Survey of marbofloxacin susceptibility of bacteria isolated from cattle with respiratory disease and mastitis in Europe

S. Kroemer, D. Galland, V. Guérin-Faublée, H. Giboin, F. Woehrlé-Fontaine

A monitoring programme conducted in Europe since 1994 to survey the marbofloxacin susceptibility of bacterial pathogens isolated from cattle has established the susceptibility of bacterial strains isolated before any antibiotic treatment from bovine mastitis and bovine respiratory disease (BRD) cases between 2002 and 2008. Minimum inhibitory concentration (MIC) was determined by a standardised microdilution technique. For respiratory pathogens, Pasteurella multocida and Mannheimia haemolytica isolates (751 and 514 strains, respectively) were highly susceptible to marbofloxacin (MIC<0.03 μg/ml for 77.39 per cent of the strains) and only 1.75 per cent of M haemolytica strains were resistant (MIC>4 μg/ml). Histophilus somni isolates (73 strains) were highly susceptible to marbofloxacin (0.008 to 0.06 μg/ml). Mycoplasma bovis MIC (171 strains) ranged from 0.5 to 4 μg/ml. For mastitis pathogens, the majority of Escherichia coli isolates were highly susceptible to marbofloxacin (95.8 per cent of 617 strains). Staphylococcus aureus and coagulase-negative staphylococci (568 and 280 strains) had a homogenous population with MIC centred on 0.25 μg/ml. Streptococcus uberis and Streptococcus dysgalactiae (660 and 217 strains) were moderately susceptible with MIC centred on 1 μg/ml. Marbofloxacin MIC for these various pathogens appeared stable over the seven years of the monitoring programme and was similar to previously published MIC results.

IN veterinary as well as human medicine, the first aim of antibiotic therapy is to eradicate the pathogens from diseased bodies. However, antimicrobial therapies also need to minimise the risk of resistance in pathogenic or commensal bacteria in order to keep these treatments of veterinary pathologies as efficacious as possible over time and to avoid the spread to human beings of antimicrobial resistance of veterinary origin through zoonotic bacteria. This is particularly true for the veterinary use of critical antibiotics such as fluoroquinolones.

As the use of antimicrobial agents is known for selecting resistance mechanisms in bacteria (Schwarz and Chaslus-Dancla 2001), resistance emergence is regularly surveyed. For many years, particularly in Europe, several national monitoring programmes have been set up to survey the emergence of antibiotic resistance, mostly in zoonotic bacteria (VAV network 2006, Wallman 2006, Hendriksen and others 2008, Agence Nationale de Sécurité sanitaire de l’Alimentation de l’Environnement et du Travail 2010, DANMAP 2010). Guidelines for harmonised monitoring and reporting of these antimicrobial resistance survey programmes have also been published by the European authorities (European Food Safety Authority 2005).

From 1994 onwards, Vétoquinol S. A. has been running a monitoring programme in Europe to survey the susceptibility to marbofloxacin of bacteria involved in porcine, bovine, feline and canine diseases sampled before any antibiotic treatment. An initial report of the 1994 to 2001 results has already been published for pets and cattle (Meunier and others 2004a, b).

Considering that the two major medical indications of injectable third-generation fluoroquinolones for adult cattle are bovine respiratory diseases (BRD) and Escherichia coli acute mastitis, an evaluation of the epidemiological data between 2002 and 2006 concerning the main bacteria isolated from these pathologies was undertaken. É. coli, Staphylococcus aureus, coagulase-negative staphylococci, Streptococcus dysgalactiae species dysgalactiae and Streptococcus uberis were isolated from cases with mastitis and Pasteurella multocida, Mannheimia haemolytica, Histophilus somni and Mycoplasma bovis were isolated from cases with BRD.

Materials and methods

Collection of bacterial strains

The epidemiosurvey network was based on a collection of the bacteria isolated from diseased cattle in eight European countries where marbofloxacin was marketed (France, Germany, UK, Belgium, Netherlands, Spain, Italy and Ireland).

Veterinary surgeons were recruited to take samples of acutely sick cattle as part of their daily practice. Respiratory samples were collected...
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by nasopharyngeal swabbing, transtracheal aspiration or by taking a lung sample at postmortem examination if the animal had recently died. For mastitis, a milk sample was collected from the infected quarter. All the samples were tested on animals before any treatment was administered in order to obtain an accurate field representation of the bacterial susceptibility (no antibiotic treatment was given in the three weeks preceding sampling). Moreover, only one sample was taken per herd in order to avoid testing epidemiologically related strains. The samples were sent to the nearest laboratory involved in the study along with the historical record forms including animal and sampling condition data.

Isolation and identification of bacterial strains

Sample cultures were performed following the standard methods. Identification of the bacterial isolates was performed by determining the following phenotypic characteristics: Gram-stained cell morphology; colony morphology and haemolytic power on Columbia agar supplemented with 5 per cent defibrinated sheep blood (bioMérieux), catalase activity for Gram-positive cocci and oxidase activity for Gram-negative bacilli. Afterwards, the isolates were identified to the species level by using API biochemical identification systems (bioMérieux).

More specifically, _M. bovis_ strains were isolated on _Mycoplasma_ agar base supplemented with G-supplement (Oxoid) incubated at 36 ± 2°C with 6 per cent CO₂ for 24 to 96 hours. _Mycoplasma_ strains were identified by colony morphology under a binocular microscope and further by dot immunoassay on membrane filtration following the method described by Poumarat and others (1991). After identification, bacterial strains were stored on cryobeads (AES) at –80°C and sent to the Vétosquinol central laboratory in France by dry-ice shipment.

Marbofloxacin minimum inhibitory concentration determination

The in vitro activity of marbofloxacin was determined by the standard microdilution broth method according to the Clinical and Laboratory Standards Institute guidelines (CLSI 2008a). Mueller-Hinton broth (bioMérieux) was used as the test medium. It was supplemented with 5 per cent sterile horse serum (bioMérieux) to test the susceptibility of streptococci and pasteurellaceae. Minimum inhibitory concentration (MIC) determinations were performed with 96-well microplates containing freeze-dried marbofloxacin solutions (Trek diagnostic systems). With the help of a Mac Farland standard, direct colony suspensions were used for inoculating microplates in order to obtain a final bacterial concentration of 10⁷ to 10⁸ CFU/ml in each well. Microplates were incubated for 18 to 24 hours at 35 ± 2°C for _E. coli_, staphylococci and streptococci and at 36 ± 2°C with 6 per cent CO₂ for _Pasteurella_ lacise isolates.

_S. aureus_ ATCC29213 (MIC range: 0.12 to 0.5 μg/ml) and _E. coli_ ATCC25922 (MIC range: 0.008 to 0.03 μg/ml) were used as reference strains for MIC quality control (CLSI 2008a).

There is no standard method for susceptibility testing of _Mycoplasma_ stains (Hannan 2000). Determination of marbofloxacin MIC for _M. bovis_ was performed according to the technique of Poumarat and Marlet (1989) using _Mycoplasma_ broth base (Oxoid) supplemented with 9.1 per cent yeast extract solution (Becton Dickinson), 9.1 per cent phenolphthalein solution (Sigma-Aldrich) and 18.2 per cent decompromised sterile horse serum (bioMérieux). A 10⁻³ dilution of a 72-hour broth culture was used to inoculate microplates in order to obtain a final bacterial concentration of 10¹ to 10² CFU/ml in each well. Microplates were incubated for a maximum of seven days at 36 ± 2°C with 6 per cent CO₂. _Mycoplasma bovis_ ATCC27748 (MIC range: 0.25 to 1.0 μg/ml) was used for MIC quality control.

The accuracy of these analytical methods is commonly considered to have a one-dilution error margin. Moreover, all the tests were performed in the same central laboratory in order to guarantee the comparability of the MIC results.

Marbofloxacin MIC interpretation

The observation of marbofloxacin MIC distributions has allowed the underlining of different resistant subpopulations. In order to interpret the MIC distribution in subpopulations in terms of likelihood of clinical success, internal breakpoints were established and validated for the aerobic pathogenic Gram-positive or negative bacteria isolated from cattle, pigs and pets, following CLSI guidelines (CLSI 2006b): resistant strains were determined as having a marbofloxacin MIC ≥ 4 μg/ml, strains that had a marbofloxacin MIC = 2 μg/ml were considered as intermediate and susceptible strains had a marbofloxacin MIC ≤ 1 μg/ml.

There are no such sample points validated for _Mycoplasma_ (Hannan and others 1997, Thomas and others 2003).

Results

Between 2002 and 2008, 1509 bacterial strains were collected from BRD cases in Europe by the epidemisurvey network: 751 _P. multocida_, 514 _M. haemolytica_, 171 _M. bovis_ and 73 _H. somni_. During the same period, 2342 bacterial strains were collected from mastitis milk samples: 660 _S. uberis_, 568 _S. aureus_, 220 coagulase-negative staphylococci and 217 _S. dysgalactiae_.

These 3851 isolates were sampled in the eight European countries targeted by the study: 2161 came from France, 413 from UK, 16 from Ireland, 68 from Belgium, 92 from the Netherlands, 51 from Germany, 183 from Italy and 103 from Spain.

Distributions of marbofloxacin MIC over the seven-year period are presented for each bacterial species in Tables 1 and 2. MIC ranges (MIC₉₀ and MIC₉₉₉₉), MIC at which 50 per cent of the isolates are inhibited (MIC₅₀) and MIC at which 90 per cent of the isolates are inhibited (MIC₉₀) are given in Tables 3 and 4. A calculated value of MIC₉₀ₙ₉₉₉ following a linear regression method, is also presented in these tables. For all the presented results, the MIC values for the control strains were within the specified limits.

Respiratory pathogens

_P. multocida_

Table 1 shows a bimodal distribution of marbofloxacin MIC for _P. multocida_. The main population of highly susceptible strains had a MIC range between 0.004 and 0.12 μg/ml, whereas a smaller population was moderately susceptible or intermediate (MIC = 0.12 to 2 μg/ml). 99.73% of the _P. multocida_ strains collected between 2002 and 2008 were classified as susceptible to marbofloxacin. During the last three years of the study (2006, 2007 and 2008), the relative size of the less susceptible population seemed to be decreasing, as indicated by the lower MIC₉₀ for this period (Table 3). Nevertheless, variations between years remained very slight.

_M. haemolytica_

A heterogeneous MIC distribution was clearly observed for _M. haemolytica_, which allowed susceptible (MIC ≤ 0.06 μg/ml), moderately susceptible (MIC = 0.12 to 1 μg/ml) and resistant (MIC ≥ 4 μg/ml) strains to be distinguished (Table 1). Between 2002 and 2008, 92.25 per cent of the _M. haemolytica_ isolates were susceptible to marbofloxacin. As observed with _P. multocida_, the relative size of the last two populations seemed to be decreasing and no resistant strain was identified in 2006, 2007 and 2008 (Table 3).

_M. bovis_

Marbofloxacin MIC for _M. bovis_ ranged from 0.5 to 4 μg/ml (Table 1), demonstrating the existence of a single homogeneous wild population. The number of _M. bovis_ collected each year was low (from 13 to 34 strains per year), especially compared with _P. multocida_ or _M. haemolytica_. Hence, the calculation of MIC₉₀ or MIC₉₉₉₉ per year for this pathogen is not relevant (Table 3).

_H. somni_

Table 1 shows only one population of _H. somni_ strains susceptible to marbofloxacin, with MIC ranging from 0.003 to 0.06 μg/ml.

Mastitis pathogens

_E. coli_

A trimodal distribution was observed for _E. coli_ marbofloxacin MIC (Table 2), with a predominant highly susceptible subpopulation (MIC = 0.008 to 0.06 μg/ml) and two smaller subpopulations with reduced susceptibility (MIC = 0.12 to 1 μg/ml) or resistance (MIC = 4 to 32 μg/ml). Overall, 98.22 per cent of the _E. coli_ mastitis strains col-

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lected between 2002 and 2008 were classified as susceptible to marbofloxacin. No significant change of MIC\textsubscript{50} and MIC\textsubscript{90} was observed over the reported period (Table 4).

**S aureus**

Over the whole considered period, 99.30 per cent of *S aureus* isolates were susceptible to marbofloxacin. MIC for these mastitis strains were all centred on 0.25 μg/ml, constituting a single population (Table 2). Three isolates in 2002 and one in 2005 were intermediate (MIC=2 to 4 μg/ml) and resistant (MIC=8 μg/ml), respectively. MIC\textsubscript{50} and MIC\textsubscript{90} did not show any evolution since 2004 (Table 4).

**Coagulase-negative staphylococci**

Coagulase-negative staphylococci strains were not targeted by the study before 2004, hence the data were only based on a five-year period. Results for coagulase-negative staphylococci isolated from bovine mastitis were very similar to those obtained for *S aureus*, but MIC\textsubscript{50} was a dilution step higher (Tables 2 and 4). Between 2004 and 2008, MIC\textsubscript{50} and MIC\textsubscript{90} did not show any significant evolution (Table 4). Overall, 99.29 per cent of the tested coagulase-negative staphylococci strains were susceptible to marbofloxacin over the five-year period.

**S uberis and *S dysgalactiae***

The patterns of marbofloxacin MIC distributions for *S uberis* and *S dysgalactiae* were similar (Table 2), with only one single moderately susceptible population (MIC=0.25 to 4 μg/ml, with a modal class of 1 μg/ml). MIC\textsubscript{50} and MIC\textsubscript{90} did not significantly change over the seven-year period (Table 4).

**Discussion**

There was no significant evolution of resistance to marbofloxacin for bacterial pathogens involved in BRD from 2002 to 2008, both year on year or compared with the previous period of the survey (Meunier and others 2004a). The most susceptible microorganism remained *H somni*, with very low MIC values, whereas *M haemolytica* continued to be the less susceptible bovine respiratory Pasteurellaceae pathogen, as commonly described.

For both *E coli* and *M haemolytica* strains, the two observed subpopulations with MIC around 0.25 to 0.50 μg/ml and higher than 2 μg/ml have been previously described for fluoroquinolones (Meunier and others 2004a, ANSES 2010). The first population corresponded to reduced susceptibility by means of a single mutation, whereas the second population corresponded to a high level of resistance, which implied multiple chromosomal mutations in the quinolone resistance-determining regions (QRDR) of the gyrA and parC genes coding for gyrase A and topoisomerase IV enzymes (Clarédes and others 2001, Katsuda and others 2009, Ozawa and others 2009). However, the decrease of the resistant subpopulations from 2006 to 2008 seems to be a confirmed trend, as it has also been reported by Hendriksen and others (2008) from the period 2002 to 2004 in Europe.

*H somni* was confirmed to be a very susceptible pathogen, as described by other studies (Aarestrup and others 2004, Meunier and others 2004a). No resistance against marbofloxacin, as for many other antimicrobial agents (Aarestrup and others 2004), has been detected for this respiratory pathogen.

Overall, 99.18 per cent of the 1338 Pasteurellaceae strains identified from respiratory samples in this European study between 2002 and 2008 were classified as susceptible (MIC≤1 μg/ml) and only 0.67 per cent were classified as resistant (MIC≥4 μg/ml). It should also be noted that there was no evolution compared with the 1994 to 2001 period as published by Meunier and others (2004a) for *M haemolytica*, *P multocida* and *H somni*.

Antimicrobial susceptibility to marbofloxacin for *M bovis* strains has not been recently studied. The latest MIC results were published by Thomas and others (2003) with a similar analysis method (Poumarat and Martel 1989). The results presented here confirmed that marbofloxacin MIC distribution against *M bovis* strains was centred on 1 μg/ml. It did not seem to have any evolution over the seven years of the study. Previous studies conducted with third-generation fluoroquinolones, such as enrofloxacin or danofloxacin (Cooper and others 1993, Hannan and others 1997), have concluded that fluoroquinolones had good activities against veterinary *Mycoplasma*, including *M bovis*, with similar MIC ranges as in this study. Enrofloxacin-resistant *M bovis* strains harbouring point mutations in the QRDR regions of the gyrA and parC genes have also been described (Lysnyansky and others 2009). The sequences of gyrA and parC QRDR should be determined for the two strains with MIC=4 μg/ml in order to verify if these strains are resistant mutants or wild isolates with elevated MIC.

There was no significant shift in susceptibility to marbofloxacin for bacterial pathogens involved in bovine mastitis from 2002 to 2006, nor evolution compared with the previous period of the survey (Meunier and others 2004a). The most susceptible microorganism was *E coli* with marbofloxacin MIC values of 0.015 to 0.05 μg/ml, followed by *Staphylococcus* species (modal class MIC of 0.25 μg/ml) and *Streptococcus* species (modal class MIC of 1 μg/ml).
### TABLE 3: MIC\textsubscript{50} and MIC\textsubscript{90} of marbofloxacin (μg/ml) calculated for bacterial species isolated from bovine respiratory pathologies in Europe

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MIC\textsubscript{50} and MIC\textsubscript{90} have not been calculated when the number of strains is lower than 20.

MIC Minimum inhibitory concentration, MIC\textsubscript{min} MIC minimal, MIC\textsubscript{max} MIC maximal, N Number of studied strains.

### TABLE 4: MIC\textsubscript{50} and MIC\textsubscript{90} of marbofloxacin (μg/ml) calculated for bacterial species isolated from cases with bovine mastitis in Europe

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<td>660</td>
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<td>Streptococcus dysgalactiae</td>
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<tr>
<td>MIC\textsubscript{50}</td>
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<td>Linear regression-MIC\textsubscript{90}</td>
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<td>46</td>
<td>42</td>
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</table>

MIC\textsubscript{50} and MIC\textsubscript{90} have not been calculated when the number of strains is lower than 20.

N Number of studied strains, MIC Minimum inhibitory concentration, MIC\textsubscript{min} MIC minimal, MIC\textsubscript{max} MIC maximal.
**E. coli** mastitis isolates showed a predominant highly susceptible subgroup with MIC = 0.015 to 0.03 μg/ml. Two other subpopulations are also shown in Table 2. 15 strains (2.43 per cent) showed a marbofloxacin MIC of 0.12 to 1 μg/ml and 11 strains (1.78 per cent) showed an MIC of 4 to 32 μg/ml over the seven-year period. These subpopulations with reduced susceptibility or resistance to marbofloxacin were commonly observed with **E. coli** from digestive origin (Meunier and others 2004a) and seemed to sporadically emerge in mastitis cases. The number of these strains did not increase significantly between 2002 and 2008, nor during the period from 1994 to 2001 (Meunier and others 2004a). Overall, the susceptibility pattern among **E. coli** isolates from cases of mastitis in adult cows was radically different from the pattern observed among **E. coli** isolates from cases of neonatal diarrhoea.

The therapeutic indication of marbofloxacin in mastitis is the treatment of severe Gram-negative bacterial infections. However, within the scope of this epidemiological survey, there was a great interest to simultaneously follow other major pathogens in order to have as complete an overview of marbofloxacin activity as possible. Hence, *Staphylococcus* and *Streptococcus* marbofloxacin susceptibility has been studied too.

*S. aureus* and coagulase-negative staphylococci strains showed a similar distribution of marbofloxacin MIC, with a susceptible population centred on 0.25 μg/ml. This level of susceptibility to fluoroquinolones is characteristic of *Staphylococcus* species.

There was also just one population corresponding to the marbofloxacin activity against *S. theriae* and *S. dysgalactiae* centred on 1 μg/ml. This reduced susceptibility is also characteristic of *Streptococcus* species.

Marbofloxacin is used in cattle practice as an injectable treatment for acute *E. coli* mastitis and BRD. As a fluoroquinolone antibiotic, its use is restricted to the treatment of individual cases and should be reserved for the treatment of clinical conditions, which have responded poorly (or are expected to respond poorly) to other antimicrobials. It is also recommended that the use of marbofloxacin is based on antimicrobial susceptibility testing. For the cattle practitioner, systematic susceptibility testing of the aetiologic agents is not always achievable. The results presented here can help to guide the veterinary surgeon’s decision making. For BRD, the susceptibility of target pathogens remains very high, especially when compared with the marbofloxacin exposure after a multiple- or single-dose treatment (AUC of 10 or 51 μg h/ml after 2 or 10 mg/kg, respectively) (Alabadi and Lees 2002, Sidhu and others 2011).

In conclusion, this comprehensive epidemiologic survey provides the cattle practitioner with a clear description of marbofloxacin susceptibility patterns in key pathogens of interest for the treatment of BRD or mastitis. At a time when the use of critical antibiotics in veterinary practice is regularly challenged, this study provides clear data on the evolution of fluoroquinolone susceptibility of pathogenic strains of adult cattle.

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