Validation of the Charm Cowside II test, a microbial inhibitor test for raw commingled milk - Report

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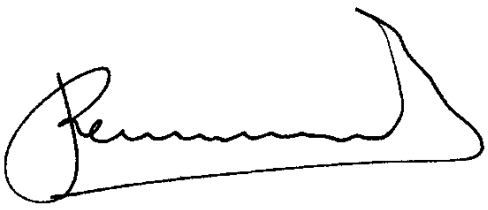

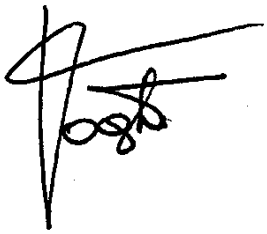

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Introduction

The CHARM Cowside II Test (Charm Sciences Inc., Lawrence, MA) is a 3 hour qualitative microbial test for testing raw milk for inhibitory substances including residues of antibiotics. The test is based on the growth inhibition of *Geobacillus stearothermophilus* var. *calidolactis* and the kit is supplied in a single sample vial format. Inhibitory substances include broad classes of antimicrobial (antibiotics) agents, such as β-lactams, tetracyclines, macrolides, aminoglycosides, and sulfonamides, used in animal care and welfare. Other inhibitors such as cleaning agents, or natural biologic inhibitors produced during udder infection, e.g. lysozyme, lactoferrin, may also cause inhibition. The CHARM Cowside II was validated at ILVO-, Flanders research institute for agriculture, fisheries and food (Melle, Belgium),on its applicability as a screening test at the interprofessional organisations, according to Commission Decision 2002/657/EC, CRL Guidelines (*Anon.*, 2010) in order to check if the test is fulfilling the FASFC (Federal Agency for the Safety of the Food Chain) acceptance criteria for screening tests (*Anon.*, 2011).

Principle of the Cowside II Test

The Cowside II detects antibiotics by inhibition of seeded bacteria in the purple agar contained in the base of the vial. If the *Geobacillus stearothermophilus* bacteria grow in the presence of a milk layer added on top of the agar (milk sample), they will produce acid that changes the purple pH indicator to yellow. If the bacteria do not grow within the 3 hours, the agar stays purple indicating there is a growth inhibitor in the milk sample. The Cowside II is a single step test of adding a milk sample and then incubating it in a 64°C incubator for approximately 3 hours. The test time is unique for each lot number of reagents. Inhibition is noticed by a visual interpretation of the color change. A color comparator aid is supplied with the kit to help interpretation based on yellow- negative; purple/blue-positive; and brownish/green-caution (positive). The test was evaluated visually using multiple brands of dry well incubators for vials: a 4 place and 20 place incubator manufactured by Charm Sciences Inc., and a 10 place Delvo® incubator supplied by DSM that is used by many farmers in EU and USA.

Length of incubation

According to the kit insert, the test should be incubated for the time specified on the reagent box, usually 2 hours and 45 minutes to 3 hours and can be verified by testing a negative control has turned yellow and a positive control stays blue/purple. The incubation time indicated by the kit manufacturer is critical to incubator temperature and the ILVO laboratory made adjustments to the supplied incubators to achieve tighter incubator temperature ranges 64 ±0.2°C than those controlled by the manufacturer. A table of the times for each lot evaluated is reported in Table 1. These times achieved the optimum detection capabilities; but it is noted that for use by farmers an additional time of 10 minutes could be added to reduce marginal caution results due to reagent variability and shipping storage conditions. This additional time will make interpretation easier but could reduce the detection capability to bacteriostatic antimicrobials such as sulfonamide drugs. One lot supplied (CSII-041B-03) did not perform according to label at 2 hours and 55 minutes and was reported to manufacturer as rejected.

Table 1. Incubation times of accepted reagents

Reagent Lot	Incubation Time
CSII-039-01	2 hours 50 minutes
CSII-040-02	2 hours 55 minutes
CSII-041-01	2 hours 45 minutes
CSII-042-06D	2hours 55 minutes

Standard material

Antimicrobials and inhibitors evaluated and their sources/lot# are supplied in Table 2.

Table 2. Chemicals used for validation of Charm Cow Side II

Product	Company	Lot
Penicillin G sodium salt	Sigma-Aldrich	054M4816V
Ampicillin sodium salt	Sigma-Aldrich	BCBL4878V
Amoxicillin	Sigma-Aldrich	SLBCO896
Oxacillin sodium salt	Sigma-Aldrich	BCBF5635V
Cloxacillin sodium salt monohydrate	Sigma-Aldrich	BCBJ1422V
Dicloxacillin sodium salt monohydrate	Sigma-Aldrich	129K0614
Nafcillin sodium salt monohydrate	Sigma-Aldrich	SLBD4037V
Ceftiofur	Fluka (Sigma)	SZBF222XV
Desfuroylceftiofur	Toronto Research Chemicals	7-GRS-90-4
Cefquinome sulfate	Sigma-Aldrich	SZBC220XV
Cefazolin	USP	L1K284
Cephapirin sodium	Fluka (Sigma)	BCBH2031V
Desacetylcephapirin	Toronto Research Chemicals	6-JGC-123-1
Cefacetrile	Toronto Research Chemicals	2202D0 0030820
Cefoperazone sodium salt	Sigma-Aldrich	SLBD1146V
Cefalexin	Fluka (Sigma)	SZBD099XV

Product	Company	Lot
Cefalonium hydrate	Fluka (Sigma)	SZBD126XV
Tetracycline hydrochloride	WHO Centrum for Chemistry	SZBD148XV
4-epimer of tetracycline	Acros Organics	A0340191
Oxytetracycline hydrochloride	Sigma-Aldrich	BCBG9599V
4-epimer of oxytetracycline	Acros Organics	A0356291
Chlortetracycline	Fluka (Sigma)	SZBB129XV
4-epimer of chlortetracycline	Acros Organics	A0356291
Spiramycin	Sigma-Aldrich	060M1392V
Tylosin A	Sigma-Aldrich	SZBE090XV
Erythromycin A dihydrate	Fluka (Sigma)	SZBB105XV
Tilmicosin	Fluka (Sigma)	SZBA088XV
Colistin sodium methanesulfonate	Sigma-Aldrich	0001419776
Marbofloxacin	Fluka (Sigma)	SZBC248XV
Danofloxacin	Fluka (Sigma)	SZBA019XV
Enrofloxacin	Fluka (Sigma)	1369030V
Flumequine	Sigma-Aldrich	SLBF5484V
Spectinomycin dihydrochloride pentahydrate	Fluka (Sigma)	SZBB166XV
Streptomycin sulfate	Sigma-Aldrich	SLBH9702V
Dihydrostreptomycin sesquisulfate	Sigma-Aldrich	BCBL8993V
Gentamicin	Sigma-Aldrich	SZBD086XV
Neomycin trisulfate salt hydrate	Sigma-Aldrich	071M0117V
Kanamycin A disulfate salt hydrate	Fluka (Sigma)	SZBB266XV
Rifaximin	Fluka (Sigma)	SZBB035XV
Thiamphenicol	Sigma-Aldrich	STBB5298
Lincomycin hydrochloride	Pfizer	030603QCS33
Pirlimycin HCl	Pfizer	090074-QCS-5
Trimethoprim, minimum 98%TLC	Sigma-Aldrich	117K0671
Baquiloprim	Sigma-Aldrich	SZBE141XV
Potassium clavulanate	Fluka (Sigma)	SZBC146XV
Bacitracin zinc salt	Sigma-Aldrich	BCBM0998V
Novobiocin sodium salt	Sigma-Aldrich	1389204 34408221
Sulfamethazine	Sigma-Aldrich	BCBM2115V
Sulfadiazine	Sigma-Aldrich	BCBF6453V
Sulfamerazine	Sigma-Aldrich	042K1582
Sulfadoxine	Sigma-Aldrich	060M1164V
Sulfadimethoxine	Sigma-Aldrich	1431982
4-Aminophenyl sulfone (dapsone)	Sigma-Aldrich	MKBF6465V
Monensin A	Sigma-Aldrich	SLBK4090V

Detection capability

Blank raw milk was spiked with antibiotics used in lactating animals and with a Maximum Residue Limit (MRL) in raw milk (Regulation (EC) No 470/2009 of the European Parliament and of the Council Commission and Commission Regulation (EU) No 37/2010 and amendments). The increment between the different antibiotic concentrations was depending on the MRL and detection concentration level, as indicated in Table 3.

Table 3. Increment between the concentrations tested.

Concentration (in µg/kg)	Increment (in µg/kg)
1-5	1
6-10	2
11-30	5
31-100	10
101-200	25
201-500	50
501-1000	100

Four batches of Cowside II tests, CSII01-039, CSII02-040, CSII01-041, CSII06-042, were evaluated as part of the detection capability validation. The blank raw milk was commingled milk originating from 4 healthy cows in mid-lactation that were not treated with antibiotics or chemotherapeutics during the last months. The number of replicates tested at each concentration was to identify the lowest level that produced a minimum of 90% positive results with 95% confidence referred to as detection capability, CCβ less than 5% (*Anon.*, 2010). Testing levels started from manufacturer supplied dose response data and increased or decrease by increments until the CCβ performance specifications were met. The lowest concentration giving at least 19 positives of 20 replicates is reported in Table 4. These sensitivities are in line to those reported in a 96 well format test, the Blue Yellow II test reported in 2011 (*Reybroeck and Ooghe*, 2011). Lower concentrations of beta-lactam and aminoglycosides could be detected with Blue Yellow II while the Cowside II is detecting the sulfonamides, the lincosamides, bacitracin, novobiocin, and most of the macrolides at a lower concentration.

Table 4. Detection capabilities (in µg/kg) of the Charm Cowside II (visual reading).

Group	Substance	MRL (µg/kg)	Detection capability (µg/kg)
Penicillins	Benzylpenicillin	4	2
	Ampicillin	4	4
	Amoxicillin	4	3
	Oxacillin	30	8
	Cloxacillin	30	20
	Dicloxacillin	30	15
	Nafcillin	30	6
	Penethamate	4	n.t.
Cefalosporins	Ceftiofur	100[a]	25(**200**)[b]
	Cefquinome	20	**80**
	Cefazolin	50	8
	Cephapirin	60[c]	5(8)[d]
	Cefacetrile	125	20
	Cefoperazone	50	25
	Cefalexin	100	100
	Cefalonium	20	15

Group	Substance	MRL (µg/kg)	Detection capability (µg/kg)
Tetracyclines	Tetracycline HCl	100[e]	60(**700**)[f]
	Chlortetracycline	100[e]	90(**900**)[g]
	Oxytetracycline	100[e]	100(**350**)[h]
Macrolides	Spiramycin	200	**250**
	Tylosin	50	25
	Erythromycin	40	**80**
	Tilmicosin	50	30
Aminoglycosides	Spectinomycin	200	**700**
	Streptomycin	200	**900**
	Dihydrostreptomycin	200	**800**
	Gentamicin	100	90
	Neomycin (+framycetin)	1,500	125
	Kanamycin	150	**>1,500**
Quinolones	Marbofloxacin	75	**>750**
	Danofloxacin	30	**>300**
	Enrofloxacin (+ciprofloxacin)	100	**>1,000 (900)**
	Flumequine	50	**>1,000**
Polymyxins	Colistin	50	**>500**
Ansamycines+ naftalene ring	Rifaximin	60	60
Thiamphenicol	Thiamphenicol	50	**1,250**
Lincosamides	Lincomycin	150	60
	Pirlimycin	100	20
β-lactamase inhibitors	Clavulanic acid	200	**2,000**
Polypeptides	Bacitracin	100	**600**
Other antibiotics	Novobiocin	50	**500**
Sulfonamides	Sulfamethazine	100	100
	Sulfadiazine	100	60
	Sulfamerazine	100	50
	Sulfadoxine	100	**125**
	Sulfadimethoxine	100	40
Diamino pyrimidinederivates	Trimethoprim	50	**70**
	Baquiloprim	30	30
Ionofors	Monensin A	2	**>20**
Other chemotherapeutics	Dapsone	---*	1

Notes: Bold and red font detection capabilities are above the drug MRL. MRL: Maximum Residue Limit, Regulation (EC) No 470/2009 and Commission Regulation (EU) No 37/2010 and amendments as of 01/07/2016. Detection capability defined as the lowest concentration tested giving a minimum of 19 positive results out of 20.

[a]: The MRL of 100 µg/kg is applied on the sum of all residues retaining the β-lactam structure expressed as desfuroylceftiofur,
[b]: desfuroylceftiofur,
[c]: the MRL of 60 µg/kg in milk is applied on the sum of cephapirin and desacetylcephapirin,
[d]: desacetylcephapirin,

[e]: the MRL of 100 µg/kg in milk is applied on the sum of the parent drug and its 4-epimer,
[f]: 4-epimer of tetracycline,
[g]: 4-epimer of chlortetracycline,
[h]: 4-epimer of oxytetracycline,
*: prohibited substance: MRL cannot be established, recommended concentration for testing = 5 µg/kg (*Anon.,* 2007).

For each substance, the testing was spread over at least three days with the use of at least two different antibiotic sources and stock solutions. Spiking was performed in different blank raw milks. An equal number of replicates from each manufactured lot were used to build lot variation robustness into the calculation. The detection capabilities are depicted graphically compared to MRL in Figures 1, 2 and 3. If the MRL circle (green) is surrounded by the sensitivity line (red), the group of drugs is detected at MRL; while if the red line falls inside the green MRL circle the drugs are not detected at MRL. Figure 1 demonstrates the beta-lactam group is primarily detected at MRL except for cefquinome and desfuroylceftiofur. Figure 2 demonstrates macrolides and tetracyclines (non-metabolic forms) are detected at MRL, while the detection of aminoglycosides is mixed with the detection at MRL of only gentamycin and neomycin. Quinolones are not detected at all. Figure 3 shows the detection of the sulfonamide and lincosamide group and the specific detection of dapsone and rifaximin.

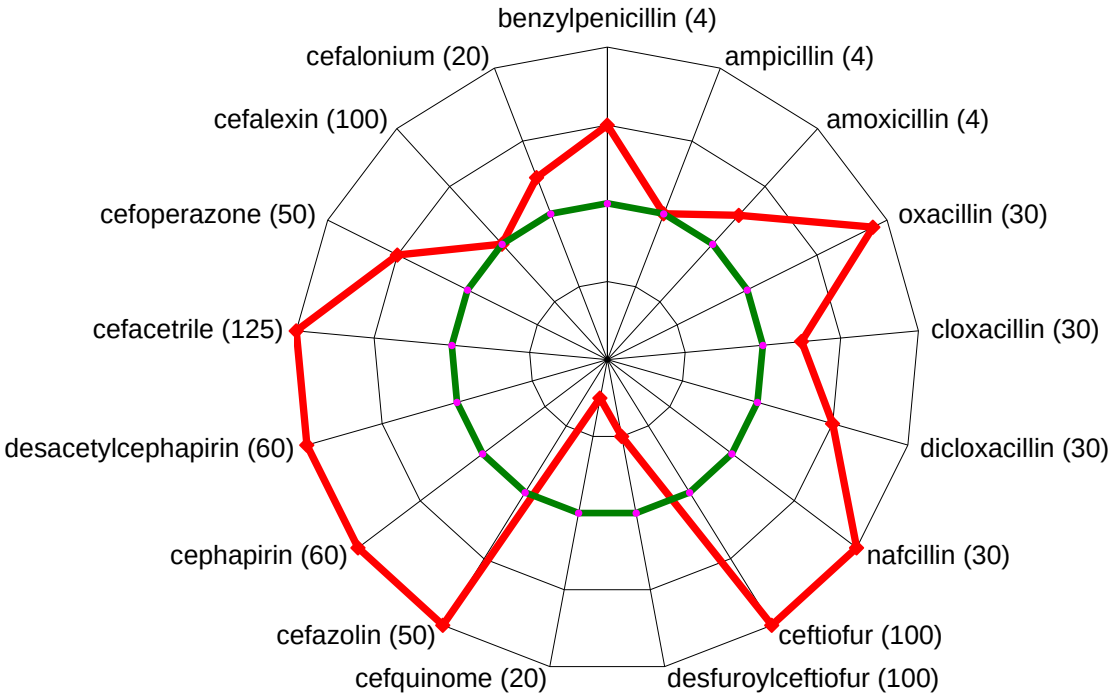

Figure 1a. Detection capability of Cowside II to Beta-lactam antibiotics.

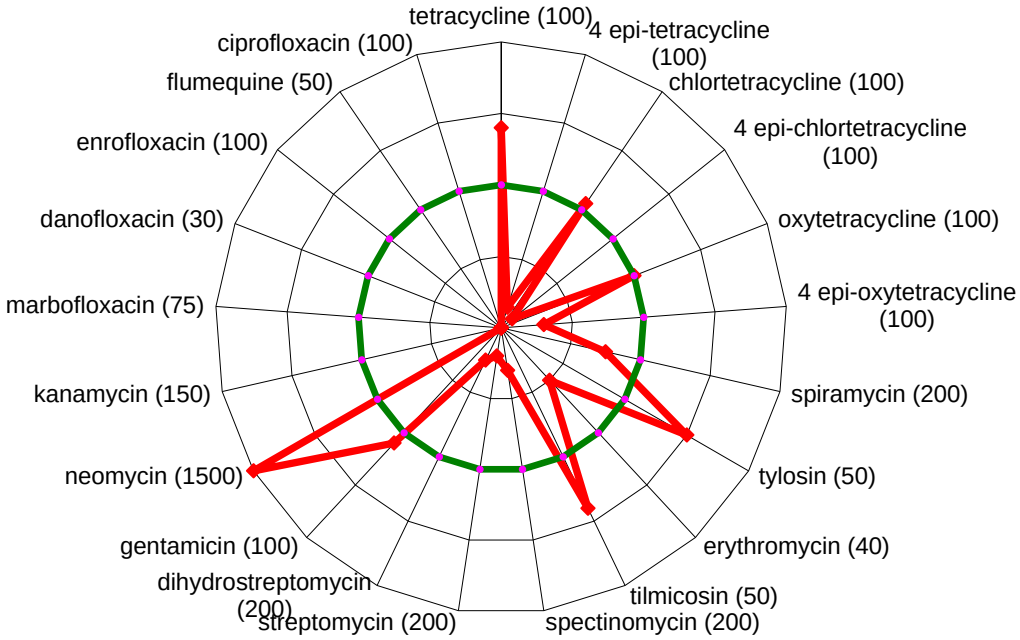

Figure 1b. Detection capability of Cowside II to tetracyclines, macrolides, aminoglycosides and quinolone antibiotics.

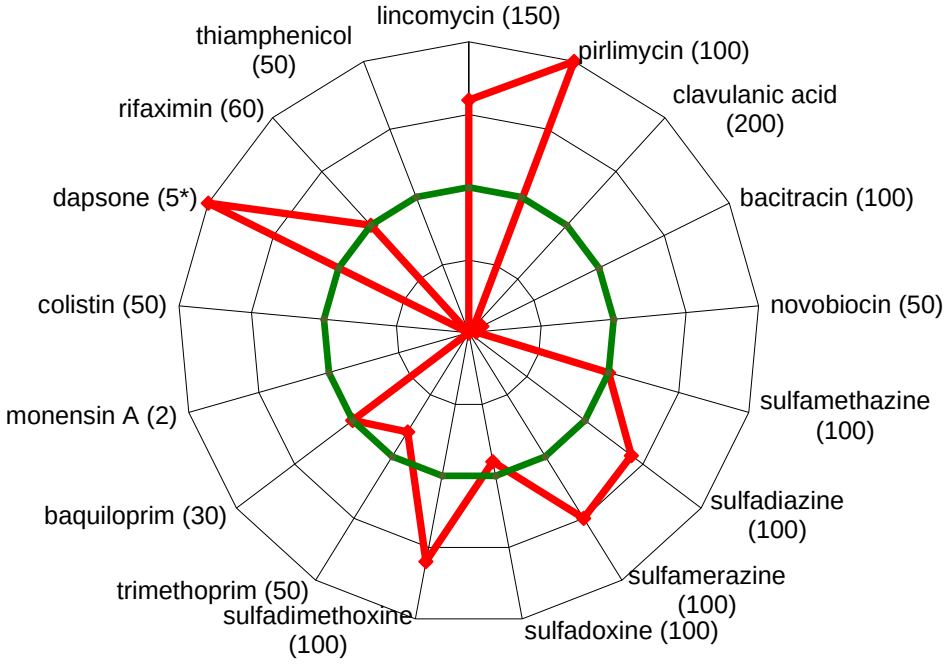

Figure 1c. Detection capability of Cowside II to sulfonamides and other antibiotics and inhibitor classes.

Figure 1. Detection capability of Cowside II related to their respective MRL (Commission Regulation (EU) No 37/2010 (situation on 01/07/2016)). Inner circle = 2 MRL; bold circle 2 = MRL; circle 3 = 0.5 MRL; outer circle 4 = 0.25 MRL. Visual color interpretation.

In Belgium criteria were set by FASFC (Federal Agency for the Safety of the Food Chain) for the approval of tests for the screening of antibiotics and sulfonamides in raw milk by the inter-branch organizations (milk control stations) (*Anon*., 2011). Some of the criteria concern the detection capabilities. In respect of the actual criteria published on the FASFC website on 09/08/2011 (*Anon*., 2011), the test must prove to be able to detect a large spectrum of antibiotics. In terms of sensitivity minimum 85 % of the following β-lactams must be detectable at their respective Maximum Residue Limit (MRL) (Commission Regulation (EU) No 37/2010 and amendments): benzylpenicillin, ampicillin, amoxicillin, cloxacillin, nafcillin, cefalexin, cefiofur, cefquinone, cefazolin, cephapirin, cefaperazone, cefalonium, desacetylcefapirine, and desfuroylceftiofur. Of the following sulfonamides (sulfadiazine, sulfadoxine, and sulfadimethoxine) and dapsone must be at least 75% detectable at their respective MRL or Recommended Concentration for Detection (*Anon*., 2007), respectively. Chlortetracycline and oxytetracycline of the tetracyclines must be detectable at two times their respective MRL in milk. Of the other families of anti-infectious agents at least 35% of the following substances must be detectable at three times their MRL: spiramycin, tylosin, spectinomycin, dihydrostreptomycin, gentamicin, neomycin, kanamycin, marbofloxacin, danofloxacin, enrofloxacin, colistin, rifaximin, lincomycin, pirlimycin, clavulanic acid, and trimethoprim.

The Charm Cowside II is fulfilling the FASFC detection capabilities criteria (Table 4): of the 14 β-lactams in the list were 12 compounds screened at MRL (85.7%). Of the 4 compounds on the FASFC sulfonamides & dapsone list, were sulfadiazine, sulfadimethoxine, and dapsone (75.0%) detected at 100 µg/kg (MRL) or 5 µg/kg (recommended concentration), respectively. Chlor- and oxytetracycline were always detected at 2× MRL (100%) (even at MRL (100%)) and of the 16 compounds of the other families of anti-infectious agents on the FASFC list there were 8 compounds detected at 3× MRL (50.0%).

Test selectivity

The Cowside II test is a broad spectrum inhibitory test and hence selective to a broad class of antimicrobial agents and inhibitors, but is nevertheless not sensitive to others (e.g. quinolones) as demonstrated in the detection capability study. It is a presence/absence test only and does not differentiate the source or chemical causing a positive result. Natural inhibitors have also been reported in milk from animals in early and late lactation and from infected (mastic) animals. Positive results from inhibition tests should be confirmed with confirmatory physico-chemical tests like LC-MS/MS to determine if inhibition is from chemical or natural sources.

Test repeatability

The test may be performed in a variety of incubators. The incubator variation was evaluated using three types of incubators, as 4 place made by Charm Sciences Inc., a 10 place made by

DSM, and a 20 place made by Charm Sciences Inc.. Also because the test is a visual interpretation two different people performed the method with a variety of antibiotic concentrations at the detection capability. As stated earlier the incubator temperatures were adjusted by the test laboratory to be 64 ± 0.2 °C as the temperatures were more variable than this as received by the manufacturer. Results are shown in Table 5. Neither the incubator types nor the person performing the method make a significant difference to the test results provided the temperature of the incubator is monitored closely to be in the range 64 ± 0.2 °C. Some slight differences in 4 place incubator more positive were seen with oxytetracycline at 70 µg/kg and sulfadiazine 70 µg/kg, but these differences do not appear to be significant.

Table 5. Repeatability of the Cowside II with different incubators and operators.

Sample	Charm 4 place incubator		Charm 20 place heater		DSM block heater	
	Operator 1	Operator 2	Operator 1	Operator 2	Operator 1	Operator 2
Blank milk	-	-	-	-	-	-
benzylpenicillin1 µg/kg	-	-	-	-	-	-
cloxacilline 10 µg/kg	-	-	-	-	-	-
cefquinome 18 µg/kg	-	-	-	-	-	-
chlortetracycline 100 µg/kg	+/-	+/-	+/-	+/-	+/-	+/-
benzylpenicillin 2 µg/kg	-	-	-	-	-	-
oxytetracycline 70 µg/kg	+	+/-	+/-	+/-	+/-	+/-
gentamycin 60 µg/kg	-	-	-	-	-	-
sulfadimethoxin 70 µg/kg	+	+	+	+	+	+
sulfadiazine 70 µg/kg	+	+	+/-	+/-	+/-	+/-
benzylpenicillin 1 µg/kg	-	-	-	-	-	-
oxytetracycline 100 µg/kg	+	+	+	+	+	+
chlortetracycline 100 µg/kg	+/-	+/-	+/-	+/-	+/-	+/-
cefquinome 18 µg/kg	-	-	-	-	-	-
oxytetracycline 70 µg/kg	+/-	+/-	+/-	+/-	+/-	+/-
cloxacillin 10 µg/kg	-	-	-	-	-	-
gentamycin 60 µg/kg	-	-	-	-	-	-
sulfadiazine 100 µg/kg	+	+	+	+	+	+
sulfadimethoxine70 µg/kg	+	+	+	+	+	+
sulfadiazine 70 µg/kg	+/-	+/-	+/-	+/-	+	+/-

Test for false positive results

The incidence of antibiotic contamination of milk farm tanks and milk bulk tanks is very low ranging 0.012-0.073% positive results in truck tanks to 0.043-0.06% in farmer samples based on various publications and public records (Reybroeck, 2010; *Anon.*, 2016). Because of the low antibiotic frequency incidence, and because the rapid screening tests are used to do a quick

determination for acceptance into dairies, it is important the tests have low false positive rates. This evaluation tested the Cowside II false positive rate with 210 farm tank milk samples, 70 blank milk samples (commingled milk from individual cows from known antibiotic free farms and used daily as negative control), and 201 tanker truck samples. Since random selected farm tank milk samples have an unknown history they also were tested with other inhibitory and rapid screening methods to determine method agreement and to determine if Cowside II results were correct, false positive or false negative. Results are summarized in Table 6. Details on the positive and false positive samples are summarized in Table 7.

Table 6. Summary of test results with different bulk farm milk and bulk milk tankers.

Samples	**Visual Reading**		
	Farm Milk	**Tanker Milk**	**Blank Milk**
Number of true positive samples	2[a]	2	0
Number of negative milk samples	148*	195	66
Number of false positive results	2*[b]	4	2*[c]
Total number of samples	152*	201	68*

Notes: *: There were 60 farm and 2 other blank milk samples evaluated with a lot that did not perform correctly in the 2 hr 55 minute incubation. 23 farm milk positives and 2 blank milk positives, respectively tested +/- or + , and are discounted as a rejected lot of tests. Additionally 37 negative farm milk samples with the rejected lot are discounted.

[a] Sample was positive on Delvotest T, after addition of beta-lactamase ES negative. Drug was not identified but high Free Fatty Acids (FFA) detected;

[b] One sample strong positive multiple times, not confirmed by other tests, high FFA; 1 sample with +/- results, not reversed with additional incubation;

[c] One sample was reversed with additional incubation.

Table 7. Summary of further examination of positive milk samples.

Sample	**Result**	**Result test repeated**	**+ES***	**LC-MS/MS**	**Conclusion**
F05	+	+	+	-	False positive High FFA 21.69
F42	+	+	+	-	False positive High FFA 21.91
F4229	+/-	+/-	-		True positive; low concentration β-lactam
F4230	+/-	+/-	-		True positive; low concentration β-lactam
T31	+/-	+/- & -			False positive (rapid tests: -)
T36	+/-	+ & -			False positive (rapid tests: -)
T217	+/-	+/- & -			False positive
T220	+	+	-	-	True positive; low concentration of β-lactam
T222	+	+	-	0.3 ppb pen G 0.9 ppb lincomycin	True positive; β-lactam
T227	+/-	-			False positive

Notes: F: farm milk; T: tanker milk; *: Charm Cowside II after addition of β-lactamase ES to the milk and a short incubation; pen G: benzylpenicillin.

If we neglect the results obtained with the rejected lot, the overall rate of false positive of the Cowside II test was 1.90%, 8 out of 421 samples. It should be noted that two false positive results were reversed with additional 10 minutes of incubation. In most cases the retest of the same replicate sample reproduced positive results. These results are consistent with other inhibition tests which can be influenced by milk constituents, like FFA and somatic cells. Positive results by inhibition methods should be verified with physico-chemical confirmatory tests.

Test robustness

The test performance under assay variance conditions was evaluated with pipet variances of high 110 µl and low 90 µl. Three replicates were evaluated using negative and positive milk doped with benzylpenicillin 2 µg/kg, oxytetracycline 100 µg/kg and sulfadiazine 100 µg/kg, which are at or near detection capability, CCβ. The qualitative positive, negative or +/- visual results are presented in Table 8. There was no negative effect seen from milk volume influence.

Table 8: Visual results obtained when testing Cowside II test with different volumes (90, 100 and 110 µl, respectively) of milk. Results obtained for blank and doped milk N=3 (benzylpenicillin 2 µg/kg, oxytetracycline 100 µg/kg and sulfadiazine 100 µg/kg).

Blank milk								
90 µl			**100 µl (reference)**			**110 µl**		
-			-			-		
-			-			-		
-			-			-		
Milk doped with benzylpenicillin 2 µg/kg, oxytetracycline 100 µg/kg and sulfadiazine 100 µg/kg								
270 µl			**300 µl (reference)**			**330 µl**		
Pen G	OTC	SDZ	Pen G	OTC	SDZ	Pen G	OTC	SDZ
+/-	+	+	+/-	+	+	+/-	+	+
+	+	+	+	+	+	+/-	+	+
+	+	+	+	+	+	+	+	+

Notes: Pen G: benzylpenicillin 2 µg/kg; OTC: oxytetracycline 100 µg/kg; SDZ: sulfadiazine 100 µg/kg.

Test for influence of the milk quality or milk composition

The influence of milk compositional components or milk quality, $>10^6$ somatic cells per ml, $>5\times10^5$ bacteria per ml, low and high fat, low and high protein, and low pH was compared with blank milk of normal quality/composition, and spiked near CCβ with benzylpenicillin 2 µg/kg, oxytetracycline 100 µg/kg and sulfadiazine 100 µg/kg, respectively. Ten replicates of compositional challenges were performed. Visual qualitative results positive, negative or +/- are shown in Table 9.

The results from the compositional/quality analysis, Table 9, show a positive somatic cell influence with blank milk, and a slight positive influence from other compositional changes of

fat and protein, but otherwise no other compositional or quality parameters influence on detection of raw milk. Positive and +/- results, when observed were replicated in duplicate so repeating the test had no change in observation. Since the Cowside II test is an inhibition test color triggered by acid production, it should be no surprise the lower pH influenced the benzylpenicillin 2 µg/kg sensitivity to become negative. Otherwise there are no significant effects from milk composition on positive results. These results suggests that a precaution be included in the manufacturer's instructions suggesting a possible influence of high somatic cells on the test and that somatic cell quality be considered with interpretation of positive results. This result and precaution is consistent with other inhibition tests that have a somatic cell influence to their results.

Table 9: Visual results obtained when testing Cowside II test with different constituents of milk. Results obtained for blank and doped milk N=3 (benzylpenicillin 2 µg/kg, oxytetracycline 100 µg/kg and sulfadiazine 100 µg/kg).

Blank Milk							
Co	SCC	TBC	HF	LF	LpH	HPr	LPr
-	+/-	-	-	-	-	-	-
-	+	-	+	-	-	-	-
-	+/-	-	-	-	-	-	-
-	+/-	-	-	+	-	-	-
-	+/-	-	-	-	-	-	-
-	+	-	-	-	-	-	-
-	-	-	-	-	-	-	+/-
-	-	-	-	-	-	+/-	-
-	+/-	-	-	-	-	-	-
-	+/-	-	-	-	-	-	-
Milk with benzylpenicillin 2 µg/kg							
Co	SCC	TBC	HF	LF	LpH	HPr	LPr
+	ND	+	+	+	-	+	+
+/-	ND	+/-	+	+	-	+	+
+/-	ND	+	+	+	-	+	+
+/-	ND	+	+	+	-	+	+
+/-	ND	+	+	+	-	+	+
-*	ND	+	+	+	-	+	+
+/-	ND	+	+	+	-	+	+
-*	ND	+	+	+	-	+	+
+/-	ND	+	+	+	-	+	+
+/-	ND	+	+	+	-	+	+

Milk with oxytetracycline 100 µg/kg							
Co	SCC	TBC	HF	LF	LpH	HPr	LPr
+	ND	+	+	+	+	+	+
+	ND	+	+	+	+	+	+
+	ND	+	+	+	+	+	-
+	ND	+	+	+	+	+	+
+	ND	+	+	+	+	+	+
+	ND	+	+	+	+	+	+
+	ND	+	+	+	+	+	+
+/-	ND	+	+	+	+	+	+
+/-	ND	+	-	+	+	+	+
+	ND	+	-	+	+	+	+
Milk with sulfadiazine 100 µg/kg							
Co	SCC	TBC	HF	LF	LpH	HPr	LPr
+	ND	+	+	+	+	+	+
+	ND	+	+	+	+/-	+	+
+	ND	+	+	+	+/-	+	+
+	ND	+	+	+	+/-	+	+
+	ND	+	+	+	+/-	+	+
+	ND	+	+	+	+	+	+
+	ND	+	+	+	+/-	+	+
+/-	ND	+	+	+	+/-	+	+
+	ND	+	+	+	+	+	+
+	ND	+	+	+	+/-	+	+

Notes: Co: control milk; SCC: Somatic milk >10^6/mL; TBC: Total bacterial count >500,000/mL; HF: high fat 6-10%; LF: low fat <2%; LpH: low pH 6.0; Hpr: high protein 4-5%; LPr: low protein <3.0%; ND: Not Determined because of positive influence on blank milk.

Test for influence of the milk types and other animal species

The Cowside II test is a test claimed for raw cows' milk only and not for other species, goat, sheep or mare. Different incubation times are provided and necessary to test finished dairy products or heat-treated milk. This evaluation looked at the influence of different types of milks: UHT, sterilized, reconstituted powder, thawed, goats', ewes' and mares' milk. In this evaluation blank (n=10) were tested and evaluated. When there was no false positive influence on the blank, the milks were spiked with benzylpenicillin 2 µg/kg, oxytetracycline 100 µg/kg and sulfadiazine 100 µg/kg (n=10), respectively and evaluated. Visual qualitative results positive, negative or +/- are shown in Table 10.

Reconstituted milk powder, goats', ewes' (sheep) and mares' milk are not appropriate for use on Cowside II and blank milk tests were positive. No additional testing with these milks was performed. UHT, sterilized milk and thawed raw milk did perform well following the test kit instructions for longer incubation for processed milk when applicable. Positive doped milk also performed consistent with detection capability using the three different antibiotics.

Table 10: Visual results obtained when testing Cowside II test with different milk types. Results obtained for blank and where applicable doped milk N=3 (benzylpenicillin 2 µg/kg, oxytetracycline 100 µg/kg and sulfadiazine 100 µg/kg).

Blank milk							
Cow	Sterile	UHT	MP	Thawed	Goat	Sheep	Mare
-	-	-	+/-	-	+	+	+
-	-	-	+/-	-	+	+	+
-	-	-	+	-	+	+	+
-	-	-	-	-	+	+	+
-	-	-	+	-	+	+	+
-	-	-	-	-	+	+	+
-	-	-	+	-	+	+	+
-	-	-	+	-	+	+	+
-	-	-		-	+	+	+
-	-	-		-	+	+	+

Doped Milk: benzylpenicillin 2 µg/kg (Pen G), oxytetracycline 100 µg/kg (OTC), sulfadiazine 100 µg/kg (SDM)								
Sterilized milk			UHT milk			Thawed milk		
Pen G	OTC	SDM	Pen G	OTC	SDM	Pen G	OTC	SDM
+	+	+	+	+	+	+/-	+	+
+	+	+	+	+	+	+/-	+	+
+	+	+	+	+	+	+/-	+/-	+
+	+	+	+	+	+	+/-	+/-	+
+	+	+	+	+	+	+/-	+	+
+	+	+	+	+	+	+/-	+	+
+	+	+	+	+	+	+/-	+	+
+	+	+	+	+	+	+/-	+	+
+	+	+	+	+	+	+	+	+
+	+	+	+	+	+	+/-	+/-	+

Notes: Cow: reference cows' milk; MP: milk powder rehydrated; UHT: UHT milk; sterile : sterilized milk; thawed: milk frozen to -18°C, stored for a week in the freezer and thawed.

Reagent influence (batch differences)

Blank and doped milk samples were tested on two different batches of Charm Cowside II reagents. The results are summarized in Table 11.

The use of Batch CSII-01-041 with 2h45 incubation is resulting in more positive results compared to Batch CSII-02-40 with an indicated incubation time of 2h55. The effect could possibly just caused by the difference in incubation time. By increasing the time of incubation it is normal to get more negative results.

Table 11. Results for blank and doped (benzylpenicillin 2 µg/kg (Pen G), oxytetracycline 100 µg/kg (OTC), sulfadiazine 100 µg/kg (SDM)) milk samples obtained with two differents lots of reagents.

Batch CSII-02-40 (2h55)				Batch CSII-01-041 (2h45)			
BL	Pen G	OTC	SDZ	BL	Pen G	OTC	SDZ
100% -	90% + 10% +/-	100% +	100% +	100% +/-	100% +	100% +	100% +

Notes: Pen G: benzylpenicillin 2 µg/kg; OTC: oxytetracycline 100 µg/kg; SDZ: sulfadiazine 100 µg/kg.

These results indicate that the incubation time specified by the kit manufacturer per batch of reagents is not always the ideal one. It is advisable to check the incubation time for each new batch of reagents by means of blank milk samples and to adapt the time if needed.

Reagents stability

Blank and doped (benzylpenicillin 2 µg/kg (Pen G), oxytetracycline 100 µg/kg (OTC), sulfadiazine 100 µg/kg (SDM)) were tested with Batch CSII-02-40 (2h55) of reagents shortly after production and just before expiry date. The results are summarized in Table 12.

Table 12. Results for blank and doped (benzylpenicillin 2 µg/kg (Pen G), oxytetracycline 100 µg/kg (OTC), sulfadiazine 100 µg/kg (SDZ)) milk samples obtained with reagents used shortly after production and just before expiry date.

Shortly after production				Just before expiry date			
BL	Pen G	OTC	SDZ	BL	Pen G	OTC	SDZ
100% -	90% + 10% +/-	100% +	100% +	100% -	100% +	100% +	100% +

Notes: Pen G: benzylpenicillin 2 µg/kg; OTC: oxytetracycline 100 µg/kg; SDZ: sulfadiazine 100 µg/kg.

For blank milk and milk doped with oxytetracycline or sulfadiazine at 100 µg/kg no differences in results were obtained by using reagents shortly after production date and just before expiry date. Only for milk doped with 2 µg/kg of benzylpenicillin, there was a slight difference: 10% of the samples tested '+/-' on reagents used shortly after production while all samples tested '+' using reagents just before expiry date. During shelf life it is possible that some spores of the test organism (*Geobacillus stearothermophilus*) lose their viability so the test becomes slightly slower what improves the detection capability.

Interlaboratory testing

Each year ILVO-T&V organizes two national ring trials regarding the detection of antibiotics in milk with microbiological and rapid tests. In April and October 2016, the Cowside II method was included with a variety of other screening tests (Ooghe and Reybroeck, 2016a and b). The results of testing eight different blind coded milk preparations are presented in Table 13 and 14.

Table 13. Results for Charm Cowside II Test in the ring trial organized by ILVO on April 21, 2016.

Sample	Compound and concentration (µg/kg)	Visual reading	Interpretation
A	cloxacillin, 30	VISPOS	+
B	doxycycline, 40	VISPOS	+
C	ceftiofur, 100	VISPOS	+
D	blank	VISNEG	-
E	amoxicillin, 4	VISPOS	+
F	neomycin,1,500	VISPOS	+
G	benzylpenicillin, 4	VISPOS	+
H	sulfadoxine, 100	VISCAUTION	+/-

Notes: VISNEG: visual negative; VISPOS: visual positive; VISCAUTION: Slightly positive

Table 14. Results for Charm Cowside II Test in the ring trial organized by ILVO on October 27, 2016.

Sample	Compound and concentration (µg/kg)	Visual reading	Interpretation
A	nafcillin, 30	VISPOS	+
B	blank	VISNEG	-
C	tylosin A, 100	VISPOS	+
D	cloxacillin, 30	VISNEG	-
E	oxytetracycline, 100	VISPOS	+
F	benzylpenicillin, 4	VISPOS	+
G	sulfadiazine, 100	VISPOS	+
H	cefalexin, 100	VISPOS	+

Notes: VISNEG: visual negative; VISPOS: visual positive; VISCAUTION: slightly positive

The results of both ring trials are consistent with drug capability determination with all doped samples testing positive and with 100 ppb sulfadoxine testing as caution with it being near the minimum detection level.

Conclusions

The Charm Cowside II test for commingled raw cows' milk is an inhibition test that detects multiple classes of antibiotics drugs in approximately 3 hours. The test is a visual test that uses bromocresolpurple, a yellow acid indicator, to detect the growth of seeded *Geobacillus calidolactis* as they grow in antibiotic negative milk at 64°C . If the milk contains antibiotic residues, the test remains blue/purple. The method meets the β-lactam drug detection criteria of the Belgium FASFC detecting 14 of the 16 compounds evaluated at MRL and only missing cefquinome and desfuroylceftiofur (metabolite of ceftiofur) at MRL. Additionally the method detects at MRL 3 tetracyclines, 5 sulfonamides, 2 macrolides, 2 lincosamides, 2 aminoglycosides, and 4 other antimicrobial drugs evaluated. The Charm Cowside II is fulfulling the Belgian FASFC acceptance criteria regarding the detection capabilities of the test for the approval of tests for the screening of antibiotics and sulfonamides in raw milk by the inter-branch organizations (milk control stations) (*Anon*., 2011).

The method was tested with 152 farm tank milk samples, 201 bulk milk tanker samples and 68 blank milk samples with 8 false positive results, a 1.9% false positive rate, that may be explained by milk variation and constituent influences. The method was evaluated for influences from compositional components or milk quality and produced false positive results for milk with somatic cells greater than 10^6 SCC/mL and from samples that had a high fatty acid content. The test is not intended and not appropriate for non-cow species milk and produced false positives with goat, ewes and mares milk. There were no influences from bacteria at 0.5 x 10^5/mL level.

The method was easily performed in multiple incubators including incubators commonly used on EU dairy farms. The temperature of the incubators 64±0.2 °C is critical to meeting the performance times specified by the manufacturer. Ten minute longer incubations give slightly less detection capability but more robustness of negative sample development and might be appropriate for individual cow and farmer use. It is advised to check for each new batch of reagents the indicated incubation time and to correct if necessary.

The method performed in two national ring trials consistent with data generated during detection capability testing.

The results indicate that the Cowside II test is appropriate for use as a screening test at dairies and farms for raw commingled cows' milk. For use in milk control stations for payment control the microplate version (Charm Blue Yellow II) is more appropriate since the color of agar in the cups of a microplate could be interpreted by automatic reading. As with all inhibition tests, initial positive tests should be confirmed with rapid screening and chemical confirmation to rule out potential milk constituent interference and to identify the antibiotic family and compound causing a positive result.

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Annex: Table. Detection performance of the Charm Cowside II at CCβ (visual reading).

Group	Substance	MRL (µg/kg)	Detection capability (µg/kg)	Test Performance at ccβ in %		
				+	+/-	-
Penicillins	Benzylpenicillin	4	2	85	10	5
	Ampicillin	4	4	20	75	5
	Amoxicillin	4	3	100	0	0
	Oxacillin	30	8	95	5	0
	Cloxacillin	30	20	90	10	0
	Dicloxacillin	30	15	100	0	0
	Nafcillin	30	6	85	15	0
	Penethamate	4	n.t.	/	/	/
Cefalosporins	Ceftiofur	100[a]	25 (200)[b]	95 95	5 5	0 0
	Cefquinome	20	80	75	25	0
	Cefazolin	50	8	90	10	0
	Cephapirin	60[c]	5 (8)[d]	60 95	40 5	0 0
	Cefacetrile	125	20	85	15	0
	Cefoperazone	50	25	55	40	5
	Cefalexin	100	100	25	70	5
	Cefalonium	20	15	100	0	0
Tetracyclines	Tetracycline HCl	100[e]	60 (700)[f]	20 60	80 35	0 5
	Chlortetracycline	100[e]	90 (900)[g]	40 10	60 85	0 5
	Oxytetracycline	100[e]	100 (350)[h]	60 55	40 40	0 5
Macrolides	Spiramycin	200	250	15	80	5
	Tylosin	50	25	100	0	0
	Erythromycin	40	80	50	50	0
	Tilmicosin	50	30	100	0	0
Aminoglycosides	Spectinomycin	200	700	55	45	0
	Streptomycin	200	900	55	45	0
	Dihydrostreptomycin	200	800	90	5	5
	Gentamicin	100	90	95	5	0
	Neomycin (+framycetin)	1,500	125	50	45	5
	Kanamycin	150	>1,500	-	-	-
Quinolones	Marbofloxacin	75	>750	-	-	-
	Danofloxacin	30	>300	-	-	-

Group	Substance	MRL (µg/kg)	Detection capability (µg/kg)	Test Performance at ccβ in %		
				+	+/-	-
	Enrofloxacin (+ciprofloxacin)	100	>1,000 (900)	- 100	- 0	- 0
	Flumequine	50	>1,000	-	-	-
Polymyxins	Colistin	50	>500	-	-	-
Ansamycines+ naftalene ring	Rifaximin	60	60	70	25	5
Thiamphenicol	Thiamphenicol	50	1,250	50	50	0
Lincosamides	Lincomycin	150	60	70	25	5
	Pirlimycin	100	20	60	35	5
β-lactamase inhibitors	Clavulanic acid	200	2,000	85	10	5
Polypeptides	Bacitracin	100	600	40	55	5
Other antibiotics	Novobiocin	50	500	30	70	0
Sulfonamides	Sulfamethazine	100	100	50	45	5
	Sulfadiazine	100	60	95	5	0
	Sulfamerazine	100	50	35	65	0
	Sulfadoxine	100	125	40	55	5
	Sulfadimethoxine	100	40	80	20	0
Diamino pyrimidinederivates	Trimethoprim	50	70	5	90	5
	Baquiloprim	30	30	30	70	0
Ionofors	Monensin A	2	>20	-	-	-
Other chemotherapeutics	Dapsone	---*	1	100	0	0

Notes: MRL: Maximum Residue Limit, Regulation (EC) No 470/2009 and Commission Regulation (EU) No 37/2010 and amendments as of 01/07/2016. Detection capability defined as the lowest concentration tested giving a minimum of 19 positive results out of 20.

[a]: The MRL of 100 µg/kg is applied on the sum of all residues retaining the β-lactam structure expressed as desfuroylceftiofur,
[b]: desfuroylceftiofur,
[c]: the MRL of 60 µg/kg in milk is applied on the sum of cephapirin and desacetylcephapirin,
[d]: desacetylcephapirin,
[e]: the MRL of 100 µg/kg in milk is applied on the sum of the parent drug and its 4-epimer,
[f]: 4-epimer of tetracycline,
[g]: 4-epimer of chlortetracycline,
[h]: 4-epimer of oxytetracycline,
*: prohibited substance: MRL cannot be established, recommended concentration for testing = 5 µg/kg (*Anon.,* 2007).