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DEVELOPMENT OF AN ANTIBIOTIC RESISTANCE MONITORING SYSTEM IN HUNGARY

Éva J. KASZANYITZKY[1*], A. TARPAI[1], SZ. JÁNOSI[1], Melitta PAPP[1], J. SKÁRE[1] and G. SEMJÉN[2]

[1]Central Veterinary Institute, H-1149 Budapest, Tábornok u. 2, Hungary; [2]Department of Pharmacology and Toxicology, Faculty of Veterinary Science, 'Szent István' University, H-1078 Budapest, István u. 2, Hungary

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Because of the rapid development and spread of antimicrobial resistance it is important that a system be established to monitor antimicrobial resistance in pathogenic zoonotic and commensal bacteria of animal origin. Susceptibility testing of bacteria from carcasses and different samples of animal origin has been carried out in veterinary institutes for a long time but by an inconsistent methodology. The disc diffusion method proposed by the National Committee for Clinical Laboratory Standards (NCCLS) was introduced in all institutes in 1997. In order to obtain a coherent view of the antimicrobial resistance of bacteria a computer system was consulted, consisting of a central computer to store all data and some local computers attached to it through the network. At these local measuring stations computers are connected to a video camera, which displays the picture of Petri dishes on the monitor, and inhibition zone diameters of bacteria can be drawn with the mouse by the inspector. The software measures the diameters, evaluates whether or not the bacteria are sensitive, and stores the data. The evaluation is based upon the data of the NCCLS. The central computer can be connected to as many local computers with measuring stations as we wish, so it is suitable for an integrated system for monitoring trends in antimicrobial resistance of bacteria from animals, food and humans, facilitating comparison of the occurrence of resistance for each circumstance in the chain. It depends on the examiners which antibiotics they want to examine. Thirty-two different antibiotic panels were compiled, taking into consideration the active ingredients of medicinal products permitted for veterinary use in Hungary, natural resistance and cross-resistance, the mechanism of resistance and the animal species, i.e. which drugs were recommended for treatment in the given animal species, and the recommendations of the OIE Expert Group on Antimicrobial Resistance. The members of the panels can be changed any time, even during the measuring process. In addition to the inhibition zone diameters of bacteria the database also includes information about bacterial and animal species, the age of animals and the sample or organ where the bacteria are from. Since January 2001 the antibiotic susceptibility of *E. coli, Salmonella, Campylobacter* and *Enterococcus* strains isolated from the colons of slaughter cows, pigs and broiler chickens has also been examined. Each of the 19 counties of Hungary submits to the laboratory three tied colon samples from a herd of the above-mentioned animals every month.

[*]Corresponding author; E-mail: juhaszne@oai.hu; Fax: +36 (1) 222 6066

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The global spread of polyresistant bacteria calls for a closer monitoring of the development of antimicrobial resistance also in Hungary. Antibiotic sensitivity testing has been conducted in several regional veterinary institutes for decades, but the applied methods lacked consistency, preventing valid nationwide comparisons of the results obtained. The Ministry of Agriculture and Regional Development entrusted the Central Veterinary Institute with the task of developing a so-called national antibiotic resistance monitoring system. The principal objective of such a system is to provide comparable results by the use of uniform methodology that meets the international expectations. During the implementation of the system we took into consideration the relevant recommendations of the Group on Antimicrobial Resistance of the Office International des Epizooties (OIE). This paper describes the essential features of the monitoring system; by the presentation of some test panels we would like to facilitate its understanding.

Method

The antibiotic sensitivity tests are carried out by the disc diffusion method described by the National Committee for Clinical Laboratory Standards (NCCLS) in document M31-A/1999, as this requires less materials, time and labour than the broth or the agar dilution method, while it provides sufficiently accurate results for routine diagnostic purposes. For determining the antibiogram of campylobacters the E-test is used (Engberg et al., 1999).

For the tests, the majority of discs are purchased from Oxoid, with the exception of ceftiofur and florfenicol originating from Becton-Dickinson, and virginiamycin, tiamulin and spectinomycin supplied by Rosco.

Information technology system

In order to obtain nationwide data suitable for comparison and adding up, besides standardisation of the method of antibiotic sensitivity testing a computer system had to be developed, which can collect and systematise data, ensures consistent evaluation and, if necessary, records and forwards images. The basis of the system is a central computer, which stores the basic data and all results of measurements. Through a network, this is connected to one or more measuring stations suitable for measuring the diameter of inhibition zones, where a video camera connected to the computer displays the image of the Petri dish on the screen. Using the mouse, the diameter of the inhibition zone can be highlighted, and then the programme in millimetres displays it. If the borders of the inhibition zones are not sharp enough on the screen (this occurs primarily if blood agar is

used), they can be rendered more visible by scratching the surface with a needle. The measurement is rendered reliable by the possibility of calibrating the system, and thus checking its accuracy, with a round object of known dimensions.

The basic data of the programme include the origin of bacteria to be tested, grouped by animal species, age group, organ or other sample, as well as the name, active ingredient content and symbol of the antibiotic discs or tablets.

Thirty-two standard panels were compiled from the active ingredients, taking into consideration the active ingredients of medicinal products that are available on the market for the different animal species in Hungary (Perényi, 1998), the natural resistance of bacteria, and the active ingredients between which cross-resistance exists (Courvalin, 1992; Kaszanyitzky et al., 1998; Greenwood, 2000; Livermore et al., 2001). We also followed the recommendations of the OIE Group on Antimicrobial Resistance. Currently the programme contains the following panels: Gram-positive and Gram-negative panel for poultry, pigs, cattle-sheep, horses, dogs and cats, rabbits and fish. There is a separate panel for *E. coli, Salmonella* and *Streptococcus* bacteria isolated from poultry, as well as for *Pasteurella* bacteria of poultry-pig-cattle-sheep, horse and milk origin. Separate panels are used for *Pseudomonas, Streptococcus* and *Staphylococcus* strains cultured from milk. An own panel can be selected for the antibiotic sensitivity testing of *Enterococcus, Campylobacter, Clostridium, Staphylococcus, Acinetobacter* strains and for that of test strains applied for quality control of the method. The panel selected for any given test can be made to appear in an instant, thus allowing the use of only those discs which correspond to the given bacterium and animal species.

For illustration, the antibiotics included in the four most commonly used panels are shown in a table. The 'poultry: Gram-negative: *E. coli, Salmonella*' panel, used for the antibiotic sensitivity testing of *E. coli* and *Salmonella* strains cultured from diseased or dead animals, contains the highest number of active ingredients. Of the penicillin derivatives it contains only ampicillin, as there is almost complete cross-resistance among members of the family. Strains whose β-lactamase can inactivate penicillin derivatives can be identified by the use of a disc containing amoxicillin/clavulanic acid (Jacoby and Archer, 1991).

Of the cephalosporins, cephalothin, which is recommended mostly against Gram-positive bacteria as it is decomposed by the broad-spectrum β-lactamases produced by Gram-negative bacteria, and ceftriaxone, which resists even the TEM- and SHV-type β-lactamases produced by Gram negatives, are not used for the treatment of farm animals in Hungary. However, on the recommendation of the OIE Group on Antimicrobial Resistance they are included in the different panels. Ceftiofur is authorised for veterinary use also in Hungary and, like ceftriaxone, it can be used against β-lactamase producing Gram-negative bacteria.

Table 1

Active ingredients of 4 panels

Poultry (Gram-negative: *E. coli, Salmonella*)	Horse (Gram-negative)	*Pseudomonas* (poultry, pig, cattle, sheep, dog)	Poultry (Gram-positive)
Amoxicillin/ clavulanic acid	Ampicillin	Ceftiofur	Penicillin
Ampicillin	Ceftiofur	Colistin	Amoxicillin/ clavulanic acid
Cephalothin	Chloramphenicol	Tetracycline	Ceftiofur
Ceftiofur	Sulphamethoxazole/ trimethoprim	Doxycycline	Tetracycline
Ceftriaxone	Sulphonamides	Apramycin	Doxycycline
Chloramphenicol	Tetracycline	Streptomycin	Apramycin
Florfenicol	Streptomycin	Spectinomycin	Gentamicin
Colistin	Spectinomycin	Neomycin	Streptomycin
Sulphamethoxazole/ trimethoprim	Neomycin	Gentamicin	Neomycin
Sulphonamides	Gentamicin	Enrofloxacin	Enrofloxacin
Tetracycline	Flumequine	Marbofloxacin	Norfloxacin
Doxycycline	Marbofloxacin		Sulphamethoxazole/ trimethoprim
Apramycin			Erythromycin
Streptomycin			Tilmicosin
Spectinomycin			Lincomycin
Neomycin			
Gentamicin			
Oxolinic acid			
Flumequine			
Enrofloxacin			
Norfloxacin			

Of agents belonging to the phenicol group, chloramphenicol has been banned for use in (food-producing) farm animals since 1988, but on the recommendation of the OIE Group on Antimicrobial Resistance it is included in the

panels to identify multiresistant strains occurring in several countries, which may carry a chloramphenicol resistance gene realised in efflux mechanism *(E. coli, Salmonella* Enterica serovar Typhimurium DT104*)* (Daly and Fanning, 2000; White et al., 2000). The use of florfenicol is spreading steadily. These two related antibiotics are included in the panel because acetyltransferase produced by resistant bacteria degrades only chloramphenicol, while a plasmid-mediated resistance supposedly based on an efflux pump mechanism causes cross-resistance between the two compounds (Cloeckaert et al., 2001).

Because of the complete cross-resistance between the active ingredients, in the panel colistin represents polymyxin B, whereas a sulphonamide and a sulphonamide/trimethoprim combination represent all active ingredients belonging to those groups.

If the bacterium produces a protein against the ribosomal binding of tetracycline, this will cause complete resistance in the group; in contrast, bacteria having an efflux mechanism based defence may remain sensitive to doxycycline and minocycline; this is why the panel includes a doxycycline disc in addition to tetracycline (Livermore et al., 2001).

The panel includes several aminoglycosides (gentamicin, neomycin, spectinomycin and streptomycin), as the target change developing towards streptomycin does not affect the other agents of the group, while in their inactivation numerous enzymes of different structure may take part; accordingly, their sensitivity patterns are varied (Livermore et al., 2001).

According to data of the literature, mutation(s) occurring at different sites of the quinolone resistance-determining region (QRDR) of bacteria raise the MIC values of all agents belonging to this group but not in the same degree (Bernard and Maxwell, 2001; Friedman et al., 2001). Depending on the genuine sensitivity of the given bacterium species, some of the fluoroquinolones may remain active despite the increased MIC value as they can attain sufficient therapeutic levels. It should be noted, however, that sensitivity is often only marginal; therefore, if a bacterial strain proves resistant to a quinolone, only in exceptional cases can the other quinolone compounds be recommended either (Livermore et al., 2001). As among Gram-negative bacteria polyresistant strains are often encountered, the testing of several quinolones is considered justified.

Certain agents (e.g. clavulanic acid, doxycycline, and fluoroquinolones with the exception of flumequine and marbofloxacin) are not recommended for use in horses because of their harmful effects on the intestinal microflora, bone and cartilage, and due to their photosensitising property. These agents were omitted from the panel, and those compounds were included which are suitable also for parenteral treatment.

Pseudomonas bacteria possess natural resistance against penicillin derivatives, their combination with β-lactamase inhibitors, first- and second-generation cephalosporins, chloramphenicol, and first- and second-generation fluoro-

quinolones. Therefore, these agents are used at most for diagnostic purposes in the testing of *Pseudomonas* organisms.

The panel compiled for the testing of Gram-positive bacteria naturally includes, in addition to the broad-spectrum active ingredients already mentioned in the case of Gram-negative bacteria, those agents which primarily act on Gram-positives. In that panel, penicillin derivatives are represented by penicillin (and in the staphylococcus panel also by oxacillin for the determination of resistance to all β-lactam antibiotics) while lincosamides by lincomycin.

In cases when an efflux mechanism representing a lower degree of resistance to macrolides develops in the bacteria, compounds having 14 lactone rings (erythromycin) lose their effect, whereas those possessing 16 lactone rings (spiramycin, tylosin, tilmicosin) do not (Cornaglia, 1999; Roberts et al., 1999; Fitoussi et al., 2001), thus both groups will be represented by one active ingredient each.

The panels can be adjusted as required by the situation, even during the measuring process itself, i.e. any active ingredient can be omitted from them or any agent included in the test system can be added.

To the active ingredients the programme can assign diameter values on the basis of which the test bacteria can be considered sensitive, moderately sensitive or resistant in the test performed according to, and in compliance with the quality requirements, of the NCCLS system. The programme can also store the results of evaluation and the lengths of the diameters measured.

An important component of the programme is the so-called bacterium tree, which starts from 'all bacteria' and then subdivides into Gram-negative and Gram-positive groups and subsequently into bacterium families and species. During measurement, the programme 'scans' the bacterium tree, starting from the species. If it does not find a value for the species, it continues the search until finally it reaches the level of 'all bacteria'. This enables the programme to evaluate e.g. the ampicillin sensitivity of staphylococci by taking into account different diameters than in the case of *E. coli*. Where there is no difference (e.g. tetracyclines, aminoglycosides), there the programme will use the 'all bacteria' value.

When the diameters of inhibition zones are measured, besides the symbol of the given active ingredient the limit values of inhibition zones typical of the given bacterium will also be displayed on the screen; thus, if we obtain values very close to these limits, we can make an even more careful evaluation. The screen shows a magnified image of the antibiotic sensitivity test, which further increases the accuracy of measurement.

The programme records not only the result of evaluation but also the data of inhibition zone diameters; thus, it is suitable for providing quantitative results. These can be printed immediately or, if we want to draw up a data communication letter, we can omit from the series any active ingredient about which we do not wish to give information in the letter (the database will naturally retain the results obtained for this omitted active ingredient). The letter can be comple-

mented with different explanatory notes, in which we can call attention e.g. to the possibility of cross-resistance.

We can perform the measurement also if at the time of evaluating the results of antibiotic sensitivity testing the only thing we know is whether the bacterium under test is Gram-negative or Gram-positive. In this case, the programme will re-evaluate the results of the previously performed measurements later, after species identification of the bacterium has been made.

The programme can be installed on several computers and is suitable for network development. Any institution performing antibiotic sensitivity testing can join it; our network includes the Veterinary Institutes of Debrecen and Kaposvár and the National Food Investigation Institute.

Origin of the bacteria to be tested

The monitoring system records not only the data of bacteria isolated from diseased or dead animals that had probably received antibiotic treatment, but – in compliance with the recommendations of international expert committees – also the antibiograms of *E. coli, Salmonella, Enterococcus* and *Campylobacter* strains cultured from the colons of clinically healthy pigs, cattle and broiler chickens slaughtered at abattoirs in the framework of normal slaughtering operations are tested. The meat or processed products of these animal species often serve as human food. Fresh meat contaminated with faeces contains bacteria normally present in the intestinal tract of animals, which bacteria may get into the intestinal tract of humans if the basic hygiene rules of food preparation are not observed. This may lead to the occurrence of zoonoses or the colonisation of the human intestine by enterococci of animal origin, which may grow there without causing any clinical signs. These bacteria especially tend to transfer the transposons and plasmids carrying the genetic code of their resistance mechanism to bacteria occurring in their environment, often also to bacteria belonging to other species. In this way the resistance mechanisms that developed in the bacteria of animals may be transferred to bacteria inhabiting the gastrointestinal tract of humans (Arthur et al., 1996). This necessitates the testing of samples taken from healthy animals already transported to the slaughterhouse.

Every month, each of the 19 counties of Hungary submits a 10–12 cm long tied colon sample from three animals each of a poultry, pig and cattle stock (from poultry the entire caecum is submitted). The samples are submitted by the local veterinarians within the shortest possible time after slaughter, and are processed in the laboratory yet on the same day. From the samples thus submitted we attempt to culture even brachyspires and campylobacters that are highly sensitive to environmental factors. The testing was started in January 2001 and is continued throughout the year. In this way we can get an overall view of the antibiotic sensitivity situation in the entire territory of the country, and a possible seasonality appearing in the development of resistance can also be revealed.

In the case of broiler chickens and growing pigs we could meet the OIE requirement that bacteria cultured from slaughterhouse samples should come from stocks of the same age; however, in the case of samples originating from cattle the age of animals varies rather widely (1–9 years). Calves are slaughtered very rarely and incidentally; therefore, samples from calves have not been included in the testing system.

Salmonellae cultured from feed samples and some of those isolated from food samples in laboratories of other institutions are also submitted to our institute for antibiotic sensitivity testing. The results of these tests provide information on the resistance mechanisms that bacteria ingested by animals have.

The majority of *Salmonella, E. coli, Enterococcus* and *Staphylococcus aureus* strains of food origin are tested in the National Food Investigation Institute. The results of these tests, along with data of antibiotic sensitivity tests performed in other institutions, are fed into the national system. These data show how much the antibiotic resistance patterns of bacterial strains of food origin resemble those of bacteria cultured from slaughter animals, and whether they carry resistance genes typical of human bacteria. Namely, during the processing of foods there are numerous opportunities even for bacteria from humans getting into foods.

The collection of data relating to the manufacture, use and method of application of antibiotics, and co-operation with laboratories performing antibiotic sensitivity tests in the public health sector represents tasks to be accomplished in the future.

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