Prevalence and antimicrobial susceptibility of udder pathogens isolated from dairy cows in Slovakia

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SUPPLEMENTARY FILE

Supplementary Material & Methods

Sampling

Between February 2015 and August 2016, a total of 633 quarter milk samples taken from 512 suspicious dairy cows with positive California Mastitis Test (CMT) were examined. The animals came from 42 Western Slovakian dairy cattle herds with conventional (non-organic) farming. The number of farms investigated represents 5% of Slovakian dairy farms of size category 50 to 500 animals. The numbers of farms investigated in this study were selected according to the frequency of distribution of the various farm sizes in Slovakia. The samples were collected aseptically into 10 ml sterilised test tubes; after cooling, they were transported into a laboratory on the day of their collection. The samples were processed within 24 to 48h.

Bacteriological culture

Milk samples (10 µl) were cultured on blood (5% bovine blood) agar plates, incubated at 37°C for 16-24 h. Growth on the plates was confirmed by additional laboratory tests. S. aureus was identified by means of typical colony morphology, and b-hemolysis, or by coagulase reaction (coagulasepositive) when typical hemolysis zones were not present. Coagulase-negative staphylococci were identified by typical colony morphology and coagulase reaction, but were

not further characterized for this paper. Streptococci were determined by colony morphology and CAMP-reaction, and 12 biochemical reactions (hippurate, aesculine, salicine, sorbitol, mannitol, raffinose, lactose, saccharose, inuline, trehalose, starch and glycerine), were used for typing to the species level. Enterococci were confirmed by Gram-staining and growth of typical colonies on SlaBa-plates (Slanetz &Bartley Medium, Oxoid Ltd., Basingstoke, England). Gram-negative bacteria with typical colony morphology, and positive for p-nitrophenyl-b-Dglucupyranosiduronic acid (PGUA) and indole were considered as E. coli. For other Gramnegative bacteria, oxidase reaction and API 20 E or API 20 NE (BioMérieux, Craponne, France) was used. Bacillus spp. was confirmed by colony morphology and Gramstaining. A milk sample was classified as positive if at least one colony-forming unit (CFU) of S. aureus or Streptococcus (Str.) agalactiae was isolated. For other agents, the presence of at least three CFUs was needed for positive classification. Samples were classified as contaminated if three or more bacterial types were isolated from one milk sample and growth of a major udder pathogen was not identified. If growth of a major udder pathogen was found in combination with contaminating species and if the CMT was high, the sample would be diagnosed as positive for growth of the major udder pathogen.

Susceptibility testing

In vitro susceptibility of the isolates against antimicrobial agents was determined by the standard disk diffusion procedure (CLSI 2008; 2013). Coagulase-negative staphylococci, *S. aureus*, *Str. uberis*, *Str. agalactiae,* and *E. coli* were tested for susceptibility to 16 antimicrobial agents from the following groups: penicillins (AMX, AMC, CLOX, PEN), cephalosporins (LEX, CEF, CEQ), tetracyclines (TET), aminoglycosides (STR, NEO, KAN), lincosamides (LCM), ansamycins (RIF), sulphonamides (SXT), quinolones (EFX), and others (NVB). The above pathogens were tested by disc diffusion method according to the CLSI manual (CLSI 2008;

2013) using the following antimicrobial discs (Oxoid Ltd., Basingstoke, England): amoxicillin AMX (25 µg), amoxicillin and clavulanic acid 2:1 AMC (30 µg), cloxacillin CLOX (5 µg), penicillin PEN (10 IU), cephalexin LEX (30 µg), ceftiofur CEF (30 µg), cefquinome CEQ (30 µg), streptomycin STR (10 µg), neomycin NEO (30 µg), tetracycline TET (30 µg), lincomycin LCM (2 µg), rifaximin RIF (40 µg), sulfamethoxazole-trimethoprim SXT (25 µg), enrofloxacin EFX (5 µg), and novobiocin NVB (30 µg). The diameters of the inhibition zones were evaluated (susceptible, intermediate, resistant) according to CLSI breakpoints. Appropriate quality control tests were performed using reference strains of *E. coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212. All isolates of staphylococci were in addition examined for presence of *mecA*-gene by polymerase chain reaction according to Strommenger *et al*. (2003).