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Charm Test II for Confirmation of Inhibitory Substances Detected by Different Microbial Assays in Herd Milk

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ABSTRACT

In Sweden, milk delivered to dairies is assayed with either the Arla microtest or the Delvo test P for the presence of inhibitory substances. A positive result has to be verified with the Delvo test P to be considered positive. In the present investigation, Charm test II was used for confirmation analysis of approximately 300 milk samples positive in the routine assays. The samples were analyzed for the presence of beta-lactams, tetracyclines, and aminoglycosides. In addition, the samples were analyzed with the Valio T 101 test. Approximately 90% of the samples, which were positive in the verification with Delvo test P, were positive for beta-lactams in the Charm test II. Of the samples, which were found positive by the Arla microtest, 37% were negative in the verification with Delvo test P. The majority of these samples were found to be positive for tetracyclines in Charm test II. The T 101 test showed a different pattern of sensitivity compared to the Delvo test P and Arla microtest. Of the samples which were positive in the verification with Delvo test P, only 68% were positive in the T 101 test, while 49% of the samples which were positive in the Arla microtest but negative in the verification with Delvo test P were positive in the T 101 test. Most of the latter samples, i.e., 95%, were positive for tetracyclines in the Charm test II. It can be concluded that the microbial assays used have quite different patterns of sensitivity toward inhibitory substances in milk and that confirmation with Charm test II gives a high degree of clarification of antibiotic substances present.

In Sweden, all milk delivered to dairies is assayed approximately once per month for the presence of inhibitory substances. The screening assays used are microbiological, i.e., the Delvo test P (10), in the northern and southern dairy regions and the Arla microtest (7,11) in the Arla dairy region. When a doubtful or positive result is obtained, the Delvo test P is always used for verification. Samples also positive in the verification are sent to the Swedish Dairies Association Central Laboratory (SMRC) for confirmation analyses. The present confirmation routine at SMRC includes Delvo test P after dilution or addition of penicillinase, Charm test I, and occasionally the Penzym test. Both the Charm test I and the Penzym test are limited to detection of beta-lactams, and evidently only this group of antibiotics can be confirmed with present methods.

In mastitis therapy penicillins have been and are still regarded to be the major chemotherapeutic used, occasionally in combination with streptomycin. Beside these drugs, sulfonamides and tetracyclines are being used to a varying degree. It has been considered important to have an inhibitor test with a high sensitivity to penicillin, while the sensitivities toward other antibiotics are not always of the same magnitude. Both the Delvo test P and the Arla microtest are very sensitive to penicillin, and consequently a high proportion of the positive samples usually contain penicillin residues.

For a certain number of samples the results in the screening assays are ambiguous, and occasionally doubts regarding the true presence of an inhibitory substance are expressed. With the limited possibilities to identify the active substances, these events present problems, with regard to both explanation and attributing responsibility. Positive reactions caused by substances other than antibiotic and chemotherapeutic residues are often called "false positive reactions". Endogenous antibacterial substances may cause these reactions (3,6,8,9), but problems in confirming a positive reaction could also arise from usage of drugs other than beta-lactams. In the Swedish system a positive reaction is only considered as "false" if the performance of the analysis was connected with errors. Accordingly, all samples which cause a positive reaction in the screening assay and in the following verification are considered as positive.

In recent years the Charm test II has been evaluated in several investigations with satisfactory results (2,4,12). With the microbial receptor technique, based on the competitive binding of radioactively labelled antibiotics, it is possible to detect seven different classes of drug residues in milk.

New microbiological assays have also become available, among them the Valio T 101 test, utilizing *Streptococcus thermophilus* as indicator organism (13). This test is claimed to be more sensitive for some antibiotics, for example streptomycin and spiramycin, than other microbiological tests on the market. Its sensitivity is also similar to that of the starter cultures used in the dairy industry. Table 1 shows the sensitivities of the Valio T 101 test,

TABLE 1. *The sensitivities[1] of the microbiological inhibitor tests Valio T 101, Delvo test P, and Arla microtest to some antibiotics used in mastitis therapy in Sweden.*

Antibiotic	T 101	Delvo test P	Arla microtest
Penicillin G, IU/ml	0,005	0,003	0,005
Dihydrostrept., µg/ml	0,3-0,5	4-6	5
Oxytetracycline, µg/ml	0,15-0,2	0,2-0,4	0,10
Spiramycin, µg/ml	0,1-0,2	1-2	1,5-2
Sulfamethazin, µg/ml	0,5-2,5	50-100	50-100

[1]According to manufacturers' information.

Delvo test P, and Arla microtest to some antibiotics used in mastitis therapy in Sweden.

This study was performed to evaluate the routine use of the Charm Test II for confirmation of samples positive in the microbiological screening assay. The T 101 test was included for a comparison with the Delvo test P and Arla microtest. Today, an increasing number of samples shows different results in Delvo test P and Arla microtest. It is not uncommon that samples are positive in the Arla microtest but negative in the Delvo test P. If this discrepancy can be explained by differences in sensitivity to inhibitors other than penicillin, the intention was to find out the identity of this substance(s) by using Charm test II.

MATERIALS AND METHODS

Milk samples

The study used 305 milk samples which had shown positive results in the microbiological screening assay with either the Arla microtest or the Delvo test P during 1989. The samples were frozen and weekly sent by mail from different milk grading laboratories in Sweden to our laboratory. Samples also positive in the verification with Delvo test P were divided into two portions. One portion was sent to SMRC for routine confirmation analyses and the second portion was sent to the Swedish University of Agricultural Sciences (SLU) (Fig. 1). The samples were usually thawed upon arrival to the SLU laboratory. They were immediately refrozen at -20°C and were never stored for more than 4 weeks before they were analyzed.

Fresh, antibiotic-free raw milk from the University research dairy farm served as negative control milk in the T 101 test and Charm test II.
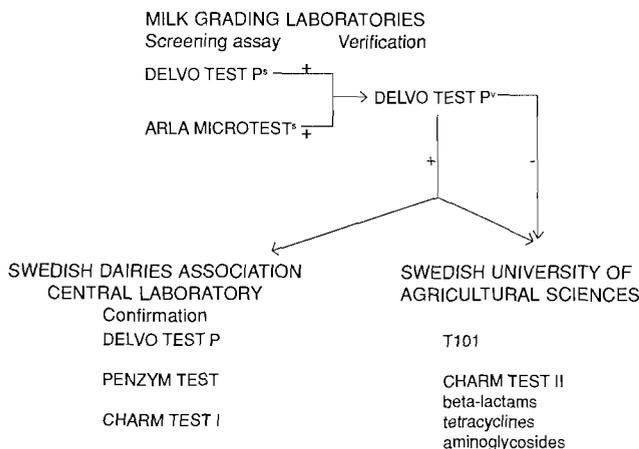

Figure 1. *Summary of tests performed at the milk grading laboratories, the Swedish Dairies Association Central Laboratory (SMRC) and the Department of Animal Nutrition and Management.*

Screening tests performed at the milk grading laboratories

The Arla microtest and the Delvo test P were performed according to the manufacturers' instructions, i.e., SMR (Malmö, Sweden) and Gist-Brocades (Delft, Netherlands), at the different milk grading laboratories. Milk samples with doubtful or positive results were verified with Delvo test P. The results from the two tests were sent to the SLU laboratory together with the milk samples.

Charm test II and T 101 performed at our laboratory

The Charm test II reagents were purchased from Penicillin Assays Inc. (Malden, MA, USA) and the test was carried out with the modifications described by Suhren and Heeschen (12). The milk samples were analyzed for the presence of beta-lactams, tetracyclines, and aminoglycosides. The control point, i.e., the count below which a sample is regarded as positive, was calculated for each antibiotic using a percentage method. The means of four negative control milk samples were multiplied by 0.80 (beta-lactams), 0.70 (aminoglycosides), and 0.50 (tetracyclines). Negative control milk samples were run with each set of analysis. No sensitivity studies were performed in this investigation. Referring to an earlier study (2), the control points used would correspond to the following approximate sensitivities for each antibiotic: penicillin G 0.002 IU/ml, dihydrostreptomycin 0.05 µg/ml, and oxytetracycline 2,5 µg/ml.

Reagents for the T 101 test were obtained from Valio (Helsinki, Finland) and the test was performed in accordance with the manufacturers' instructions.

Statistical analysis

Students t-test was carried out on a MacIntosh personal computer using the Statworks™ program.

RESULTS

Charm test II

Samples analyzed with Delvo test P in the screening assay

Of the samples which were positive in the screening assay with Delvo test P, the majority were also positive in the verification with the same method (Fig. 2). Most of these samples were beta-lactam positive in the Charm test II, often in combination with an aminoglycoside. Some samples were positive for tetracycline in combination with either beta-lactams or aminoglycosides, or with both. One sample which was positive in the verification with Delvo
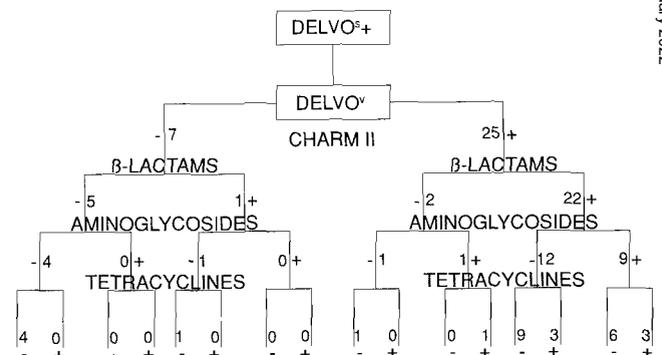

Figure 2. *Results in Charm test II for milk samples positive in the screening assay with Delvo test P with regard to their results in the verification with Delvo test P.[1]*

[1]*Because some samples were not tested with all tests, the sums with positive and negative results do not always add up to the total samples tested.*

test P was negative for all three antibiotics tested with Charm II.

All except one of the samples which were negative in the verification with Delvo test P were also negative for the three antibiotics in the Charm test II. The remaining sample was positive in the beta-lactam test.

Samples analyzed with Arla microtest in the screening assay

Of the samples which were positive in the screening assay with Arla microtest, 37% were positive in the verification with Delvo test P (Fig. 3). The majority of these
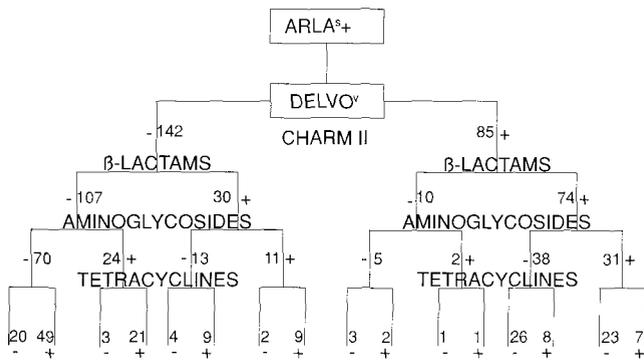

Figure 3. *Results in Charm test II for milk samples positive in the screening assay with Arla microtest with regard to their results in the verification with Delvo test P.*[1]

[1]*Because some samples were not tested with all tests, the sums with positive and negative results do not always add up to the total samples tested.*

samples were beta-lactam positive in Charm test II, often in combination with aminoglycosides. Some samples were positive for all three antibiotics.

Among the samples, which were negative in the verification with Delvo test P, 22% were positive for beta-lactams in Charm test II. There were significant differences (p<0.001) in the Charm test II results between samples which were positive and negative in the verification with Delvo test P, and according to Charm test II, were positive for beta-lactams. The Delvo test P negative samples had higher relative counts in the beta-lactam test, indicating less beta-lactam in the sample, and lower relative counts in the tetracycline test, indicating more tetracycline in the sample, than the Delvo test P positive samples. Many Delvo test P negative samples were positive in only the tetracycline test; however, samples positive for tetracyclines in combinations with the other antibiotics were also frequent. Among the Arla microtest positive and Delvo test P negative samples, 75% were positive in the tetracycline test, in comparison with 25% among the Arla microtest and Delvo test P positive samples.

Samples analyzed with the T 101 test
Samples positive in the screening assay with Delvo test P and Arla microtest.

Many samples which were positive in the screening assay with Delvo test P were negative in the T 101 test (Fig. 4). Charm test II analysis of the T 101 positive samples indicated the presence of beta-lactams in all samples, the majority in combination with aminoglycosides. In the analysis
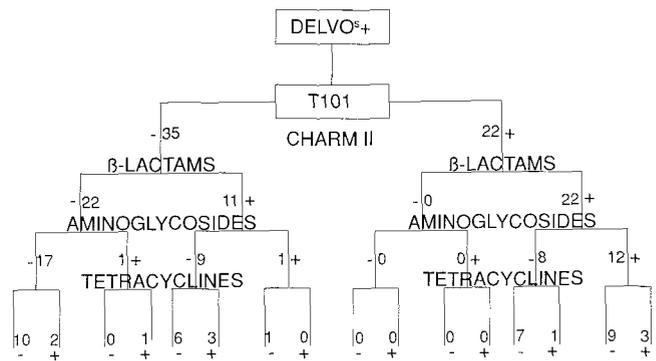

Figure 4. *Results in Charm test II for milk samples positive in the screening assay with Delvo test P with regard to their results in the T 101 test P.*[1]

[1]*Because some samples were not tested with all tests, the sums with positive and negative results do not always add up to the total samples tested.*

of the T 101 negative samples, 33% were beta-lactam positive; however, 9 out of 10 beta-lactam positive samples were aminoglycoside negative. In 43% of the Delvo test P positive but T 101 negative samples, Charm test II did not detect any of the three antibiotics.

Of the milk samples which were positive in the screening assay with Arla microtest, 57% were positive in the T 101 test (Fig. 5). The Charm II beta-lactam test was positive for 59% of the T 101 positive samples and more than 50% of these samples were also positive for aminoglycosides. A high proportion of the T 101 positive samples was positive for tetracycline (57%), often in combination with beta-lactams and/or aminoglycosides. In the analyses of the Arla microtest positive but T 101 negative samples, 35% were positive in the beta-lactam test. The majority of these samples were negative in the aminoglycoside test. Approximately 30% of the T 101 negative samples were positive for only tetracycline in Charm test II. There were differences in relative Charm test II counts between samples which were positive and negative in the T 101 test and, according to Charm test II, were positive for only tetracyclines. The T 101 positive samples had significantly lower relative counts (p<0.01) in the tetracycline test, indicating more tetracycline residues.
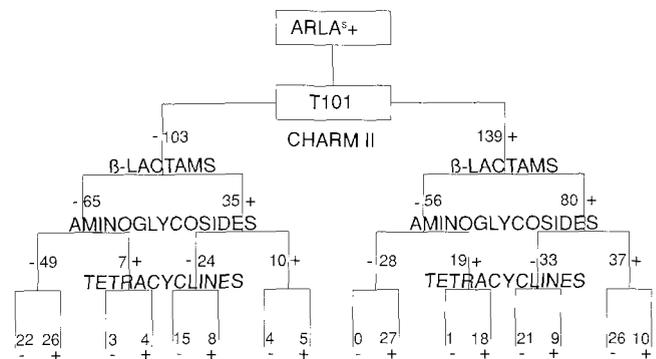

Figure 5. *Results in Charm test II for milk samples positive in the screening assay with Arla microtest with regard to their results in the T 101 test P.*[1]

[1]*Because some samples were not tested with all tests, the sums with positive and negative results do not always add up to the total samples tested.*

Samples positive in the verification with Delvo test P. Among the samples which were positive in the verification with Delvo test P, 68% also showed positive results in the T 101 test (Fig. 6). Almost all T 101 positive samples were positive in the Charm II beta-lactam test and more
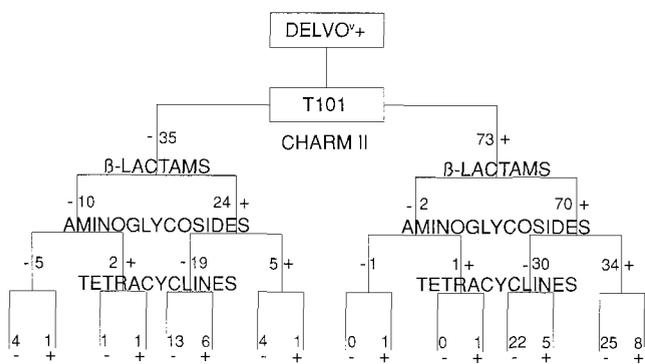
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                    DELVOʸ+
                       │
                     T101
              -│35           73│+
                   CHARM II
        β-LACTAMS              β-LACTAMS
     -│10      24│+          -│2       70│+
     AMINOGLYCOSIDES         AMINOGLYCOSIDES
   -│5   2│+  -│19   5│+   -│1  1│+  -│30    34│+
     TETRACYCLINES            TETRACYCLINES
   4 1  1 1  13 6  4 1    0 1  0 1  22 5  25 8
   - +  - +  -  +  - +    - +  - +  -  +  - +
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Figure 6. *Results in Charm test II for milk samples positive in the verification with Delvo test P with regard to their results in the T 101 test P.*[1]

[1]*Because some samples were not tested with all tests, the sums with positive and negative results do not always add up to the total samples tested.*

than 50% of these samples were also positive in the aminoglycoside test. Charm test II analyses of the T 101 negative samples indicated the presence of beta-lactams in 71% of the samples. There were differences (p<0.01) in relative Charm test II counts between the samples which were positive or negative in the T 101 test and positive in the Charm II beta-lactam test. The samples which were positive in the T 101 test had lower relative counts in the beta-lactam test, indicating higher concentrations of beta-lactam residues.

Samples negative in the verification with Delvo test P. Of the samples which were negative in the verification with Delvo test P, approximately 49% were positive in the T 101 test (Fig. 7). Among the T 101 positive samples, Charm test II indicated the presence of tetracyclines in 95% of the samples and in 48% of the samples only tetracycline. Beta-lactam positive samples were found among the T 101 positive as well as the negative samples. Approximately 36% of the T 101 negative samples were negative in the Charm test II analyses, while another 36% were positive for only tetracyclines. There were differences in relative Charm test II counts between samples which showed positive or negative results in the T 101 test and were positive for only tetracyclines in Charm test II. The T 101 positive samples had lower relative counts than the negative samples (p<0.01), indicating higher concentrations of tetracycline residues.

DISCUSSION

The use of two different screening assays for detection of milk inhibitors in Sweden seems to have revealed a change in the use of antibiotics and chemotherapeutics. In the 1960's when penicillin was used almost exclusively, results in the Delvo test P and the Arla microtest were usually overlapping. In recent years the results seem to
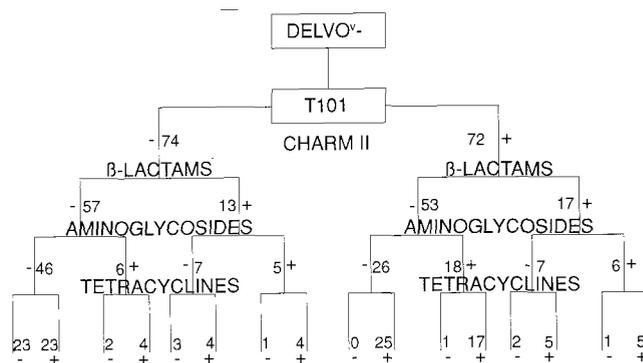
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                     DELVOʸ-
                        │
                      T101
              -│74            72│+
                   CHARM II
        β-LACTAMS              β-LACTAMS
     -│57     13│+          -│53      17│+
     AMINOGLYCOSIDES        AMINOGLYCOSIDES
   -│46  6│+  -│7   5│+   -│26  18│+  -│7    6│+
     TETRACYCLINES            TETRACYCLINES
   23 23  2 4  3 4  1 4   0 25  1 17  2 5  1 5
   -  +   - +  - +  - +   -  +  -  +  - +  - +
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Figure 7. *Results in Charm test II for milk samples negative in the verification with Delvo test P with regard to their results in the T 101 test P.*[1]

[1]*Because some samples were not tested with all tests, the sums with positive and negative results do not always add up to the total samples tested.*

have become more divergent. A sample which is positive in the screening assay with Arla microtest but negative in the verification with Delvo test P is, with present regulations, considered as negative. Results in this investigation indicated that certain samples which seem to contain antibiotic residues will not be detected with the present inhibitor control.

The results showed a marked difference in the sensitivities of the Delvo test P, the Arla microtest, and the Valio T 101 test. The result in a microbiological assay is the reaction against all inhibitory factors present, while Charm test II measures the presence of one specific drug family at a time. Therefore, the correlation between two microbiological assays as well as the correlation between a microbiological assay and Charm test II will decrease when the samples contain combinations of inhibitory substances.

Milk samples which were positive in the screening assay with Delvo test P were usually beta-lactam positive in Charm test II. Approximately 90% of the samples, which were positive in the verification with Delvo test P and according to regulations are considered as positive, were beta-lactam positive in Charm test II (Fig. 2 and 3). Due to the somewhat lower penicillin sensitivity of the T 101 test, a number of Delvo test P positive samples were negative in the T 101 test (Fig. 4). Some of these samples were negative for all three antibiotics tested with the Charm test II. It is likely that some of the unconfirmed positive reactions were obtained for samples which were positive in the screening but negative in the verification with Delvo test P. Another explanation may be that the samples contained low concentrations of inhibitors (e.g., penicillin) which lose their activities during transport to the SLU laboratory.

Most samples which were positive in screening assay with the Arla microtest were negative in the verification with Delvo test P (Fig. 3). Although negative in the Delvo test P, a surprisingly high number of these samples were positive for beta-lactams. The statistical analyses of the counts in the Charm II beta-lactam and tetracycline tests suggested that the additive effect of tetracycline in combination with very low penicillin concentrations caused inhibition in the Arla microtest but not in the Delvo test

P. In general, a high proportion of the Arla microtest positive samples was positive for tetracyclines in Charm test II. Considering that the Arla microtest and the T 101 test have similar sensitivities to tetracyclines, a surprisingly high number of the Arla positive samples were negative in the T 101 test (Fig. 5). A statistical analysis of the relative counts for samples which were positive or negative in the T 101 test, and positive for just tetracycline in the Charm test II, indicated slightly higher tetracycline concentrations in the positive samples. It is uncertain if the slight differences in test sensitivities to tetracyclines could cause the difference in frequency of positive samples. In addition, 25% of the samples which were negative in the T 101 test were negative for all three antibiotics tested for with Charm test II. Due to the fact that the milk samples usually thawed during the transport to the SLU laboratory, the results in the tetracycline test should be considered with caution. The tetracycline test is very susceptible to changes occurring when the milk ages (S. Charm & E. Zoomer, personal communication). The sometimes very high count reductions in the tetracycline test were not likely to be due to contamination only but also to dissociation of milk components which interfered with the test. However, the fact that all samples were positive in at least one microbiological test before they were frozen cannot be explained by such compositional changes. It is therefore most likely that a high number of the tetracycline positive samples actually contained tetracycline residues.

Samples which were positive in the verification with Delvo test P were often positive in the T 101 test (Fig. 6). Almost all of the T 101 positive samples were positive for beta-lactams and many (52%) were also positive for aminoglycosides. Of the samples which were negative in the T 101 test but positive for beta-lactams, only 21% were also positive for aminoglycosides. The T 101 sensitivity toward aminoglycosides is approximately 10 times higher than that with the Delvo test P or Arla microtest (13). The additive effect of an aminoglycoside seems, especially at low penicillin concentrations, to be important to cause inhibition in the T 101 test. Samples which are positive in Delvo test P due to low concentrations of only penicillin would therefore normally be negative if only the T 101 test was used for screening. On the other hand, approximately the half of the samples which were negative in the verification with the Delvo test P were positive in the T 101 test (Fig. 7). Most of these samples originated from the screening assay with the Arla microtest and were positive for tetracycline in Charm test II.

The overall high frequency of samples which were positive in the Charm tetracycline test was unforeseen. It was also surprising to find that almost 8% of the samples were positive for tetracyclines in combination with beta-lactams and aminoglycosides. In an American survey of commercial milk samples, using the Charm test II, Brady and Katz (1) found that tetracyclines and sulfonamides were the most predominant residues. Tetracyclines as well as sulfonamides were present in 40% of the milk samples, and approximately 3% of the milk samples contained the combination of beta-lactam, tetracycline, and streptomycin. In a similar study, Collins-Thompson et al. (5) sur-

veyed U.S. and Canadian consumer milk samples with the Charm test II. Tetracyclines and sulfa drugs were again the most frequently detected residues, in 28% and 44% of the samples. Compared to these figures, the frequency of positive samples in our investigation must be considered rather low, bearing in mind that these samples were first identified from a large number of samples by the microbiological screening assays.

With the Charm II tests for beta-lactams, tetracyclines, and aminoglycosides, it was possible to verify the inhibitory substance(s) in most milk samples. The residues found varied with the screening method used, demonstrating a difference in sensitivity between the microbial assays, and thus in the expected number of positive samples to be found in different regions of Sweden. The results raise the question whether there has been a change in the therapeutic use of antibiotics and chemotherapeutics in the treatment of bovine mastitis. Tetracycline residues seem to be found more often today, and the lower tetracycline sensitivity of the Delvo test P compared to the Arla microtest results in a less effective control. The Valio T 101 test seems to exhibit qualities differing from both the Arla microtest and the Delvo test P. It was not as sensitive as the Delvo test P with regard to beta-lactams, but on the other hand detected many samples which were positive in the Arla microtest but negative in the Delvo test P. The detected substances might also have been other than those three analyzed with the Charm test II.

The present control system for milk inhibitors has deficiencies and should be revised. A new control system requires screening methods capable of detecting low levels of sulfonamides and other new, important drugs. In addition, the handling routines of the milk samples during transport from one laboratory to another must be improved. The low sensitivities of both Arla microtest and Delvo test P to sulfa drugs were the reason for not including this group of chemotherapeutics in our investigation. These substances will be covered in a future study. Provided the appropriate screening methods are employed, Charm test II is a useful method for confirmation of positive samples. However, depending on the handling and the nature of a milk sample, it may contain factors which interfere with the test and further comparative studies are needed.

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