The Use of Different Microbial Assays in Combination with the Charm II Test in the Detection of Antibiotic Residues in Herd Milk

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ABSTRACT

Three microbial assays, i.e. Delvo test SP, BR-test AS and Valio T101, were used to screen approximately 40 000 milk samples for inhibitory substances at ten different milk grading laboratories. Samples found positive by any of the assays were then analysed by the Charm II test for the presence of \(\beta\)-lactams, tetracyclines, aminoglycosides, macrolides and sulfa drugs. Positive samples were also analysed by the screening assays used routinely at Swedish dairies, i.e. Delvo test P and Arla microtest. The results revealed considerable differences between the methods regarding their sensitivity to various antibiotics. \(\beta\)-Lactams were the prevailing type of antibiotics found, irrespective of the microbial assay method used. A surprisingly large number of samples were positive for tetracyclines and/or macrolides. These substances were best detected by Arla microtest and Valio T101, while \(\beta\)-lactams were somewhat better detected by the Delvo tests. A future control system with an alternative use of more than one microbial screening assay in combination with the Charm II test is suggested.

INTRODUCTION

Veterinary drug residues, being the most important inhibitory substances in milk, constitute a potential health risk and may interfere with the manufacture of fermented dairy products. In Sweden, the present control scheme is based on two microbial screening assays, the Arla microtest and the Delvo test P. The test organisms, *Bacillus subtilis* and *Bacillus stearothermophilus*, respectively, exhibit high sensitivities to penicillin G, which is still the most frequently used antibiotic in mastitis therapy. There are, however, distinct differences in sensitivity between the two methods with regards to other substances, and during the last few years this has been expressed in an increasing number of samples which are positive in the Arla microtest but negative in the Delvo test P.

In a previous study, the identity of the substance(s) causing the divergence between the two methods was investigated (Carlsson & Björck, 1991). Samples which were positive in the screening assay by either the Arla microtest or the Delvo test P were analysed by the Charm II test for the presence of β-lactams, tetracyclines and aminoglycosides, which are commonly used antibiotics in Sweden. A third microbial inhibitor test, the Valio T101, was included for comparison. The results indicated the presence of tetracyclines in a surprisingly large number of the samples which were positive in the Arla microtest, while predominantly β-lactams were found in the samples which were positive in the Delvo test P. The Valio T101 test gave a pattern of detection which was complementary neither to the results in the Delvo test P, nor to those in the Arla microtest. These results raised the question whether additional substances would be detected if microbial assays other than the Arla microtest and Delvo test P were to be used in the screening. In the present follow-up study, three new microbial screening assays were assessed. The primary objective was to compare the frequencies of positive samples as well as identify the inhibitory substances detected by the different tests.

MATERIALS AND METHODS

Methods

The Arla microtest (Swedish Dairies Association, Lund, Sweden), the Delvo test P and the Delvo test SP (Gist-Brocades, Delft, Netherlands), the Valio T101 test (Valio, Helsinki, Finland) and the BR-test AS (Enterotox, Krefeld, Germany) were all conducted in accordance with the manufact-
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### TABLE 1

Detection Limits* (μg/ml) for some Antibiotics/Sulfa Drugs of the Microbiological Inhibitor Tests Used in the Study

<table>
<thead>
<tr>
<th>Antibiotic/sulfa drug</th>
<th>Arla micro test</th>
<th>Delvo test P</th>
<th>Delvo test SP</th>
<th>BR-test AS</th>
<th>Valio T101 test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzylpenicillin</td>
<td>0.004</td>
<td>0.001-0.003</td>
<td>0.001-0.003</td>
<td>0.002</td>
<td>0.003</td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td>0.1</td>
<td>0.2-0.4</td>
<td>0.2-0.4</td>
<td>0.2-0.25</td>
<td>0.15-0.2</td>
</tr>
<tr>
<td>Dihydrostreptomycin</td>
<td>5</td>
<td>4-6</td>
<td>4-6</td>
<td>5-6</td>
<td>0.3-0.5</td>
</tr>
<tr>
<td>Sulfamethazine</td>
<td>50-100</td>
<td>50-100</td>
<td>0.4-0.8</td>
<td>0.08-0.1</td>
<td>0.5-2.5</td>
</tr>
<tr>
<td>Spiramycin</td>
<td>1.5-2</td>
<td>1-2</td>
<td>1-2</td>
<td>1</td>
<td>0.1-0.2</td>
</tr>
</tbody>
</table>

*Detectable concentrations as stated by the manufacturers except in the case of spiramycin, for which the concentrations were determined by the authors.

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The Charm II test (Charm Sciences Inc., Malden, MA, USA) was conducted on defatted milk, with the modifications described by Suhren & Heeschen (1987a). Milk fat was separated by centrifugation for approximately 5 min at 2500 × g. Milk samples were analysed for the presence of β-lactams, tetracyclines, aminoglycosides, sulfa drugs and macrolides. Control points, i.e. the count below which a sample is classified as positive, were calculated for each antibiotic by multiplying the mean of six negative control milk samples by 0.80 (β-lactams), 0.60 (tetracyclines), 0.70 (aminoglycosides), 0.30 (sulfa drugs) and 0.70 (macrolides). The corresponding sensitivities, determined by adding different concentrations of the respective antibiotic to negative control milk, were for penicillin G approximately 0.002 μg/ml, oxytetracycline 0.5 μg/ml, dihydrostreptomycin 0.04 μg/ml, sulfamethazine 0.05 μg/ml and spiramycin 0.15 μg/ml.

Fresh, antibiotic-free milk from the University research dairy farm served as negative control milk in the microbial assays as well as the Charm II test.

Collecting and testing of milk samples at the milk grading laboratories

Milk samples were collected during the months of February through May, 1990. Approximately 40 000 milk samples were analysed at 10 different milk grading laboratories located throughout Sweden. Each microbial method was used as the only screening assay at three different
laboratories. At the tenth laboratory, milk samples were screened with all three assays concurrently.

Milk samples with positive results in one screening assay were confirmed by the same test. They were also analysed by the two new microbial assays which had not been used as screening assays at the particular laboratory. In addition, positive samples were analysed by the Delvo test P and, in most laboratories, the Arla microtest. Samples positive in the confirmation analysis were frozen (-20°C) and sent weekly to our laboratory. During the experimental period, a total of 130 milk samples were received for further analysis.

Analysis of milk samples at the University laboratory

On arrival at our laboratory, milk samples were immediately refrozen. The samples were then re-analysed by the different microbial methods within 1 month and also analysed by the Charm II test. Occasionally, the sample volume was not sufficient to run all Charm II analyses. In these cases, the results in the microbial assays would usually provide enough information to make it possible to give priority to certain Charm II analyses.

RESULTS

Although samples were positive in the screening assay, their results in the other microbial methods varied. Table 2 shows the results in the different microbial methods when the collected samples were re-analysed at our laboratory. Occasionally, a sample which was positive at the milk

<table>
<thead>
<tr>
<th>Method</th>
<th>Number of samples analysed</th>
<th>Positive results Numbers</th>
<th>Positive results %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Valio T101</td>
<td>124</td>
<td>56</td>
<td>45.2</td>
</tr>
<tr>
<td>Arla microtest</td>
<td>92</td>
<td>38</td>
<td>41.3</td>
</tr>
<tr>
<td>Delvo test SP</td>
<td>125</td>
<td>45</td>
<td>36.0</td>
</tr>
<tr>
<td>Delvo test P</td>
<td>52</td>
<td>16</td>
<td>30.8</td>
</tr>
<tr>
<td>BR-test AS</td>
<td>89</td>
<td>14</td>
<td>15.7</td>
</tr>
</tbody>
</table>
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Fig. 1. Results when re-analysing samples positive in the screening assays by other microbial methods. A set of bars represents samples positive in a certain microbial method, while the individual bars within each set show the results for these samples (percentage positive samples) in the other microbial methods. The figure above each bar indicates the total number of samples which were re-analysed by a particular method. Samples positive in: ■, Arla microtest; □, Delvo test P; △, Delvo test SP; ☐, BR-test AS; ☐, Valio T101.

ggrading laboratory, was negative by the same assay method upon re-analysis at our laboratory.

Figure 1 shows how samples positive on re-analysis by a certain microbial method reacted in the other microbial methods. Each set of bars represents samples positive in a certain microbial method while the individual bars illustrate the results for these samples in the re-analyses by the other microbial methods.

The collected samples were analysed by the Charm II test for verification of the inhibitory substance(s). Table 3 shows the occurrence of

| Antibiotic/Sulfa Drug Residues as Determined by the Charm II Test in Samples Positive in the Microbial Screening Assays |
|---|---|---|
| Assays | Number of samples analysed | Positive results Numbers % |
| | | | |
| β-Lactams | 93 | 48 | 51.6 |
| Tetracyclines | 97 | 30 | 30.9 |
| Macrolides | 85 | 18 | 21.2 |
| Sulfa drugs | 110 | 19 | 17.3 |
| Aminoglycosides | 50 | 8 | 16.0 |
Fig. 2. Occurrence of antibiotic/sulfa drug residues in samples positive in the microbial screening assays. A set of bars represents samples positive in the re-analysis by a certain microbial method, while the individual bars within each set show the residues present in these samples as determined by the Charm II test (percentage positive samples). The figure above each bar indicates the total number of samples which were analysed for a particular type of residues. Samples positive for: ■, β-lactams; □, tetracyclines; ▶, aminoglycosides; ▶, sulfa drugs; □, macrolides.

antibiotic/sulfa residues in samples as determined by the Charm II test.

Figure 2 shows the results in the Charm II test for samples positive in the re-analysis by different microbial methods. Each set of bars represents samples positive in a certain microbial method and the individual bars show the occurrence of antibiotic/sulfa drug residues in these samples as determined by the Charm II test.

The Charm II test frequently indicated the presence of more than one type of antibiotic/sulfa drug residues, which is illustrated in Fig. 3. A set of bars represents milk samples which were positive in the analysis for a

Fig. 3. Combinatory presence of antibiotic/sulfa drug residues in samples positive in the microbial screening assays. A set of bars represents samples positive in the Charm II test for a certain type of drug residues, while the individual bars within each set show additional residues in these samples as determined by the Charm II test (percentage positive samples). The figure above each bar indicates the total number of samples which were analysed for a particular type of residues. Samples positive for: ■, β-lactams; □, tetracyclines; ▶, aminoglycosides; ▶, sulfa drugs; □, macrolides.
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Fig. 4. Results in the various microbial methods for samples positive in the Charm II test. A set of bars represents samples positive for a certain type of drug residues, while the individual bars within each set show the results for these samples (percentage positive samples) in the re-analysis by the microbial methods. The figure above each bar indicates the total number of samples which were analysed by a particular microbial method. Samples positive in: ■. Arla microtest; □. Delvo test P; ▣. Delvo test SP; ☑. BR-test AS; ☑. Valio T101.

certain type of antibiotic/sulfa drug residues by the Charm II test, while the individual bars show the concurrent presence of other residues in these samples.

The results in the microbial methods for samples positive in the analyses for different types of antibiotic/sulfa drug residues by the Charm II test are shown in Fig. 4. Each set of bars represents samples which were positive for a certain type of residues, and the individual bars show the results for these samples in the different microbial methods.

DISCUSSION

A considerable difference in sensitivity between different microbial methods was found when samples positive in the screening assay were re-analysed by the other microbial methods (Fig. 1). As expected, the Delvo test P and SP showed a similar pattern of detection. Samples positive in the BR-test AS were also positive in the Delvo test P and SP, while only 40–50% of the samples positive in either Delvo test were positive in the BR-test AS. The Valio T101 and the Arla microtest had a similar pattern of detection, although Valio T101 detected slightly more of the samples with a positive result in Delvo test P/SP and BR-test AS. This difference may be attributed to the somewhat higher penicillin sensitivity of the Valio T101 test as compared to the Arla microtest.

The samples positive in the microbial screening assays were also analysed by the Charm II test in order to verify the specific residue, or the
combinations of antibiotic residues, causing inhibition in the microbial methods. β-Lactam antibiotics were the prevailing type of residues found, irrespective of microbial method (Table 2). In samples positive in the Delvo test P/SP and BR-test AS, β-lactams were detected in 90-100% of the samples (Fig. 2). Samples positive in the Arla microtest and the Valio T101 test, on the other hand, contained a more even distribution of the different types of antibiotic/sulfa drug residues as determined by the Charm II test.

More than a third of the β-lactam positive samples, i.e. 38%, were also found positive for aminoglycosides (Fig. 3). This is in accordance with the frequent treatment of mastitis with a combination of penicillin G and dihydrostreptomycin (Wierup et al., 1989). The presence of tetracyclines and macrolides was indicated in 30.9% and 21.2% of the samples (Table 3), occasionally in combination (Fig. 3). Also sulfa drugs, detected in 17.3% of the samples, often appeared in combination with tetracyclines and/or macrolides. These results may reflect persistent infections, where different successive treatments were applied to the cow.

Two of the microbial tests used for screening, i.e. the Delvo test SP and BR-test AS, are designed to achieve increased sensitivity to sulfa drugs as compared to other microbial tests. It is therefore somewhat surprising that, in this investigation, samples positive for sulfa drugs in the Charm II test were detected best by the Arla microtest and the Valio T101 test (Fig. 4). One plausible explanation is that the samples, in addition to sulfa drug residues, contained antibiotics. The inhibition observed in the microbial method was, consequently, not only an effect of the sulfa drug residues, but also the presence of other antibiotic residues.

The presence of sulfa drug residues in milk has received considerable attention in recent investigations (Brady & Katz, 1988; Charm et al., 1988; Collins-Thompson et al., 1988; Larocque et al., 1990). Suhren et al. (1990) investigated the incidence of inhibitory substances in consumption milk in nine European countries. A total of 337 milk samples were collected and analysed by different microbial methods and the Charm II test. An indistinct distribution of the sulfonamide results made an evaluation impossible with respect to these substances. Certain unspecific factors are known to interfere with the sulfa drug determination by the Charm II test (Suhren & Heeschen, 1987a, 1990; Charm et al., 1988) and positive results should consequently be confirmed by other, more specific techniques. Also, the tetracycline determination is highly influenced by variations in milk composition. Suhren & Heeschen (1987b) found high correlations \((r = 0.6-0.9)\) between results in the Charm II tetracycline test and milk pH, somatic cells and state of lactation. The large counting range of antibiotic-free milk samples influences the limit of detection,
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and a larger count decrease is necessary to set the control point in the tetracycline assay. Tetracyclines form chelates with divalent cations like calcium and bind to proteins. By pretreating samples with ammonium oxalate these bonds were split, and the test sensitivity increased (Suhren & Heeschen, 1990). Samples positive for tetracyclines as determined by the Charm II microbial receptor test should, ultimately, be verified by a specific analysis, e.g. HPLC. This was not feasible in our present investigation, and the large number of tetracycline positive samples must, therefore, be regarded with some apprehension.

From our results, it is evident that by using the Delvo test P as screening assay, milk samples containing predominantly β-lactam antibiotics will be detected. The Arla microtest unquestionably fails to detect samples containing low concentrations of penicillin. It will, on the other hand, detect milk samples with tetracycline residues at levels which would pass the Delvo test P. This is also valid for the Valio T101 test, which has approximately the same sensitivity to tetracyclines as the Arla microtest. In addition, the Valio T101 test detects dihydrostreptomycin and spiramycin at levels 10 times lower than the other microbial tests (Table 1). Bearing the test sensitivities in mind, it is remarkable that samples which were positive for macrolides were efficiently detected by the Arla microtest, but not at all by the Delvo tests (Fig. 4), although their sensitivities to spiramycin are equal. This phenomenon again accentuates the need for comparative analyses with chromatographic methods, to confirm the true presence of different antibiotics.

Many factors must be considered when the most suitable screening assay for inhibitory substances in milk is to be selected (Senyk et al., 1990). The types of antibiotic preparations known or expected to be used by veterinarians, the need for sensitivity, special requirements of regulatory agencies and costs, are some of the more important factors that have to be considered. In addition to the microbial assays, there are today an increasing number of rapid tests, often based on immunological principles, which are commercially available. However, the high specificity of these methods to a certain extent restricts their use as screening assays. The microbial assays, on the other hand, have a broader spectrum of detection, which is a prerequisite in many countries where the inhibitor control is to be a control for inhibitory substances in general and not for the presence of one or a few specific antibiotic substances.

The results in our previous investigation (Carlsson & Björck, 1991) and the results presented here indicate an increase in the use of antibiotic preparations other than β-lactams in Sweden in recent years. By using an assay which is sensitive predominantly to β-lactam antibiotics as rejection
test, this change has not been evident. Although the detection limits of the microbial methods can be improved, a method in which high sensitivities to all inhibitors which may occur in milk are combined will probably never exist. It may therefore be necessary to have a concurrent use of more than one microbial screening method with complementary patterns of detection. These methods can be used at the same time, which has the drawback of increased costs, or alternatively of an intermittent scheme. Changes in the present situation, for example, the introduction of new antibiotic preparations, can in such a system be encountered by replacing a certain method with another having a more appropriate pattern of detection. It is important to point out that any system should be based on control at the individual farm level to be efficacious.

The results of the present investigation indicate that a combined system of two or more different microbial screening methods in combination with the Charm II test for verification of the antibiotic/sulfa drug residue would be an efficient model for controlling inhibitory substances in farm milk. In certain cases, however, it may be necessary to use chromatographic methods for confirmation of the results in the Charm II test.

REFERENCES


