Proficiency testing of microbial inhibitor tests[2]

Gertraud Suhren and J. Reichmuth

Federal Dairy Research Centre, Institute for Hygiene, Hermann-Weigmann-Str. 1, 24103 Kiel, Germany

Abstract

Seven microbial inhibitor tests (BR-Blue Star, BR-AS, BR-EC, BR-6, Charm AIM-96, Delvo SP and *B.cereus* microtitre test with indicator) were examined in 8-9 participating laboratories with respect to the detection of 5 antimicrobials (oxytetracycline, sulfadimidine, sulfadimethoxine, gentamicin, spiramycin). Surprisingly high were the differences in readings of the test results between the participating laboratories. Including all tests the extreme values between laboratories were 10% and 40% positive results respectively. Causes of this different interpretation might be subjective interpretation of colour and/or adjustment of correct reading time. Defining the sensitivities of this proficiency study as those concentrations were at least 80% of the results were indicated as positive the following antimicrobials can be detected at ≤ MRL level: Oxytetracycline (*B.cereus*), gentamicin (Charm AIM-96), sulfadimethoxine (BR-Blue Star, BR-AS, BR-EC, Charm AIM-96, Delvo SP). None of the included inhibitor tests is suitable for the detection of sulfadimidine and spiramycin.

Introduction

In the context of the checks provided for in Article 14 of the EU-Milk Hygiene Directive 92/46 the EU Member States have to ensure that tests are carried out to detect residues of antimicrobial substances exceeding maximum residue limits (MRLs) which are fixed according to EC-Regulation 2377/90. Within the IDF (International Dairy Federation) integrated system for the detection of residues of antimicrobials, which comprises the application of different methods and the definition of shared responsibilities of the parties of concern, microbial inhibitor tests play an important role as screening methods which is demonstrated in **fig. 1** (1). The interpretation of the graph is that milk samples are screened by microbial inhibitor tests and in dependency on further need - quality payment, self control in the dairy, food inspection - positive samples are further analysed by more sophisticated methods which allow identification and quantification. As there are not available microbial inhibitor tests providing satisfying detection limits for all antimicrobials in question, e.g. chloramphenicol, it is necessary to start examinations with specific and sensitive tests to detect those residues.

Factors and procedures which have to be considered when evaluating microbial inhibitor tests and consistently when interpreting test results are summarized in an IDF Standard (2). One item is the evaluation of tests within collaborative studies. The purpose of the proficiency study presented here was to evaluate the sensitivities of several microbial inhibitor tests for the detection of antimicrobials other than ß-lactam-antibiotics when analysed in different laboratories.

[2] The experimental work was initiated by a group of experts at the Federal Institute for Health Protection of Consumers and Veterinary Medicine, Berlin/DE. The authors are obliged to the members of that group for their participation in this study.

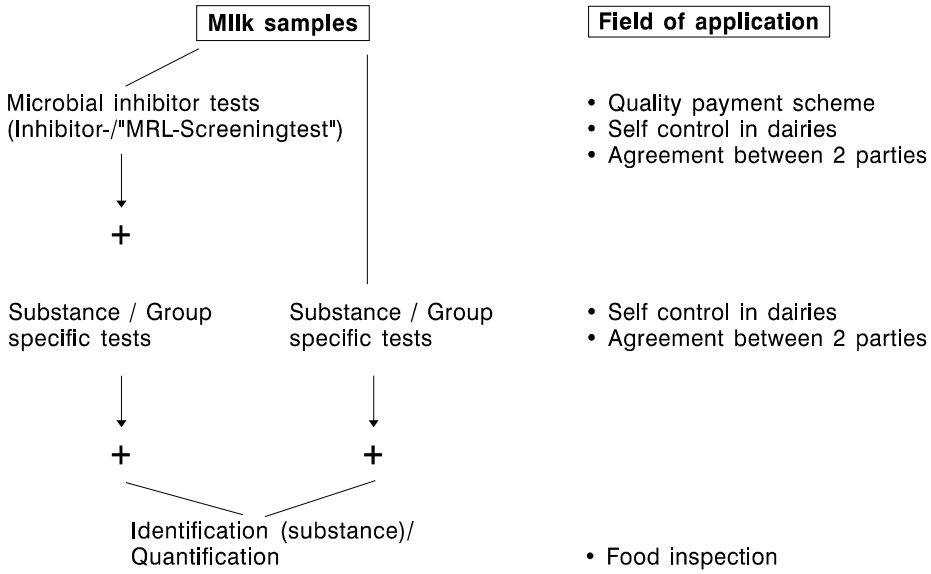

Figure 1: Position of microbial inhibitor tests within an integrated detection system

Experimental design

Three different batches each of the following microbial inhibitor **tests**[3] were included: BR-Blue Star[4], BR-AS[3], BR-EC[3], BR-6[3], Charm AIM-96[5], Delvo SP[3] and *Bacillus cereus* microtitre test (3).

For the preparation of the **test samples** raw milk from the experimental herd of the Federal Dairy Research Centre, which was not treated with antimicrobials in the preceding 4 weeks was skimmed by centrifugation. 3.3 ml portions of negative and test samples with the antimicrobials/concentrations listed in **table 1** were dispensed into tubes and lyophilized. The test samples for the 3 trials were prepared at the same time; they had to be reconstituted by 3 ml water on the day of use.

The lyophilized test samples coded at random, test kits and test protocols were mailed individually for each of the 3 kit batches to 8-9 participating laboratories. Due to the experimental design 4 results per substance/concentration and test and trial for every participating laboratory were obtained. The participating laboratories had to use their own negative/positive control samples and to indicate the test results as negative or positive.

[3] We thank the companies which provided us with the commercially available test kits free of charge.
[4] Gist Brocades, Delft/NL;
[5] Charm Sciences, Inc., Malden, USA.

Table 1: Antimicrobial/concentration combinations (µg/kg) of the test samples and MRLs and FDA safe/tolerance levels (µg/kg)

Substance	Supplier	Concentrations tested	EU-MRL[1)]	Codex MRL[2)]	FDA safe/ tolerance[3)]
Oxytetracycline	Serva 31357	30, 100, 150, 200	100	100	30/0
Sulfadimidine	Serva 35635	10, 100, 150, 200	100	25	10/0
Sulfadimethoxine	Sigma S7385	10, 100, 150, 200	100	–	10/10
Gentamicin	Serva 22185	30, 100, 200, 400	100	100	30/0
Spiramycin	Sigma S9132	75, 150, 200, 300	200	100	–
Negative milk	–	–	–	–	–

1) EU-Regulation 2377/90 ff
2) Codex Committee on Residues of Veterinary Drugs in Food
3) CFR 21 and CVM correspondence

Results

The **variation between test kit batches/trials** expressed in percentage of positive results within test and antimicrobial is summarized in **table 2**. From this table it becomes obvious that the variation between test kit batches depends on the kind of test and antimicrobial under study and has therefor to be evaluated individually for each antimicrobial/test combination. The greatest variation between batches were observed on the following combinations:

Oxytetracyclin: BR-EC
Gentamicin: Delvo SP
Sulfadimidine: BR-EC
Sulfadimethoxine: Charm AIM-96.

With regard to the total results it is striking that in the first trial/batch the lowest and in the third batch the highest number of positive results were indicated.

Table 2: Percentage of positive results within test and antimicrobial (n=144*) with respect to the test kit batch/trial

Antimicrobial Test	**Oxytetra-cycline**			**Genta-micin**			**Spira-mycin**			**Sulfa-dimidine**			**Sulfadi-methoxine**			**Total**		
Batch/Trial No.	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
BR-Blue Star	10	0	31	5	14	14	0	0	5	27	47	40	72	68	68	23	26	33
BR-AS	8	2	8	6	5	8	0	1	0	28	31	38	74	64	73	23	20	25
BR-EC	**26**	**38**	**51**	17	17	24	10	9	14	**43**	**59**	**40**	72	74	78	34	39	41
BR-6	56	60	63	0	0	0	1	0	0	1	0	4	0	0	0	12	12	13
Charm AIM-96	26	34	35	70	79	74	1	1	0	29	20	28	**35**	**50**	**51**	32	37	38
Delvo SP	14	8	19	**22**	**20**	**49**	5	3	11	60	60	66	76	75	76	35	33	46
B.cereus	75	69	72	8	6	3	10	8	4	8	9	5	10	10	6	23	21	18
Total	31	30	40	17	19	24	4	3	5	28	33	32	49	49	52	26	27	31

*) Charm AIM n = 128

The **differences** in indicated positive results **between** the **participating laboratories** were evident for all substances and tests under study. The results are summarized in **table 3**.

Table 3: Extreme values of positive results (%) in participating laboratories with respect to the inhibitor test (n = 240/test and lab)

Test	Min	Max	Lab.No.
BR-Blue star	7	42	6/2
BR-AS	6	37	6/9
BR-EC	13	50	6/9
BR-6	4	14	6/7
Charm AIM-96	13	51	6/9
Delvo SP	17	52	6/3
B.cereus	14	44*	6/3

* Probably problems with test procedure as numerous negative samples were evaluated as positive by lab 3

The detection of oxytetracycline, gentamicin, sulfadimidine and sulfadimethoxine is demonstrated in **fig. 2** in form of dose (µg/kg) - response (% positive results) curves and the **detection limits** are summarized in **table 4**. Negative samples were evaluated as positive in some cases; this proportion was relatively high in the case of *B.cereus* test and lab. 3. The detection limits for this proficiency study were defined as the intersections of 80% and 90% positive results lines respectively and concentration. The spiramycin concentrations tested were not detected by any test. The demands according the EU and/or Codex MRLs (see **table 1**) were fulfilled for the following combinations of antimicrobial and test:

Oxytetracycline: B.cereus

Gentamicin: Charm AIM-96

Sulfadimidine: –

Sulfadimethoxine: BR-Blue Star, (BR-AS), BR-EC and Delvo SP

Spiramycin: –

Table 4: Detection limits* (µg/kg) of oxytetracycline, gentamicin, sulfadimidine and sulfadimethoxine by various inhibitor tests

	Oxytetracycline		**Gentamicin**		**Sulfadimidine**		**Sulfadimethoxine**	
	80%	**90%**	**80%**	**90%**	**80%**	**90%**	**80%**	**90%**
BR-Blue Star	>200	>200	>400	>400	>200	>200	**85**	**100**
BR-AS	>200	>200	>400	>400	>200	>200	**90**	125
BR-EC	>200	>200	>400	>400	>200	>200	**85**	**100**
BR-6	135	150	>400	>400	>200	>200	>200	>200
Charm AIM-96	>200	>200	**90**	**100**	>200	>200	>200	>200
Delvo SP	>200	>200	>400	>400	135	200	**80**	**90**
B.cereus	**80**	**90**	>400	>400	>200	>200	>200	>200

* Detection limits within this proficiency study are defined as the intersections of concentration and 80 and 90% positive results lines respectively

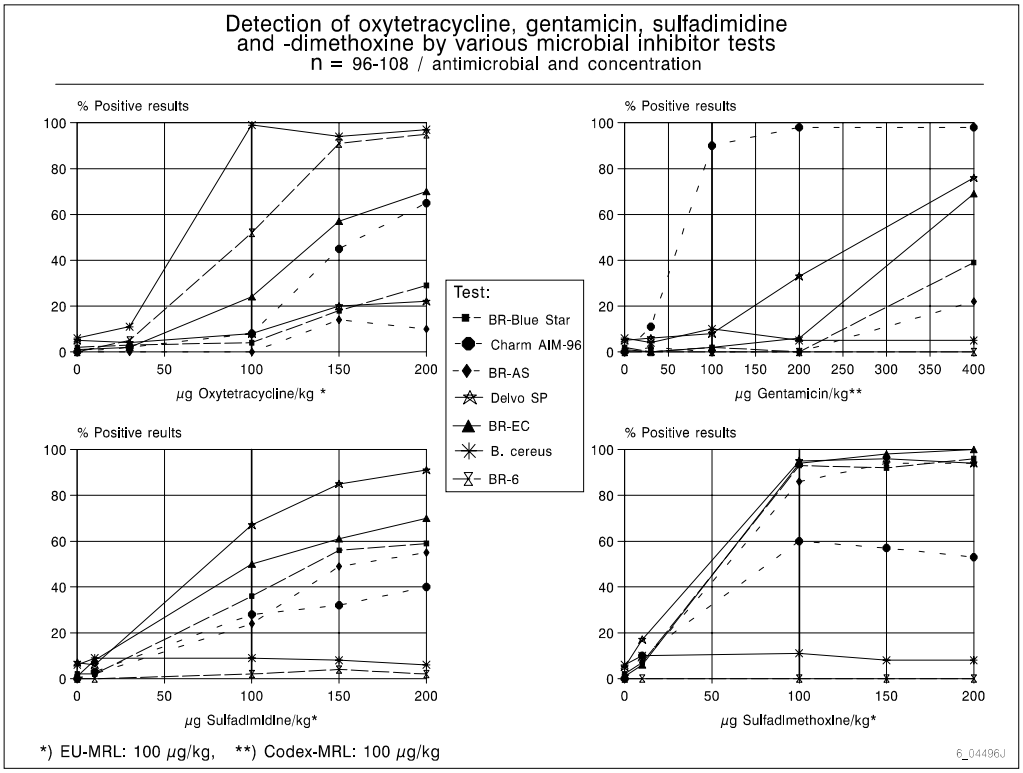

Figure 2

Conclusions

In interpreting the results it has to be kept in mind that the antimicrobials and concentrations included are more difficult to detect by microbial inhibitor tests with *B. stearothermophilus* as for example ß-lactam-antibiotics. Further it has to be considered that most often positive control samples containing penicillin are used and that due to the different mode of action of antimicrobials on the test microorganisms the colour change might differ.

Within the 3 trials the percentage of samples indicated as positive increased in the overall evaluation. Reasons might be

- different sensitivities of the test kit batches
- stability of the test samples and/or
- "learning effect“ of the participating people.

The variation between test kit batches/trials proved to be dependent on the test and antimicrobial under study. For some combinations the difference between the percentage of positive results of the 3 trials were in an order of magnitude of >20%. These results emphasize the need to check the stability between test kit batches with various antimicrobials.

The variation in test interpretation between the participating labs was surprisingly high. The differences between the maximum and minimum percentage of positive results were - with exception of BR-6 with only a low number of positive results - ≥30 %. The minimum figures were indicated in the same lab for all tests applied. Reasons of the different test interpretation might be

- adjustment of the correct incubation period and/or
- interpretation of the colour.

These unsatisfying reproducibilities of test results stress the need for better and feasible possibilities to standardize the test procedure including test interpretation by objective readings as for example ELISA reader (4, 5).

Defining the detection sensitivities of this proficiency study as those concentrations where at least 80 and 90% of the results were indicated as positive respectively (see **table 4**) the following tests are suitable with respect to the detection of the MRL level (EU and/or Codex):

Oxytetracycline:	*B. cereus*
Gentamicin:	Charm AIM-96
Sulfadimethoxine:	(BR-AS), BR-Blue Star, BR-EC and Delvo SP.

The test kits under study detect different kinds of antimicrobials with the required sensitivities. Taking into account only positive test results all tests failed to detect sulfadimidine and spiramycin.

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