Antimicrobial consumption on dairy herds and its association with antimicrobial inhibition zone diameters of non-*aureus* staphylococci and *Staphylococcus aureus* isolated from subclinical mastitis

M. Stevens,*¹ S. Piepers,* K. Supré,[†] and S. De Vliegher*

*M-team and Mastitis and Milk Quality Research Unit, Department of Reproduction, Obstetrics and Herd Health, Faculty of Veterinary Medicine, Ghent University, 9820 Merelbeke, Belgium

[†]Milk Control Centre Flanders, 2500 Lier, Belgium

ABSTRACT

The main objectives of this study were to quantify the consumption of antimicrobials on a convenience sample of dairy herds and to determine the association between herd-level antimicrobial consumption and inhibition zone diameters (IZD) of non-*aureus* staphylococci and *Staphylococcus aureus* isolates from subclinical mastitis cases. Also, the association between the IZD of non-*aureus* staphylococci and *Staph. aureus* isolates within a herd was studied. Antimicrobial consumption data on 56 Flemish dairy farms were obtained between 2013 and 2014 by so-called garbage can audits and expressed as antimicrobial treatment incidence (ATI), with the unit of ATI being the number of defined daily doses animal (DDDA) used per 1,000 cow-days. The average total ATI in adult dairy cattle for all active substances was 18.73 DDDA per 1,000 cow-days and ranged from 6.28 to 42.13 DDDA between herds. The ATI of critically important (for human health) antimicrobials was 6.91 DDDA per 1,000 cow-days; that is, 37% of total antimicrobial consumption. The average ATI for intramammary therapy of (sub)clinical mastitis, intramammary dry-cow therapy, and systemically administered therapy was 5.20, 6.70, and 6.73 DDDA, respectively. The IZD of 239 non-*aureus* staphylococci and 88 *Staph. aureus* isolates originating from milk samples from cows with subclinical mastitis collected on selected dairy herds were determined using Kirby-Bauer disk diffusion and ranged between 6 and 42 mm. Because only a limited number of clinical breakpoints (Clinical and Laboratory Standards Institute) and epidemiological cut-off values (European Committee on Antimicrobial Susceptibility Testing) are available for mastitis-causing bacteria in bovine, IZD were used as a proxy for antimicrobial resistance. Inhibition zone diameters of

non-*aureus* staphylococci for cefquinome, a critically important β -lactam antibiotic, were negatively associated with the ATI of critically important β -lactam for systemically administered therapy and positively with the ATI for intramammary therapy of (sub)clinical mastitis of critically important β -lactam antimicrobials. Only for neomycin was a positive association between the IZD of non-*aureus* staphylococci and *Staph. aureus* isolates within the same herd observed.

Key words: antimicrobial consumption, staphylococci, subclinical mastitis, inhibition zone diameters

INTRODUCTION

Acquired antimicrobial resistance is a growing global concern in both human and veterinary medicine because it can result in treatment failure. The 2 main factors involved in the development of antimicrobial resistance in bacteria are the presence of resistance genes and selection pressure caused by the use of antimicrobials (Levy, 1997; Acar and Moulin, 2006; Silbergeld et al., 2008; Chantziaras et al., 2014). In dairy cattle, most antimicrobials are being used for the prevention and control of mastitis (Mitchell et al., 1998; Pol and Ruegg, 2007b; Menéndez González et al., 2010; Stevens et al., 2016). Potential associations between antimicrobial consumption on dairy herds and antimicrobial susceptibility of bovine mastitis pathogens have been studied, but not in Europe and not in much detail (Rajala-Schultz et al., 2004; Pol and Ruegg, 2007a; Saini et al., 2012b).

Over the last 10 yr, non-*aureus* staphylococci, also referred to as CNS, have become the most common cause of subclinical mastitis worldwide (Chaffer et al., 1999; Rajala-Schultz et al., 2004; Piepers et al., 2007; Vanderhaeghen et al., 2014; Sztachañska et al., 2016), particularly in heifers (De Vliegher et al., 2012). They form a heterogeneous group of more than 50 species, of which approximately 10 have been associated with IMI in dairy cattle (Vanderhaeghen et al., 2014) and are abundantly present in bulk milk (De Visscher et al., 2017). Non-*aureus* staphylococci are also present in

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¹Corresponding author: Marina.Stevens@Ugent.be

other bovine-associated habitats such as air, housing, bedding material, milking equipment, and body parts such as teat apices, hair coat, nares, and the vagina (White et al., 1989; Nagase et al., 2002; Paduch and Kroemker, 2011; Piepers et al., 2011; Piessens et al., 2012; Braem et al., 2013; De Visscher et al., 2014, 2016). Their omnipresence, especially on the body of the cows, implies that non-*aureus* staphylococci are generally more exposed to antimicrobial treatments than other mastitis pathogens, which could explain the higher levels of antimicrobial resistance of non-*aureus* staphylococci compared with the more pathogenic *Staphylococcus aureus* (Owens and Watts, 1988; Taponen and Pyorala, 2009; Schmidt et al., 2015). It has been suggested that non-*aureus* staphylococci might act as a potential reservoir for resistance genes that can be transferred and integrated into the genome of *Staph. aureus* (Holmes and Zadoks, 2011; Otto, 2013; Vitali et al., 2014).

One of the most widely used methods for measuring antimicrobial susceptibility of bacteria is the Kirby-Bauer disk diffusion test, in which radial zones of bacterial growth inhibition are cross-referenced to breakpoints to determine whether an organism is susceptible or resistant to therapy with a particular antimicrobial drug (Traub and Leonhard, 1994). Still, the required clinical breakpoints to correctly interpret the results are often unavailable for a specific combination of pathogen and antimicrobial per host species (CLSI, 2008, 2015; http://www.eucast.org/ast_of_veterinary_pathogens/). This is the case particularly for non-*aureus* staphylococci isolated from bovine milk, partly because they are generally considered minor pathogens and typically not treated with antimicrobials. Using inhibition zone diameters (IZD) for non-*aureus* staphylococci and for *Staph. aureus* to determine the presence of (acquired) genetic resistance elements as a proxy for potential antimicrobial resistance, rather than relying on (clinical) breakpoints, avoids these issues.

The main objectives of this study were (1) to quantify the use of antimicrobials on a convenience sample of dairy herds, (2) to determine the association between the herd-level antimicrobial consumption and the IZD of non-*aureus* staphylococci and *Staph. aureus* isolates, respectively, and (3) to determine the association between the IZD of the non-*aureus* staphylococci and *Staph. aureus* isolates originating from the same herds.

MATERIALS AND METHODS

Herds and Cows

Data collected for this study originated from a convenience sample of 56 Flemish dairy herds. Herd inclusion

criteria were (1) participation in the DHI program in Flanders with an interval of 4 to 6 wk between 2 test-days, and (2) record keeping of all treatments in adult cattle (date of occurrence, identification number of animal, treatment protocol applied). More details about the included herds are described elsewhere (Stevens et al., 2016).

From March to April 2014, approximately 10 lactating cows per herd with subclinical mastitis (defined as a test-day SCC >150,000 cells/mL and >250,000 cells/mL for heifers and multiparous cows, respectively) were randomly selected for milk sampling for bacteriological culturing.

Antimicrobial Consumption Data

Antimicrobial consumption data were retrieved by so-called garbage can audits, as described in a previous paper reporting on the antimicrobial consumption of the herds between January 2012 and February 2013 (Stevens et al., 2016). For the current paper, empty antimicrobial receptacles collected from March 2013 to February 2014 were used. Product name, volume, and number of receptacles were registered using a datasheet that included herd identification number and start and end date of collection for that particular herd. The quantification of the herd-level antimicrobial consumption was defined by the antimicrobial treatment incidence (ATI) as described previously (Stevens et al., 2016) and expressed as the number of defined daily doses animal (DDDA) used per 1,000 cows per day.

Classification of Antimicrobials

Route of Administration and Indication. Antimicrobials registered for intramammary use were divided into intramammary injectors registered for dry-cow therapy and injectors registered for the intramammary treatment of (sub)clinical mastitis cases. All systemically injectable antimicrobials used for the therapy of various diseases (including mastitis) are hereafter referred to as “systemically administered therapy.” Intrauterine use of antimicrobials was very limited and therefore not taken into account in further analyses, as was done previously (Stevens et al., 2016).

Strata of Antimicrobials. Four strata were generated based on classification as β -lactam and non- β -lactam and their importance in human health: critically important (i.e., third- and fourth-generation cephalosporins and fluoroquinolones) or not critically important, according to the Office International des Épizooties (OIE) classification: stratum 1: critically important β -lactam antimicrobials (i.e., third- and fourth-generation cephalosporins); stratum 2: critically

important non- β -lactam antimicrobials (i.e., fluoroquinolones); stratum 3: not critically important β -lactam antimicrobials (i.e., aminopenicillins, aminopenicillin-clavulanic acid combination, aminopenicillin-polymyxin combination, first-generation cephalosporins, first-generation cephalosporin-aminoglycoside combination, penicillins, penicillin-aminopenicillin combination, and the penicillin-aminoglycoside combination); and stratum 4: not critically important non- β -lactam antimicrobials (i.e., aminoglycosides, lincosamides, lincosamide-aminoglycoside combination, macrolides, polymyxins, rifaximins, sulfonamide-trimethoprim combination, and tetracyclines).

Collection of Milk Samples

Quarter foremilk samples were collected aseptically by either the farmer or the first author and colleagues of the research group at milking (or between successive milkings on herds equipped with an automatic milking system) following the guidelines of the National Mastitis Council (Oliver et al., 2004). Sample materials were provided to the farmers and (aseptic) sampling procedures were explained. Sampling date, cow identification number, and quarter position were recorded. The quarter milk samples were transported under cooled conditions to the Flemish Milk Control Centre (MCC, Lier, Belgium), where routine bacteriological culturing was performed.

Bacteriological Culturing

Bacteriological culturing was performed following the National Mastitis Council guidelines (NMC, 1999). From each sample, 10 μ L of milk was spread on a quadrant of an esculin blood agar plate (Oxoid, Basingstoke, UK) and incubated aerobically at 37°C for 24 to 48 h. Samples were considered to be culture-positive if one or more colonies were observed (≥ 100 cfu/mL). Identification of bacteria was done by Gram staining, inspection of colony morphology, and biochemical testing. The catalase test was used to distinguish staphylococci (positive reaction) from the *Streptococcus-Enterococcus* group (negative reaction). *Staphylococcus aureus* were differentiated from non-*aureus* staphylococci by colony morphology, hemolysis patterns, and DNase tests. A DNase test was considered positive if the clearing zone was 3 times the width of the incubation streak and negative/intermediate positive when the zone was less than 3 times the width of the streak. An isolate was considered as *Staph. aureus* when showing a complete or double hemolytic pattern after 48 h of incubation and a positive DNase test. All other catalase-positive isolates (thus, isolates showing no or single hemolysis

and a negative/intermediate DNase test) were defined as “non-*aureus* staphylococcus”). Testing of DNase activity has a sensitivity of 97% and a specificity of 83% after a 24-h incubation for *Staph. aureus* (Graber et al., 2009). Samples yielding 2 different bacterial species were considered mixed samples and were, in case of staphylococci, included in the analyses. Samples yielding 3 or more different bacterial species were considered contaminated, and *Staphylococcus* species from these samples were not further examined.

Inhibition Zone Diameters

All isolates identified as non-*aureus* staphylococci and *Staph. aureus* isolates were submitted to agar disk diffusion testing (Bauer et al., 1966) on Mueller-Hinton agar (i2a, Perols, France) as described previously (Supré et al., 2014). In short, with the InoClic system (i2a), used as described by the manufacturer, a clearly separate colony of the bacteria of interest was picked and suspended in 5 mL of saline solution, resulting in 0.5 McFarland. The suspension was used for flooding the abovementioned agar plates (i2a) and the excess solution was discarded. Antibiotic-impregnated paper disks were applied with a dispenser.

The panel consisted of fourth-generation cephalosporin (cefquinome, 30 μ g), fluoroquinolone (marbofloxacin, 5 μ g), the amoxicillin-clavulanic acid combination (20/10 μ g), aminopenicillin (ampicillin, 10 μ g), first-generation cephalosporin (cephalonium, 30 μ g), macrolide (erythromycin, 15 μ g), lincomycin (15 μ g), oxacillin (1 μ g), aminoglycoside (neomycin, 30 μ g), rifaximin (40 μ g), the sulfonamide-trimethoprim combination (23.75/1.25 μ g), and tetracycline (30 μ g). Disks were purchased from i2a, except for cefquinome, cephalonium, and rifaximin (Mast Group, Merseyside, UK). After an overnight incubation at $35 \pm 2^\circ\text{C}$, plates were read with the SIRscan Micro reader (i2a). The reference strain *Staph. aureus* ATCC 25923 was used for quality control. Oxacillin was used for detecting methicillin-resistant *Staph. aureus*, because no structural difference is observed between oxacillin and cefoxitin testing for this bacterium in the Flemish Milk Control Centre laboratory (Lier, Belgium; data not shown). No further investigation was performed to confirm methicillin resistance.

Statistical Analyses

Before statistical analyses, all outcome and independent variables were transformed into a normal distribution. Because the conventional ways of transformation (e.g., $\log_{10}$, ln, inverse, square root, quadratic) were not sufficient to obtain normally distributed variables, a

2-step approach as described by Templeton (2011) was applied. The first step involved transforming the variable into a percentile rank, which resulted in uniformly distributed probabilities. The second step applied the inverse-normal transformation to the results of the first step to form a variable consisting of normally distributed z -scores. The mean and standard deviation of the original variable were retained to improve the interpretation of the results.

After normalization of the data, the association between stratum-specific herd-level antimicrobial consumption (i.e., strata 1 to 4) and the IZD (in mm) of individual non-*aureus* staphylococci and *Staph. aureus* isolates, clustered on the herd level, was evaluated by fitting several separate linear mixed regression models with the IZD of different active substances (cefquinome, marbofloxacin, amoxicillin-clavulanic acid, ampicillin, erythromycin, cephalonium, lincomycin, oxacillin, neomycin, rifaximin, sulfonamide-trimethoprim, and tetracycline) for non-*aureus* staphylococci and *Staph. aureus*, respectively, as continuous outcome variables. Per outcome variable, a univariable model was first fit with the stratum-specific herd-level total ATI as continuous independent variable, and then a multivariable model was fit including the stratum-specific herd-level ATI for intramammary therapy of (sub)clinical mastitis, ATI for intramammary dry-cow therapy, and ATI for systemically administered therapy as continuous independent variables.

Second, the association between the IZD of individual non-*aureus* staphylococci and the IZD of individual *Staph. aureus* isolates originating from the same herds was studied by fitting univariable linear mixed regression models with the IZD of different active substances (cefquinome, marbofloxacin, amoxicillin-clavulanic acid, ampicillin, cephalonium, erythromycin, lincomycin, neomycin, oxacillin, rifaximin, sulfonamide-trimethoprim, and tetracycline) for the non-*aureus* staphylococci as continuous outcome variables, and the calculated average herd-level IZD of *Staph. aureus* for each antimicrobial substance as continuous independent variable.

All models contained herd as a random effect to adjust for clustering of isolates within a herd and were fit using PROC MIXED in SAS 9.4 (SAS Institute Inc., Cary, NC). The goodness-of-fit measures included $-2 \times \log$ -likelihood, Akaike information criterion, and Bayesian information criterion, using PROC MIXED in SAS 9.4 (SAS Institute Inc.). The conditional Pearson residuals were evaluated graphically and graphed against the normal values and predicted values to check whether the assumptions of normality and homogeneity had been fulfilled, respectively. Also, plots of standardized residuals versus the dependent variables were gen-

erated to check whether the assumption of linearity had been fulfilled. No problems were detected. Significance was assessed at $P \leq 0.05$. As multiple associations were investigated, the false discovery rate was calculated using SPSS 24.0 (SPSS Inc./IBM Corp., Chicago, IL).

RESULTS

Total Antimicrobial Consumption

The average total ATI was 18.73 DDDA per 1,000 cow-days (Table 1), with a large variation between herds, ranging from 6.28 to 42.13 DDDA. β -Lactam antimicrobials, both critically important and not critically important, were used in large amounts in the dairy herds. Third- and fourth-generation cephalosporins and penicillins were used on 47, 50, and 51 herds out of the 56 herds, respectively (Table 1). Fourth-generation cephalosporins had the highest ATI (3.57 DDDA per 1,000 cow-days) followed by penicillins (3.23 DDDA) and third-generation cephalosporins (2.95 DDDA).

Classification of Antimicrobials

Route of Administration and Indication. Antimicrobials administered intramammary had the highest ATI (11.90 DDDA per 1,000 cow-days) followed by systemically administered antimicrobials (6.73 DDDA; Table 1). The use of antimicrobials administered by other routes (e.g., intrauterine) was very low (0.10 DDDA) and therefore not included in this analysis, as mentioned previously. The ATI for intramammary dry-cow therapy was 6.70 DDDA and that for intramammary therapy of (sub)clinical mastitis cases 5.20 DDDA. Dry-cow injectors containing fourth-generation cephalosporins had the highest ATI (1.92 DDDA), followed by first-generation cephalosporins and penicillins (1.68 and 1.30 DDDA, respectively). For the intramammary treatment of (sub)clinical mastitis, the combination first-generation cephalosporins-aminoglycosides had the highest ATI (1.63 DDDA). A large majority of the herds used fourth-generation cephalosporins and the combination of first-generation cephalosporins with aminoglycosides ($n = 39$ and $n = 36$, respectively) for the treatment of (sub)clinical mastitis.

The average ATI for systemically administered therapy was 6.73 DDDA of which third-generation cephalosporins had the highest ATI (2.66 DDDA), followed by penicillins (1.92 DDDA) and macrolides (0.93 DDDA). Penicillins, third-generation cephalosporins, and macrolides were used systemically on 47, 46, and 40 herds, respectively.

Strata of Antimicrobials. The average ATI of critically important antimicrobials was 6.91 DDDA

(range 0.00–26.34 DDDA) whereas that for not critically important antimicrobials was 11.83 DDDA (range 0.00–24.76 DDDA; Table 1).

Critically important β -lactams were used on the majority of the herds, with an average ATI of 6.52 DDDA (range 0.00–26.34 DDDA). Critically important non- β -lactam antimicrobials, in contrast, were only used on 29 herds, with a low average ATI (0.39 DDDA; range 0.00–2.54 DDDA). A similar distribution was observed for the not critically important antimicrobials, where the average ATI of β -lactam antimicrobials was higher than that of non- β -lactam antimicrobials (10.12 and 1.71 DDDA, with a range of 0.52–24.76 and 0.00–6.43 DDDA, respectively).

Distribution of IZD

A total of 239 non-*aureus* staphylococci and 88 *Staph. aureus* isolates originating from 47 and 33 herds, respectively, were submitted to agar disk diffusion testing. The number of tested isolates per herd varied from 1 to 13 and from 1 to 14 for non-*aureus* staphylococci and *Staph. aureus*, respectively. The distribution of IZD of non-*aureus* staphylococci and *Staph. aureus* is presented in Table 2. The median IZD of the non-*aureus* staphylococci and *Staph. aureus* isolates were 33.0 and 34.0 for cefquinome, 30.0 and 29.0 for marbofloxacin, 39.0 and 40.0 for amoxicillin-clavulanic acid, 34.0 and 40.0 for ampicillin, 35.0 and 40.0 for cephalonium, 28.0 and 27.5 for erythromycin, 30.0 and 31.0 for lincomycin, 24.0 and 21.0 for neomycin, 23.0 and 27.0 for oxacillin, 35.0 and 31.0 for rifaximin, 27.0 and 29.0 for sulfonamide-trimethoprim, and 32.0 and 31.0 mm for tetracyclines, respectively.

For some isolates, growth was barely inhibited by the presence of the antimicrobial disk (IZD of 6 mm) in the agar disk diffusion testing, specifically 10 and 1 isolate(s) for the non-*aureus* staphylococci-erythromycin and *Staph. aureus*-erythromycin combination, respectively; 18 and 3 isolates for the non-*aureus* staphylococci-lincomycin and *Staph. aureus*-lincomycin combination, respectively; 3 and 2 for the non-*aureus* staphylococci-marbofloxacin and *Staph. aureus*-marbofloxacin combination, respectively; 5 and 2 isolates for the non-*aureus* staphylococci-sulfonamide-trimethoprim and *Staph. aureus*-sulfonamide-trimethoprim, respectively; 4 isolates for the *Staph. aureus*-oxacillin combination and 2 isolates for the non-*aureus* staphylococci-tetracyclines combination (Table 2).

Associations Between ATI and IZD. Associations between herd-level ATI (i.e., ATI overall, ATI for intramammary therapy of (sub)clinical mastitis, ATI for intramammary dry-cow therapy, and ATI for systemically administered therapy, respectively) and the

IZD of non-*aureus* staphylococci and *Staph. aureus* obtained by Kirby-Bauer disk diffusion are presented in Figure 1.

For non-*aureus* staphylococci, the ATI for systemic therapy of critically important β -lactam antimicrobials was negatively associated with the IZD for cefquinome, whereas the ATI for intramammary therapy of (sub)clinical mastitis of critically important β -lactam antimicrobials was positively associated with the IZD for cefquinome ($P = 0.04$ and $P = 0.05$, false discovery rate = 84 and 51%, respectively).

Association Between IZD of Non-*Aureus* *Staphylococcus* and *Staph. aureus*. Only for neomycin was a positive association between the IZD of non-*aureus* staphylococci and *Staph. aureus* isolates within the same herd observed ($P < 0.01$; false discovery rate: 0.12%).

DISCUSSION

Besides precisely quantifying antimicrobial consumption on several commercial dairy herds, we investigated the association between herd-level antimicrobial consumption and IZD, used as a proxy for the presence of antimicrobial resistance, focusing on non-*aureus* staphylococci (frequently found in milk samples from dairy cows) and *Staph. aureus* (an important major mastitis pathogen).

The average total ATI in this study was 18.73 DDDA per 1,000 cow-days, which is high compared with the 14.35, 3.56, 16.05, 14.28, and 4.66 DDDA per 1,000 cow-days obtained in studies in Canada (Saini et al., 2012a), Austria (Obritzhauser et al., 2016), the Netherlands (Kuipers et al., 2016), Argentina (González Pereyra et al., 2015), and New Zealand (McDougall et al., 2016), respectively. Similar to the data obtained in Austria, critically important (for human health) antimicrobials accounted for a large part of the total antimicrobial consumption (Obritzhauser et al., 2016). However, differences in the calculation of antimicrobial consumption hamper a straightforward comparison of the results between the studies (Collineau et al., 2017). The total ATI on the same herds during 2012 and 2013 was 20.78 DDDA per 1,000 cow-days (Stevens et al., 2016), which is slightly higher than described in this article. Because the current study included data obtained as part of a larger 2-yr project, half of the herds included received mastitis management input on a 4- to 6-weekly basis (intervention group) and 28 herds did not receive mastitis management input but were requested to collect and record the data (control group). In accordance with Kuipers et al. (2016), the expected effect of mastitis management input on a dairy herd's antimicrobial consumption over 2 yr is

Table 1. Antimicrobial treatment incidence (ATI; defined daily doses animal per 1,000 cow-days) of 4 strata of antimicrobials (i.e., critically important β -lactams, critically important non- β -lactams, not critically important β -lactams, and not critically important non- β -lactams), and route of administration and indication used across a convenience sample of 56 Flemish dairy herds from 2013 to 2014

Antimicrobial strata and substances	Route of administration ¹ and indication	No. of herds ²	ATI	Percentile				
				Minimum	25th	50th	75th	Maximum
Total	Overall	56	18.73	6.28	13.13	17.63	22.22	42.13
Critically important β -lactams ³	IMM (sub)clinical mastitis therapy	55	5.20	0.00	2.97	4.49	7.56	12.79
	IMM dry-cow therapy	56	6.73	0.18	5.51	7.31	7.89	11.05
	Systemically administered therapy	56	6.73	0.20	2.75	5.92	8.79	25.03
	Overall	54	6.52	0.00	2.65	5.24	9.55	26.34
	IMM (sub)clinical mastitis therapy	40	1.65	0.00	0.00	0.88	2.38	9.30
	IMM dry-cow therapy	33	1.92	0.00	0.00	0.64	2.87	8.22
	Systemically administered therapy	50	2.95	0.00	0.40	2.11	3.64	20.86
	Overall	47	2.95	0.00	0.45	2.26	3.67	20.86
	IMM (sub)clinical mastitis therapy	5	0.30	0.00	0.00	0.00	0.00	9.30
	IMM dry-cow therapy	—	—	—	—	—	—	—
Cephalosporins (third generation)	Systemically administered therapy	46	2.66	0.00	0.39	1.43	3.51	20.86
	Overall	50	3.57	0.00	0.91	2.57	5.48	19.47
	IMM (sub)clinical mastitis therapy	39	1.38	0.00	0.00	0.55	1.76	7.75
	IMM dry-cow therapy	33	1.92	0.00	0.00	0.64	2.87	8.22
Critically important non- β -lactams ⁴	Systemically administered therapy	19	0.30	0.00	0.00	0.00	0.16	4.55
	Overall	29	—	—	—	—	—	—
	IMM (sub)clinical mastitis therapy	—	—	—	—	—	—	—
	IMM dry-cow therapy	—	—	—	—	—	—	—
Not critically important β -lactams	Systemically administered therapy	29	0.39	0.00	0.00	0.08	0.60	2.54
	Overall	56	10.12	0.52	7.34	9.86	12.06	24.76
	IMM (sub)clinical mastitis therapy	48	3.48	0.00	1.43	3.28	5.10	9.90
	IMM dry-cow therapy	46	4.52	0.00	1.77	4.74	7.53	9.59
	Systemically administered therapy	52	2.10	0.00	0.51	1.37	2.48	13.43
	Overall	13	—	—	—	—	—	—
Aminopenicillins	IMM (sub)clinical mastitis therapy	—	—	—	—	—	—	—
	IMM dry-cow therapy	—	—	—	—	—	—	—
	Systemically administered therapy	13	0.09	0.00	0.00	0.00	0.00	1.16
	Overall	1	—	—	—	—	—	—
Aminopenicillin-clavulanic acid	IMM (sub)clinical mastitis therapy	—	—	—	—	—	—	—
	IMM dry-cow therapy	—	—	—	—	—	—	—
	Systemically administered therapy	—	—	—	—	—	—	—
	Overall	1	0.00	0.00	0.00	0.00	0.00	0.03
Aminopenicillin-polymyxin	IMM (sub)clinical mastitis therapy	2	—	—	—	—	—	—
	IMM dry-cow therapy	—	—	—	—	—	—	—
	Systemically administered therapy	—	—	—	—	—	—	—
	Overall	—	—	—	—	—	—	—
Cephalosporins (first generation)	IMM (sub)clinical mastitis therapy	2	0.00	0.00	0.00	0.00	0.00	0.09
	IMM dry-cow therapy	40	2.97	0.00	0.00	1.12	5.96	11.90
	Systemically administered therapy	27	1.29	0.00	0.00	0.00	1.91	6.76
	IMM (sub)clinical mastitis therapy	19	1.68	0.00	0.00	0.00	2.54	8.75
	IMM dry-cow therapy	19	1.68	0.00	0.00	0.00	2.54	8.75
	Systemically administered therapy	4	0.01	0.00	0.00	0.00	0.00	0.29
Cephalosporin (first generation)-aminoglycoside	Overall	36	—	—	—	—	—	—
	IMM (sub)clinical mastitis therapy	36	1.63	0.00	0.00	0.79	2.55	9.20
	IMM dry-cow therapy	—	—	—	—	—	—	—
	Systemically administered therapy	—	—	—	—	—	—	—
Penicillins	Overall	51	3.23	0.00	0.95	1.89	4.96	14.65
	IMM (sub)clinical mastitis therapy	2	0.01	0.00	0.00	0.00	0.00	0.70
	IMM dry-cow therapy	23	1.30	0.00	0.00	0.00	0.97	7.74
	Systemically administered therapy	47	1.92	0.00	0.31	1.01	2.45	13.33

Continued

Table 1 (Continued). Antimicrobial treatment incidence (ATI; defined daily doses animal per 1,000 cow-days) of 4 strata of antimicrobials (i.e., critically important β -lactams, critically important non- β -lactams, not critically important β -lactams, and not critically important non- β -lactams), and route of administration and indication used across a convenience sample of 56 Flemish dairy herds from 2013 to 2014

Antimicrobial strata and substances	Route of administration ¹ and indication	No. of herds ²	ATI	Percentile				
				Minimum	25th	50th	75th	Maximum
Penicillin-aminopenicillin	Overall	21	0.60	0.00	0.00	0.00	0.49	8.35
	IMM (sub)clinical mastitis therapy	14	0.35	0.00	0.00	0.00	0.03	4.47
	IMM dry-cow therapy	9	0.26	0.00	0.00	0.00	0.00	8.35
	Systemically administered therapy	—	—	—	—	—	—	—
Penicillin-aminoglycoside	Overall	33	1.59	0.00	0.00	0.19	2.05	10.00
	IMM (sub)clinical mastitis therapy	8	0.24	0.00	0.00	0.00	0.00	3.33
	IMM dry-cow therapy	19	1.28	0.00	0.00	0.00	1.75	7.97
	Systemically administered therapy	16	0.08	0.00	0.00	0.00	0.13	0.78
Not critically important non-β-lactams	Overall	50	1.71	0.00	0.54	1.14	2.15	6.43
	IMM (sub)clinical mastitis therapy	7	0.07	0.00	0.00	0.00	0.00	2.14
	IMM dry-cow therapy	12	0.27	0.00	0.00	0.00	0.00	3.86
	Systemically administered therapy	44	1.29	0.00	0.16	0.70	1.61	5.07
Aminoglycosides	Overall	3	—	—	—	—	—	—
	IMM (sub)clinical mastitis therapy	—	—	—	—	—	—	—
	IMM dry-cow therapy	—	—	—	—	—	—	—
	Systemically administered therapy	3	0.01	0.00	0.00	0.00	0.00	0.39
Lincosamides	Overall	4	—	—	—	—	—	—
	IMM (sub)clinical mastitis therapy	4	0.01	0.00	0.00	0.00	0.00	0.20
	IMM dry-cow therapy	—	—	—	—	—	—	—
	Systemically administered therapy	—	—	—	—	—	—	—
Lincosamide-aminoglycoside	Overall	20	0.13	0.00	0.00	0.00	0.13	2.14
	IMM (sub)clinical mastitis therapy	5	0.06	0.00	0.00	0.00	0.00	2.14
	IMM dry-cow therapy	—	—	—	—	—	—	—
	Systemically administered therapy	15	0.06	0.00	0.00	0.00	0.05	0.80
Macrolides	Overall	40	—	—	—	—	—	—
	IMM (sub)clinical mastitis therapy	—	—	—	—	—	—	—
	IMM dry-cow therapy	—	—	—	—	—	—	—
	Systemically administered therapy	40	0.93	0.00	0.00	0.48	1.03	5.07
Polymyxins	Overall	3	—	—	—	—	—	—
	IMM (sub)clinical mastitis therapy	—	—	—	—	—	—	—
	IMM dry-cow therapy	—	—	—	—	—	—	—
	Systemically administered therapy	3	0.00	0.00	0.00	0.00	0.00	0.06
Rifaximins	Overall	12	—	—	—	—	—	—
	IMM (sub)clinical mastitis therapy	12	—	—	—	—	—	—
	IMM dry-cow therapy	—	—	—	—	—	—	—
	Systemically administered therapy	12	0.27	0.00	0.00	0.00	0.00	3.86
Sulfonamide-trimethoprim	Overall	16	—	—	—	—	—	—
	IMM (sub)clinical mastitis therapy	—	—	—	—	—	—	—
	IMM dry-cow therapy	—	—	—	—	—	—	—
	Systemically administered therapy	16	0.13	0.00	0.00	0.00	0.12	1.55
Tetracyclines	Overall	17	—	—	—	—	—	—
	IMM (sub)clinical mastitis therapy	—	—	—	—	—	—	—
	IMM dry-cow therapy	—	—	—	—	—	—	—
	Systemically administered therapy	17	0.23	0.00	0.00	0.00	0.07	3.52

¹IMM = intramammary.

²The number of herds using the specific antimicrobial active substance.

³According to the World Organisation for Animal Health (OIE, Paris, France).

⁴Critically important other than β -lactam.

Table 2. Proportion of non-*aureus* staphylococci (NAS) and *Staphylococcus aureus* (SA) isolates per inhibition zone diameter (IZD, mm) for cefquinome (CFQ), marbofloxacin (MAR), amoxicillin-clavulanic acid combination (AMC), ampicillin (AMP), cephalonium (CEPH), erythronium (ERY), lincomycin (LIN), neomycin (NEO), oxacillin (OXA), rifaximin (RIF), sulfonamide-trimethoprim combination (T/S), and tetracyclines (TET)

Item	CFQ		MAR		AMC		AMP		CEPH		ERY		LIN		NEO		OXA		RIF		T/S		TET	
	NAS	SA	NAS	SA	NAS	SA	NAS	SA	NAS	SA	NAS	SA	NAS	SA	NAS	SA	NAS	SA	NAS	SA	NAS	SA	NAS	SA
No. of isolates	239	88	239	88	239	88	239	88	239	88	239	88	239	88	239	88	239	88	239	88	239	88	239	88
IZD																								
6	0.0	0.0	1.3	2.3	0.0	0.0	0.0	0.0	0.0	0.0	4.2	1.2	7.5	3.4	0.0	0.0	0.0	4.5	0.0	0.0	2.1	2.3	0.8	0.0
7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0	1.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.0
8	0.0	0.0	0.0	1.1	0.0	0.0	0.0	0.0	0.0	0.0	1.3	0.0	1.7	1.1	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.7	2.3
9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.9	1.1	0.0	0.0	0.8	0.0	0.0	0.0	0.4	0.0	2.9	1.1
10	0.0	0.0	0.0	0.0	0.0	0.0	0.4	1.1	0.0	0.0	0.4	0.0	2.9	1.1	0.0	1.1	0.0	0.0	0.0	0.0	0.0	0.0	3.8	1.1
11	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.8	1.1	0.4	0.0	0.8	0.0	0.0	0.0	0.4	0.0	1.7	0.0
12	0.0	0.0	0.4	0.0	0.0	0.0	0.0	2.3	0.0	0.0	0.0	0.0	5.0	1.1	0.0	0.0	0.8	0.0	0.0	0.0	0.4	0.0	1.7	1.1
13	0.0	0.0	0.0	0.0	0.0	0.0	1.3	0.0	0.0	1.1	0.8	0.0	2.1	1.1	0.0	0.0	0.8	1.1	0.0	0.0	0.8	0.0	0.0	3.4
14	0.0	0.0	0.0	0.0	0.0	0.0	0.4	1.1	0.0	0.0	0.4	0.0	1.7	0.0	0.0	0.0	2.9	1.1	0.0	0.0	0.4	0.0	0.0	0.0
15	0.0	0.0	0.0	2.3	0.0	1.1	0.8	1.1	0.0	0.0	0.0	1.2	0.4	1.1	0.0	0.0	1.3	0.0	0.0	0.0	0.4	0.0	0.0	0.0
16	0.0	0.0	0.0	0.0	0.0	0.0	3.8	1.1	0.0	0.0	0.4	0.0	0.4	0.0	0.0	1.1	1.3	0.0	0.0	0.0	0.4	0.0	0.0	0.0
17	0.0	0.0	0.0	0.0	0.0	2.3	4.6	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0	4.5	2.1	1.1	0.0	0.0	0.0	0.0	0.0	0.0
18	0.4	0.0	0.0	0.0	0.0	0.0	5.0	1.1	0.0	0.0	0.4	0.0	2.5	0.0	1.3	9.1	3.3	0.0	0.0	0.0	0.0	0.0	0.4	0.0
19	0.0	0.0	0.0	0.0	0.0	1.1	3.4	0.0	0.0	1.1	0.0	0.0	0.8	1.1	1.7	11.4	5.0	0.0	0.8	0.0	0.8	0.0	0.0	0.0
20	0.0	0.0	0.4	0.0	0.0	1.1	4.6	1.1	0.0	1.1	0.0	0.0	0.0	0.0	4.2	13.6	6.3	5.7	0.4	0.0	0.8	1.1	0.0	0.0
21	0.0	0.0	0.8	0.0	0.0	0.0	5.0	0.0	0.8	2.3	0.4	0.0	0.4	1.1	5.9	25.0	6.3	3.4	0.0	1.1	3.3	2.3	0.0	0.0
22	0.8	2.3	2.1	1.1	0.0	0.0	3.8	0.0	0.4	0.0	1.7	1.2	2.1	1.1	11.7	15.9	10.0	5.7	0.4	0.0	3.3	1.1	0.0	0.0
23	0.4	2.3	1.7	0.0	1.3	1.1	0.8	1.1	0.4	1.1	0.0	4.9	1.3	0.0	13.0	6.8	8.4	3.4	1.3	1.1	3.8	2.3	0.0	0.0
24	1.3	1.1	2.1	0.0	4.6	2.3	2.5	0.0	2.9	1.1	1.3	6.1	2.1	0.0	12.6	2.3	6.7	9.1	0.0	1.1	4.2	0.0	0.0	0.0
25	1.3	0.0	1.3	4.5	2.1	1.1	0.0	0.0	0.8	4.5	5.4	6.1	0.4	3.4	17.6	1.1	7.1	4.5	1.3	1.1	7.5	1.1	0.8	1.1
26	0.4	0.0	3.3	13.6	5.4	0.0	1.7	1.1	4.2	1.1	10.0	14.6	0.8	1.1	10.9	3.4	3.8	6.8	0.4	6.8	13.8	1.1	0.4	3.4
27	3.8	1.1	6.3	12.5	6.3	2.3	1.3	0.0	3.8	0.0	10.0	14.6	1.3	2.3	9.6	1.1	3.3	5.7	4.2	10.2	9.6	9.1	1.3	3.4
28	3.3	3.4	12.6	9.1	5.0	2.3	0.8	0.0	4.6	1.1	15.5	20.7	6.7	6.9	2.5	0.0	4.2	8.0	1.7	11.4	7.5	17.0	2.9	2.3
29	3.3	1.1	17.2	9.1	2.9	2.3	0.8	0.0	5.9	4.5	7.1	11.0	3.8	4.6	1.3	1.1	2.1	3.4	2.9	6.8	11.3	14.8	4.2	8.0
30	6.3	5.7	13.0	5.7	2.9	4.5	0.8	0.0	7.5	3.4	8.4	6.1	4.6	12.6	0.4	0.0	1.7	5.7	4.2	9.1	10.0	9.1	9.6	14.8
31	7.1	6.8	11.3	11.4	7.1	1.1	1.7	3.4	6.3	1.1	5.9	2.4	6.3	16.1	0.8	0.0	6.7	4.5	6.7	5.7	4.6	10.2	8.8	9.1
32	8.8	12.5	5.4	9.1	4.2	1.1	2.9	3.4	2.5	0.0	5.4	6.1	8.8	6.9	0.0	0.0	0.8	6.8	5.4	5.7	5.0	12.5	13.4	9.1
33	15.5	6.8	3.3	3.4	2.5	0.0	2.5	0.0	4.2	1.1	2.9	0.0	7.5	13.8	0.4	0.0	1.7	3.4	9.2	4.5	2.9	11.4	9.2	6.8
34	11.7	12.5	1.7	0.0	0.8	1.1	1.3	0.0	3.8	5.7	1.3	0.0	5.9	3.4	0.4	0.0	1.3	0.0	6.7	4.5	1.3	1.1	11.3	13.6
35	6.7	10.2	2.1	1.1	1.3	1.1	1.3	3.4	5.4	2.3	0.4	0.0	5.9	3.4	0.4	0.0	0.0	1.1	7.5	9.1	0.4	1.1	7.5	9.1
36	6.7	11.4	0.4	0.0	0.8	0.0	4.6	2.3	0.4	5.7	0.8	0.0	3.8	2.3	0.0	0.0	0.0	0.0	3.8	0.0	0.4	0.0	4.6	4.5
37	6.7	9.1	0.4	0.0	0.4	0.0	1.7	3.4	3.3	4.5	0.8	0.0	0.8	2.3	0.4	0.0	0.4	0.0	1.7	0.0	0.4	0.0	5.0	3.4
38	3.8	6.8	0.4	0.0	1.7	1.1	6.3	3.4	3.3	4.5	0.4	0.0	0.8	0.0	0.0	0.0	0.0	0.0	2.1	0.0	0.0	0.0	1.3	0.0
39	2.1	4.5	0.0	0.0	0.8	0.0	7.6	3.4	3.8	1.1	0.0	0.0	0.0	1.1	0.0	0.0	0.0	0.0	2.9	0.0	0.0	0.0	0.0	0.0
40	9.6	2.3	12.6	13.6	49.8	72.7	28.2	63.6	35.1	51.1	4.2	3.7	6.7	2.3	4.2	2.3	10.0	14.8	36.4	21.6	3.3	2.3	6.3	2.3
41	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	9.6	0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
42	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.1	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Figure 1. Visualization of associations between inhibition zone diameters (IZD, mm) (CEFQ, cefquinome; MAR, marbofloxacin; AMC, amoxicillin-clavulanic acid; AMP, ampicillin; CEPH, cephalonium; OXA, oxacillin) of (A) non-*aureus* staphylococci, and (B) *Staphylococcus aureus* from cows with subclinical mastitis and the herd-level antimicrobial consumption expressed as the antimicrobial treatment incidence (ATI) in defined daily dose animal (DDDA) per 1,000 cow-days for 3 strata of antimicrobials: (top) critically important β -lactams (third- and fourth-generation cephalosporins), (middle) critically important non- β -lactams (fluoroquinolones), (bottom) not critically important β -lactams (aminopenicillins, aminopenicillin-clavulanic acid, aminopenicillin-polymyxin, first-generation cephalosporins, first-generation cephalosporin-aminoglycoside, penicillins, penicillin-aminopenicillin, penicillin-aminoglycoside). The horizontal bars represent the β (regression factor) originating from univariable models for ATI overall and from multivariable models for herd-level ATI for intramammary therapy of (sub)clinical mastitis, ATI for intramammary dry-cow therapy, and ATI for systemically administered therapy, respectively (see Materials and Methods). *For non-*aureus* staphylococci, the ATI for systemic therapy of critically important β -lactam antimicrobials was negatively associated with the IZD for cefquinome ($P = 0.04$ with a false-discovery rate of 84%) and the ATI for intramammary therapy of (sub)clinical mastitis of critically important β -lactam antimicrobials was positively associated with the IZD for cefquinome ($P = 0.05$ with false discovery rate of 51%). (1) The β of “ATI overall” is equal to the β of “ATI for systemically administered therapy.” In Belgium, Marbofloxacin is only available for systemic use.

limited. The latter assumption appears to be reinforced by the preliminary results obtained in our work in progress. In fact, antimicrobial consumption and changes in antimicrobial consumption on dairy herds seem to be more influenced by changes in mastitis management over time independently of the group to which they were assigned rather than by whether the farm received mastitis management input from the first author. Given that, we concluded that the effect of the farm-based intervention on the results described in this study and their extrapolation is expected to be very limited or nonexistent. Still, we hypothesize that the establishment of a national center of expertise on antimicrobial consumption and resistance in animals (AMCRA, 2014) in 2012 because of political interest in emerging antimicrobial resistance might have played a role as it evolved into several nationwide awareness campaigns targeting farmers and veterinarians, among other projects that were initiated during the time our study was executed.

The Kirby-Bauer disk diffusion method, performed at the Flemish MCC (Lier, Belgium) as part of routine analyses, has a moderate to high diagnostic accuracy and categorical agreement for most udder pathogen-antimicrobial combinations (Saini et al., 2011), particularly if plates are read by using the SIRscan Micro (i2a) as was done in this study. Because only a limited number of clinical breakpoints (CLSI) and epidemiological cut off values (EUCAST) are available for mastitis-causing bacteria, we refrained from using cut-off values to stratify isolates into susceptible and resistant populations. Rather, we opted to report IZD and use them as a proxy for antimicrobial resistance when looking for associations with antimicrobial consumption. Still, for most *Staph. aureus* and non-*aureus* staphylococci-antimicrobial active substance combinations, a bimodal distribution was observed, indicating the presence of acquired resistance in some isolates. Corresponding to the work of Supré et al. (2014), separation of 2 populations based on the curve was less obvious for the *Staph.*

aureus-ampicillin combination. This indistinctness was also observed for the non-*aureus* staphylococci-oxacillin combination.

The negative association between ATI for systemic therapy of critically important β -lactam antimicrobials and IZD of non-*aureus* staphylococci for cefquinome might be explained by the (partial) commensal character of non-*aureus* staphylococci. Because of that commensal character, non-*aureus* staphylococci are more likely to be exposed to whatever (systemically administered) antimicrobial therapy. The negative association between ATI for systemic therapy of critically important β -lactam antimicrobials and IZD of non-*aureus* staphylococci for cefquinome (representing the critically important β -lactams) is, in this respect, not that surprising and, in fact, confirms selection pressure, because the critically important β -lactam antimicrobials represent about one-third of the total antimicrobial consumption (6.52 DDDA, of which almost 50% was used as systemically administered therapy). Still, caution is needed in the interpretation of the data. Although statistically significant associations between IZD and ATI were found in the present study, this does not prove causality. When studying larger data sets with many variables and associations as in the present study, the probability of finding associations by chance increases substantially (Dohoo et al., 1997). Given that, in theory, for every 20 comparisons, a significant difference will be found purely by coincidence without having a real difference (Dewulf et al., 2009), the associations between ATI for systemic therapy of critically important β -lactam antimicrobials and IZD of cefquinome and the herd-level ATI for intramammary therapy of (sub)clinical mastitis of critically important β -lactam antimicrobials on the one hand and the IZD of non-*aureus* staphylococci for cefquinome on the other hand might be due to chance, as reflected by the high false discovery rates. In addition, factors that potentially influence both IZD and herd-level ATI could act as confounders. Still, including herd as a random effect, as done in this study, takes into account the association between isolates within the same herd, and thus controls for any confounding factor at the herd level (Dohoo et al., 2003).

The most common resistance mechanism in staphylococci is the production of β -lactamase, which results in resistance to penicillin G and aminopenicillins (Persson Waller et al., 2011; Preethirani et al., 2015; Schmidt et al., 2015). In our results, the trend to resistance, as reflected by a smaller IZD for penicillin G and aminopenicillins, was not pronounced, which is in accordance with findings of Sawant et al. (2009). Still, we are cautious in drawing conclusions about antimicrobial resis-

tance in our study, because IZD were used as a proxy for antimicrobial resistance only, and small IZD in the absence of cut-offs and clinical breakpoints are only an indication for the presence of antimicrobial resistance. Moreover, comparison between different studies is likely not legitimate, because different sampling strategies, different susceptibility testing methods (Kirby-Bauer disk diffusion versus broth dilution method), and different interpretive criteria have been used to categorize isolates as either susceptible or resistant. Also, because we did not identify non-*aureus* staphylococci to the species level and susceptibility to antimicrobials varies between staphylococcal species (Sawant et al., 2009; Bal et al., 2010; Persson Waller et al., 2011; Schmidt et al., 2015; Raspanti et al., 2016), part of the large variation might be explained by differences in IZD between non-*aureus* staphylococci species.

The positive association in IZD between non-*aureus* staphylococci and *Staph. aureus* for neomycin could be explained by the fact that many of the genes encoding neomycin resistance reside on mobile genetic elements, which can be exchanged between bacteria of the same or different species occupying the same ecological niche (Lyon and Skurray, 1987; Lindsay and Holden, 2006).

CONCLUSIONS

The average ATI on a convenience sample of Flemish dairy herds was high, with a large between-herd variation. Most antimicrobials were used for intramammary therapy. Fourth-generation cephalosporins, critically important for human health, had the highest ATI. A decrease in the antimicrobial susceptibility of non-*aureus* staphylococci with increasing systemic administration of critically important β -lactam antimicrobials was suggested. In contrast, the use of critically important β -lactam antimicrobials for intramammary treatment of (sub)clinical mastitis did not seem to affect the antimicrobial susceptibility of non-*aureus* staphylococci. The IZD of non-*aureus* staphylococci and *Staph. aureus* were positively associated for neomycin.

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