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Microbial screening for quinolones residues in cow milk by bio-optical method

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ABSTRACT

The use of antibiotics on lactating cows should be monitored for the possible risk of milk contamination with residues. Accordingly, Maximum Residue Levels (MRLs) are established by the European Commission to guarantee consumers safety. As pointed out by Dec 2002/657/EC, screening is the first step in the strategy for antibiotic residue control, thus playing a key role in the whole control procedure. However, current routine screening methods applied in milk chain still fail to detect residues of quinolones at concentrations of interest.

This paper reports the findings of a new bio-optical method for the screening of quinolones residues in bovine milk, based on *E. coli* ATCC 11303 growth inhibition.

The effect of blank and spiked cow milk samples (aliquots equivalents to 0.8%, v/v) is evaluated in Mueller Hinton Broth (MHb) and MHb enriched with $MgSO_4$ 2% (MHb-Mg) inoculated with the test strain at the concentration of 10^4 CFU/mL.

The presence of quinolones inhibits the cellular growth in MHb, while this effect is neutralized in MHb-Mg allowing both detection and presumptive identification of quinolones.

Growth of the test strain is monitored at 37 °C in a Bioscreen C automated system, and Optical Density (OD) at 600 nm is recorded every 10 min after shaking for 10 s. Growth curves (OD *vs.* time) of *E. coli* ATCC 11303 are assessed in milk samples, with and without quinolones, and their differences in terms of ΔOD ($\Delta OD_{600nm} = OD_{MHb\text{-}Mg} - OD_{MHb}$) are calculated.

The presence of quinolones is detected by the cellular growth inhibition (OD *vs* time, none increase in the value OD) and presumptively identified through the increase of the slope of ΔOD_{600nm} curve (ΔOD *vs.* time), after about 3 h of incubation.

The detection limit for ciprofloxacin and enrofloxacin is at the level of MRL, for marbofloxacin is at 2-fold the MRL whereas for danofloxacin is at 4-fold the MRL. Although the sensitivity of the method could be further improved and the procedure automated, it is a promising step forward to integrate screening assays into the control process and, in particular, to fill in the gap for quinolones; moreover, these technological developments contribute to the One Health perspective through the monitoring of safe and correct use of veterinary antibiotics.

1. Introduction

Council Regulation (EEC) No. 2377/90 [1] defines the residues of veterinary medicinal products as *pharmacologically active substances which remain in foodstuffs from treated animals*. Moreover, it lays down a community procedure for the establishment of Maximum Residue Limits (MRLs), defined as the *maximum residue concentrations legally permitted and recognized as acceptable in a food in accordance with recognized principles of safety assessment*. Among veterinary drugs, antibiotics are, broadly speaking, the most important group: for instance in dairy cows, treatment of mastitis and, to a less extent, of foot infections requires antibiotic

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use; substances and treatment protocols are variable, depending on the infectious agents involved as well as on the presence of antibiotic resistance. Notwithstanding the undisputed usefulness of antibiotics to protect farm animal health, the presence of veterinary antibiotics' residues in foods of animal origin is a matter of concern for public health [2]. While some antibiotic groups show also specific target organ toxicity at higher dose levels (e.g., ototoxicity and nephrotoxicity for aminoglysides, bone toxicity for fluoroquinolones) the main health effects used for the safety assessment of antibiotics are generally the elicitation of allergic reactions and, most important the effects related to antimicrobial activity, namely: altered gut flora and increased pressure selection towards antibiotic resistance [3]. The latter, in particular, currently is the problem considered most alarming [4]. The inclusions of antibiotic resistance in the modern and extended view of zoonoses [5,6] originates from the wide use of similar active principles in humans and food producing animals; this is related to the steadily growing number of antibiotic-resistant bacterial strains and the subsequent decrease of therapeutic usefulness of antibiotics to treat animal and human diseases. Quinolones are one major group of veterinary antibiotics; quinolones (albeit different compounds) are also used to treat a variety of bacterial infections in humans. As in the case of other antibacterial agents, the rise in quinolone resistance threatens the human clinical utility of this important class and, through cross-resistance to other antimicrobial classes as well. The level of public health attention toward the issue of antibiotic resistance has led WHO to define fluoroquinolones, 3rd and 4th generation cephalosporins, and macrolides as critically important antibiotics for which risks management measures are urgent [7].

According to the European Report for 2012 on the results from the monitoring of veterinary medicinal product residues in foods of animal origin [8], the overall presence of samples with non-compliant residues of antibacterials is low (0.18%) and decreasing in Europe: in bovine tissues the non-compliant samples are 0.24%, while in milk are 0.05% only. These figures seem to indicate the widespread achievement of a high level of safety, which has to be supported and maintained, also by developing new and cost-effective tools [9] but, in the meantime, pose the question on the real rate of false compliant samples. Actually, the use of "routine screening" methods that fail to detect some classes of antibiotics at the level of interest, implies a possible underestimation of non-compliant samples.

Residue analysis is a very broad area, encompassing registered veterinary medicinal products for which the MRLs have been fixed as well as banned substances. The analytical strategy for antibiotic residues control is based on the combined use of screening and confirmatory methods, whereas the concept of routine methods and reference methods has been superseded by criteria approach, in which performance criteria and procedures for the validation of screening and confirmatory methods are established [2]. A screening method should be able to detect the presence of a substance or class of substances at the level of interest in a given matrix, as well as have the capability for a high sample throughput. For consumer health protection, screening methods are specifically designed to avoid false compliant results, i.e., sensitivity is more important than specificity. In the case of a suspected non-compliant result, this shall be confirmed by a confirmatory method to obtain full or complementary information enabling identification and eventually quantification. In a food safety perspective, it should not be overlooked that the microbiological tests do provide a biologically relevant signal, i.e., reveal the presence of a concentration able to exert an inhibitory effect on the growth of sensitive bacteria. Indeed, it is currently envisaged that the analytical limits for non-allowed veterinary drugs (e.g., the antibiotic chloramphenicol) are integrated by limits based on safety-relevant endpoints [10]. Moreover, a positive response to the microbiological tests might account for the combined effect of two or more antibacterials, albeit each might be present at concentrations below the MRL; in the field of pesticide residues, the European Food Safety Authority has already pointed out that different substances having the same effect should be considered together in risk assessment [11]. The development of up-to-date tests based on biologically relevant endpoints is highly relevant both to achieve a "compliant–not compliant" outcome and in food chain self-management [9,12,13]: even screening methods with detection limit higher than the MRL may be still useful to address risk management measures by the food business operator, e.g., as a tool for the early detection of potential hazards, as suggested by the EU White Paper on Food Safety [13], Tests based on biological endpoints have to cope with the background noise of such matrices as milk: for instance, a rate of false positives close to 30% has been observed for a carboxypeptidase-biosensor for the analysis of beta-lactams in milk [14], which would make the assay unsuitable as a decision-making tool. Nowadays, commercially available screening methods for the detection of inhibitors/antibiotic residues in milk lists dozens tests. These essentially consist in microbial tests or enzymatic, immunological and receptor based assays [15]. Commercial microbial tests for milk screening normally are designed to guarantee an easy use in routine conditions and to deliver results in about 3 h. They are based on one microbial strain, i.e., *Geobacillus stearothermophilus* or *Streptococcus thermophilus*, both characterized by a spectrum of sensitivities which, albeit wide, is not complete: in fact, these strains are unfit for the detection at the levels of interest of some relevant antibiotic families like quinolones.

A wider spectrum of detectable antibiotic residues combined with better sensitivities can be achieved by the use of additional sensitive strains as test microrganisms. This is usually obtained in non-commercial systems which can use up till 18 plates. The plate for quinolone detection is normally prepared with a suspension of *E. coli* ATCC 11303 in an agar medium at different pH values as reported by different authors: [16–20]. In particular, the latter method reported has been validated in milk for 10 antibiotic groups; within quinolones, it allows the detection of enrofloxacin, ciprofloxacin, marbofloxacin and danofloxacin at concentrations $\leq$ MRLs and of flumequine at concentrations > 4 MRLs. Notwithstanding the good sensitivity, these methods are rather cumbersome and produce results in at least 18 h, resulting unsuitable for routine milk control that needs shorter response times. With regard to the response time, a novel microbiological system in microtitre plates for the detection in 4–6 h of beta-lactams, tetracyclines, sulfonamides and quinolones in milk was recently proposed [21]. The improved response time and the introduction of quinolones in the spectrum of detectable antibiotics at the level of interest make this method very efficient and handy to use in the routine practice. Very time-effective responses, combined with good sensitivities, can be achieved by EIA methods and ROSA (Rapid One Step Assay) technology-based methods. Unfortunately these are rather expensive for routine control and cover a limited range of substances; therefore, the advantage of the short response time is lost when these methods have to be used in combination with microbial kits to control the sample for a wider range of potential antibiotic residues.

Based on the need to update tools for safety management of antibiotics in animal production, and keeping in mind the link between the veterinary use of quinolones and the selection of resistant strains of pathogens such as *Salmonella* and *Campylobacter* [4,7], the present work aimed to develop a method for the detection of different fluoroquinolones in milk at concentrations of interest (i.e., corresponding to MRL) and with response times comparable to those of commercial microbial kits. This method was intended to integrate the current screening control for antibiotic residues, acting also as deterrent for the intentional improper use of quinolones.

2. Materials and methods

2.1. Method concept and overview

The method is a microbial inhibitor test in which the growth of the quinolone-sensitive strain *E. coli* ATCC 11303 is evaluated in broth media in presence of milk samples. Bioscreen C turbidimetric analyzer is used to monitor the cell growth kinetics by bio-optical method, i.e., turbidimetric spectrophotometry, in appropriate culture media at 37 °C. This automated system allowed the *in-continuum* registration of the OD (due to optical cell absorption) at 600 nm *versus* time, so to build the growth curves of the test strain. A delay in *E. coli* ATCC 11303 growth indicates the presence of an inhibitory substance in the milk sample.

The parallel use of a medium containing $MgSO_4$ to neutralize the quinolones inhibitory activity, allows also the simultaneous presumptive identification of the quinolone inhibitor when actually present.

2.2. Antibiotic agents

Analytical grade powders of ciprofloxacin (CIPRO), enrofloxacin (ENRO), marbofloxacin (MAR), danofloxacin (DANO), flumequine (FLU), benzylpenicillin (PEN), tetracicline (TC), oxytetracycline (OTC), sulfadiazine (SDZ), streptomycin (STR) and dihydrostreptomycin (DHS) were purchased from Sigma–Aldrich (Milan, Italy). The stock solutions were prepared at active substance concentration of 1 mg/mL. Aliquots were stored at −20 °C and subsequently diluted with ultra-pure deionized water (Millipore, Bedford, MA, USA) to 10 fold the final concentrations to be tested in milk.

2.3. Samples preparation

Raw milk samples were provided by an organic farm in the Lazio region (Italy), from cows that had never been never treated with antibiotics, in frozen aliquots of 50 mL. Pasteurized milk samples were purchased from a local (Lazio) market and refrigerated and the absence of antibiotics was tested for comparison with a lyophilized blank pasteurized milk. This was the blank reference material prepared in occasion of a Proficiency Test for the detection of antibiotic residues in milk, organized by the Italian National Reference Laboratory for veterinary antimicrobial residues in food from animal origin. Samples thawing and spiking were performed on the same day of testing. Ciprofloxacin, enrofloxacin, marbofloxacin, danofloxacin and flumequine were spiked in milk at the final concentrations of 0.2–8 folds the respective MRL in milk while benzylpenicillin, tetracycline, oxytetracycline, sulfadiazine, streptomycin and dihydrostreptomycin were added at the final concentration of 5–10 folds MRL for the specificity assay.

The above mentioned MRLs are reported in Table 1 [22].

Table 1
Molecules tested in the study and respective EU-MRLs in milk.

Pharmacologically active substance	MRL in milk (μg/kg)
Benzylpenicillin	4
Danofloxacin	30
Dihydrostreptomycin	200
Enrofloxacin	100 (sum of enrofloxacin and ciprofloxacin)
Flumequine	50
Marbofloxacin	75
Oxytetracycline	100
Streptomycin	200
Sulfadiazine	100
Tetracycline	100

In all the cases, the volume of the antibiotic working solutions added to milk never exceeded the 10% of the final volume of the sample put in culture media.

In order to eliminate the interference of natural flora in raw milk, and considering the thermal stability of quinolones [23], blank and spiked raw milk samples were heated in water bath at 83 °C for 5 min, immediately cooled down and kept at 4 °C until the assay. Blank and spiked pasteurized milk samples were kept at 4 °C until the assay.

2.4. Culture media

The culture media were purchased from Oxoid (Milan, Italy), and prepared and sterilized according to the supplier indications. Mueller Hinton Broth (MHb) and MHb enriched with $MgSO_4$ 2% (w/v) (MHb-Mg) were respectively used for the detection and presumptive identification of quinolones due to the quinolone-neutralizing effect of $MgSO_4$ described in solid detection media [19,24].

Plate count agar (PCA) was used as non selective medium for growth and enumeration of *E. coli* ATCC 11303.

2.5. Test microorganism and culture conditions

The test microorganism *E. coli* ATCC 11303 was stored at −80 °C in Microbank® beads (ProLab Diagnostics, Canada) according to the manufacturer's instructions. Before each experiment, one cryovial bead was streaked on the surface of a plate of PCA medium and incubated at 37 ± 1 °C for 18–24 h. The inoculum was prepared from an isolated colony previously checked for purity [25] and aseptically inoculated into 10 mL of sterile MHb. After incubation for 18–24 h at 37 °C, the culture was diluted in MHb to the OD of 0.1 at 600 nm (Smart SpecPlus spectrophotometer; Bio-Rad, Munich, Germany).

Serially dilutions of the standardized cell suspension were performed in sterile saline solution 0.85% (w/v) and the approximate concentration (CFU/mL) was verified by viable cell counts on PCA.

2.6. Protocol of bioscreen C analysis

The *E. coli* ATCC 11303 growth in presence of blank and spiked milk samples inoculated in MHb and MHb-Mg was monitored by Bioscreen C (Oy Growth Curves AB Ltd., Helsinki, Finland). Bioscreen C is a turbidimetric analyzer for disposable 100-well honeycomb plates allowing the control of up till 200 samples simultaneously.

Previously optimized conditions for the experimental plan finalized to quinolones detection in milk resulted equivalent to: total volume/well = 400 μL (86.7%, v/v of test medium, 0.8%, v/v of milk sample and 12.5%, v/v of diluted cell suspensio at the well concentration of about 10^4 CFU/mL).

The assay temperature was set to 37.0 ± 1 °C and a preheating step of 10 min was included.

$OD_{600\ nm}$ values were recorded at 10 min intervals after a 10 s shaking and for a total run length of almost 5 h.

All the recorded data ($OD_{600\ nm}$) were collected in a MS Excel workbook automatically generated in a protected mode by the Research Express software. For each experiment, data were elaborated in a spreadsheet (Excel 2007): the averages of the 5 replicates were used to generate turbidity versus time graphs (growth curves) relative to the test strain in the two test media, in presence of blank or spiked milk sample.

2.7. Growth curves interpretation

In presence of quinolones spiked samples, the growth curves in MHb and in MHb-Mg showed different slopes. Conversely, in

presence of blank samples the microorganism's growth curves were very close but not parallel. Hence, a criterion for standardized interpretation of the growth curves in the system MHb/MHb-Mg turned out as necessary to distinguish between blank and spiked milk samples.

For all the samples tested, the deviations between the two growth curves (in MHb and MHb-Mg) were quantified and plotted in $\Delta OD_{600\,nm}$ versus time graphs: $\Delta OD_{600\,nm}$ values were calculated as the difference between the $OD_{600\,nm}$ data recorded in MHb-Mg and those recorded in MHb. The trend of $\Delta OD_{600\,nm}$ values for blank and spiked milk samples was obtained under repeatability conditions.

Routine microbial kits are based on the evaluation of the test microorganism growth through a pH or redox indicator. The change of the colour at the reading time means the absence of inhibitory substances at the detection level. A control sample (blank) is required to define the optimal reading time before losses in sensitivity due to the progress of the strain growth.

The absence of a blank sample exactly equal to the sample under analysis implies the comparison of the microorganism growth in different conditions. On the basis of these considerations, two threshold curves for raw and pasteurized milk blank samples were elaborated. The confidence intervals which characterized the ΔOD_{600nm} dispersion for blank raw and for blank pasteurized milk samples, were calculated with a 95% probability. The blank threshold curve was used as internal calibration to compensate for the $MgSO_4$ effect exerted on the growth of *E. coli* ATCC 11303 in presence of raw or pasteurized blank milk samples.

The availability of a blank curve calculated from averaged results of a number of different samples minimizes the mentioned bias.

3. Results and discussion

Sensitivity towards quinolones makes *E. coli* ATCC 11303 an appropriate microorganism, used for the microbial screening of residues by multiplate assays [26]. Hence the *E. coli* ATCC 11303 has been chosen as test microorganism for the development of a bio-optical method for the detection of fluoroquinolones in raw and pasteurized milk.

3.1. Definition of parameters for optimal detection conditions

The effect exerted by milk samples spiked with fluoroquinolones at concentrations of interest on *E. coli* ATCC 11303 was evaluated simultaneously in two liquid media (MHb and MHb-Mg) respectively for the detection and presumptive identification. Preliminary tests were performed to determine the optimal volume of milk under our assay conditions, considering its optical interference (opacity). As the test relies on turbidimetric measures, the initial turbidity of the sample is by itself a limiting factor: milk aliquots, with and without the fluoroquinolone ciprofloxacin, were added to the test medium in percentages ranging 0.6–50% (v/v) to evaluate the optical interference. Each aliquot was tested in presence of different final concentrations of *E. coli* ATCC 11303 (10^3–10^5 CFU/mL). Microorganism concentration equivalent to 10^4 CFU/mL combined with 0.8% (v/v) of milk resulted the optimal analytical condition for the detection of ciprofloxacin at levels of interest starting from 3 h of incubation. In fact, tests realized with the concentrations of 10^3 CFU/mL and 10^5 CFU/mL affected response times and sensitivities respectively. Final percentages of milk >1% caused interferences severely affecting the optical signals whereas percentages <0.8% led to an excessive dilution of the sample causing a loss in sensitivity.

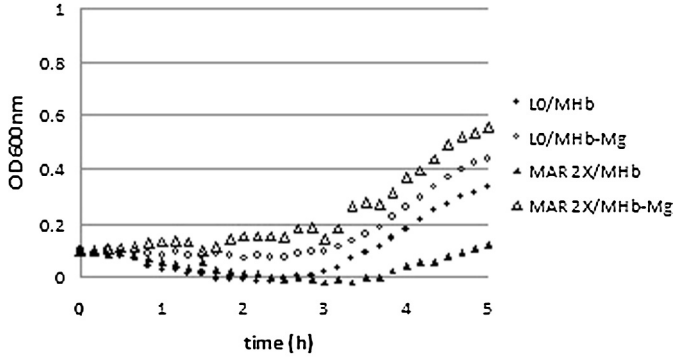

Fig. 1. *E. coli* ATCC11303 growth curves in the system MHb/MHb-Mg: mean turbidity values (OD_{600nm}; %RSD ≤ 5%) in presence of 10 blank raw milk samples (L0) and 10 raw milk samples spiked with marbofloxacin (MAR) 2 × MRL. Experimental results (mean values, 5 replicates) are plotted versus time.

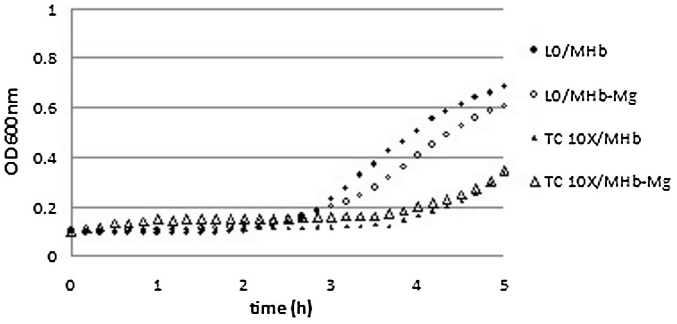

Fig. 2. *E. coli* ATCC11303 growth curves in the system MHb/MHb-Mg: mean turbidity values ($OD_{600\,nm}$; %RSD ≤ 5%) in presence of 10 blank pasteurized milk samples (L0) and 10 pasteurized milk samples spiked with tetracycline (TC) 10 × MRL. Experimental results (mean values, 5 replicates) are plotted versus time.

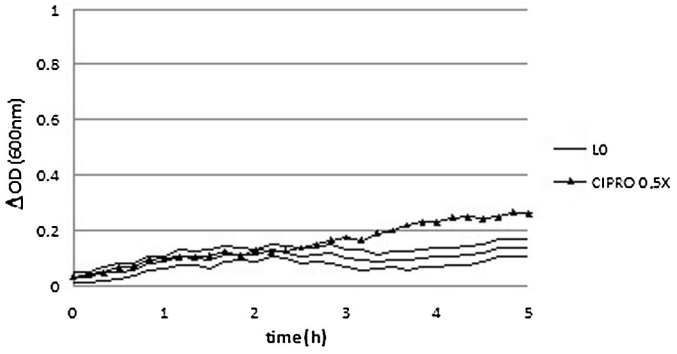

Fig. 3. *E. coli* ATCC11303 ΔOD_{600nm} curve in presence of 10 raw milk samples spiked with ciprofloxacin (CIPRO) 0.5 × MRL. The threshold curve elaborated for 10 raw milk samples (L0) and the respective confidence interval are shown. ΔOD_{600nm} values (mean values, 5 replicates) are plotted versus time (%RSD ≤ 5%).

3.2. Growth curves in MHB

For each Bioscreen C experiment, the averaged turbidity values ($OD_{600\,nm}$) of 5 replicates were plotted *versus* time in growth curves graphs (Figs. 1–5). %RSD resulted ≤5% for all data. Results proved that the growth of *E. coli* ATCC 11303 in MHB was strongly delayed in presence of milk samples spiked with CIPRO and ENRO (1 MRL), MAR (2 MRL) and DANO (4 MRL), thus proving the ability to detect residues of fluoroquinolones in milk.

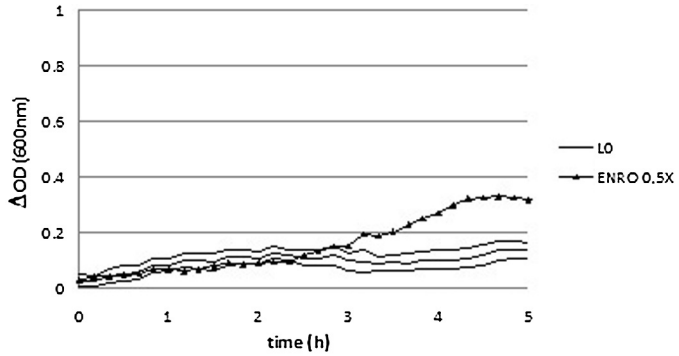

Fig. 4. *E. coli* ATCC11303 ΔOD_{600nm} curve in presence of 10 raw milk samples spiked with enrofloxacin (ENRO) 0.5 × MRL. The threshold curve elaborated for 10 raw milk samples (L0) and the respective confidence interval are shown. ΔOD_{600nm} values (mean values, 5 replicates) are plotted versus time (%RSD ≤ 5%).

3.3. Method's specificity towards quinolones: the neutralizing effect of $MgSO_4$

The perspective to define a specific method to take advantage in addressing the chemical confirmation required in cases of non-compliant samples [2] led us to investigate the applicability of $MgSO_4$ as quinolones neutralizing agent [19,24] also in broth media to run their presumptive identification.

Growth rates of *E. coli* ATCC 11303 in MHb supplemented with different concentrations of $MgSO_4$ ranging 0–4% (w/v) were monitored in presence of blank milk samples. Under these conditions, the microorganism growth was inhibited in MHb enriched with $MgSO_4$ at concentrations ≥4% (w/v), whereas the 2% concentration produced a growth curve overlapping the one in MHB, with maximum deviation in OD of about 0.1. $MgSO_4$ (2%) was hence added to MHb to obtain a test medium for the presumptive identification tests.

The simultaneous assay of milk samples spiked with fluoroquinolones in the system MHb/MHb-Mg evidenced the microorganism growth delay only in MHb. The different trend produced a typical "fork effect" between the spiked sample's growth curves in the two media (MHb and MHb-Mg), as shown in Fig. 1 for samples spiked with marbofloxacin 2 MRL. Therefore, $MgSO_4$ resulted effective to neutralize the inhibitory effect of fluoroquinolones.

Tests run in presence of benzylpenicillin, oxytetracycline, tetracycline, sulfadiazine, dihydrostreptomycin and streptomycin (spiked in concentrations up to 10 MRL) showed that the growth curves produced in the system MHb/MHb-Mg were totally

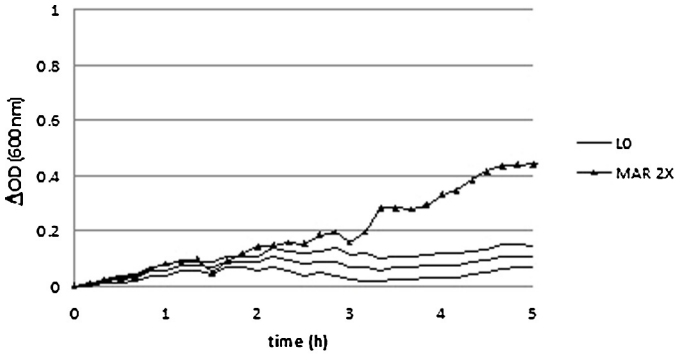

Fig. 5. *E. coli* ATCC11303 ΔOD_{600nm} curve in presence of 10 raw milk samples spiked with marbofloxacin (MAR) 2 × MRL. The threshold curve elaborated for 10 raw milk samples (L0) and the respective confidence interval are shown. ΔOD_{600nm} values (mean values, 5 replicates) are plotted versus time (%RSD ≤ 5%).

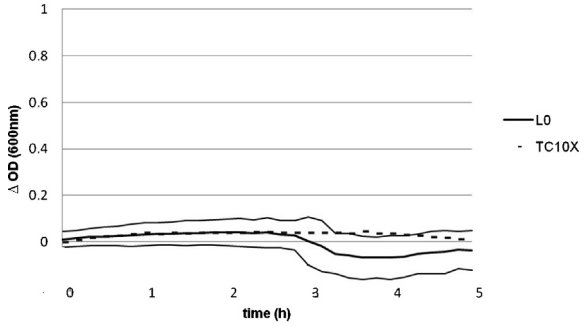

Fig. 6. *E. coli* ATCC11303 ΔOD_{600nm} curve in presence of 10 pasteurized milk samples spiked with tetracycline (TC) 10 × MRL. The threshold curve elaborated for 10 pasteurized milk samples (L0) and the respective confidence interval are shown. ΔOD_{600nm} values (mean values, 5 replicates) are plotted versus time (%RSD ≤ 5%).

coincident and equal to those reported in Fig. 2 for pasteurized milk samples spiked with tetracycline.

These results reasserted the $MgSO_4$ specific activity on fluoroquinolones respect to other antibiotic families tested in the present experimental conditions.

3.4. ΔOD_{600nm} versus time graphs

The fork effect described above (see Section 3.3) for fluoroquinolones spiked milk samples resulted variable as a function of the molecule activity. In particular, the more the molecule was active on the microorganism, the more the fork effect was evident.

As shown in Figs. 3–5 the ΔOD_{600nm} calculated for blank samples remained below approximately 0.1 whereas those calculated for fluoroquinolones spiked samples, after 3 h incubation, were always above this value.

In our conditions, a sample was screened as potential positive for the presence of quinolones when, starting from 3 h incubation, its ΔOD_{600nm} curve exceeded the threshold one.

Although ciprofloxacin *per se* is only approved for use in humans, it is detectable in milk as major and active metabolite of enrofloxacin and over a longer period from administration than enrofloxacin. Accordingly, the EU-MRL for fluoroquinolones in milk was set at 100 μg/kg considering the total amount of both enrofloxacin and ciprofloxacin as the marker residue [22]. Since ciprofloxacin accounts for about 90% of the total residue, we tested this molecule at concentrations below the MRL [27].

Satisfactory results were obtained for the 0.5 MRL concentration proving no negative results on a total of 10 different samples, replicated 5 times each. The detection level for enrofloxacin and ciprofloxacin resulted both at 0.5 MRL: this is regarded as a favorable outcome of our study, because the EU-MRL is set for the sum of both, Moreover screening methods, have mainly to minimize the rate of false compliant, rather than that of false non-compliant [2].

The ΔOD_{600nm} curve for marbofloxacin spiked raw milk samples exceeded the threshold curve starting from 1.5 MRL and resulted fully satisfactory for the 2 MRL concentration, proving no negative results on a total of 10 different samples, replicated 5 times each.

In the case of experiments on milk samples spiked with danofloxacin, no negative results on a total of 10 different samples, replicated 5 times each were obtained starting from the concentration of 4 MRL; however, a false compliant rate of >5% was still observed for flumequine at the concentration 8 MRL.

Fig. 6 reports ΔOD_{600nm} versus time graphs for pasteurized milk samples blank and spiked with tetracycline 10 MRL. The results of the spiked samples show a trend not exceeding the threshold curve calculated for pasteurized blank milk, thus demonstrating the usefulness of a blank threshold for different kinds of milk. The same results were obtained for benzylpenicillin tetracycline,

oxytetracycline, sulfadiazine, streptomycin, thus supporting the specificity of the system MHb/MHb-Mg for fluoroquinolones detection.

The absence of cross specificity for the other antimicrobial classes tested (beta lactams, tetracyclines, sulfonamides, aminoglicosides) is consistent with data reported for (multi) plate systems using *E. coli* ATCC 11303 as quinolones sensitive strain [28,29]. In fact, the concentration of oxytetracycline reported to cross react in an *E. coli* ATCC 11303 system actually corresponds to approximately 30 × MRL, far exceeding a probable level of contamination of an incurred milk sample. In any case, the possibility of cross specificity in our system would be excluded by the no-effect of $MgSO_4$ (absence of fork effect), even in case of cephalexin at the concentration of about 2 × MRL [28], and colistin 10 × MRL and trimetoprim 10 × MRL [29].

3.5. Preliminary data validation

Owing to the current unfitness of EIA methods for routine screening, the systematic quality control at the farm and dairy industry levels does not include adequate methods for the detection of quinolones in milk; as a consequence, the presence of quinolone residues in milk may remain unchecked. In addition, due to quinolones high resistance to heat treatments up to 120 °C for 20 min [23], the risk associated to the presence of their residues in milk after heat treatment cannot be excluded; as a consequence, quinolone residues might persist along the processing of dairy products, such as cheeses. Hence the applicability of the test was also studied in whole and partially skimmed pasteurized milk samples, frozen or refrigerated. The method resulted fully applicable to all the matrices considered.

4. Conclusion

In conclusion, performance parameters obtained in the preliminary validation study proved the method able to detect ciprofloxacin + enrofloxacin at the MRL concentration, whereas marbofloxacin and danofloxacin at 2 MRL and respectively 4 MRL. Performance on flumequine is till unsatisfactory being >4 MRL. The method provesdto be specific for quinolones with absence of false positive results when used on blank milk samples as well as on samples spiked with beta-lactams, sulfonamides, aminoglycosides, tetracyclines. In addition it provides responses in times comparable to those of the traditional routine microbial methods (starting from 3 h of incubation) and last but not least, it is applicable to different kinds of milk. For all this, and for its equivalence to routine methods for sample throughput and cost-effectiveness, the use of these methods in combination could efficiently improve the quality of residue screening control for milk enlarging the number of detectable antribiotics.

The test presented in this paper offers a good compromise between the desirable characteristic of both the chemical and the biological assays. The assay is specific for quinolones, but it is based on the effect on a target microorganism, *E. coli* ATCC 11303, and it may reveal an effect due to the combined presence of residues of different quinolones. Thus, it may represent a group-specific screening test. A successful achievement is observed for two compounds, ciprofloxacin and enrofloxacin, for which the biologically active concentration revealed by the test is equal to the MRL. The sensitivity of the test has to be improved for other quinolones; however, the detected biologically active concentration is ≤4 folds (i.e., less than one magnitude order) higher than the respective MRL. Thus, further refinements could bring sensitivity to a level consistent with MRL for all the investigated quinolones. Another successful achievement is that the method works in the milk matrix. Milk is a complex mixtures of different proteins, fat globules, salts, vitamins, as well as bioactive substances carried on from feeds and pastures; thus, milk can provide a substantial background noise to detection tools such as biosensors. Thus, albeit further trials in field situations are required, we regard as a promising step forward the specific response to quinolones in the milk matrix observed in laboratory tests. Under the respect of further refinements of the test, it is noteworthy that user-friendly information management graphical tools, e.g., the 'Naji plot', may be applied, allowing the assessment and comparison of the constituents of accuracy (bias and precision) of results [30].

Refinements of the test are, therefore, to enhance its relevance in different applications, beyond the mere legal requirements of official control, e.g., providing early alerts in food chain self-management, pharmacosurveillance of proper antibiotic treatment, and risk assessment by detecting the presence of active concentrations of quinolones.

Beyond refinements of accuracy, the next major development of the bio-optical test is the integration, as a biosensor probe, in systems aimed at the at-line monitoring of cow's milk at milking like the patent (*Bio*)*Sensors' system in Food Safety* [*BEST*] of the Italian national Institute of Health [9]. In this case, the signals provided by the probe would integrate the signals from other probes; while the bio-optical test results are significant *per se*, as they concern substances regulated by MRLs, the integrated array of signals could build up an in-house control charting depicting the time-course of the impact on milk quality by the farm management, including treatments with veterinary drugs. Under this view, screening methods may provide biomarkers of One Health, i.e., contributing to the integrated protection of human, animal and environmental health.

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