PERGAMON

Food Control 10 (1999) 35–39

FOOD CONTROL

A simple test for the detection of antibiotics and other chemical residues in ex-farm milk

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Received 26 June 1998; revised 22 September 1998; accepted 5 October 1998

Abstract

A simple Yoghurt Culture Test (YCT) for the routine detection of antibiotic and other residues in raw milk was developed. It involved acidifying a sample of the milk to pH 6.0, adding Chlorophenol Red as an indicator and inoculating the milk (4%, v/v) with a culture containing a balanced mixture of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* sub-sp. *bulgaricus*. In the absence of inhibitory substances, incubation at 42°C for 2.5 h gave rise to sufficient acid production for the milk to gel and the indicator to change colour (PASS), but low levels of the antibiotics tested increased the incubation time beyond 4 h (FAILURE). A survey of 618 samples of raw milk collected from three dairies in Jordan identified a failure rate of 15% with the YCT at 2.5 h whereas, due to differences in sensitivity, the Delvotest-P failed only 12.3% of the milks. It was concluded that the YCT was a reliable and inexpensive approach for testing ex-farm milks for inhibitory residues.

Keywords: Milk; Antibiotics; Testing

1. Introduction

Antibiotics are essential for the control of mastitis and other bacterial problems in dairy herds (Jones and Seymour, 1988; Oliver et al., 1990), and their passage into milk can cause major problems for a receiving dairy. Thus, if the milk is to be processed for direct retail sale or into milk powder or some other product, it would be regarded in many countries as 'adulterated' and a public health risk (Albright et al., 1961). This intolerance of antibiotic residues stems from the fact that some adults are hypersensitive to penicillins, and allergic reactions and even death can result from ingestion (Seymour et al., 1988). In addition, any exposure of the intestinal microflora of humans to antibiotics may lead to an increase in the numbers of antibiotic-resistant species present and, if some of these are pathogenic, then their possible spread within a community could have dire consequences (McGrane et al., 1996). It is for this reason that the Federal Drug Agency in the USA have specified a 'zero tolerance' for antibiotics in milk and milk products, i.e. no higher than the lowest concentration that can be detected by prevailing techniques (Kornfield, 1977; IDF, 1991).

For the manufacturers of cheese or fermented milks, the situation is slightly different, for the presence of antibiotics or other inhibitory materials in milk can lead to total or partial failure of the starter culture. If the failure is total, then an entire vat of milk may be wasted, while if acid development is slow or inadequate, consumer complaints may arise if poor product reaches the market or, in the case of certain cheeses, there may be a risk of a serious incident of food-borne disease (Keceli and Robinson, 1997). In order to avoid the financial losses that may be associated with the use of contaminated milk, both the dairy industry and Regulatory Authorities have come to rely on routine programmes of testing (Shearer, 1995; Robinson and Wilbey, 1998). For this purpose, a number of microbial inhibitor tests approved by the AOAC (1990) or other Bodies are available, such as the Cylinder Plate Assay

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0956-7135/98/$ — see front matter
PII: S0956-7135(98)00154-6

and the Disc Assay(s) (Marshall, 1992), the Delvotest-P and Delvotest-SP (Anon, 1997), and the Charm AIM-96 Test (Marshall, 1992; McGrane et al., 1996; Suhren and Heeschen, 1996). In general, these broad spectrum tests take > 2.5 h to complete but, more recently, a number of rapid (< 10 min) ELISA systems have been introduced which are highly specific for β-lactam residues (Bell et al., 1995; Scannella et al., 1997). In the case of penicillin G, most of these procedures can detect between 0.004 and 0.006 IU ml^{-1} of milk, but the responses of the microbial inhibitor tests to other antibiotics or inhibitory residues varies with the compound in question. As far as the fermentation sector of the dairy industry is concerned, these figures compare favourably with the known sensitivity of *Streptococcus thermophilus* (Tamime and Deeth, 1980), a species that is amongst the most susceptible of starter bacteria to inhibition.

In Europe, North America and Australasia, dairy factories can, therefore, monitor their milk supplies on a routine basis and, given the speed of the ELISA tests, are in a position to reject contaminated tanker loads in advance of discharge into a silo. However, this use of an antibiotic residues test as a 'reception test' is a comparatively recent innovation and, as most recognised tests take > 2.5 h, their use is intended, at least in part, to avoid vat failure through the prior isolation of any heavily contaminated supplies. In the less developed parts of the world, the non-availability of imported commercial kits and the cost of each test tends to preclude even this essential monitoring (Jurdi and Asmar, 1981). Nevertheless, these restrictions do not mean that testing should not be implemented, and hence the aim of this project was to:

(i) examine the possibility of employing a simple, standardised growth test to check milk supplies for inhibitory materials within the same time-span as the widely used microbial inhibitor tests; and
(ii) compare the performance of the test against an internationally-accepted method — the Delvotest-P.

2. Materials and methods

Three yoghurt cultures — each containing equal mixtures of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* sub-sp. *bulgaricus* — that are in regular use in dairy plants in Jordan were selected for the project. Each culture was prepared by mixing 1 g of well-mixed, fresh yoghurt with 99 ml of skim-milk (10% dry milk solids, w/v) that had been heat treated at 95°C for 5 min. After incubation at 42°C for 4 h, the cultures were stored at 5°C. Fresh sub-cultures were prepared in the same manner, and were always used on the following day.

2.1. Establishment of the procedure

Fresh, raw milk was drawn from a single cow known to be free from any form of medication, and the sample was transported to the laboratory in an ice-box; the maximum journey time was around 1 h. Appropriate volumes of the milk were then dispensed into four clean jars and, while one jar was held as a control, the pHs of the other samples were adjusted to 6.4, 6.2 and 6.0 using 1 N HCl. The milk in each jar was then divided into four equal portions and, after warming in a domestic microwave oven (Toshiba ER 692) for a period of time known to give a temperature of 45°C, the four portions were inoculated with either 2, 3, 4, or 5% (w/w) of one of the three available cultures. This procedure was repeated for each pH treatment and control, and with each of the three cultures. After thorough mixing, 10 ml aliquots from each portion were transferred to each of 10 test tubes, and the tubes placed in a water bath at 42°C. Duplicate measurements of pH were made immediately using a Hana Instruments pH meter (Model HI 8416), and after 1.5, 2.0 and 2.5 h incubation; the titratable acidities of the same samples were measured using the procedure of Case et al. (1985).

As an alternative to measurements of acidity, the use of pH indicators was examined in a further trial by adding 0.1 ml of either Bromocresol Green (0.2% in 50% ethanol), Methyl Red (0.2% in 50% ethanol), Chlorophenol Red (0.2% in 50% ethanol) or Bromocresol Purple (0.2% in 50% ethanol) to the milk either before or after incubation.

2.2. Sensitivity of the test to different antibiotics

Stock solutions of each antibiotic, namely penicillin, chloramphenicol, oxytetracycline, tetracycline, ampicillin, erythromycin and cloxacillin, were prepared in deionised water at 10 times the maximum concentration to be tested. Appropriate aliquots were then added to standard volumes of fresh, raw cow's milk to give the following concentrations:

Penicillin — 0.1, 0.06, 0.03, 0.006, 0.003 and 0.0006 IU ml^{-1} of milk
Chloramphenicol — 10.0, 5.0, 4.0, 3.0, 2.0 and 1.0 μg ml^{-1} of milk
Oxytetracycline — 0.50, 0.25, 0.20, 0.15, 0.10 and 0.05 μg ml^{-1} of milk
Tetracycline — 0.80, 0.60, 0.40, 0.30, 0.20 and 0.10 μg ml^{-1} of milk
Ampicillin — 0.20, 0.15, 0.10, 0.075 and 0.05 μg ml^{-1} of milk

Erythromycin — 0.60, 0.50, 0.40, 0.30 and 0.20 $\mu g\ ml^{-1}$ of milk
Cloxacillin — 0.40, 0.35, 0.30, 0.25 and 0.20 $\mu g\ ml^{-1}$ of milk

The pH value of each sample of milk was then adjusted to 6.0 with 1 N HCl, and the sample warmed to 45°C in the microwave. A fresh starter culture was then used to inoculate the contaminated milks, along with a control sample (zero antibiotic), at a rate of 4%, and Chlorophenol Red was added as an indicator. After mixing well, 10 ml amounts of each milk were dispensed into test tubes, and the tubes incubated at 42°C. Measurements of pH and acidity were made at 0, 1.5, 2.0, 2.5 and 4.0 h, and the colour changes were recorded as well.

2.3. Market test of the YCT procedure

Over a period of 3 months, a total of 618 samples of milk supplied to three largest dairy plants in Jordan were examined using the Yoghurt Culture Test (YCT). On each occasion, the dairy operator produced a composite sample from the milks of each supplier, and 100 ml from this composite sample were transferred to a screw-capped bottle. The sample, stored in ice at 2–5°C, was returned to the laboratory within 1 h and the pH measured.

Each sample of milk (around 100 ml) was then poured into an individual polyethylene yoghurt carton (200 g capacity), and sufficient 1 N HCl added to give a pH of 6.0. Four grams of starter culture and 1.0 ml of Chlorophenol Red were added and, after mixing, the carton was placed in an incubator at 42°C. A control made up in antibiotic-free skim milk was run in parallel, and the colour change/curd formation was recorded at 2.5 and 4.0 h; samples without any change in colour/curd formation were suspected of being contaminated with antibiotics and were considered positive. As an additional check, each of the samples that failed to form a coagulum after 2.5 or 4 h was tested with a Delvotest-P kit obtained from Gist-brocades, Delft, The Netherlands, and ampoules that were entirely or partially purple at the end of 2.5 h were recorded as positive.

3. Results and discussion

The preliminary trials confirmed that the optimum conditions to achieve curd formation — pH 4.8 and titratable acidity of 0.5% (as lactic acid) — within 2.5 h involved an inoculation rate of 4.0% and an initial pH of the milk of 6.0. However, only one of the three starter cultures was able to achieve this level of performance on a repeatable basis, and hence the other two were eliminated from the trial. It was found also that Chlorophenol Red was the most appropriate indicator, as the change in colour from light violet to beige–yellow was easy to note during routine observations.

Table 1
Minimum concentrations of antibiotics detected by the YCT as measured by the time for the culture to lower the pH of the milk to 4.8, i.e. achieve minimum coagulum formation

Antibiotic	Coagulum formation ($\mu g\ ml^{-1}$)	
	2.5 h	4.0 h
Penicillin	0.03 (0.05 IU)	0.06 (0.1 IU)
Chloramphenicol	2.0	4.0
Oxytetracycline	0.1	0.25
Tetracycline	0.2	0.4
Ampicillin	0.1	0.15
Erythromycin	0.3	0.4
Cloxacillin	0.3	0.35

The minimum concentrations of the different antibiotics giving positive results in the YCT, i.e. no curd formation or colour shift in the presence of Chlorophenol Red, are shown in Table 1. The sensitivity of the test to penicillin was rather disappointing because, at 2.5 h, the YCT appeared 10 times less sensitive than some of the results published for the Charm or Delvotest-P techniques, and it may be that the *Lac. delbrueckii* sub-sp. *bulgaricus* component of the culture was not affected immediately by the inhibitor; Tamime and Deeth (1980) suggest that some strains of *Lac. delbrueckii* sub-sp. *bulgaricus* can tolerate 0.10 IU ml^{-1} of penicillin. It has also been reported that mixed cultures of *Str. thermophilus* and *Lac. delbrueckii* sub-sp. *bulgaricus* are less sensitive than the individual species growing alone, and this effect might have altered the results as well (Robinson and Tamime, 1990). The sensitivity of the YCT to chloramphenicol, oxytetracycline, tetracycline and erythromycin at 2.5 h was better than that reported for Delvotest-P (Jones and Seymour, 1988), but the result for ampicillin revealed a lower sensitivity.

The reduced sensitivities of the YCT at 4 h is a reflection of the fact that the concentrations that cause a 'failure' at 2.5 h leave a percentage of cells of one or both organisms unaffected. Consequently, sufficient acidity has been generated at the end of 4 h to form a coagulum, and a higher concentration is needed to ensure that too few cells survive to lower the pH to 4.8 or below. Nevertheless, the results at 4 h were useful for developing the following protocol:

Failure to change indicator in 2.5 and 4 h
— unacceptable level of inhibitory substances in the milk

Failure to change indicator in 2.5 h, but change after 4 h
— marginal level of inhibitory substances in the milk
Change of indicator in 2.5 h
— inhibitory substances below level of detection

This pattern is broadly comparable with that proposed for detection of penicillin G with the Delvotest-P (Anon, 1997) with readings after 2.5 h incubation, i.e. (i) total colour change of the medium containing Bromocresol Purple from purple to yellow — residues of penicillin G <0.002 IU ml^{-1} and comparable situation with other inhibitory agents; (ii) medium appears purple/green colour — concentration of penicillin G of 0.002–0.005 IU ml^{-1}; and (iii) entire medium appears purple — concentration of penicillin G >0.005 IU ml^{-1}.

Clearly, the disadvantage of the YCT is that it is not so sensitive to β-lactam antibiotics as some of the commercial kits, but this criticism does not alter the value of the YCT as a practical method of assessment for a dairy. In other words, as long as the working culture can coagulate the milk in 2.5 h, the dairy can use the milk with confidence for a fermentation process, whereas, if the milk fails at 4 h, it is totally unsuitable for a fermentation process. Whether or not the 'marginal' category is helpful is open to debate, but at least the result alerts the dairyman to an impending problem. In addition, the YCT is simple, employs an in-house culture, needs little skill to perform and is inexpensive, so that its use as a routine screen of supplies could find wide application. Consequently, it was decided to use the YCT for a market survey of selected supplies of raw milk in Jordan, with the Delvotest-P being employed to confirm any positive results; it was assumed that negative results would be negative with the Delvotest-P as well.

One of the notable features of the survey was that care of the milk appeared to be variable, in that, at the time of testing in the laboratory, 24% of the samples had a pH <6.4 and 13% had a value <6.2; 6% of the samples were below the target pH of 6.0 set for the YCT. These figures suggest that some of the milk is not being adequately cooled after collection and, for some processing procedures, these low values could cause problems.

The results of the antibiotic tests are shown in Table 2, and it is important that, whereas after incubation for 2.5 h only 81% of the samples detected as 'positive' for inhibitory substances by the YCT were later confirmed as 'positive' by the Delvotest-P, samples that failed the YCT after 4 h were all confirmed as containing a high level of one or more inhibitory substances by the Delvotest-P. The practical significance of this pattern lies in the facts that:

(i) if it is assumed that the results of the Delvotest-P were 'correct', then the YCT at 2.5 h gave 19% 'false positives', i.e. any 'failures' were on the side of caution;
(ii) the sensitivities to the two tests are not identical. For example, a failure with the YCT at 2.5 h could indicate the presence of inhibitory levels of sulphonamides, a group of antibiotic materials that are only detected by Delvotest-P at levels of 50–100 μg ml^{-1}. Thus, it could be that the results referred to above as 'false positives' could, in reality, be providing a true reflection of the quality of the milk; and
(iii) the YCT can be carried out with the culture that is employed in the dairy for the manufacture of yoghurt, so that the result at 2.5 h gives an immediate indication of the suitability of the test milk for processing.

Overall, it would appear that the YCT employing sensitive strains of *Str. thermophilus* and *Lac. delbrueckii* sub-sp. *bulgaricus* provides a test for inhibitory substances in milk that is broadly comparable in response to the Delvotest-P. Obviously, the YCT would not be suitable for use in the laboratory of a Regulatory Authority where the priority is to protect consumers from extremely low levels of β-lactam residues, but use of the YCT could be encouraged in countries where the testing of milk supplies for antibiotics is not mandatory.

Table 2
Numbers of milk samples from three different dairy plants which gave positive results in the YCT, i.e. no colour change and/or coagulation within the times indicated, and the numbers which were then confirmed by the Delvotest-P

Source	Number	Positives identified by YCT		Positives identified by YCT and confirmed by Delvotest-P	
		at 2.5 h	at 4 h	at 2.5 h	at 4 h
Dairy 1.	184	34	6	27	6
Dairy 2.	311	29	7	21	7
Dairy 3.	123	30	0	28	0
Total	618	93	13	76	13

What emerged also from the survey was that the workload on a dairy laboratory could become excessive in a country like Jordan where individual farmers deliver the milk direct to a dairy, and consideration should, perhaps, be given to the establishment of collecting centres where milks could be tested, bulked and then delivered to dairy plants in the vicinity. The attraction of such an approach is that it might serve to: (a) reduce the number of farmers attempting to market contaminated milk — 2.1% of supplies tested in the survey contained unacceptable levels of inhibitory substances, including probably antibiotics of the β-lactam group; and (b) improve the chilled storage capacity in the area, and so avoid the build-up of acidity that was noted in many of the individual samples.

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