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Microbiological detection of residues of ten different quinolone antibiotics and its application to artificially contaminated poultry samples

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In order to examine if microbiological inhibition tests commonly used for detection of antibiotic residues are suited for routine screening for residues from all commonly used quinolones, the limits of detection (LOD) of 10 different quinolones and fluoroquinolones were determined. Two media were tested, one at pH 6 and one at pH 8, and each was seeded with one of the following test strains: *Bacillus subtilis*, *Escherichia coli*, or *Bacillus cereus*. The LODs of the 10 substances were highest on the plates seeded with *B. cereus*, intended for selective detection of tetracycline residues. The patterns of the zones on the 4 other plates differed for the targeted quinolones: flumequine and oxolinic acid were detected at lower concentrations at pH 6, while the LODs of ciprofloxacin, enrofloxacin, danofloxacin, marbofloxacin, sarafloxacin and norfloxacin were lower at pH 8. Nine of the 10 quinolones were detected easier with *E. coli*, but the LOD of difloxacin was lower with *B. subtilis*. Finally, the three most sensitive media were selected, and fluid from chicken meat spiked with 8 quinolones, at or around MRL levels was analysed on each of the plates. The plate seeded with *E. coli* at pH 8 detected 5 of the 8 quinolones at levels of interest, but an additional *E. coli* plate at PH 6 was necessary for detection of flumequine in species other than poultry and fish. None of the plates detected

oxolinic acid and difloxacin at levels equal to the maximum residue limit (MRL) in muscle tissue.

Keywords: fluoroquinolone, quinolone, antibiotics, residues, screening, meat, agar diffusion tests.

Introduction

Quinolones are a group of synthetic molecules used as antibacterials in human and animal medicine. Nalidixic acid, the first quinolone used as an antibacterial agent, was introduced in 1962. During the following decades, different chemical alterations were made to the quinolone nucleus, resulting in lower toxicity, enhanced antibacterial activity and more advantageous pharmacokinetic properties (Papich and Riviere, 2001).

Several quinolones have been approved for use in food producing animals. In poultry, they are almost always administered via the drinking water. During the last decades however, fluoroquinolone resistance in zoonotic pathogens such as *Campylobacter* has become more frequent, and therefore the approval for veterinary use has been criticized (Pérez-Trallero and Zigorraga, 1995; Nachamkin et al., 2002). Maximum residue limits (MRL) have been established in the European Community for seven quinolones: danofloxacin, difloxacin, enrofloxacin, flumequine, marbofloxacin, oxolinic acid and sarafloxacin. Ciprofloxacin, a quinolone approved for use in human medicine, is also an active metabolite of enrofloxacin, and the sum of both the parent molecule and metabolite should be measured instead of enrofloxacin alone. Sarafloxacin does not have an MRL for muscle tissue in poultry, only in liver and “skin + fat”, while the MRL in “muscle + skin in natural proportions” in salmonidae is $30 \mu\text{g kg}^{-1}$ (Anonymous, 2004; Van Hoof et al. 2005). The six other products have MRLs in muscle ranging from $100\mu\text{g kg}^{-1}$ to $600\mu\text{g kg}^{-1}$, depending on the quinolone and on the animal species. A summary of the MRLs in muscle tissue from different food producing species can be found in Table 1.

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[Insert Table 1 about here]

Animal tissues are often screened for antibacterials using microbiological inhibition tests. They rely on the common property of all antibacterials, namely inhibition of growth of susceptible bacteria, and are thus considered as not specific. In practice, however, such tests do not detect residues belonging to all groups of antibiotics, mainly because the different test strains are not equally sensitive to sufficiently low levels of each antibiotic (Korsrud et al., 1998). For example, a widely used method based on inhibition of growth of *Bacillus stearothermophilus* is able to detect several sulfonamides at levels near to the MRL, β -lactam antibiotics are detected at levels far below the MRL, but the test is not suited for quinolones (Stead et al. 2005). Methods based on the inhibition of *Bacillus subtilis* are also intended for detection of different antibiotics and find lower levels of the quinolones, but methods using *Escherichia coli* as a test strain are considered the best choice for this antibiotic family (Ellerbroek 1991; Okerman et al. 2001; Gaudin et al. 2004).

However, few data obtained with quinolone and fluoroquinolone antibiotics on *B. subtilis* and *E. coli* seeded media have been published. These methods were first considered for analysis of flumequine, enrofloxacin and ciprofloxacin in meat, (Ellerbroek 1991, Okerman et al. 2001). More recently, Gaudin et al. (2004) developed a discriminating method for 66 antibiotics, including 5 quinolones, in milk. They used 5 different media, and found that the *E. coli* seeded plate at pH8 was suited for enrofloxacin, ciprofloxacin, danofloxacin and marbofloxacin, while flumequine was not detected at MRL levels on any of the plates. Media seeded with *E. coli* or with *B. subtilis* at pH6 were not included in the screening method.

In the present study, test media of pH 6 or pH 8, seeded with *B. subtilis* or *E. coli*, were compared for detection of quinolone and fluoroquinolone antibiotics. The LODs of the 10 substances were also determined with media seeded with *Bacillus cereus* at both pH levels, because this test strain is used for detection and presumptive identification of

tetracyclines (Okerman et al. 2001, Gaudin et al. 2004). We found that the quinolones did not behave in a similar way, and that the inhibition patterns of some of them were different from the others, making a presumptive identification of the quinolone antibiotic family more complicated. The practical consequences of these findings are discussed.

Materials and Methods

Media and strains

Six different test media were prepared. Antibiotic Test Agar pH6 and Antibiotic Test Agar pH8 (Merck, Darmstadt, Germany) were constituted and sterilized as prescribed by the manufacturer, and cooled to 45-50°C. One of the following test bacteria was added: *B. subtilis* BGA spore suspension (Merck, 1.10649.0001), *B. cereus* spore suspension (Difco, Detroit, USA; not available any more), or a suspension of *E. coli* strain Bayer 14 (kindly provided by Bayer, Leverkusen, Germany) prepared as described in a previous paper (Okerman et al. 2001). The final concentration of the test strains was in each case between 10^4 – 10^5 colony forming units per mL medium. 90mm plates were filled either with 5mL of the seeded media, for determination of LODs of quinolones dissolved in 10 μ l water, or with 14 mL, for analysis of 100 μ L of spiked meat fluid. Plates were stored before use at 4°C for 7 days maximum.

Antibiotics

The antibiotics enrofloxacin, ciprofloxacin, and nalidixic acid were obtained from ICN biomedicals (Irvine, USA), flumequine, norfloxacin and oxolinic acid from Sigma (St Louis, USA), marbofloxacin from Vetoquinol (Lure, France), sarafloxacin from Solvay Duphar (Weesp, The Netherlands), difloxacin from Fort Dodge Animal Health (Naarden, the Netherlands), and danofloxacin from Pfizer (Sandwich, U.K.). Stock solutions of 1 mg mL⁻¹ were prepared in 0.1N NaOH, divided into aliquots, frozen and kept at -20°C for maximum 2 months.

Determination of LODs

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After thawing, the stock solutions were diluted in distilled water. Series of twofold concentrations were tested, from 8000 ng mL⁻¹ to 62.5 ng mL⁻¹, when necessary. Paper disks with a diameter of 6 mm were laid upon the thin agar layers, and impregnated with 10 µL of the appropriate dilutions. Each dilution that produced zones between 6 mm (the diameter of the paper disk) and 25 mm was tested fourfold on each of the 6 media. The plates were incubated for 16-20h at 30°C, and then controlled for zones of complete inhibition. The lowest concentration that gave at least 3 zones of 12 mm diameter around the 4 paper disks, paper disk included, was considered as the LOD, and was expressed as ng per paper disk.

Preparation and analysis of fortified meat fluid

Frozen fillets from chickens that had not been treated with antibiotics were thawed at room temperature in a clean recipient, and the fluid was collected in tubes. 10% of a solution of one of the quinolones enrofloxacin, ciprofloxacin, flumequine, marbofloxacin, danofloxacin, difloxacin, sarafloxacin or oxolinic acid were added to 90% of this meat fluid. The final concentrations expressed in ng per mL meat fluid were equal to their respective MRLs in µg kg⁻¹. When necessary, concentrations corresponding with the double of the MRLs were also tested. Holes with a diameter of 8mm were punched into the 2mm agar layer and the wells were filled with 100 µL of the artificially contaminated meat fluid. The plates were incubated overnight at 30°C. The diameters of the rings of inhibition, from the edges of the wells till the first visible colonies, were measured. At least 20 observations were done, and they were obtained on two or three different occasions.

Results

Table 2 shows the LODs of the 10 quinolones and fluoroquinolones tested on the 6 different media. All but one of the 10 substances were better detected with *E. coli* than with *B. subtilis*. The LOD of difloxacin was two to four times higher with *E. coli* than with *B. subtilis*, at pH6 as well as at pH8. Plates seeded with *B. cereus* were least sensitive to the 10 quinolones tested.

[Insert Table 2 about here]

The pH did not markedly influence the detection of marbofloxacin, while sarafloxacin, norfloxacin, enrofloxacin, danofloxacin and ciprofloxacin were optimally detected at pH8. On the other hand, difloxacin, oxolinic acid and especially flumequine had lower LODs on the pH 6 media. Nalidixic acid was not detected on any of the plates at levels lower than 40ng per disk.

Figures 1 and 2 illustrate a comparison between the two plates seeded with *E. coli*, and between the *E. coli* pH 8 plate and the *B. subtilis* pH 6 plate. The lines between the LODs of the different quinolones are not concentric on these figures. This means that an optimal pH, that detects all quinolones at the lowest concentration possible, could not be determined, and that neither of the two microorganisms is more sensitive to all quinolones.

[Insert figure 1 about here]

[Insert figure 2 about here]

The results of the artificially contaminated chicken fluid are presented in Table 3. Plates seeded with *B. subtilis* did not detect any of the quinolones at MRL levels in 100µl fluid from chicken muscle. Difloxacin, however, was found at twice the MRL on the pH6 medium seeded with *B. subtilis* (86% positive results), while this concentration was not detected on the plates seeded with *E. coli*.

Best results were obtained on *E. coli* plates for most quinolones: the pH8 medium detected enrofloxacin, ciprofloxacin, danofloxacin and flumequin at MRL levels for poultry, and enrofloxacin, ciprofloxacin, danofloxacin and marbofloxacin at MRL levels for porcine and bovine muscle. The pH6 medium detected flumequine at 400ng per mL

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1 fluid, corresponding with the MRL for poultry, and in 90% of the cases at 200ng per mL,
2 corresponding with the MRL for slaughter animals other than poultry and fish.

3 [Insert Table 3 about here]

4
5 **Discussion**

6
7 Two important conclusions can be drawn from the results obtained with aqueous solutions
8 of quinolones as well as with chicken fluid, spiked with quinolones at levels
9 corresponding with the MRL:

- 10
11 1. The detection capability of the plates differs depending on the quinolone,
12 2. Discrimination between quinolones and other antibiotic families is not possible when
13 only plates seeded with *E. coli* and *B. subtilis* are used.

14
15 The implications of these findings in the field of residue testing can be described as
16 follows.

17
18 *Choice of a plate capable to detect quinolone antibiotics*

19 The LODs of the ten quinolones were highest on plates seeded with *B. cereus*. But, the
20 determination of LODs diluted in distilled water demonstrated different sensitivities of
21 the quinolones to *E. coli* and *B. subtilis*. Moreover, the optimal pH was dependent on the
22 substance. This means that none of the plates can be selected as best choice. Indeed, In
23 order to obtain the lowest LODs of the seven quinolones, a combination of three plates
24 was needed: two seeded with *E. coli*, at pH6 and at pH8, and one seeded with *B. subtilis*,
25 at pH6. The pH8 plate seeded with *E. coli* detected enrofloxacin, ciprofloxacin,
26 flumequine, and danofloxacin in meat fluid spiked at levels equal to the MRL in chicken
27 muscle. Marbofloxacin does not have a MRL for poultry, but levels corresponding with
28 the MRL for bovine and porcine muscle tissue, 150µg kg⁻¹, were detected easily.
29 Flumequine however, with a MRL of 200 µg kg⁻¹ in all animal species other than fish and
30 poultry, was only detected at that level at pH6,

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2 Only three approved quinolones and fluoroquinolones were not detected at sufficiently
3 low levels with a combination of agar diffusion tests: difloxacin, sarafloxacin and
4 oxolinic acid. The pharmacokinetic properties of difloxacin and sarafloxacin suggest that
5 residues in muscle are not at issue, because only in the first days or hours after treatment
6 very low levels of these substances can be found. Moreover, difloxacin is partly
7 metabolised into sarafloxacin *in vivo* (Barrón et al. 2002). Sarafloxacin does not have a
8 MRL in muscle from poultry or from other species except fish, where the MRL relates to
9 skin + muscle in normal proportions. Residues of difloxacin and sarafloxacin remain
10 present for a longer time in liver tissue or in poultry skin and fat. On the other hand, high
11 residue levels occur in all tissues, including muscles, after oral administration of oxolinic
12 acid to broilers.

13
14 Quinolones may also be detected with immunological or with chromatographic methods,
15 such as liquid chromatography coupled to mass spectrometry (Hernandez-Arteseros et al.
16 2002). Chromatographic methods are particularly intended for identification and
17 quantification of separate compounds, and are not considered first choice for screening
18 large numbers of samples, most of which are not likely to contain any residues.
19 Immunological methods are preferred for screening and post screening, as they are
20 specific for one group. Still, these methods are much more labour-intensive and
21 expensive than the classical inhibitor tests. Enzyme immuno assay (EIA) kits have been
22 commercialised by different manufacturers for detection of quinolone residues (for
23 example, Euro-diagnostica, Arnhem, the Netherlands; Randox, Crumlin, Co Antrim,
24 Northern Ireland; CER, Marloie, Belgium). Cross reactivities between different
25 quinolones are however not optimal in most cases. Especially the case of flumequine is
26 problematic. This substance which is used more often to broilers in Belgium and the
27 Netherlands than any other quinolone or fluoroquinolone (Okerman et al. 2004), is not
28 detected with most commercial tests, unless the tests are selective for this antibiotic. The
29 use of two EIA kits for detection of one group is expensive, compared to microbiological
30 screening or even chromatographic detection.

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Discrimination between quinolones and other antibiotic families

Microbiological screening of slaughter animals and poultry for antibiotic residues in general requires the use of more than one medium and/or test bacterium (Korsrud et al.1998). Profit can be taken from this apparent complication to attempt presumptive identifications (Okerman et al. 2001). These provide guidance in the selection of appropriate confirmatory tests. A *B. subtilis*- seeded medium at pH6 can be used for tetracyclines (Okerman et al. 2004), while the present results suggest the use of a pH8 medium seeded with *E. coli* for detection of as many quinolones as possible. However, the fact that some quinolones are better detected with *B. subtilis* seeded pH6 media has consequences on the presumptive identification of antibiotic families in inhibitor positive samples. It is expected that samples contaminated with tetracyclines will produce larger zones on the *B. subtilis* seeded pH6 medium, and samples contaminated with quinolones on *E. coli* seeded pH8 medium (Gaudin et al. 2004). But this is not true for flumequine, difloxacin and sarafloxacin (figure 2). Although the STAR method, as described by Gaudin et al. (2004), requires an incubation of the *E. coli* plate at 37°C, while in the present investigation all incubations were carried out at 30°C, it is highly improbable that the inhibition patterns of quinolones will change at a higher temperature. Media seeded with *B. cereus*, on the other hand, are applied for the presumptive identification of tetracyclines, and the high LODs that we found earlier with enrofloxacin, ciprofloxacin and flumequine on these plates were now confirmed with the other quinolones. Unfortunately, the commercial *B. cereus* spore suspension is no more available, and has to be prepared with own techniques, which is not convenient for many routine residue labs. The *B. cereus* based method can be replaced by a group specific test for tetracyclines, such as a receptor assay (Okerman et al. 2004).

Practical conclusions

The addition of a pH8 medium seeded with *E. coli* Bayer 14 in a microbiological screening system allows the detection of 5 fluoroquinolones at MRL levels in muscle tissue from poultry. A second plate with a pH6 medium, seeded with the same test

bacterium, is necessary to detect flumequine at MRL levels in species other than poultry and fish. All samples producing zones on one or two of the *E. coli* plates should be considered as suspect for the presence of quinolones. Samples producing zones on a pH6 medium seeded with *B. subtilis* are most likely to contain tetracyclines, but the presence of fluoroquinolones cannot be excluded.

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Table 1. MRLs ($\mu\text{g kg}^{-1}$) of quinolones in muscle tissue of different food producing animals.

	Bovine	Ovine, caprine	Porcine	Poultry	Rabbits	Fish	Other species
Danofloxacin	200	200	100	200	100	100	100
Difloxacin	400	400	400	300	300	300	300
Enro + ciprofloxacin	100	100	100	100	100	100	100
Flumequine	200	200	200	400	200	600	200
Marbofloxacin	150		150				
Sarafloxacin						30 *	
Oxolinic acid			100	100		100	

* MRL refers only to salmonidae, muscle and skin in natural proportions

Table 2. LODs (ng/paper disk) of 10 quinolones dissolved in water on 6 media.

	<i>E. coli</i> Bayer 14		<i>B. subtilis</i> BGA		<i>B. cereus</i>	
	pH6	pH8	pH6	pH8	pH6	pH8
Enrofloxacin	1.25	0.625	20	10	>40	40
Ciprofloxacin	2.5	1.25	20	10	40	40
Difloxacin	40	>40	10	20	>40	>40
Flumequine	5	40	20	>40	>40	>40
Sarafloxacin	20	5	20	10	>40	40
Marbofloxacin	2.5	2.5	10	10	>40	40
Norfloxacin	10	5	40	40	>40	>40
Danofloxacin	5	1.25	10	2.5	>40	40
Nalidixic acid	40	>40	>40	>40	>40	>40
Oxolinic acid	5	20	10	>40	40	>40

Table 3. Detection of 8 quinolones in fluid from chicken muscle spiked at levels of interest with 4 agar diffusion tests.

		Range of zones (number detected/number analysed)			
		<i>E. coli</i> pH6	<i>E. coli</i> pH8	<i>B. subtilis</i> pH6	<i>B. subtilis</i> pH8
Enrofloxacin	100 ng mL ⁻¹	ND *	3.1-5.1 (25/25)	ND	ND
Ciprofloxacin	100 ng mL ⁻¹	1.2-6.3 (25/25)	4.7-5.5 (25/25)	ND	ND
Difloxacin	300 ng mL ⁻¹	ND	ND	ND	ND
Difloxacin	600 ng mL ⁻¹	ND	ND	0-5.7 (26/30)	ND
Flumequine	200 ng mL ⁻¹	0-1.6 (18/20)	ND	ND	ND
Flumequine	400 ng mL ⁻¹	1.6-4.1 (25/25)	1.1-2.4 (25/25)	ND	ND
Sarafloxacin	100 ng mL ⁻¹	ND	ND	ND	ND
Sarafloxacin	200 ng mL ⁻¹	ND	0-2.6 (10/20)	ND	ND
Marbofloxacin	150 ng mL ⁻¹	0-5.1 (23/25)	4.1-5.8 (20/20)	ND	ND
Danofloxacin	100 ng mL ⁻¹	ND	4.2-5.2 (20/20)	ND	ND
Danofloxacin	200 ng mL ⁻¹	ND	4.5-7.5 (25/25)	ND	0-4,3 (19/20)
Oxolinic acid	100 ng mL ⁻¹	ND	ND	ND	ND
Oxolinic acid	200 ng mL ⁻¹	0-3.7 (19/20)	0.2-2.5 (25/25)	ND	ND

* ND = not detected: no zones were observed in more than 50% of the cases.

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Figure 1

Figure 2

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	<i>E. coli</i> pH6	<i>E. coli</i> pH8		<i>B.subtilis</i> pH6
SARX	20	5	SARX	20
ENRX	1.25	0.625	ENRX	20
CIPX	2.5	1.25	CIPX	20
DANX	5	1.25	DANX	10
MARX	2.5	2.5	MARX	10
OXOL	5	20	OXOL	10
FLUM	5	40	FLUM	20
DIFX	40	40	DIFX	10

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<i>E. coli</i> pH8	MRL
5	3
0.625	10
1.25	10
1.25	10
2.5	15
20	10
40	20
40	30

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