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Detection and Identification of β-Lactam Residues in Milk Using a Hybrid Biosensor

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A novel application of a hybrid biosensor is here employed as an analytical method for the detection and presumptive identification of β-lactam residues in milk. The method is based on measurements of carbon dioxide (CO_2), the production of which is related to the microbial growth of the test microorganism *Bacillus stearothermophilus* var. *calidolactis*. The presence of β-lactams in milk inhibits microbial growth and, consequently, the CO_2 production rate. The analysis is based on the variation of CO_2 between a milk sample spiked with β-lactams and a twin milk sample containing β-lactams plus a broad spectrum β-lactamase, using an electrochemical device of biosensor. A blank milk sample is included as control. The result is obtained starting from the first 120 min. Moreover, the ability to recognize all of the β-lactams speeds the total time of analysis when chemical identification and quantification are required. The analytical method appears to be adequate for milk control for qualitative screening purposes, complying with the requirements stated in Decision 2002/657/EC.

KEYWORDS: β-Lactams; milk; antibacterial residues; biosensor

INTRODUCTION

In current zootechnical practice, raising animals for food production largely depends on the use of pharmacologically active substances. Antibiotics are widely applied to control, to treat, and to prevent bacterial infections with the benefits of maintaining animal welfare and to reduce the spread of infectious diseases on farms. Those antibiotics used in milk production are mostly related to mastitis therapy. At present, β-lactams are the prevalent family of antibacterials used in dairy cattle owing to their effectiveness in this therapy (*1–4*).

Adverse effects of the presence of antibiotic residues in foodstuff produced from animals treated with veterinary medicinal products can be related to (a) allergenic or toxicological responses in consumers, (b) selective pressure for antibiotic-resistant strains, and, eventually, (c) technological problems in the production of fermented foods (*5, 6*). To meet the above considerations, Regulation 2377/90 states the maximum residue limit (MRL) of a number of veterinary drugs in foodstuffs of animal origin (*7*). To guarantee consumers safe and high-quality products, raw ex-farm milk is regularly analyzed for the presence of antibiotics (*8*). Many microbial inhibitor methods have been developed for the detection of antibacterial drug residues in animal foods (*5, 9–15*), and several kit assays have been commercially developed for milk (Delvo SP and BRtest by DSM, The Netherlands; Copan test by Copan, Italy; Charm Farm test and Charm AIM-9 by Charm Science, United States; ECLIPSE by Zeu-Immunotec, Spain; and Kalidos by Euroclone, Italy) on the same principle of the old inhibitor screening reference method (*15*), thus becoming routine methods for testing antibacterial residues in milk. *Bacillus stearothermophilus* var. *calidolactis* is the common test microorganism due to its relatively high sensitivity to a broad spectrum of inhibitory substances (*15*). All of these microbial methods are generally cheap and easy to perform: in about 3 h they produce binary results (presence/absence) referred to a generic "inhibitor". In the case of a noncompliant result (*16*), a second step is possible for the presumptive identification of penicillins only and/or sulfonamides.

In recent years, the need of a faster control has promoted the development of receptor enzyme assays (ROSA MRL by Charm, United States; Delvo-X-Press by DSM, The Netherlands; IDEXX SNAP by Snap IDEXX, Unites States; Tetrasensor and Twinsensor by Unisensor, Belgium), competitive enzyme immunoassays (Parallux by Medexx, Korea), radioimmunoassay (Charm MRL by Charm, Unites States), or enzymatic assays (Penzym S by UCB, Belgium). All of these methods are specific for the different families of antibiotics and share the common feature of a very short time of analysis but at a relatively high cost. Owing to their specificity, when a wide spectrum control is needed, more tests need to be undertaken separately, with obvious economic disadvantage. Among the "last generation" methods, biosensors represent an interesting approach potentially able to solve several problems related to food control (*17–25*).

The present paper describes a new screening method for the detection and presumptive identification of β-lactams in raw

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milk using a hybrid biosensor (*23*). The method is based on measurements of carbon dioxide (CO_2), the production of which is related to the microbial growth of *B. stearothermophilus* var. *calidolactis* used as test microorganism. The presence of microbial inhibitors in milk prevents the microbial growth and, consequently, the CO_2 production rate. The hybrid biosensor registers this variation with respect to a control milk sample. The presumptive identification is carried out by means of a broad spectrum β-lactamase to selectively inactivate β-lactams. This pilot study has been carried out on β-lactams because they represent the prevalent type of antibiotic residues in milk, due to the fact that they are the most used antibiotics for mastitis treatment. For the same reason, most of the methods developed to date were targeted to β-lactams.

MATERIALS AND METHODS

Gas Reference Materials (GRMs). Five GRMs of CO_2 (SOL SpA, Rome, Italy) at concentrations of 348, 1253, 1745, 2881, and 6600 μL of CO_2 L^{-1} of synthetic air were employed for sensor calibration.

Microorganisms. The microorganism used throughout this work was *B. stearothermophilus* var. *calidolactis* (1.11499, Merck, Milan, Italy). Before the analysis, an appropriate volume of the commercial spore suspension was diluted with Mueller−Hinton broth (Oxoid, Milan, Italy) at a final concentration of 5×10^4 cfu mL^{-1}.

Antibiotic Standards. Standards of six penicillins (penicillin G, ampicillin, amoxicillin, oxacillin, cloxacillin, and dicloxacillin) and two cephalosporins (cefoperazone and cefazolin) were all supplied by Sigma Chemical (Sigma-Aldrich, Milan, Italy). Standard stock solutions in distilled water (10 mg L^{-1}) of each antibiotic were prepared and stored at −18 °C and used in 1 week. Working standard solutions of all drugs were freshly prepared by diluting suitable volumes of each standard stock solution with distilled water (*26*).

Enzymes. A broad spectrum β-lactamase mixture (SR 0113, Oxoid, Milan, Italy) was dissolved in distilled water according to the manufacturer's instructions. This β-lactamase is a broad spectrum mixture of β-lactamase I and β-lactamase II and was used as confirmatory solution to counteract the activity of β-lactams.

Equipment. The hybrid biosensor (HyBS) employed in this study is derived from an already described prototype based on the use of a high-performance noninvasive specific carbon dioxide sensor (CO_2-S) coupled to a bacterial culture such as sensitive cells (*23, 25*). Briefly, the electrochemical device was a galvanic cell composed of a reference electrode (RE) dipped in a working electrolytic solution (WS) and connected to an indicator electrode (IE) by means of a silk filament (hydrophilic material) that acts as a sensor membrane (SM). The IE is a metallic corrosion electrode (usually stainless steel), whereas the RE is either a conventional one or a corrosion electrode like the IE. The WS is an immobilized solution; its composition fits the CO_2 gaseous concentration (y_{CO2}, μL L^{-1}) and avoids interferences from other gaseous chemical species. In this study the WS was 0.1 m KCl in water, a strong base, that is, Na_2CO_3, in a concentration ranging from 10^{-4} to 10^{-2} m, and CO_2 to solution saturation. The sensor is contained in a case-box that protects it from electrical straw currents and drafts. All components of the CO_2 sensor system (carrier gas source included) can be assembled in a box of 8 × 25 × 28 cm (height × width × length). The case-box is connected by steel tubes with the CG source (air compressed in cylinder), and the analytical glass tubes specifically realized. The HyBS is connected to an emf-meter connected in series with a computer for data acquisition and elaboration. Instrumentation employed and working conditions adopted are shown in **Table 1**.

Sample Preparation. Blank raw milk was received from an experimental farm in the central part of Italy, where cows were housed in controlled and monitored conditions. Neverthless, before use, the milk was tested for the absence of inhibitors by the Delvo SP test. An aliquot of blank raw milk was spiked with β-lactam and allowed to stand overnight at 4 °C to allow protein binding. Before the assay, an aliquot of the spiked milk was treated for 15 min at room temperature with the β-lactamase mixture in the proportion 10:1. Samples for each analytical cycle were prepared by adding to three analytical glass tubes containing 4.5 mL of *B. stearothermophilus* spore suspension, prepared as described above, 0.5 mL of raw milk (control), 0.5 mL of raw milk spiked with β-lactam, and 0.5 mL of raw milk spiked with β-lactam plus β-lactamase mixture, respectively. Eight antibacterial drugs were tested at respective MRL levels. Moreover, considering that the possibility of a presumptive identification of the β-lactams in milk is based on their enzymatic neutralization, the suitability of the method was also evaluated in the case of very high concentrations as in the case of milking during β-lactam administration.

Table 1. Instrumentation and Working Conditions for Hybrid Biosensor (HyBS)

Instrumentation	
galvanic cell	reference electrode dipped in electrolytic solution and connected to a stainless steel indicator electrode by means a silk filament that acts as sensor membrane
linearity range	from 10 to 10^4 μL L^{-1}
k value	about 20 mV
resolution	0.5 μL L^{-1} at 400 μL L^{-1} level
signal noise	1–2 μV
thermal drift	about 20 μV $centigrade^{-1}$
lifetime	about 1500 detections
analytical tube	glass tube height = 15 cm; i.d. =2 cm
potentiometer	multimeter Hewlett-Packard 34401A
thermostatic water bath	GFL 1002 Digit
stripping gas source	compressed air in cylinder
software	Agilent Intui Link, Hewlett-Packard
Working Conditions	
stripping gas flow	0.1 mL s^{-1}
gas reference materials (GRMs)	CO_2 at concentrations of 348, 1253, 1745, 2881, and 6600 μL L^{-1} in synthetic area
biological recognition element and grown conditions	*Bacillus stearothermophilus* var. *calidolactis* in Mueller−Hinton broth at 63 ± 1 °C
β-lactam standards	penicillin G, ampicillin, amoxicillin, oxacillin, cloxacillin, dicloxacillin, cephaperazone, cephazolin
incubation time	up to 180 min
reading time	1 min

Analysis. The three analytical glass tubes containing the samples prepared as above-described were dipped into a thermostatic water bath set a 63 °C for up to 180 min. Afterward, the carrier gas (CG) was diverted in succession through the three analytical tubes. The CO_2 produced by microorganisms was stripped and carried to the electrochemical device, which measured the potential difference correlated to the different y_{CO2}. Analytical cycles for a total length of 5 min were realized as follows: 1 min of measurement and 4 min for the sensor re-equilibration. The total time of instrumental analysis (three samples) was 15 min for each of the chosen intervals of time (0, 60, 120, and 180 min).

Statistical Analysis. To evaluate the significance of the differences noted among the different concentrations of antibiotics versus blank milk, the one-way analysis of variance (ANOVA test) was applied. The SPSS software (version 14.0 for Windows) was used for processing. Data are reported in **Tables 2−4**.

RESULTS AND DISCUSSION

Today, depending on the control, there are basically two types of rapid test available for screening: (a) results achievable in <10 min through immune-receptor principle or enzymatic reaction (i.e., Charm MRL or IDEXX SNAP test) or (b) results obtained in 2.5–4 h (i.e., Delvo test, Copan test, and BR test) known as the routine inhibitor tests. The rapidity of the first methods is generally combined with the specificity for an antibacterial or for a family of antibacterials. In this case, the result of the control is partial and can be applied only when no

Table 2. ΔE and Percentage Decrease at 180 min as a Function of Microbial Inhibition Related to the Presence of β-Lactams in Milk at LMR Value without or with Addition of β-Lactamase[a]

β-lactam	MRL concn (μg L^{-1})	ΔE blank milk (control)	ΔE milk with β-lactam at LMR level	inhibition[b] (%)	milk with β-lactam at MRL level and β-lactamase
penicillins					
amoxicillin	4	3.753 ± 0.20	2.116 ± 0.17	44	3.546 ± 0.19
ampicillin	4	5.241 ± 0.27	3.025 ± 0.23	42	4.952 ± 0.22
cloxacillin	30	3.369 ± 0.28	1.933 ± 0.16	43	3.092 ± 0.19
dicloxacillin	30	5.460 ± 0.28	2.975 ± 0.15	45	5.216 ± 0.30
oxacillin	30	4.555 ± 0.97	2.753 ± 0.62	40	3.916 ± 0.62
penicillin G	4	5.389 ± 0.24	2.663 ± 0.27	51	5.300 ± 0.25
cephalosporins					
cephazolin	50	4.392 ± 0.21	2.215 ± 0.20	50	4.334 ± 0.36
cephaperazon	50	3.502 ± 0.29	2.027 ± 0.21	42	3.239 ± 0.35

[a] Values are mV, mean ± SD for $n = 5$ independent samples. [b] $P < 0.001$ (as found by ANOVA spiked samples vs correlated control) for all β-lactams except the oxacillin ($P < 0.05$).

Table 3. ΔE and Percentage Decrease at 180 min as a Function of Microbial Inhibition Related to the Presence of β-Lactams at Very High Level without or with Addition of β-Lactamase[a]

β-lactam	concn (μg L^{-1})	ΔE blank milk (control)	ΔE milk with β-lactam at highest level	inhibition (%)	milk with β-lactam at highest level and β-lactamase
penicillins					
amoxicillin	50	4.051 ± 0.21	2.061 ± 0.11	49	3.895 ± 0.13
ampicillin	50	4.245 ± 0.14	2.053 ± 0.10	52	4.082 ± 0.15
cloxacillin	15000	4.144 ± 0.28	2.191 ± 0.21	47	3.932 ± 0.28
dicloxacillin	15000	4.251 ± 0.29	2.105 ± 0.16	50	3.945 ± 0.30
oxacillin	15000	4.008 ± 0.17	2.211 ± 0.14	45	3.818 ± 0.21
penicillin G	300	4.182 ± 0.20	2.002 ± 0.10	52	3.987 ± 0.26
cephalosporins					
cephazolin	5000	4.302 ± 0.65	2.046 ± 0.33	52	4.121 ± 0.12
cephaperazon	50000	4.585 ± 0.09	2.465 ± 0.18	46	4.472 ± 0.18

[a] Values are mV, mean ± SD for $n = 5$ independent samples.

Table 4. Statistical Significance of ΔE Measured Differences

		blank milk (control) versus milk with β-lactam			milk with β-lactam and β-lactamase versus milk with β-lactam			blank milk (control) versus milk with β-lactam and β-lactamase		
β-lactam	concn (μg L^{-1})	t stat	t critical two-tail	probability	t stat	t critical two-tail	probability	t stat	t critical two-tail	probability
amoxicillin	4[a]	13.82	5.04	0.001	12.32	5.04	0.001	1.66	1.86	>0.1
	50	16.08	5.04	0.001	23.70	5.04	0.001	1.41	1.86	>0.1
ampicillin	4[a]	14.00	5.04	0.001	13.59	5.04	0.001	1.83	1.86	>0.1
	50	28.37	5.04	0.001	25.08	5.04	0.001	1.79	1.86	>0.1
cloxacillin	30[a]	9.76	5.04	0.001	10.31	5.04	0.001	1.81	1.86	>0.1
	15.000	12.47	5.04	0.001	11.07	5.04	0.001	1.20	1.86	>0.1
dicloxacillin	30[a]	17.45	5.04	0.001	14.88	5.04	0.001	1.31	1.86	>0.1
	15.000	14.47	5.04	0.001	12.29	5.04	0.001	1.64	1.86	>0.1
oxacillin	30[a]	3.49	2.31	0.05	2.95	2.31	0.05	1.24	1.86	>0.1
	15.000	18.48	5.04	0.001	14.45	5.04	0.001	1.58	1.86	>0.1
penicillin G	4[a]	16.93	5.04	0.001	15.97	5.04	0.001	0.58	1.86	>0.1
	3.000	21.48	5.04	0.001	15.70	5.04	0.001	1.32	1.86	>0.1
cephazolin	50[a]	16.87	5.04	0.001	11.52	5.04	0.001	0.31	1.86	>0.1
	5.000	6.92	5.04	0.001	13.13	5.04	0.001	0.61	1.86	>0.1
cephaperazon	50[a]	9.26	5.04	0.001	6.67	5.04	0.001	1.30	1.86	>0.1
	50.000	23.73	5.04	0.001	17.44	5.04	0.001	1.24	1.86	>0.1

[a] MRL concentration.

wide-spectrum screening is required (in the self-control after the administration of a known antibacterial or, in general, when the control is intentionally targeted to a specific antibacterial). On the contrary, microbial inhibitor tests share the common characteristic of being sensitive to a wide spectrum of antibacterials at the level of, or close to, MRLs, making it statistically probable that the MRL for a given antibiotic will be exceeded in the case of a noncompliant result. Unfortunately, to date no rapid test capable of detecting all possible antibiotic residues in milk has ever been realized.

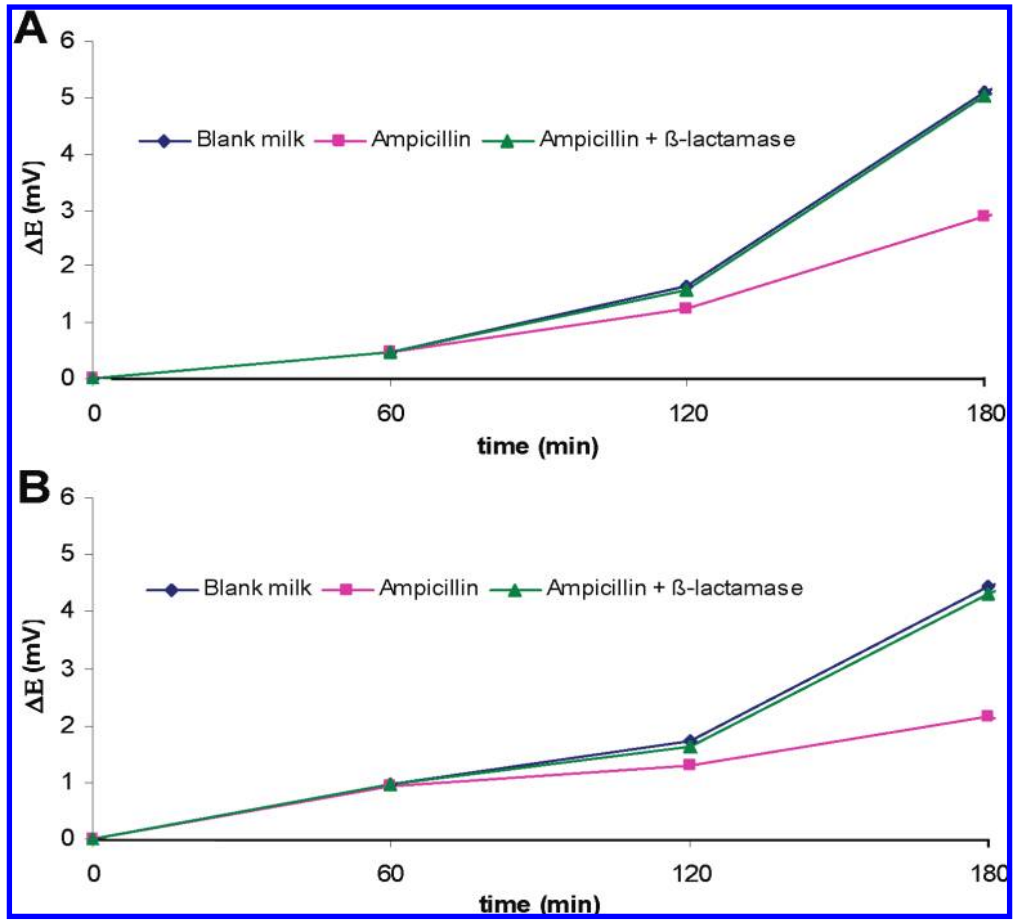

Figure 1. ΔE variation related to CO_2 production up to 180 min: (**A**) milk spiked with ampicillin at 4 ppb (MRL); (**B**) milk spiked with ampicillin at 50 ppb.

The performances of the present method are intermediate with respect to those of the above-mentioned methods: in a full time of 120–180 min it offers a complete investigation, presumptive identification inclusive, in the case of presence of β-lactams.

Analytical determinations of GRMs showed good accuracy with values varying between 97 and 106%. Precision was more than satisfactory, with relative coefficients of variation (CV %) between 0.8 and 2.4%. The calculated calibration curve showed a good linearity in the whole range of tested concentrations (348–6600 μL of CO_2 L^{-1} of synthetic air) with a correlation coefficient (r^2) equal to 0.9991. The resulting equation was $y = 0.0011x + 1.6173$, where y is the potential difference (ΔE, mV) and x is the analyte concentration (μL of CO_2 L^{-1} of synthetic air).

In general, it was possible to detect all of the molecules tested at MRLs starting from 120 min. **Figure 1** shows that the differences of CO_2 production detected in milk samples spiked with ampicillin at 4 ppb (**A**) and 50 ppb (**B**) could be appreciated starting from 120 min and that the inhibition degree continued to increase in the time producing reductions of 24.6 and 23.8% with respect to the control samples at 180 min. After this time, there were not significant variations of ΔE. Analytical results of biosensorial analysis for all β-lactams investigated are shown in **Tables 2** and **3**.

In terms of percent ΔE decrease, the β-lactam showing the highest inhibitory effect at MRL concentration (**Table 2**) after 180 min was penicillin G (−51%), whereas the lowest inhibition was displayed by ampicillin and cefoperazon (−42%). At the highest concentrations penicillin G (300 μg L^{-1}) (**Table 3**) confirmed the maximum effect (−52%), whereas oxacillin (15000 μg L^{-1}) exhibited the minimum inhibitory activity. It is noteworthy that β-lactams at highest concentrations gave rise to ΔE decreases not much greater than those related to MRLs.

In any case, all β-lactams tested produced a statistically significant decrease of ΔE both at the highest concentrations and at MRL levels. The ANOVA test proved significant differences between the means at $P < 0.001$ for all tested antibiotics and concentrations with the only exception of oxacillin at MRL ($P < 0.05$).

The present method is a combination of the classical microbial methods for antibacterial screening in milk with an electrochemical method of detection and reading. Despite the method being specific for the identification of β-lactams, thanks to the same microorganism test routinely used in inhibitory screening methods, it can detect the presence of different inhibitors demonstrating, in the meanwhile, the performance of a broad spectrum screening. Results can be obtained starting from 120 min with a medium advantage of 45–60 min of time with respect to routine methods. Incubation of longer than 60–120 min does not affect the results, whereas other microbial inhibitor tests can lose sensitivity after just an additional 15 min of incubation, giving false-negative results. The numerical result obtained through an electrochemical detection is not affected by a subjective interpretation as can, in some cases, happen when the reading of the results relies on the change of color through the pH indicator (bromocresol purple) or the redox indicator (brilliant black) of the routine inhibitor tests.

A further improvement with respect to traditional methods is represented by the use of a broad spectrum β-lactamase mixture that totally counteracts the tested penicillins and cephalosporins and produces highly specific results. Traditional methods take advantage of penicillinase for the identification

of penicillins, but this is not always effective on cephalosporins. The enzyme amount chosen for this study was effective in neutralizing very high β-lactam concentrations, as could happen if milk were illegally collected during an antibacterial treatment.

In addition, an eventual presence of inhibitory substances in the milk vial sample that is not counteracted in the vial with β-lactamase is indicative of the presence of an inhibitory substance different from β-lactams. This can be considered advantageous with respect to very specific methods that can produce noncompliant results only in case of the presence of their specific target analyte, regardless of the presence and the concentration of any other antibiotic. These methods work well and are intentionally projected to be specific, but allow no general judgment on the antibiotic presence of the sample. It is important to stress that when a sample passes the screening step, no other controls are run. This method fulfils all of the performance criteria stated for screening methods by Decision 2002/657/EC (*16*): percentage of false-compliant rate $<5\%$ at the level of interest, specificity, and applicability to milk. According to the same Decision (*16*), in the case of official control, the chemical analysis is required for all of the suspected noncompliant results to the screening step. The possibility to start the chemical step after only 120 min can effectively reduce the whole time necessary to obtain the complete results. In the case of the presence of cephalosporins, the usual system of neutralization based on penicillinase is not always effective for the neutralization. In this case, the uncertainty of the result can delay the chemical analysis, requiring a frozen storage of the sample, the length of which, owing to the low stability of β-lactams at frozen storage condition, at least in the case of low concentrations, could call into question the quality of the screening (*27*).

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