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Antimicrobial susceptibility and distribution of inhibition zone diameters of bovine mastitis pathogens in Flanders, Belgium

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ABSTRACT

In dairy farms, antimicrobial drugs are frequently used for treatment of (sub)clinical mastitis. Determining the antimicrobial susceptibility of mastitis pathogens is needed to come to a correct use of antimicrobials. Strains of *Staphylococcus aureus* ($n = 768$), *Streptococcus uberis* ($n = 939$), *Streptococcus dysgalactiae* ($n = 444$), *Escherichia coli* ($n = 563$), and *Klebsiella* species ($n = 59$) originating from routine milk samples from (sub)clinical mastitis were subjected to the disk diffusion method. Disks contained representatives of frequently used antibiotics in dairy. A limited number of clinical breakpoints were available through CLSI, and showed that susceptibility of *Staph. aureus*, *E. coli*, and *Klebsiella* was moderate to high. For streptococcal species however, a large variation between the tested species and the different antimicrobials was observed. In a next step, wild type populations were described based on epidemiological cut off values (EUCAST). Because of the limited number of official cut off values, the data were observed as a mastitis subpopulation and self-generated cut off values were created and a putative wild type population was suggested.

The need for accurate clinical breakpoints for veterinary pathogens is high. Despite the lack of these breakpoints, however, a population study can be performed based on the distribution of inhibition zone diameters on the condition that a large number of strains is tested.

1. Introduction

Mastitis, in its clinical or subclinical form, remains the most costly disease on dairy farms over the world. A survey on dairy herds in Flanders, the northern part of Belgium, revealed in 2003 that 41% of the cows had a subclinical intramammary infection (Piepers et al., 2007). The incidence of clinical mastitis in Belgium is hardly studied. In the Netherlands (Barkema et al., 1998) and Canada (Riekerink et al., 2008), respectively 26 and 23 cases per 100 cows per year were registered. Along with the disease, farmers are confronted with production losses, animal discomfort, disturbance in the milking routine, and treatment costs.

In Flanders, the most frequently isolated major pathogens from subclinically infected quarters are *Streptococcus uberis* and *Staphylococcus aureus*, and *Escherichia coli*, *Strep. uberis*, *Staph. aureus*, and *Streptococcus dysgalactiae* from clinical mastitis cases (MCC, 2012). Investigating the antimicrobial resistance pattern of a bacterium is frequently performed by means of the disk diffusion method in routine veterinary labs due to the practical and economic factors. To inform the farmer and/or veterinarian on the most appropriate therapy choice, clinical breakpoints are required however often unavailable for the particular combination of the pathogen/antimicrobial per

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host species. As an alternative for the division into susceptible, intermediate susceptible, or resistant, a bacterial population can be divided into "wild type" and "non-wild type" population based on the distribution of inhibition zone diameters.

In this paper, a description of the antimicrobial resistance profile of the most prevalent pathogens from bovine mastitis is presented according to both clinical and epidemiological criteria.

2. Materials and methods

2.1. Laboratory

Analyses were performed at the laboratory of the Milk Control Centre Flanders (MCC). This organization is authorized to perform the official analysis of bulk milk samples of the Flemish dairy herds, on milk quality and composition. Besides that, MCC runs a routine lab for bacteriological culturing of milk samples.

2.2. Milk samples

Quarter milk samples presented at the lab during a one-year period (September 2012 until September 2013) were taken into account. The samples were send to the lab on voluntary basis by farmers or their veterinarian, and originated from (sub)clinical intramammary infections. If multiple samples from the same farm were submitted on the same day, this set of samples was referred to as one 'dossier'.

2.3. Bacteriological culturing

For standard culturing, the guidelines of the National Mastitis Council were followed (NMC, 1999). Briefly, a 0.01 mL loop of milk was spread on a quadrant of an aesculin blood agar plate (Oxoid, Basingstoke, UK) and incubated aerobically at 37 °C for 36 ± 12 h. Milk samples showing abnormal milk were plated also onto a McConckey agar plate (Oxoid). A quarter was considered culture-positive when growth of 1 or more colonies was detected. Phenotypic features were examined after 24 and 48 h. Growth characteristics, Gram-staining and/or presence of growth on the McConckey plate were used to distinguish Gram-negative from Gram-positive bacteria. The catalase test was used to distinguish staphylococci (positive reaction) from the *Streptococcus–Enterococcus* group (negative reaction). Within the staphylococci, the DNase activity permitted to distinguish *Staph. aureus* (positive) from other staphylococci (DNase negative or intermediately positive). Isolates from the *Streptococcus–Enterococcus* group were divided based on the aesculin reaction. Within the aesculin-negative cocci, the CAMP-test allowed to distinguish *Strep. dysgalactiae* from *Streptococcus agalactiae*. Within the aesculin-positive cocci, the growth characteristics (including color), bile aesculin agar and NaCl 6.5% were used to differentiate *Strep. uberis* from other aesculin-positive cocci (enterococci, lactococci, and aerococci). If no conclusive identification could be made, the API® strep was carried out as prescribed by the manufacturer. Within Gram-negative bacteria, appearance on McConckey agar, KOH reaction, indol production, and the triple sugar iron test were used to differentiate between *Escherichia coli*, *Klebsiella* species, and others. Other bacteria including *Corynebacterium bovis*, yeast, fungi, Prototheca species, *Bacillus* species, and *Trueperella* (*Arcanobacterium*) *pyogenes* were identified through their appearance on aesculin agar and/or morphology on Gram-staining. A milk sample was defined as contaminated if >2 different colony types were present.

2.4. Antimicrobial susceptibility testing

Not all strains of the species of interest (*Staph. aureus*, *Strep. uberis*, *Strep. dysgalactiae*, *E. coli*, and *Klebsiella*) were subjected to susceptibility testing. Of each dossier (this is the set of milk samples from one farm arriving in the lab at the same day), one strain per species of interest was selected, namely the strain isolated in the quarter with the highest somatic cell count (data on somatic cell count not presented). Selected strains were subjected to the Kirby-Bauer disk diffusion method. In short, with the InoClic® system (i2a, Perols, France) used as described by the manufacturer, a clearly separate colony of the pathogen of interest was picked and suspended in 5 mL saline solution, resulting in 0.5 McFarland. The suspension was used for flooding the Mueller Hinton agar plates (i2a), and the redundant solution was discarded. Streptococci were examined on Mueller Hinton agar plates supplemented with 5% horse blood and 20 mg/L NAD (Biomerieux, France). Antibiotic impregnated paper disks were combined in two panels, for Gram-positive and Gram-negative bacteria, respectively, and disks were applied with a dispenser. The antimicrobial agents were selected according to their occurrence in commercially available products for mastitis treatment and/or dry-cow therapy. The Gram-positive panel (for *Staph. aureus* and streptococci) consisted of oxacillin, cefoxitin, ampicillin, amoxicillin/clavulanic acid, cefquinome, tetracycline, neomycin (not for streptococci), lincomycin, erythromycin, marbofloxacin, trimethoprim/sulfamethoxazole, and rifaximin. The Gram-negative panel (for *E. coli* and *Klebsiella* species) consisted of ampicillin (not for *Klebsiella*), amoxicillin/clavulanic acid, cefquinome, tetracycline, neomycin, marbofloxacin, and trimethoprim/sulfamethoxazole. Disks were purchased from i2a, except for cefquinome and rifaximin (Mast Group, Merseyside, UK). Disks contents were indicated in Table 1. After an overnight incubation at 35 ± 2 °C, plates were read with the SIR scan Micro (i2a). The reference strains *Staph. aureus* ATCC 25923 and *E. coli* ATCC 25922 were used for quality control.

2.5. Evaluation of the inhibition zone diameters

Inhibition zone diameters were first evaluated by clinical breakpoints as provided by the Clinical and Laboratory Standards Institute (CLSI) to determine resistant strains. When veterinary breakpoints (CLSI, 2008) were not available for a certain pathogen/antimicrobial combination, human breakpoints were used (CLSI, 2012). If neither of them was available, no clinical interpretation was performed. Secondly, the distribution of the inhibition zone