Available online at www.sciencedirect.com
ScienceDirect
Analytica Chimica Acta 586 (2007) 275–279
ANALYTICA CHIMICA ACTA
www.elsevier.com/locate/aca

Experiences with an identification and quantification program for inhibitor-positive milk samples

Claudia Kress, Caroline Seidler*, Bianca Kerp, Elisabeth Schneider, Ewald Usleber

Dairy Sciences, Institute of Veterinary Food Science, Justus-Liebig-University, Ludwigstr. 21, 35390 Giessen, Germany

Received 19 June 2006; received in revised form 6 October 2006; accepted 10 October 2006
Available online 17 October 2006

Abstract

Beta-lactam antibiotics (penicillins, cephalosporins) are still the most commonly used antibiotics for dairy cows in Germany. In routine milk testing, according to the German milk quality regulation, a positive result obtained for bulk tank milk by microbiological inhibitor tests needs no further confirmation, but results in reduced milk payment of €0.05 kg^{-1} for one month. In some cases, however, further identification of the causative agent can be of interest, either if antimicrobial drugs have not knowingly been used recently, or if improper use of such drugs is denied. As a service for milk producers, our laboratory offers further analyses of violative milk samples, aiming at the identification and quantification of the inhibitor(s). In this program, a panel of microbiological inhibitor tests, receptor tests, and enzyme immunoassays (EIA) is used in a step-by-step analysis, which primarily focusses on β-lactams, but also includes other compounds such as sulfonamides or tetracyclines, respectively. Here we report results for violative milk samples ($n = 63$) analysed between 2003 and 2005. In most cases (95%), β-lactam antibiotics could be identified, although not always at levels exceeding the respective MRL values. Penicillin G (mostly together with benzylpenicilloyl metabolites) could be identified in 74.6% of all samples. Other compounds identified were, in decreasing order, ceftiofur (11%), ampicillin/amoxicillin (6.3%), isoxazolyl penicillins (3.2%), and sulfonamides (1.6%). The results indicate that penicillin G is still the predominant antibiotic responsible for violative bulk tank milk samples as detected during regulatory control.

Keywords: Milk; β-Lactum antibiotics; Pencillin G; Ceftiofur; Residue

1. Introduction

Beta-Lactam antibiotics (penicillins and cephalosporins) are still the predominant cause of violative drug residues in bulk tank milk in Germany. For example, out of a total of about 2.5–3 million tests for antimicrobial inhibitors in bulk tank milk performed annually in the state of Bavaria during the last years, typically 4–5 samples per 10,000 tested samples were positive, which adds up to 1000–1500 inhibitor-positive samples per year. About 90–95% of these were caused by penicillinase-labile β-lactams [1]. Other antimicrobials seem to occur only rarely in bulk tank milk or in drinking milk [2], although recent data are not available. In routine testing according to the German milk quality regulation scheme (milk recording), a positive result (obtained by microbiological inhibitor tests) results in reduced milk payment (€0.05 kg^{-1}) for one month. Since this is a major economic loss for the milk producer, in some cases further information on the nature of the causative agent would be of interest. As there is still no convenient and practicable reference method covering all relevant compounds, such information is not obtained easily. Because a large number of structurally non-related compounds with widely varying maximum residue limits (MRL, European Union Regulation 2377/90 [3]) could theoretically be present in violative tank milk samples, the primary requirement of a suitable analytical strategy is the ability to systematically narrow the number of candidate compounds and to efficiently trace the causative substance(s). Currently, the combination of several methods is the only practical approach here.

Besides classical non-specific microbial inhibitor tests, several receptor tests are available for the selective detection of β-lactam antibiotics. Some of these are suitable for routine control [4], and are useful to confirm positive results of microbial inhibitor tests, but they do not differentiate between β-lactam antibiotics. Several chromatographic methods for penicillins

* Corresponding author. Tel.: +49 641 9938965; fax: +49 641 9938959.
E-mail address: Milchwissenschaften@vetmed.uni-giessen.de (C. Seidler).

0003-2670/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.aca.2006.10.013

Table 1
Coverage of individual β-lactam antibiotics by the EIA test systems used for residue identification

EIA test system	Penicillin G	Penicillin V	Ampicillin	Amoxicillin	Oxacillin	Cloxaxillin	Dicloxacillin	Nafcillin	Ceftiofur	Cefquinom	Cefalexin
Penicillins generic	+	+	+	+	+	+	+	+	−	−	−
Isxazolyl penicillins	−	−	−	−	±	+	+	−	−	−	−
Ampicillin/amoxicillin	−	−	±	±	−	−	−	−	−	−	−
Penicillin G metabolites	(+)[a]	−	−	−	−	−	−	−	−	−	−
Ceftiofur	−	−	−	−	−	−	−	−	+	−	−
Cefquinome	−	−	−	−	−	−	−	−	−	+	−
Cefalexin	−	−	−	−	−	−	−	−	−	−	+

+, Compound detectable below or at MRL; ±, compound detectable but at >5–10 × MRL; −, compound not detectable.
[a] Detectable only after enzymatic cleavage with penicillinase.

have been developed [e.g., 5–8], but none of these has been found broader use in routine analysis so far.

In such a situation, a simple and versatile approach is offered by combining antimicrobial inhibitor tests, receptor tests, and enzyme immunoassays (EIA) into an analytical multimethod strategy. Since 2001 our laboratory offers, as a service for milk producers, further analysis of inhibitor-positive milk samples using this multimethod strategy, aiming on identification and quantification of the causative drug. In this paper, we report results obtained for tank milk samples from Hessen, Germany, between 2003 and 2005.

2. Experimental

2.1. Test systems

The microbiological inhibition test (Brillant Black Reduction test, BRT) was obtained from AIM GmbH, Munich, Germany. This test detects all β-lactam antibiotics at or below the MRLs, but other antimicrobial residues as well. The receptor assays used were SNAP β-lactam test (IDEXX GmbH, Wörrstadt, Germany) and b.e.t.a. Star (Chr. Hansen Nienburg, Germany). This test detects all β-lactam antibiotics at levels of the MRL ± 50% [4]. All these tests were used according to the instructions of the manufacturer.

A generic EIA for penicillins [9] with improved sensitivity through high-affinity antibodies [10] was used to detect penicillins. The detection limit for penicillin G in milk was 2 ng mL^{-1}, pencillin V, ampicillin, oxacillin, cloxacillin, dicloxacillin, and nafcillin were all detected at 0.5 × MRL to 1.0 × MRL; the detection limit for amoxicillin (6–8 ng mL^{-1}) was slightly above the MRL. For detection of isoxazolyl penicillins, a EIA for cloxacillin, as described by Dietrich et al. [11], was used. With this assay, oxacillin, cloxacillin, and dicloxacillin were all detected in milk well below the MRL, although oxacillin has lower relative cross-reactivity of about 10%. Ampicillin and amoxicillin at levels of >50 ng mL^{-1} in suspect samples were confirmed by a EIA with specificity for these amphoteric penicillins as described by Dietrich et al. [12]. To indirectly confirm the prescence of penicillin G, a EIA specifically detecting penicillin G metabolites (benzylpenicilloyl metabolites, BPO) was used [13]. This assay detects BPO at 3 ng mL^{-1} in incurred milk samples, but also gives positive results for penicillin G after enzymatic cleavage with penicillinase. For the detection of ceftiofur, two EIAs were used, one detecting only ceftiofur, the other detecting both ceftiofur and its metabolite, desfuroylceftiofur. The detection limits for ceftiofur and desfuroylceftiofur were 1–3 and 50–100 ng mL^{-1}, respectively [14]. Other EIAs for cefalexin [14] and cefquinome were recently developed in our laboratory. Table 1 summarizes how the individual β-lactam antibiotics are covered by the tests systems used in the integrated detection system. In case of a positive result for penicillin G, samples were in some cases further analysed by an EIA for streptomycin/dihydrostreptomycin [15], since many veterinary drug formulations used for dairy cows contain both compounds. In suspect samples, tetracyclines were additionally analysed by EIA [16].

2.2. Sample material

Bulk tank milk samples which had been scored as inhibitor-positive in routine milk quality control were sent to our laboratory via the responsible milk control laboratory if requested by the milk producer ($n = 60$). A few milk samples from individual cows were directly sent by farmers because of repeated inhibitor-positive results ($n = 3$).

2.3. Analytical procedure

The three steps of the integrated system to differentiate and quantitate β-lactam antibiotics, and the information concerning the nature of the residue, obtained by microbiological and receptor tests, are shown in Fig. 1. If the results from microbiological and receptor assays demonstrated the presence of a β-lactam in a milk sample, further differentiation was achieved by EIA as shown in Fig. 2. The number of differentiation tests necessary to achieve a result was dependent on the nature of the inhibiting substance.

For analysis by the microbiological inhibitor test and the receptor test, milk was used without any sample extract preparation. For enzyme immunoassays, milk samples were defatted by centrifugation, and the skim milk was analysed directly or after dilution with reconsituted skim milk powder (10 g skim milk powder dissolved with 90 mL distilled water).

For cleavage of penicillins by β-lactamase [penicillinase, Penase Concentrate (Difco, Sparks, USA, product number 234620)] with a specific activity of 10 million units mL^{-1} was used. Twenty microliter of the concentrate were added