of mobile genetic elements such as resistance (R) plasmids, transposons and integrons (Hall and Collis, 1995; Lucey *et al.*, 2000). Dissemination of resistance is further mediated through the interactions between micro-ecological systems in different animal hosts (including humans) and the environment, which may enhance the transfer of resistant bacteria and their resistance genes (van den Bogaard and Stobbringh, 2000; Witte, 2000). Animal husbandry systems specifically promote the housing of large numbers of livestock thereby ensuring the genetic interconnection between large populations of bacteria and hence interspecies transfer of antimicrobial resistance encoding genes (On *et al.*, 1998). To properly elucidate the scope and magnitude of anti-bacterial resistance in a given area, the rates of changes in the susceptibility of specific antibiotics of organisms isolated from both humans, animals and their environment are needed.

We, therefore suggest that protocols for appropriate epizootiological investigation of antimicrobial resistance among farm animals bacterial pathogens should include data on resistance patterns in both pathogenic bacteria from diseased livestock and others from interacting micro-ecological systems in the area. Furthermore, we argue that the effects of varying temperatures, precipitation and humidity ranges experienced during the different seasons in the tropics which may influence the incidence and bionomics of not only the resistant bacteria but also other interacting hosts and vectors in the farming environment are poorly understood.

The application of molecular biological techniques alongside other conventional diagnostic methods in the epizootiological investigation of antimicrobial resistance tends to elucidate in greater details the genetic

background of bacterial resistance (Vahaboglu *et al.*, 1996). Such resistance gene characterization will not only help to generate information on the diversity of drug resistant genes in animal pathogens but also highlight the sources and routes of dissemination of resistance.

Interactions between micro-ecological systems in different animal hosts and environment

Resistant genes have been shown to spread among a variety of different microbial species and over long distances, for example, tetM, in staphylococci, enterococci, clostridia, listeria and enteric bacteria (Roberts, 1996) and TEM-I B-lactamases in enteric bacteria, haemophilus spp., *Neisseria gonorrhoeae* (Bush, 1997). Broad host range plasmids such as those in the incQ group of gram –ve bacteria have been shown to be capable of transferring resistance determinants to a variety of species (Tietze, 1998). Dramatic increase in induced antibiotic susceptibility in salmonella from travellers returning to Europe from third world countries have also been reported (Hakanen *et al.*, 2001; Davis *et al.*, 1999).

Hospitals, human communities and animal husbandry units remain powerful foci of antimicrobial selective pressure. Reservoirs of antibiotic resistance in these foci can interact through a number of routes which may include foods of animal origin, water, plant food and other terrestrial and aquatic environments (Witte, 2000).

In most developing countries, poor and unhygienic meat handling methods predispose meat products to faecal contamination (Okoli *et al.*, 2002). Although hygienic standards of meat processing may be high in most developed countries, faecal contamination moreover, remains a serious problem (Witte, 2000). The role of such contaminated meat product in the transfer of *E coli* harbouring antibiotic resistance genes has been elaborated for sat-mediated resistance to streptothricin antibiotics in coliform bacteria and for vanA mediated glycopeptide resistance in enterococci (Tschape, 1994; Klare *et al.*, 1995).

Resistance plasmid transfer in aquatic environments has also been demonstrated by Dahlberg *et al.* (1998). Although the gut is the natural ecosystem of enteric bacteria, they are able to survive in different environment (Witte, 2000). Massive presence of antimicrobial resistant enterics in sewage treatment plants and their outlets with sewage of human and animal origin has also been reported (Marcinek *et al.*, 1998). In most developing countries, humans and animals may acquire antibiotic resistance via untreated drinking water or water used for recreational activities.

Sewage and surface water are frequently used for watering vegetables in urban and peri-urban centres of developing and even developed countries. Studies from Europe reported the detection of antibiotic resistant coliform bacteria on plants 2 weeks after watering with sewage (Osterbald *et al.*, 1999).

Soil naturally contains large population of micro-organisms. Enteric bacteria are known to survive in soil and to compete

with soil's microflora. Such enteric bacteria from the intestinal flora of humans and animals are spread to the soil by manure or waste water. There is also evidence that Pseudomomads and Acinetobacter spp. might play an important role in the spread of resistant genes in the rhizo and phytospheres (Sikoski *et al.*, 1998). These resistant genes might probably re-enter the human microflora via food plants such as vegetables.

Because antimicrobial resistant genes are usually located on conjugative plasmids, conjugation had previously been assumed to be the most important transfer mechanism. However, current evidence tend to suggest that transduction and transformation are equally very important (Kidambi *et al.*, 1994) and may account for transfer events in low populations of enteric bacteria and pseudomonads found in aquatic environment where they grow in more concentrated fashion in bio-films.

Antibacterial chemotherapeutic agents belonging to different classes have been detected in sewage treatment plants and their outlets. While concentrations measured are far below the minimum inhibition concentration (MIC), for relevant species, low concentrations of oxytetracycline have been reported to stimulate conjugative transfer of Tn916 in anaerobes (Salyers and Shoemaker, 1996).

Epizooticology of antimicrobial resistance

Studies performed in developed countries highlighted the emergence and selection of resistance in bacteria from animals (van den Bogaard *et al.*, 1997b; van den Bogaard and Stoberingh, 2000). Linton *et al.* (1985) found a significant increase in the prevalence of resistance against tylosin and bacitracin in faecal enterococci of pigs and poultry fed these drugs. Ohmae *et al.* (1983), noticed an increase of resistance against carbadox in faecal *E. coli* isolates of pigs after its introduction as animal growth promoter.

After a ban was placed on the usage of animal growth promoters in Sweden in 1986, the prevalence of resistance against animal growth promoters or related compounds in faecal samples of Swedish pigs in 1997 was found to be significantly lower them that of Dutch pigs (van den Bogaard *et al.*, 1997b). Similarly, in U.S.A. where the growth promoting drug, avoparcin has never been used, no high level of vancomycin resistant enterococci (VRE) has been found in faecal sample of food animals or healthy humans outside hospitals (Coque *et al.*, 1996). Again, after the ban of avoparcin in Denmark the occurrence of vancomycin resistant *E. faecium* in faecal sample of broilers declined significantly from more than 80% in 1995 to less than 5% in 1998. However the prevalence in pig faecal samples remained constant at approximately 20% (Bager *et al.*, 1999).

From the foregoing it can be concluded that the use of APP-like veterinary antibiotics select for resistance among susceptible micro-organisms, not only in pathogens but also in bacteria belonging to the normal flora of animals such as enterococci and *E. coli* (van den Bogaard and Stobberingh, 2000).

Most investigations on the transfer of resistant bacteria from animals to humans concern gram negative food infections caused by bacteria such as *Salmonella spp., Campylobacter spp.* and *Yersinia spp*. Transfer of resistant salmonella from animals to man has been described (Macdonald *et al.*, 1997). Humans become infected with salmonella from animals, animal faeces and most importantly through food products of animal origin (Jones, 1992). Asymptomatic salmonella infections and carriers are common in food animal husbandry. Salmonella from the intestinal tract of such animals contaminate meat products during slaughtering and infect humans through them (Wierup, 1996).

S. enteritidis is the most frequently isolated salmonella serotype from human infection in most EU member states and USA but does not produce serious clinical symptoms in poultry from where it is extensively disseminated (Henzler *et al.*, 1994). An inverse relationship has been reported between the incidence of *S. gallinarum* infection in chickens and egg associated *S. enteritidis* infections in humans and has prompted a hypothesis that *S. enteritidis* filled the ecologic niche vacated by eradication of *S. gallinarum* from domestic fowls in Europe and U.S.A. (Rabsch *et al.*, 2000).

The primary reservoirs of *S. typhimurium* are domestic animals including poultry. Because these strains cause serious disease in animals, these animals are treated with antibiotics and as a result of the selection pressure, they tend to become multi-resistant (Wray, 1997). Commercial feeds and fed ingredients have also been implicated as major potential vectors of salmonella and coliform bacterial infections in poultry (Garland, 1996). It has been reported that such organisms found in commercial feeds form major sources of transfer of antimicrobial resistance to poultry pathogens (Durand *et al*, 1987).

Antibiotic usage has also been shown to disrupt the colonization resistance (CR) of the intestinal flora of animals exposed to certain antibiotics (Vollaard and Clasener, 1994). The increased excretion of both pathogenic or resistant bacteria that results from this considerably enhance dissemination of salmonella or other resistant bacteria and contamination of carcasses with such bacteria during slaughter (Gustafson *et al.*, 1989; Vollaard and Clasener, 1994).

The level of resistance against antibiotic among bacteria belonging to the normal intestinal flora of humans and animals may increase due to exposure to antibiotics (van den Bogaard and Stobberingh, 2000). These bacteria which constitute an enormous reservoir of resistance genes for potentially pathogenic bacteria may serve as major indictors for selection pressure exerted by antibiotic use in a given animal or human population (Murray, 1992). Investigation of prevalence of resistance in such bacteria, especially *E. coli* and enterococci make it possible to understand the prevalence of resistance in different animal populations and to detect a possible transfer of resistant bacteria from animals to humans and vice versa (van den Bogaard and Stobberingh, 2000). Nijsten *et al.* (1994) for example, found significantly more resistant *E coli* in the faecal flora of pig farmers than in faecal sample of

pig slaughters and urban residents. Again, a comparison of prevalence of ciprofloxacin resistant *E. coli* in faecal samples of turkeys and turkey farmers with pigs and pig farmers, strongly indicate transfer of ciprofloxacin resistant *E. coli* strains from turkeys to turkey farmers (van den Bogaard *et al.*, 1997a).

Levy *et al.* (1976), observed that in chicken fed tetracycline, there was transfer of tetracycline resistant genes between chicken *E. coli* strain, from chicken to chicken and from chicken to humans. The relationship between the usage of an antibiotic and the dissemination of bacteria resistance from animals to humans have been described. Studies by Chaslus-Dancla *et al.* (1991) have shown that even when an antibiotic was restricted to veterinary use alone, resistance genes which are co-transferred have not only been found in animal isolates or zoonotic bacteria isolates from humans, but also from enterobacteriaceae in the environment, the intestinal flora of farmers and hospital isolates.

Sampling methods

Veterinary monitoring of bacterial susceptibility has a different structure and emphasis when compared with similar surveys in man. Usually a national surveillance programme must include a large number of animal species (ruminant livestock, poultry, pigs, horses, companion and wild animals). As a consequence, veterinary surveillance are frequently directed to detect resistance level in bacteria isolated from animals and animal-derived foods, to evaluate the risk of use of antimicrobials in animals and quantify the impact of these findings on human health (Caprioli *et al.*, 2000). Furthermore, it has been shown that surveillance of antimicrobial resistance should include different bacterial species such as bacteria causing clinical infections of animals (Veterinary pathogens), bacteria isolated from animals but capable of causing human infections (zoonotic bacteria) and commensal bacteria isolated from healthy animals (FAIR- CT97-3654).

Sampling is therefore usually organized to include not only clinical cases but also human populations in the farm, other animals sharing the environment such as dogs, cats, mice, rats, insects, birds etc. Similarly sampling of water used in medicating the animals, litter and farmhouse dust have also been recommended (Engvall, 1994). Studies in Sweden show that most salmonella positive samples originate from the raw materials, section of the feed mills, while fewer isolated were found in finished feed (Haggblom, 1993). This is probably because most materials undergo heat treatment during processing.

It has been demonstrated that the most likely source of salmonella are materials of animal origin such as meat-meal, bone-meal, feather, blood, poultry offal and fish meals (Wilson, 1990). Studies in UK by the Ministry of Agriculture Fisheries and Food have also shown that vegetable protein and vegetable bye products could be major sources of salmonella in the feed industry.

Susceptibility testing

Testing the susceptibility or resistance to antimicrobial agents was intrinsic to the

discovery and development of antimicrobials (Fleming, 1929). Susceptibility testing serves the purposes of providing meaningful results to the prescriber and helps to monitor changes in susceptibility of microbial populations (Greenwood, 2000). Several methods of susceptibility testing have been developed over the years. In general, these can be subdivided into agar diffusion, (Bauer *et al.*, 1966), broth or agar dilution, breakpoint methods and genetic approaches (Singleton, 1997; Caprioli *et al.*, 2000).

The agar diffusion tests rely on the application of disks containing the antibacterial agent on a semi-confluent lawn of the test isolate on an agar plate. During incubation, the antimicrobial diffuses around the disc and leads to a zone of inhibition, the diameter of which depends on susceptibility of the isolates (Caprioli *et al.*, 2000). The agar dilution method on the other hand depends on growth of the microbes in the medium containing serial 2-fold dilutions of the drug. The first concentration in which there is no visible growth defines the minimal inhibitory concentration (MIC) (Ericsson and Sherris, 1971).

Both the disc diffusion and agar dilution methods have a number of drawbacks, therefore their performance relies on careful control and standardization of the experimental condition such as the incubation temperature, the batch and storage of media, varying staff, absence of a control strain and the final interpretation of the results which is based on breakpoint definitions and settings (Caprioli *et al.*, 2000). Generally, breakpoints which are usually species-specific, highlight clinical efficiency based on pharmacokinetics and clinical data. However, in the veterinary field, breakpoints are often arbitrary since they are frequently based on pharmacokinetics and clinical data obtained from human medicine.

The disc diffusion method has the advantage of being long and widespread in use, easy to perform, being reproducible within one laboratory and allowing the testing of large number of isolates at moderate costs. On the other hand, it has the drawback of being mainly qualitative and not being easily correlated with MIC values and needs a high level of standardization and quality control. Out dated discs, or those stored at the wrong temperature or those exposed to moisture can bring out methodological difficulties. Furthermore, agar plates errors such as being too deep, too shallow or out of date may occur. The inhibition zone can also vary strongly depending on the inoculum's size (Singleton, 1997; Caprioli *et al.*, 2000; Greenwood, 2000).

The E-test is a diffusion test which can give an MIC and is based on a plastic strip carrying the antibiotic in concentration gradients on the side placed in contact with a solid medium, while the uppermost side is graduated with the MIC scale. After incubation, the test is read by noting the lowest concentration of antibiotic which prevents the growth (Brown, 1994; Singleton, 1997).

The agar dilution method gives quantitative MIC values, is highly reproducible and can be automated. It is however more expensive and may also experience some of the drawbacks listed

for the disc diffusion method especially as it relates to media pH and inoculums density (Caprioli *et al.*, 2000).

The approved standard M31A (NCCLS, 1999), the consensus document of the NCCLS sub-committee on veterinary antimicrobial susceptibility testing, recommends the use of the M2 (disk diffusion) and M7 (broth dilation) approved standards taking into account that these methods are widely recognized to work well with rapidly growing facultatively anaerobic and aerobic organisms such as staphylococci and enterobacteriaceae. Furthermore, a recent study (Engberg *et al.*, 1999), compared the results obtained using different techniques and suggested that reliable results can be achieved with agar dilution, disc diffusion or E-test strips.

Quality control procedures and the panel of reference strains are also provided in the M31A document and include the ATCC strain *E. coli* 25922 and 35218 among others for dilution method and *Staph. aureus* 25923 for disc diffusion. In general, susceptibility data is recorded quantitatively by measuring the size of the diameters of inhibition zones or the MIC values and may be converted to qualitative categories such as resistant, intermediate or sensitive for clinical purposes

Bacterial cultural and typing methods

Enterobactericeae diagnostic work is usually based on traditional cultivation, isolation and serotyping of the particular specie. The actual principles of cultivation and isolation from routine include the following; pre-enrichment (16-24hours), selective enrichment (18-24hours), selective cultivation on agar plates (18-24hours) and identification and serotyping (Gunnarsson, 1994).

According to a summary of the subject by Aiello and Mays (1998), culture for bacterial pathogen and determination of antimicrobial sensitivity requires a stock of dated media, reagents, equipment and experienced personnel. Cultures may be started in appropriate media such as thioglycollate broth, which support both aerobic and anaerobic bacterial growth. Culture of GIT samples can be started on selective medium such as MacConkey's agar. Amies transport medium can be used for shipment of un-refrigerated swab samples directly to the diagnostic laboratory. Initiated cultures can be submitted directly to a lab. or incubated for 24 hours at 37°C and if growth occurs it can be gram stained and sub-cultured on blood agar or Mueller-Hinton medium for antibiotic sensitivity testing.

In bulk samples, the sparse population of micro-organisms such as salmonella in feed or faeces, antibody coated beads (Dynabeads) have been found to be a very promising tool for the concentration of micro-organisms (Luk and Linberg, 1991) as well as for immunomagnetic separation of salmonella from foods (Skjerve and Olsvik, 1991) and as an alternative to enrichment broths for salmonella detection (Mansfield and Forsythe, 1993).

Methods for demonstration of salmonella or *E. coli* are based on enzyme-linked immunosorbent assay, immuno-immobilisation, DNA-probe, hydrophobic grid membrane filtration, conductance, generic biochemistry, genus specific virus and latex

agglutination (Gunnarsson, 1994). The reduction of time is usually 12-24hours. However, kits are relatively more expensive when compared to traditional cultural methods. The most common methods of sub-typing salmonella are bio-typing, phage typing and antimicrobial susceptibility testing (Lillengen, 1948; Callow, 1959; Threlfall and Frost, 1990).

Molecular typing methods

Molecular typing involves fingerprinting the genomic DNA of an organism according to the technique of Cockerill (1999). Genomic DNA is digested with various enzymes according to the manufacturers recommendations. Enzymes are selected based on published reports as well as current experience within the typing section. Two enzymes may be used for typing to ensure the presence or absence of clonality (Peterson and Noskin, 2001). DNA fragments are separated into patterns by running them through agarose gel at constant field electrophoresis. Usual run time is about 16-24 hours and the resultant gels are then stained with a nucleic acid bonding fluorescent agent and visualized with uv illumination. Gels are thereafter imaged with a photo documentation system in such a way that the molecular weight marker extends 6 cm to 7 cm in the image. Similarities between the new and reference types are scored by visual comparison of each 1mm segment of the top 60 mm of the DNA band pattern. A similarity index is then calculated from the number of identical 1mm segment expressed as a percentage of the total number of 1mm segments measured.

The most widely used molecular typing methods include plasmid profiling restriction endonuclease analysis of plasmid and genomic DNA, Southern hybridisation analysis using specific DNA probes and chromosomal DNA profiling using either pulsedfield gel electrophoresis (PFGE) or Polymerase chain reaction (PCR)-based methods (Courvalin, 1991). Other modern molecular method such as multilocus-enzyme, and electrophoresis of allo-enzymes (Kapperud *et al.*, 1989) are also in use.

Molecular typing the microtitre plate system (the pheno plate (PhP) system) has also been used for sub-typing the most common serovars of a salmonella in an environment. This is based on computerized biochemical fingerprinting method that measures the kinetics of biochemical reactions of bacteria grown in liquid medium in microtitre plates (Katouli *et al.*, 1992). Plasmid profiling has equally been used to demonstrate the environmental persistence despite proper cleaning procedures of *Salmonella enterica* in successive flocks on broiler parent stock farms (Brown *et al.*, 1992; Baggensen *et al.*, 1992) thus enabling the identification of beetles as the likely vector. Similarly, restriction endonuclease analysis and antibiogram typing were used to confirm the clonal relationship of a number of *S. typhimurium* strains, isolated on poultry farms in the UK. (Brown *et al.*, 1991).

Using ribo-typing during investigation of an outbreak in Europe, German and Danish isolates of *S. typhimurium* were shown to have the same rare ribo-type clearly

indicating an epidemiological connection. However, a difference in one electrophoretic band was observed in the pulsed field gel electrophoresis pattern of strains from the two countries (Christensen *et al.*, 1994). In a similar manner chromosomal DNA from one isolate can be cleaved by restriction endonuclease and the resulting fragmentation pattern compared with that of other strains (Olsen *et al.*, 1994). The resulting patterns are usually very complex and pattern recognition involves the analysis of hundreds of fragments. Pulsed field gel electrophoresis increases the resolution of the bands within predefined size range and has been used in the investigation of an out break of *S. gallinarum* in Denmark (Christensen *et al.*, 1994) and out break of *S. blockley* in Greece (Tossios *et al.*, 2000).

Heterogeneity in chromosomal DNA fragmentation patterns can also be demonstrated by the use of DNA probes such as random DNA fragments or sequence-specific (ribo-typing) (Grimont and Grimont, 1986).

Polymerase Chain Reaction (PCR): the enzymatic amplification of specific DNA sequences based on the use of specific systemic primers) is increasingly finding wide application in the detection of specific gene sequence that are specie-specific. The specificity of the fragment that is amplified is dependent on the specificity of the short oligonucleotide sequence used to prime the polymerase enzyme (Olsen *et al.*, 1994). PCR-based methods for the specific detection of salmonella have been published (Aabo *et al.*, 1993). For non- specific cases with many possible sites of hybridization, a number of DNA fragments are usually amplified and by comparing the sizes and number of fragments produced, a typing scheme could be constructed. This method (RAPD-Random Amplification of polymorphic DNA) has been used to type *Listeria* species and could be used to sub-type salmonella serovars (Mazurier *et al.*, 1992). Another method (AFLP - Amplified Fragment Length Polymorphism) based on the amplification of the fliC gene together with restriction enzyme digestion of amplified product has been used to distinguish H-antigen groups in salmonella (Kilger and Grimont, 1993).

Choice of microbiological technique

Although these molecular methods can rapidly detected antimicrobial drug resistance and have contributed to the understanding of the spread and genetics of resistance, conventional broth- and agar-based susceptibility testing methods provide a phenotypic profile of the response of a given microbe to an array of agents (Pfaller, 2001). The conventional methods are however slow, too variable and labour-intensive.

Despite its numerous advantages, molecular typing methods will not likely replace conventional broth- and agar-based methods for detecting anti-microbial drugs resistance in the near future. Molecular methods for resistance detection may be applied directly to clinical specimen providing both the detection and identification of the pathogen plus resistance characterization (Cockerill, 1999; Pfaller, 2001). However, because of their high specificity, molecular methods may not detect new emerging resistance

mechanisms and may also not be useful in detecting resistance genes in species where the gene has not been observed earlier (Courvalin, 1991). Furthermore, the presence of a resistant gene does not necessarily mean that the gene will be expressed and the absence of a known resistance gene does not exclude the possibility of resistance from another mechanism (Pfaller, 2001). Phenotypic methods on the other hand allow the testing of many organisms and detection of newly emerging as well as established resistance patterns.

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