SPECIAL GUEST EDITOR SECTION

Validation of the Charm MRL-3 for Fast Screening of -Lactam Antibiotics in Raw Milk

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The biochemicals utilized in the Charm MRL -Lactam test (8 min test) were applied to faster flowing lateral components to create a new 3 min, one-step -lactam test called Charm MRL-3 (Charm Sciences Inc., Lawrence, MA). This new test was validated at T&V-ILVO according to Commission Decision 2002/657/EC. The following analytical parameters were checked: test specificity, detection capability, and test robustness (impact of deviation of the test protocol, and impact of the milk composition, batch differences of reagents). Further, the suitability of the Charm MRL-3 to screen heat-treated milk or milk from animal species other than the cow was also tested. Finally, the test was integrated in the monitoring of dairy samples to check the occurrence of false-negative or false-positive results, and the test was also included in a national ring trial and an international proficiency study. The results proved that the Charm MRL-3 is a fast, simple, and reliable cows' milk test that can be used at the farm level in order to prevent tanker milk contamination, or at the entrance of the dairy plant to screen tanker milk for the presence of -lactam antibiotics.

The group of -lactam antibiotics consists of penicillins and cefalosporins because of their common -lactam ring structure. -Lactam antibiotics act as bactericides by inhibiting the synthesis of the bacterial cell wall (1). -Lactam antibiotics are the most frequently administered drugs in parenteral and intramammary therapy in dairy cattle, in most cases to treat mastitis (2). All antimicrobial drugs administered to cows enter the milk to some degree. A residue can be the drug itself or its metabolite. Testing for antimicrobial drug residues in milk is necessary for ethical, health, and technological reasons (3). The dairy industry is screening milk for antimicrobials in order to prevent inhibition of dairy starter cultures used in the production of cheese and yogurt (4, 5). Antimicrobial residues could also mean a risk for consumer health through toxicological effects, allergies, or antimicrobial resistance of pathogenic bacteria. Therefore, in the European Union (EU), maximum residue limits (MRLs) were fixed in bovine milk for 16 -lactam compounds ranging from 4 to 125 g/kg (6, 7).

Inhibitory substances are screened routinely in farm milk samples as part of the regulatory quality program. A positive result normally leads to a penalty for the responsible farmer. In 2009 in Belgium, 872 (0.06%) out of 1 374 801 analyzed farm milk samples were found positive by the milk control stations. In most cases, residues of -lactam substances were the main reason for bulk tank milk failure. Since the result of the routine testing of farm milk for antimicrobials by the milk control stations is only known after the milk is processed, the dairy industry performs additional tests in order to prevent technological problems in the production of fermented dairy products, and to avoid problems with consumption of noncompliant milk. In most cases, milk is checked for the presence of -lactam residues at the entrance of the dairy plant by a rapid test. Several rapid screening tests are on the market for that purpose (8–13). Results can be obtained in less than 10 min. Most rapid tests are designed for a group-specific detection of -lactam residues, but recently, tests for the simultaneous detection of -lactams and tetracyclines also became available. Instead of entrance control, some dairy companies check the milk in the production tank by means of a broad-spectrum microbiological inhibitor test before starting production.

Due to very strict legislation, in most countries rejected milk needs to be destroyed. This results in high costs for the transport, incineration, and the milk itself. The dairy industry

Guest edited as a special report on "Analytical Methods for the Determination of Veterinary Drug Residues in Food Products of Animal Origin" by Badar Shaikh.
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is, therefore, interested in testing at the farm before collection of the milk, hence placing more responsibility on the farmer. However, in such a strategy, a short test time is very important due to the number of tests involved.

Regarding fast tests for antimicrobial residue testing in milk, there is a tendency to shorten test times or to test more groups of compounds in a single run. The biochemicals utilized in the Charm MRL -Lactam Test (8 min test) were applied to faster flowing lateral components to create a new 3 min, one-step -lactam test called Charm MRL-3. This new test was validated at the Technology and Food Science Unit of the Institute for Agricultural and Fisheries Research according to Commission Decision 2002/657/EC (14). The specificity, detection capability, and ruggedness of the assay were demonstrated to meet the criteria required by the EU Commission Decision. Some of the results of this evaluation study were presented in 2008 at the EuroResidue VI Conference on Residues of Veterinary Drugs in Food (15).

Experimental

Reagents, Standards, and Apparatus

Amoxicillin (A8523), cefazolin (C 5020), cefoperazone (C4292), cephapirin (C8270), cloxacillin (C9393), dicloxacillin (D9016), oxacillin (O10002), nafcillin (N3269), and penicillin G (PENNA) were all from Sigma-Aldrich (Bornem, Belgium). Ampicillin (9930212) was from the WHO Collaborating Centre for Chemical Reference Substances (Kungens Kurva, Sweden). Cefalexin (33989) and ceftiofur (34001) were from Riedel-de Haën (Bornem, Belgium). Cefacetrile (22020D000) was from Novartis Animal Health Inc. (Basel, Switzerland), cefalonium (2629) from Schering-Plough (Levallois-Perret, France), cefquinome (Batch 01-01) from Intervet International GmbH (Unterschlei heim, Germany), clavulanic acid from DSM Anti-Infectives (Delft, the Netherlands), desfuroyl-ceftiofur (D289980) from Toronto Research Chemicals Inc. (Ontario, Canada), and penethamate (PE-0708004) from Deltapharma s.a. (Barcelona, Spain). The antibiotic standards were dissolved in water except for ceftiofur, cefalonium, and cefazolin (acetonitrile–water 50 + 50, v/v). Acetonitrile (01207802) was from Biosolve B.V. (Valkenswaard, the Netherlands).

Standard stock solutions of the antibiotic standards (100 mg/L) were made in water and kept below 4 C for a maximum of 1 week. Dilutions of 1 and 0.1 mg/L were freshly prepared daily. To differentiate nonsynthetic penicillins from the group of synthetic penicillins and cefalosporins, 25 L penase solution (1.2 10^5 units/mL; BD Difco Penase Concentrate, Becton, Dickinson, and Co., Sparks, MD) was added to 1 mL milk and incubated for 10 min at 37 C.

The Charm MRL-3 kits were from Charm Sciences Inc. (Lawrence, MA). All sensitivity tests were performed with Lot 009001 (Expiration July 2007) = Lot 009A (Expiration September 2007, same lot packed on a different day). For the study of batch-to-batch differences, Lot 008003 was used. The reagents were stored in a cool room at 4 ± 2 C.

The eta s.t.a.r. kits were from Neogen Corp. (Lansing, MI), the Charm MRL -Lactam from Charm Sciences Inc., and the Delvotest SP-NT 5-PACK kits from DSM-Food Specialties (Delft, the Netherlands).

To check both the reader and reagents, a reconstituted Charm standard (Charm Sciences Inc.) was used. A tablet was dissolved in 5 mL blank raw milk to obtain a milk solution containing cloxacillin 30 g/kg and penicillin G 4 g/kg. A mixture of raw milk, aseptically collected from four individual cows, was used as blank milk. Cows in mid-lactation were selected on the basis of not being treated with veterinary drugs during the last months and giving milk with a low number of somatic cells (<2 10^5/mL). The blank milk was always tested before use with a Delvotest SP-NT 5-PACK.

For the incubation of the strips, a dry-block heater type ROSA with an integrated timer (Charm Sciences Inc.) was used. For the interpretation of the color formation on the Charm MRL-3 strips, a three line reader system (ROSA Pearl Reader, Charm Sciences Inc.) was used. A low and a high calibration strip (Charm Sciences Inc.) were used to check the performance of the reader system.

Test Procedure and Interpretation of the Results

For raw milk no sample pretreatment was required, while milk powder was reconstituted with distilled water. Charm MRL-3 test strips were placed in a 3 min timed ROSA incubator at 56 ± 1 C with the flat side facing up. The tape was peeled back, 300 L milk was pipetted into either side well of the sample pad compartment, and the strips were resealed. The lid of the incubator was closed; a solid timer was automatically started. After 3 min incubation the strips were removed from the incubator and the results were read on the ROSA Pearl Reader within 3 min.

As milk flows through the device, a line is formed in the X (cloxacillin) and T (test) position when the sample contains no -lactams. A weaker intensity X or T line is formed when -lactam antibiotics are present in the sample. The X and T lines are compared to the C (control) line. If both the X and T lines are darker than or equal to the C line, the sample is free of -lactams (negative). If either or both the X and T line are lighter than the C line, or the X and/or T line does not form, the milk is contaminated (positive). If the C line does not form, the test is invalid and must be repeated.

The reader measured the color formation at both test lines and the control line position and converted the line comparison into a reading. Milk giving a reader value 0 was considered as free from -lactam antibiotics (negative), while milk giving a reader value >0 was considered as suspect of presence of -lactam antibiotics (positive). If the control line is missing or smeared, or the color is unevenly developed, or if sample is obscuring either the C, T, or X lines, the reader will indicate 'INVALID', and the sample must be retested.

The performance of the reader system is checked daily by a low and a high calibration strip and by testing a negative (residue-free raw milk) and a positive control standard prior to testing samples.

Visual interpretation is not easy since, most of the time, three lines are present, and the color differences are not always very pronounced. So the use of a reader system able to read strips at three positions is recommended.

Test and Reader Repeatability

To calculate the repeatability of the ROSA Pearl Reader, negative and positive strips were measured twice. The repeatability of the reader was calculated for different reader value levels. Blank and spiked milk samples were analyzed, and the strips were each measured twice with the ROSA Pearl Reader. The repeatability of the test was calculated at different reader value levels by analyzing and measuring blank and positive milk samples in duplicate.

Test Selectivity

The selectivity of the Charm MRL-3 was investigated by spiking residue-free raw milk with a substance belonging to other groups of antibiotics or chemotherapeutics at 10x MRL and testing in duplicate. One representative substance was chosen from each of the most important groups: oxytetracycline (tetracyclines), sulfadiazine (sulfonamides), enrofloxacin (quinolones), neomycin (aminoglycosides), erythromycin (macrolides), lincomycin (lincosamides), clavulanic acid (-lactamase inhibitors), colistin (polymyxins), and trimethoprim (diamino pyrimidine derivatives). The forbidden compounds chloramphenicol and dapsone, spiked at 3 and 50 g/kg, respectively, were also tested. Non- -lactam compounds testing positive were spiked in different concentrations in milk to test the minimal concentration causing positive results.

Detection Capability

The detection capability (CC) was determined for all -lactams mentioned in the list of MRLs in milk (6, 7). Therefore, starting from the CC concentrations indicated by the kit manufacturer, blank milk was spiked with the -lactams investigated at different concentrations in various ranges in different increments: in the range 1–10 g/kg, 1 g/kg; 10–20, 2 g/kg; 20–50, 5 g/kg; 50–100, 10 g/kg; 100–250, 25 g/kg; 250–500, 50 g/kg; 500–1000, 100 g/kg; and 1000–5000, 500 g/kg. The spiked samples were blind-coded before analysis. Each concentration was tested 20 times, in a time period of at least 3 days. For each -lactam investigated, the lowest concentration giving 19 positive results out of 20 total test results was determined, interpreting with the ROSA Pearl Reader.

Test Robustness

(**a**) *Length of incubation.*—The impact of the length of incubation on the test result was studied. The incubation time was modified between 150 s and 4 min. Each situation was tested with four blank milk samples and with four milk samples spiked with penicillin G (3 g/kg) or cloxacillin (30 g/kg).

(**b**) *Influence of waiting time on reader results.*—Blank milk samples and samples spiked with penicillin G (3 g/kg) or cloxacillin (30 g/kg) were analyzed, and the strips were read with the ROSA Pearl Reader directly after the incubation and after 0.5, 1, and 3 min.

Milk Influences

(**a**) *Milk quality and composition.*—The impact of the milk quality (somatic cell count, total bacterial count) and composition (fat and protein content, pH) was tested by comparing the performance of the Charm MRL-3 for milk

Table 1. Repeatability of the reader and of the Charm MRL-3 test at different levels

Milk	Compound; concn, g/kg	No. of samples	Mean level	Repeatability (s_r)[a]	Repeatability %
		Reader repeatability			
Blank milk		10	–1336	25	1.9
Positive milk	Penicillin G; 3	10	1133	24	2.1
	Penicillin G; 5	10	1477	30	2.0
	Cloxacillin; 25	10	1091	30	2.7
	Cloxacillin; 45	10	1485	38	2.6
		Test repeatability			
Blank milk		30	–1008	507	50.3
Positive milk[b]		30	1218	186	15.3
Positive milk[c]		20	1830	109	6.0

[a] s_r = Standard deviation of repeatability.
[b] Reader level 0–1500.
[c] Reader level >1500.

with a normal quality and composition with milk with a high somatic cell count (34 samples) or a high total bacterial count (36 samples). A comparison of the test performance was also executed on 10 different spiked milk samples with a normal and an abnormal composition. Milk of normal and abnormal composition was analyzed with and without spiking with penicillin G (3 g/kg) or cloxacillin (16 g/kg). For each different milk type, the average, highest, and lowest reader values were calculated.

Milk samples with a high number of somatic cells (>10^6/mL) were selected at the milk control station based on Fossomatic 5000 (Foss, Hillerød, Denmark) measurements. Milk samples with a high total bacterial count (>5 10^5 CFU/mL) were obtained by keeping normal milk samples for 4–6 h at room temperature. The final bacterial count was determined by performing a spiral plate count (Eddy Jet, IUL sa, Barcelona, Spain) on plate count agar plates after 3 days incubation at 30 C. Milk samples with a low fat content (<2 g/100 mL) were obtained by removal of the fat layer by centrifugation (3050 *g*, 10 min, at 5 C). Milk samples with a high fat content (>6 g/100 mL) and a low (<2.5 g/100 mL) and a high (>4 g/100 mL) protein content were natural milk samples with extreme fat or protein content that were selected at the milk control station based on IR spectroscopic results (MilcoScan 4000, Foss). To prepare samples with an abnormal pH, normal milk was initially adjusted to pH 6.0 and 7.5 with 1 M HCl or 1 M NaOH, respectively; then the pH was further adjusted with the addition of either 0.1 M HCl or 0.1 M NaOH.

(**b**) *Type of milk and animal species.*—Ultra high temperature processing (UHT) milk, sterilized milk, reconstituted milk powder, thawed milk, goats' milk, ewes' milk, and mares' milk were also tested to determine if the Charm MRL-3 was a suitable test for these types of milk. Ten different samples of each milk type were tested, with the exception that only three samples were tested for thawed milk. The aim was not only to investigate if certain milk types interfere and cause false-positive results but also to test if the detection capability was or was not hampered. Therefore, penicillin G (3 g/kg) and cloxacillin (30 g/kg) were spiked into raw cows' milk, milk of other types, or milk from animal species other than cow.

Test for False-Positive/False-Negative Results

Twenty-two farm milk samples, 22 truck milk samples, 11 consumer milk samples, and eight milk powders were analyzed with the Charm MRL-3 as part of a monitoring program. The same samples were also tested by the Delvotest SP-NT, *Bacillus cereus*-test, *Escherichia coli*-test, and Charm MRL -Lactam Test. Also, special sampled milk samples were analyzed with Charm MRL-3 to verify the rate of false-positive results. The special sampling concerned 41 individual cow milk samples, 300 farm milk samples, and 300 tanker milk samples. Positive samples were further analyzed with other microbiological and immunological antimicrobial residue tests.

For testing the rate of false-negative results and to verify if the test capacity for penicillin G in incurred samples is comparable to the value determined in spiked milk samples, 82 incurred milk samples originating from 27 individual cows treated with a veterinary drug containing penicillin G and neomycin were analyzed with the Charm MRL-3 and with other microbiological and -lactam receptor screening tests. Sampling started at the end of the withholding period. The exact concentration of penicillin G present in the milk samples was determined by HPLC-MS/MS in an external laboratory.

Reagent Influence (Batch Differences)

To study the differences of different batches of reagents, blank and spiked milk samples were analyzed at the same time with two different batches of Charm MRL-3 reagents [Lot 009001 (Expiration July 2007) = Lot 009A (Expiration Sept. 2007 and Lot 008003)]. Besides spiking with penicillin G (3 g/kg) or cloxacillin (30 g/kg), 20 milk samples were also spiked with 3 g/kg cefalonium to obtain reader values closer to the cutoff value of 0.0. In the area around the cutoff, any change in intensity of the test line can be quickly noted. The stability of reagents during shelf life was also checked. Blank and spiked standards were tested with reagents of Lot 009001 shortly after the production date and just before the expiration date.

Interlaboratory Testing

Twice a year, T&V-ILVO organizes a national ring trial for the Belgian dairy industry regarding the detection of residues of antibiotics in milk by microbiological and rapid tests. Since 2007, laboratories using Charm MRL-3 participated.

In 2007, AFFSA Fougères, Community Reference Laboratory for Antimicrobial Residues in Food of Animal Origin, organized an international proficiency study for the analysis of -lactam residues in raw milk. T&V-ILVO participated with the Charm MRL-3 test.

Daily Control Samples

During the study, the reader was checked daily with a low and a high calibration strip. To check the reagents and the reader, blank milk and control samples were analyzed daily. Blank raw milk (once daily to check the reader and four additional times), a positive standard prepared by reconstitution of a Charm tablet containing penicillin G (3 g/kg) and cloxacillin (12 g/kg), raw milk spiked with penicillin G (3 g/kg, twice daily), and a reconstituted lyophilized Charm standard of cloxacillin (30 g/kg, four times daily) were used as control samples.

Results and Discussion

Test and Reader Repeatability

All repeatability results are shown in Table 1. The repeatability of the Charm ROSA Pearl Reader was very good; very low SDs of repeatability (s_r) values were obtained.

Table 2. Detection capability of the Charm MRL-3 instrumental reading with a cutoff reader value of 0.0 in comparison with Charm MRL

Compound	MRL, g/kg[b]	Detection capability[a]	
		Charm MRL-3	Charm MRL[c]
Penicillins			
Penicillin G	4	3	2
Ampicillin	4	4	3
Amoxicillin	4	4	3
Oxacillin	30	18	30
Cloxacillin	30	14	25
Dicloxacillin	30	12	25
Nafcillin	30	90	45
Penethamate	4	200	ND[d]
Cefalosporins			
Ceftiofur	100	4 (6[e])	6
Cefquinome	20	14	14
Cefazolin	50	16	10
Cephapirin	60	3	5
Cefacetrile	125	9	6
Cefoperazone	50	4	4
Cefalexin	100	10	15
Cefalonium	20	3	3

[a] Detection capability defined as the lowest concentration tested giving a minimum of 19 positive results out of 20.

[b] MRL = Maximum residue limit [Commission Regulation (EU) 37/2010 as of January 21, 2010 and amendments as of April 1, 2009].

[c] Ref. (10).

[d] ND = No data available.

[e] Desfuroyl-ceftiofur (metabolite).

There is a difference in repeatability of the test between testing blank and positive results. An SD of repeatability of 507 for negative milk samples is too high; values up to 250–300 are acceptable. The high s_r value was mainly caused by five samples giving a negative and a positive result at the same time. For these five samples, the difference between the first and the second reading ranged between 1294 and 1964. Five false-positive results were encountered in this repeatability test. The repeatability for positive samples was better and is acceptable. The best repeatability was found for the samples with the highest reader result level.

Test Selectivity

The Charm MRL-3 is very selective for the detection of penicillins and cefalosporins. A real interference was caused only by clavulanic acid, a -lactamase inhibitor, at 175 g/kg and above. Positive results for enrofloxacin (quinolones) and colistin (polymyxins) were also obtained. A larger number of replicates showed that these positive results were false-positive results instead of a real interference.

Despite a special test line for cloxacillin and related compounds, the test is not able to differentiate between cloxacillin and the other -lactams. Also, within the -lactam group, the test is not specific for any particular -lactam, but nonsynthetic penicillins (penicillin G, ampicillin, and amoxicillin) could be differentiated from the group of synthetic penicillins and cefalosporins after pretreatment of the milk with penase (data not shown).

Detection Capability

A summary of the detection capabilities is given in Table 2. The Charm MRL-3 detected all -lactams with an MRL in milk (Commission Regulation 37/2010 and amendments; 6) at their respective MRL excepted for nafcillin (MRL = 30 g/kg) and penethamate (MRL = 4 g/kg), which were respectively detected at 90 and 200 g/kg and above. However, from a practical perspective, the high LOD of 100 g/kg for penethamate in relation to the MRL is of no significance since penethamate is not stable in milk and is rapidly and completely hydrolyzed to penicillin G and diethylaminoethanol (16).

Most cefalosporines were detected very sensitively; concentrations far below the respective MRL values caused a positive result. Concentrations like 12 and 14 g/kg cloxacillin sometimes gave a higher color intensity for the cloxacillin test line compared to the intensity of the control line. Nevertheless, the ROSA Pearl Reader converted the line comparison to a positive reading.

When performing the Charm MRL-3 instead of the classic Charm MRL test (8 min; 10) nearly the same detection capabilities were obtained for the group of natural penicillins and cefalosporins. The Charm MRL-3 was more sensitive for cloxacillin and related compounds due to a separate test line for cloxacillin on Charm MRL-3 strips. Only for nafcillin was there a real loss in sensitivity: the detection capability shifted from 45 to 90 g/kg.

Test Robustness

Length of incubation.—All data obtained when changing the length of incubation are summarized in Table 3. Performing the Charm MRL-3 protocol with the different incubation times tested had no significant impact on the reader values obtained for blank milk or positive spiked milk samples. Even when the incubation differed from the standard 3 min, within the limits tested, correct and acceptable results were still obtained, proving that a strict adherance to timing was not a critical point.

Influence of waiting time on reader results.—All data obtained when delaying the reading of the strips are summarized in Table 4. If the reading of the strips after incubation was delayed, the reader values did not change for blank milk samples, while positive reader values had the tendency to increase. So delaying the reading did not cause incorrect results, but slightly improved the detection capability.

Table 3. Values obtained when testing blank and spiked milk samples (four replicates) after incubations of different lengths of time

	Incubation time		
Reader	3 min	2 min 30 s	4 min
	Blank milk		
Mean value	–1564	–1320	–1330
Lowest value	–1942	–1531	–1442
Highest value	–1418	–1012	–1258
	Milk spiked with penicillin G at 3 g/kg		
Mean value	1259	1153	1125
Lowest value	1131	1126	937
Highest value	1306	1175	1413
	Milk spiked with cloxacillin at 30 g/kg		
Mean value	1159	1019	1209
Lowest value	840	828	1033
Highest value	1420	1281	1419

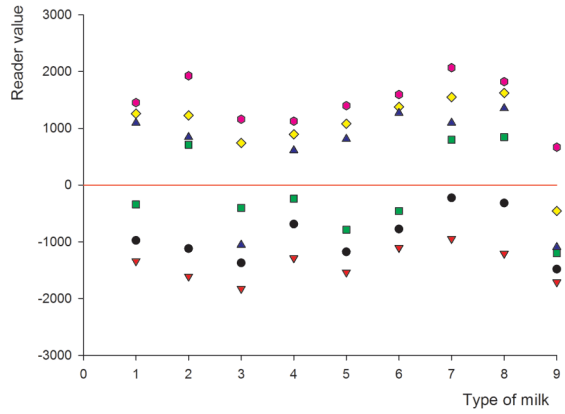

Figure 1. Reader values for normal and abnormal blank milk (●, mean; ▼, lowest; ■, highest) and normal and abnormal milks containing 3 g/kg penicillin G (◆, mean; ▲, lowest; ⬢, highest). Milks were (1) of normal composition or with (2) high somatic cell count; (3) high bacterial count; (4) low fat content; (5) high fat content; (6) low protein content; (7) high protein content; (8) low pH; or (9) high pH. The horizontal line at a value of 0.0 gives the cutoff between a negative and a positive result.

Milk Influences

Milk quality and composition.—With respect to testing the impact of the milk quality and composition (somatic cell count, total bacterial count, fat and protein content, and pH), the mean, the highest reader value, and the lowest reader value for each milk type are given in Figures 1 and 2.

The milk quality and composition had some influence on the performance of the Charm MRL-3 when testing blank milk; most blank milk samples were clearly negative with reader values below 0.0. However, positive reader values were obtained for blank milk with a high somatic cell count (one out of 34), for milk with a high protein content (three out 10), and for milk with a low pH (four out of 10). Also, some influence of milk quality and composition on the Charm MRL-3 results was noticed when testing spiked milk samples. In milk with a high pH, the detection of penicillin G was

Table 4. Values obtained when measuring four strips directly after incubation and after 0.5, 1, and 3 min

	Delay after incubation before reading, min			
Reader	0	0.5	1	3
	Blank milk			
Mean value	–1564	–1484	–1546	–1526
Lowest value	–1942	–1640	–1638	–1678
Highest value	–1418	–1399	–1459	–1361
	Milk spiked with penicillin G at 3 g/kg			
Mean value	1060	1102	1127	1230
Lowest value	1026	1016	1062	1177
Highest value	1122	1150	1164	1321
	Milk spiked with cloxacillin at 30 g/kg			
Mean value	1091	1117	1181	1298
Lowest value	840	819	872	978
Highest value	1420	1510	1632	1694

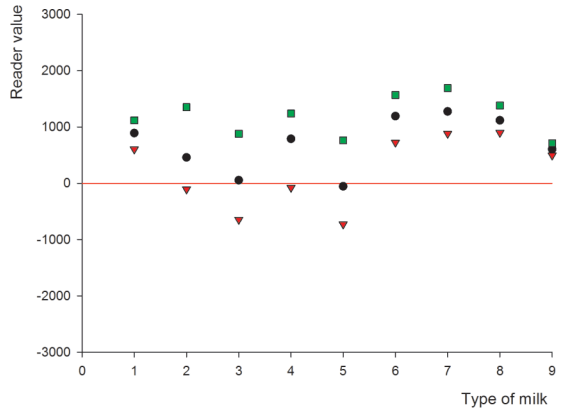

Figure 2. Reader values for normal and abnormal milks containing 16 g/kg cloxacillin (●, mean; ▼, lowest; ■, highest). Milks were (1) of normal composition or with (2) high somatic cell count; (3) high bacterial count; (4) low fat content; (5) high fat content; (6) low protein content; (7) high protein content; (8) low pH; or (9) high pH. The horizontal line at a value of 0.0 gives the cutoff between a negative and a positive result.

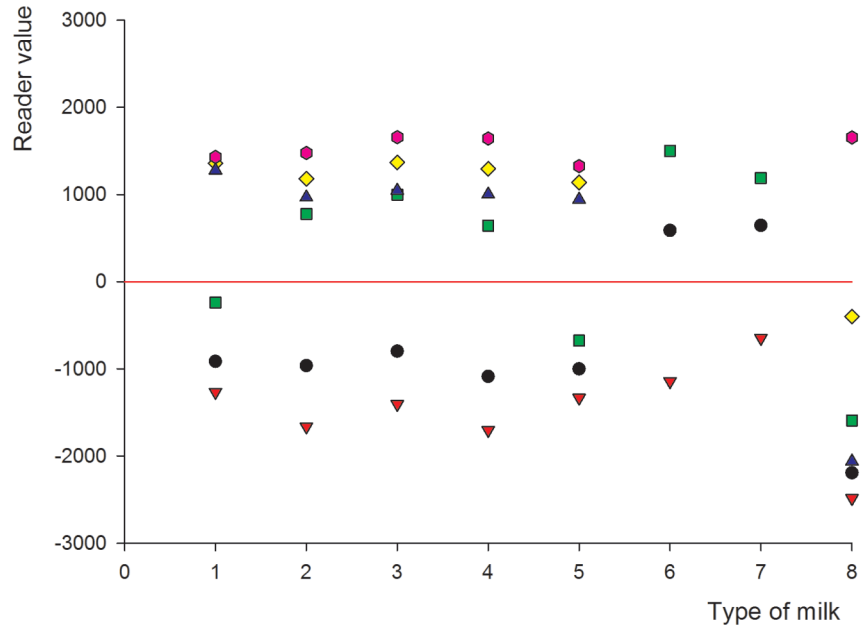

Figure 3. Reader values for blank milk (●, mean; ▼, lowest; ■, highest) and different milks containing 3 g/kg penicillin G (◆, mean; ▲, lowest; ⬢, highest). (1) Raw cows' milk compared with (2) UHT milk; (3) sterilized milk; (4) reconstituted milk powder; (5) thawed milk; (6) goats' milk; (7) ewes' milk; and (8) mares' milk. The horizontal line at a value of 0.0 gives the cutoff between a negative and a positive result.

hampered; seven out of 10 samples tested negative. In five out of 10 milk samples with a high fat content, 16 g/kg cloxacillin tested negative. The same result was obtained for two out of 10 milk samples with a high somatic cell count and for five out of 12 milk samples with a high bacterial load. Also, one negative result was obtained for milk with a high bacterial load spiked with penicillin G at 3 g/kg. The production of bacterial -lactamase in some milk samples with a high bacterial load could not be excluded. Lower reader values were obtained when testing milk with a high fat content spiked with cloxacillin at 16 g/kg. A hampered flow of milk with a high fat content on the strip could be the reason for the low results.

A high pH can occur in milk of individual cows due to the presence of subclinical mastitis, but it is unlikely that an entire bulk collection of milk will be affected. Further, it must also be recognized that the test is qualitative rather than quantitative and is used only to discriminate between -lactam residue-free milk and milk containing such residues.

Type of milk and animal species.—The results of the testing of UHT milk, sterilized milk, reconstituted milk powder, thawed milk, goats' milk, ewes' milk, and mares' milk are presented in Figures 3 and 4. Charm Sciences Inc. is only claiming the Charm MRL-3 as a test for raw cows' milk. Dairy companies are mainly interested in testing raw milk from incoming tankers or at the farm before collection. When testing blank UHT milk, sterilized milk, and reconstituted milk powder, false-positive results (respectively, two out of 20, two out of 15, and two out of 18) were obtained. No significant differences were obtained in testing different milk types spiked with penicillin G at 3 g/kg or cloxacillin at 30 g/kg. So Charm MRL-3 is not only a raw milk test for the dairy industry, but it could also be used by other laboratories to test UHT milk, sterilized milk, reconstituted milk powder, or thawed milk (monitoring samples are often kept frozen

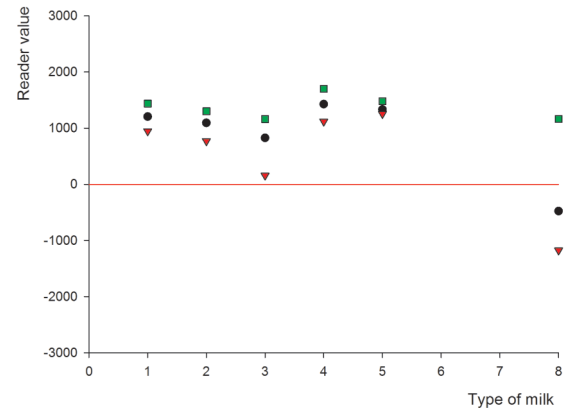

Figure 4. Reader values for different milks containing 30 (situation 1 to 5) or 16 (situation 8) g/kg cloxacillin (●, mean; ▼, lowest; ■, highest). Raw cows' milk (1) compared with (2) UHT milk; (3) sterilized milk; (4) reconstituted milk powder; (5) thawed milk; (6) goats' milk; (7) ewes' milk; and (8) mares' milk. The horizontal line at a value of 0.0 gives the cutoff between a negative and a positive result.

Table 5. Values obtained and number of positive (pos) and negative (neg) milk samples when testing the same blank and the same spiked milk samples with Charm MRL3 reagents from different batches or with reagents of a different age

	Lot 009001 just after production					Lot 008003					Lot 009001 just after production					Lot 009001 just before expiration				
	Ratio			Number		Ratio			Number		Ratio			Number		Ratio			Number	
Tested	Mean	Min[a]	Max[b]	Pos	Neg	Mean	Min	Max	Pos	Neg	Mean	Min	Max	Pos	Neg	Mean	Min	Max	Pos	Neg
Blank milk	–1207	–1753	–627	0	20	–1116	–1661	–530	0	20	–1207	–1753	–627	0	20	–230	1597	838	10	10
Milk spiked with penicillin G at 3 g/kg	1173	785	1576	24	0	986	–853	1627	23	1	1173	785	1576	24	0	1412	1252	1604	24	0
Milk spiked with cloxacillin at 12 g/kg	1421	1239	1681	20	0	939	–190	1386	19	1	1421	1239	1681	20	0	1444	1124	1939	20	0
Milk spiked with cefalonium at 3 g/kg	905	–688	1364	19	1	238	–1271	1220	14	6	905	–688	1364	19	1	1106	898	1379	20	0

[a] Min = Minimum.
[b] Max = Maximum.

during transport and storage) on the condition that positive results are further tested with a different antibiotic test.

When testing blank goats' and ewes' milk, false-positive results (respectively, six out of eight and 10 out of 12) were obtained. Since such a high percentage of false-positive results were obtained for blank milk from these animal species, no testing of spiked samples was performed. Blank mares' milk gave three invalid readings out of 10, while spiked mares' milk testing gave fewer positive, and even false-negative, results. The Charm MRL-3 is, therefore, not a suitable test to screen milk from animal species other than cow (goat, ewe, or mare).

Test for False-Positive/False-Negative Results

Throughout the evaluation study, false-positive results were obtained when testing blank raw milk. Out of the special sampling of 41 individual cow's milk samples, 300 farm milk samples, and 300 tanker milk samples, the percentage of false-positive results can be estimated as 2.4, 0.7, and 2.7%, respectively. Samples giving false-positive results were retested (five replicates). The replicates always gave a negative Charm MRL-3 result. So it is recommended to retest a positive sample to confirm the presence of -lactam antibiotics.

In legislation for screening methods there is only a norm of <5% (-error) for the false compliant rate at the level of interest (14). There is no norm for the rate of false-positive results; for logistic and economic reasons, this rate should be as low as possible. In the same legislation (14), it is stipulated that a suspected noncompliant result shall be confirmed by a confirmatory method.

No false-negative results were obtained when farm milk, truck milk, consumption milk, and milk powders were tested as part of a monitoring program. However, false-positive Charm MRL-3 results were also obtained in this monitoring program.

For testing the rate of false-negative results, incurred milk samples originating from individual cows treated with a veterinary drug containing penicillin G and neomycin were analyzed with the Charm MRL-3. From a penicillin G content 1.2 g/kg, all 32 incurred milk samples tested positive. These data confirm that the detection capability for penicillin G in spiked milk (3 g/kg, Table 2) is also valid for the detection of penicillin G in incurred milk samples. In this study of incurred milk samples originating from individual cows, false-positive Charm MRL-3 results were also obtained. Twenty-four out of 50 samples with a penicillin G content 1.0 g/kg tested positive on Charm MRL-3. If just the group of milk samples with a penicillin G content below the CC from the HPLC-MS/MS determination (0.3 g/kg) is considered, still, 12 out of 33 samples gave a positive Charm MRL-3 result. It is difficult to indicate the reason for this high rate of false noncompliant results. Milk of individual cows is more likely to have an anomalous fat or protein content, although this was not the case in the depletion study. Some milk samples causing false-positive results were centrifuged and decanted. On the bottom of some test tubes, debris was

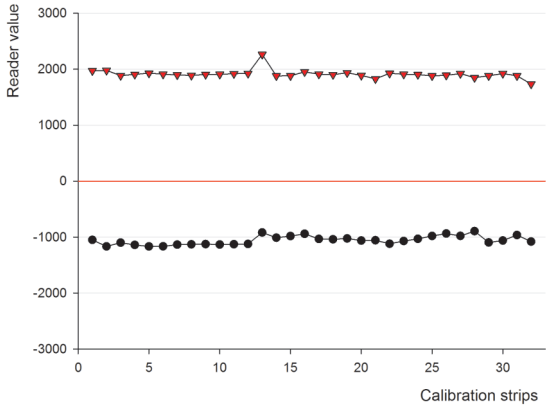

Figure 5. Values obtained for 32 daily checks of the reader with a low (●) and a high (▼) calibration strip. The horizontal line at a value of 0.0 gives the cutoff between a negative and a positive result.

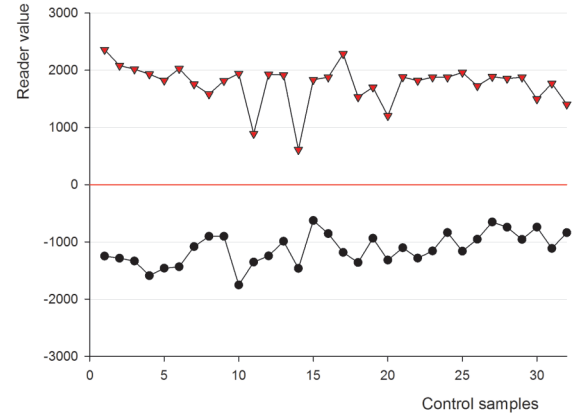

Figure 6. Values obtained for 32 daily checks of the reader with a negative (●) and a positive (▼) control. The horizontal line at a value of 0.0 gives the cut off between a negative and a positive result.

present, microscopically identified as particles of hair and dust. Such small particles could possibly hamper the lateral flow of milk on the dipstick. The presence of small particles in the milk samples of the depletion study is more likely since this milk was unfiltered; milk is normally passed through a filter fitted in the milk tubes before entering in the milk silo at the farm.

Reagent Influence (Batch Differences)

A summary of the results of the testing of spiked milk samples with two different batches of Charm MRL-3 reagents is given in Table 5. Only small differences in test capability were found between different batches of reagents of Charm MRL-3. Blank milk gave essentially the same reader values. Batch 008003 was somewhat less sensitive when residues were detected in the spiked samples containing penicillin G at 3 g/kg, cloxacillin at 12 g/kg, or cefalonium at 3 g/kg, but the difference is of no importance when the kit is used for the discrimination of positive from blank milk.

The stability of reagents during shelf life was also checked. Blank and spiked standards were tested with reagents of Lot 009001 shortly after the production date and again shortly before the expiration date. In general, comparable results were obtained for the spiked milk samples, but 10 out of 20 blank milk samples tested positive (false-positive results) with the reagents just before the expiration date.

Interlaboratory Testing

Twice a year, T&V-ILVO organizes a national ring trial for the Belgian dairy industry regarding the detection of residues of antibiotics in milk by microbiological and rapid tests. Since 2007, laboratories could also participate with the Charm MRL-3. Each time eight blind-coded milk samples were distributed to the laboratories.

In the five ring trials organized since spring 2007, Charm MRL results were reported by two laboratories. No false-negative results were obtained; however, in four ring trials seven (out of 20) false-positive results were generated. Details of the results are given in separate reports (17–21).

In the international proficiency study organized by AFSSA Fougères, six milk samples were distributed among the participating laboratories (22). The blank milk was analyzed as positive by T&V-ILVO with Charm MRL-3 (reader value 761), while the five spiked milk samples, containing cefquinome at 50 g/kg, cloxacillin at 40 g/kg, cefalonium at 20 g/kg, and penicillin G at 6 g/kg (in duplicate) all tested positive.

Daily Control Samples

During the study, a low and a high calibration strip, a negative control sample (blank raw milk), a positive standard sample [reconstituted Charm tablet

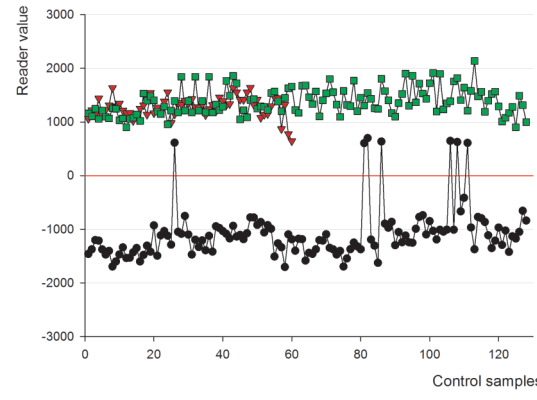

Figure 7. Values obtained for 128 control samples with blank raw milk (●), 60 control samples containing 3 g/kg penicillin G (▼), and 128 control samples containing 30 g/kg cloxacillin (■). The horizontal line at a value of 0.0 gives the cutoff between a negative and a positive result.

containing penicillin G (3 g/kg) and cloxacillin (12 g/kg)], four blank raw milk samples, two raw milk samples spiked with penicillin G (3 g/kg), and four reconstituted lyophilized Charm standards of cloxacillin (30 g/kg) were analyzed daily. The results are shown in Figures 5–7. Over 32 working days, the control samples gave very constant reader values. For the entire period, the following average reader values were obtained: blank raw milk: –1087 ± 482; milk spiked with 3 g/kg penicillin G: 1279 ± 195; and milk spiked with 30 g/kg cloxacillin: 1377 ± 254. It is worth noting the influence of some false-positive results for some blank milk samples on the SD.

Conclusions

With a total test time of 3 min, the Charm MRL-3 is presently one of the fastest tests on the market for the detection of -lactam residues in milk. The short test time and the very easy, one-step test protocol enable the use of the test at the farm before collection in order to prevent tanker milk contamination. A drawback is the recommendation of the use of a reader system for the interpretation of the color formation on the dipsticks. The same reagents and test protocol could also be used at the entrance of the dairy plant to check the incoming milk for the presence of -lactams. It is recommended that initial positive samples as indicated by the kit manufacturer be retested, as false-positive results could occur.

Acknowledgments

We appreciate the valuable work performed by Veronique Ottoy and Katleen Vander Straeten, and thank Charm Sciences Inc. for kindly providing Charm MRL-3 test reagents, MelkControleCentrum Vlaanderen for providing part of the raw cows' milk samples with a special composition or quality and for the MilcoScan 4000 and Fossomatic 5000 measurements, and Siegrid De Baere (UGent) for the incurred milk samples used for testing of false-negative results and for the determination of penicillin G content of these incurred samples.

References

(1) *Martindale: The Complete Drug Reference* (2005) 34th Ed., S.C. Sweetman (Ed.), Royal Pharmaceutical Society of Great Britain, Pharmaceutical Press, London, UK, pp 1–2756

(2) Pyörälä, S. (1995) in *The Bovine Udder and Mastitis*, M. Sandholm, T. Honkanen-Buzalski, L. Kaartinen, & S. Pyörälä (Eds), University of Helsinki, Finland, Faculty of Veterinary Medicine, pp 143–148

(3) Honkanen-Buzalski, T., & Reybroeck, W. (1997) in *Monograph on Residues and Contaminants in Milk and Milk Products*, I.D.F. (International Dairy Federation), Brussels, Belgium, pp 28–34

(4) Suhren, G. (1996) *Kieler Milchwirtschaftl. Forschungsber.* **48**, 131–149

(5) Grunwald, L. (2002) *Untersuchungen zur Analytik und zum Einfluss Technologischer Prozesse auf Penicillinrückstände in Milch*, Dissertation: Wuppertal, Bergische Universität Wuppertal, Fachbereich Chemie, pp 1–169

(6) European Commission Regulation (EU) No. 37/2010 (2010) *Off. J. Eur. Comm.* **L15**, 1–72

(7) European Council Regulation (EC) No. 470/2009 (2009) *Off. J. Eur. Comm.* **L152**, 11–22

(8) Kroll, S., Usleber, E., Zaadhof, K.-J., Schneider, E., & Märtlbauer, E. (2000) *Proceedings of the Euroresidue IV Conference on Residues of Veterinary Drugs in Food*, Veldhoven, the Netherlands, May 8–10, pp 693–697

(9) Reybroeck, W. (2004) *Le Point Vétérinaire*, **No. 242**, 52–57

(10) Reybroeck, W., & Ooghe, S. (2004) in *A Farm-to-Table Approach for Emerging and Developed Dairy Countries*, Proceedings IDF/FAO International Symposium on Dairy Safety and Hygiene, Cape Town, Republic of South Africa, 2–5 March, pp 157–161

(11) Quandt, B. (2006) *Identifizierung van antimikrobiellen Rückständen in Milch mittels Schnelltestsystemen*, Inaugural-Dissertation zur Erlangung der Tiermedizinischen Doktorwürde der Tierärztlichen Fakultät der Ludwig-Maximilians-Universität München, Deutschland, Germany, pp 1–123

(12) Žvirdauskiené, R., & Šalomskiené, J. (2007) *Food Control* **18**, 541–547

(13) Reybroeck, W. (2008) in *Bulletin of the IDF 424/2008 "Advances in Analytical Technology*," pp 13–16

(14) European Commission Decision (EC) No. 2002/657 (2002) *Off. J. Eur. Comm.* **L 221**, 8–36

(15) Reybroeck, W., & Ooghe, S. (2008) *Proceedings of the Euroresidue VI Conference on Residues of Veterinary Drugs in Food*, Egmond aan Zee, the Netherlands, May 19–21, pp 787–792

(16) Committee for Veterinary Medicinal Products (2000) *Penethamate (Summary Report)*, The European Agency for the Evaluation of Medicinal Plants, Veterinary Medicines Evaluation Unit, London, UK, pp 1–3

(17) Ooghe, S., & Reybroeck, W. (2007) *Rapport Ringonderzoek Antibiotica 24 Mei 2007: Microbiologische Testen en Sneltesten*, T&V-ILVO, Melle, Belgium, pp 1–23

(18) Ooghe, S., & Reybroeck, W. (2007) *Rapport Ringonderzoek Antibiotica 18 Oktober 2007: Microbiologische Testen en Sneltesten*, T&V-ILVO, Melle, Belgium, pp 1–17

(19) Ooghe, S., & Reybroeck, W. (2008) *Rapport Ringonderzoek Antibiotica 24 April 2008: Microbiologische Testen en Sneltesten*, T&V-ILVO, Melle, Belgium, pp 1–21

(20) Ooghe, S., & Reybroeck, W. (2008) *Rapport Ringonderzoek Antibiotica 23 Oktober 2008: Microbiologische Testen en Sneltesten*, T&V-ILVO, Melle, Belgium, pp 1–17

(21) Ooghe, S., & Reybroeck, W. (2009) *Rapport Ringonderzoek Antibiotica 23 April 2009: Microbiologische Testen en Sneltesten*, T&V-ILVO, Melle, Belgium, pp 1–20

(22) *Final Report of Proficiency Study for the Analysis of Beta-Lactam Residues in Raw Milk* (2008) AFSSA, Fougères, France, pp 1–43