Journal of Pharmaceutical and Biomedical Analysis xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

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

Brunella Appicciafuoco[a], Roberto Dragone[b,*], Chiara Frazzoli[c], Giuseppe Bolzoni[d], Alberto Mantovani[a], Anna Maria Ferrini[a]

[a] Department of Veterinary Public Health and Food Safety, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Roma, Italy
[b] Institute of Nanostructured Materials, Consiglio Nazionale delle Ricerche, c/o Department of Chemistry, 'Sapienza' University of Rome, Piazzale Aldo Moro 5, 00186 Roma, Italy
[c] External Relations Office, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Roma, Italy
[d] National Reference Centre for Bovine Milk Quality, Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Via Bianchi 9, 25124 Brescia, Italy

ARTICLE INFO

Article history:
Received 18 August 2014
Received in revised form 9 November 2014
Accepted 18 November 2014
Available online xxx

Keywords:
One Health
Farm animals
Veterinary drugs
Food safety
Milk
Quinolones

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

* Corresponding author: Tel.: +39 06 4991 3374; fax: +39 06 490321.
E-mail address: roberto.dragone@uniroma1.it (R. Dragone).

http://dx.doi.org/10.1016/j.jpba.2014.11.037
0731-7085/

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.

Please cite this article in press as: B. Appicciafuoco, et al., Microbial screening for quinolones residues in cow milk by bio-optical method, J. Pharm. Biomed. Anal. (2014), http://dx.doi.org/10.1016/j.jpba.2014.11.037