An appraisal of methods used in the examination of retail samples of cows milk

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SUMMARY

The use of the methylene blue test for the examination of cows milk was investigated in an inter-laboratory survey. A poor relationship was found between results of these tests and total viable counts. Coliforms were detected in a large number of pasteurized milks, indicating frequent post-pasteurization contamination. No relationship was found between the results of the methylene blue test and the presence of coliforms.

Results from this survey highlight the need for reappraisal of the methylene blue test as a statutory method for the examination of milk. A total viable count and coliform test are suggested for providing information regarding dairy hygiene and the quality of the product at the point of retail sale.

INTRODUCTION

Methods for examining samples of cows milk and cream in public health laboratories are laid down in a number of Statutory Instruments (SI No. 1033, Great Britain, 1977; SI No. 1508 and 1509, Great Britain, 1983; SI No. 722, Great Britain, 1986). Among the tests described are the methylene blue test and a coliform test. The methylene blue test is a dye reduction test which was introduced over 50 years ago (Wilson *et al.* 1935) as a rapid, inexpensive test to indicate poor quality milk likely to be unacceptable to the consumer after overnight storage without refrigeration. It is a statutory test for both pasteurized and untreated milk. The length of time required for bacterial dehydrogenases to reduce the dye and decolourize it is used as an index of the bacterial load of the test sample. In non-refrigerated milk there is a consistent relationship between the bacterial count and the dye reduction time (Lück, 1981). In recent years the dairy industry has embraced the concept of low temperature storage before and after pasteurization. This is normally maintained in the retail chain up to the point of sale and beyond. Increased use of domestic refrigerators coupled with changes in the dairy industry allows extended storage of milk with concomitant changes in the bacterial flora. The predominant organisms in refrigerated milk are

psychrotrophs, which have a low dehydrogenase activity, and there is less correlation between dye reduction time and the bacterial count than there is with non-refrigerated milk (Lück 1981).

Most public health laboratories apply only the statutory tests to milk samples. The dairy industry in the UK uses colony counts and the presence of coliforms for quality control. Standards set by other countries vary, but mainly require a colony count of less than 10^5/ml and the absence of coliforms and *Escherichia coli* in freshly pasteurized milk. A directive of the Council of the European Communities (85/397/EEC) lays down requirements for heat-treated milk intended for intra-Community trade, to come into force in January 1989. Among other standards, in order to be eligible to receive heat treatment, untreated milk must have a plate count (30 °C) of less than 3×10^5/ml. Pasteurized milk must have a plate count less than 5×10^4/ml and the coliform count must be less than 5/ml when sampling checks are carried out at the treatment premises. The use of the coliform test as described in SI 1509 is restricted to cream samples taken not later than the day following pasteurization at the premises where heat treatment took place. Pasteurization eliminates the majority of coliforms and the test is used as a measure of post-pasteurization contamination (Nelson, 1981). There is at present no statutory requirement in this country to test for the presence of coliforms in milk, other than in milk-based drinks (SI 1508).

An inter-laboratory survey has examined the use of the methylene blue test and the relationship between the results of this test and total viable counts found in cows milk at the point of retail sale. In addition, a coliform test based on that described in SI 1509 has been used to assess post-pasteurization contamination. Samples were also examined for the presence of Enterobacteriaceae and *Yersinia* spp.

MATERIALS AND METHODS

Sampling

Samples of cows milk were collected mainly from retail outlets but also direct from dairies by members of the Environmental Health Department. All samples were transported in insulated containers without artificial cooling to the local public health laboratory and examination commenced on the same say as collection. A total of 430 pasteurized milk and 79 untreated milk samples were obtained between March and November 1985. These samples originated from 33 dairies, 10 of which were small farm dairies.

Methylene blue test

The methylene blue test for examining milk samples was carried out in accordance with SI 1033 (Great Britain, 1977). Briefly, this involved storing the sample at atmospheric shade temperature from the time of arrival of the sample in the laboratory until the time of testing on the following day. If shade temperature exceeded 21 °C, the methylene blue test was not performed. During March, April and November, overnight (17·00–09·30 hours) storage was carried out in a waterbath at 18·3 °C. Standard methylene blue solution (1 ml) was then added to 10 ml of milk, and the sample incubated at 37 °C for 30 min. Samples in which the methylene blue was not decolourized were regarded as satisfactory.

Total viable counts

Decimal dilutions of milk samples were prepared in 0·1 % peptone solution. Viable counts were performed on milk agar (Oxoid CM21) by the spiral plate method (Gilchrist *et al.* 1973) or the surface drop method (Miles & Misra, 1938, modified according to Thatcher & Clark, 1968). Plates were incubated at 30 °C for 3 days before enumeration.

Detection of coliforms

The examination of milk samples for the presence of coliforms was carried out using a Most Probable Number method based on the method laid down for the detection of coliforms in cream samples in SI 1509 (Great Britain, 1983). Ten ml amounts of the 10^{-1} dilution were added to each of three tubes containing 10 ml double-strength brilliant green lactose bile broth (BGLBB; Oxoid CM31) and a Durham fermentation tube. In addition, three aliquots of 1 ml and three aliquots of 0·1 ml of the decimal dilution were each added to 10 ml single-strength BGLBB containing a Durham tube. All tubes were incubated at 30 °C for 48 h, and then examined for gas production. The number of tubes showing gas production was used to compute the most probable number of coliforms per ml using tables (Jacobs & Gerstein, 1960).

Some samples were also examined for the presence of coliforms using violet red bile agar (VRBA; Oxoid CM107). Two laboratories applied 1 ml of the decimal dilution of sample to the surface of VRBA plates (Greenwood *et al.* 1984). The third laboratory distributed 10 ml of the undiluted milk samples into three Petri dishes and 1 ml into a fourth dish. Four plates were then prepared using molten, cooled VRBA (Hartmann & LaGrange, 1985). All plates were incubated overnight at 37 °C, and typical colonies enumerated.

Detection of Enterobacteriaceae

The medium used for the detection of Enterobacteriaceae was BGLBB with the addition of 10 g/l glucose (single strength broth). The test was performed as for the coliform test.

Detection of Yersinia *spp.*

Twenty-five ml of sample was added to 225 ml of 1 % buffered peptone water (Oxoid CM 509), incubated at 4 °C for 2–3 weeks and then subcultured to Yersinia selective agar (Oxoid CM 653 plus SR 109). The plates were incubated at 30 °C for 20–24 h and suspect colonies were confirmed by methods described previously (Greenwood & Hooper. 1985). Strains of *Yersinia* spp. were sent to the reference facility at Leicester Public Health Laboratory for biotyping and serotyping by the method of Wauters (1970).

RESULTS

Total viable counts

Total viable counts obtained from samples of pasteurized and untreated milk are shown in Fig. 1. Two-thirds (67 %) of all samples of pasteurized milk had viable counts below 10^4/ml, 85 % had counts less than 10^5/ml and 6·5 % had
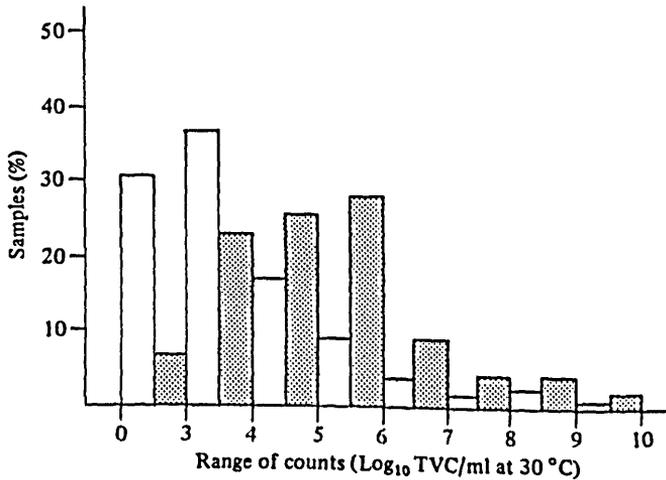
Fig. 1. Total viable counts (TVC) obtained from cows milk. ☐, Pasteurized milk; ☐, untreated milk.

Table 1. *Relationships of methylene blue (MB) test results to total viable counts in pasteurized cows milk*

Log_{10} TVC/ml	No. of samples	No. of MB failures	No. of samples not tested*
<3	132	—	18
3–3·99	158	1	27
4–4·99	73	1	12
5–5·99	40	3	9
6–6·99	15	2	1
7–7·99	5	3	—
8–8·99	7	4	—
Total	430	14	67

* Samples not tested because shade temperature exceeded 21 °C.

counts exceeding 10^6/ml. Only 54% of untreated milk samples had total counts less than 10^5/ml. Counts exceeded 10^6/ml in 18% of raw milk samples.

Methylene blue test

Only 14 (3·3%) samples of pasteurized milk and 9 (11·4%) samples of untreated milk failed the methylene blue test. However, 67 tests of pasteurized milk were omitted because the shadebox temperature exceeded 21 °C, that is, one-fifth of all samples tested in the months May–October. One laboratory found that all 27 samples in this category satisfied the methylene blue test when pre-incubation was performed in a waterbath at 18·3 °C.

Relationship of methylene blue test results to total viable counts

The relationship of the methylene blue test results to total viable counts obtained on receipt of the samples is shown in Tables 1 and 2. One-fifth of

Table 2. *Relationship of methylene blue test results to total viable counts in untreated cows milk*

Log_{10} TVC/ml	No. of samples	No. of MB failures
<3	5	—
3–3·99	18	—
4–4·99	20	—
5–5·99	22	2
6–6·99	7	3
7–7·99	3	3
8–8·99	3	1
9–9·99	1	—
Total	79	9

pasteurized milk samples and one-quarter of untreated milk samples with viable counts exceeding 10^5/ml failed the methylene blue test.

Comparison of total viable counts obtained after pre-incubation of pasteurized milk samples in the shadebox with those obtained in the 18·3 °C waterbath

Pre-incubation for the methylene blue test was carried out at 18·3 °C in a waterbath in parallel with the atmospheric shadebox in 147 samples of pasteurized milk. The lines of regression obtained by comparing total viable counts after pre-incubation by each method for each laboratory are shown in Fig. 2. Differences in total counts obtained by the two methods were not significant ($P > 0·5$). However correlation coefficients were poor ($r = 0·70$–$0·73$). In addition, 34% of results differed by more than one logarithmic cycle and 12% by more than two logarithmic cycles; over 90% of these were greater after pre-incubation in the waterbath than in the shadebox.

Coliforms

The three tube MPN test using brilliant green bile lactose broth detected coliforms in 55% of pasteurized milk samples and 90% of untreated milk samples (Fig. 3). Coliforms were found in the pasteurized milk of all but five dairies. The incidence and level of coliform contamination in pasteurized milk samples increased as total viable counts increased, but the relationship was not linear ($P < 0·0001$). No relationship was found in untreated milk samples. The detection of coliforms in pasteurized milks was more frequent in the months July–September, and this was accompanied by an increase in the numbers found.

The MPN method for detecting coliforms was compared with direct enumeration using VRBA plates (Table 3). MPN values exceeded counts obtained by the direct method by more than one logarithmic cycle in 14 samples of pasteurized milk. The reverse was found in two samples of milk.

Coliforms v. Enterobacteriaceae

Examination for the presence of coliforms and Enterobacteriaceae was carried out in parallel in 164 samples of pasteurized milk. Comparison of results obtained for coliforms and Enterobacteriaceae by the MPN methods showed little difference
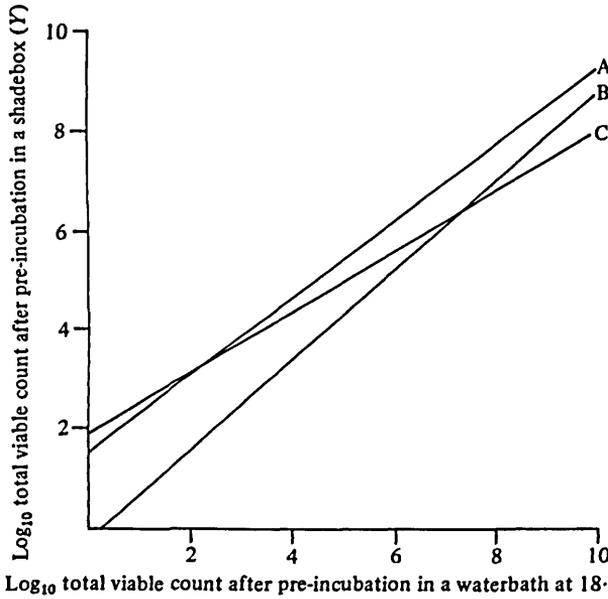
Fig. 2. Lines of regression obtained after pre-incubation for the methylene blue test. Regression of viable counts after incubation at 18·3 °C in a waterbath (X) on corresponding results obtained after incubation in a shadebox (Y). Laboratory A: $Y = 1·5 + 0·75X$, Laboratory B: $Y = 0·91X - 0·15$. Laboratory C: $Y = 1·84 + 0·6X$.
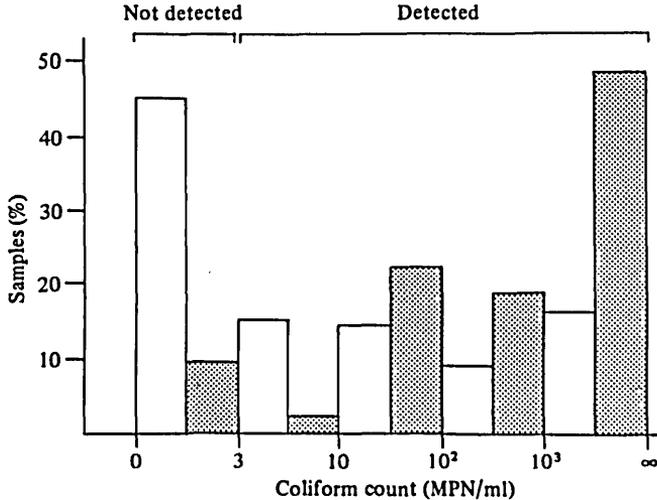
Fig. 3 Coliforms in cows milk. □, Pasteurized milk; ▣, untreated milk.

between counts for the two groups of organisms (Table 4). Enterobacteriaceae counts exceeded coliform counts in 23 milk samples. Differences of more than one logarithmic cycle were found in three milk samples only.

Yersinia *spp.*

Yersinia spp. were isolated from 22 pasteurized milk samples and 3 untreated milk samples. Biotypes and serotypes are shown in Table 5. Most strains belonged to *Y. enterocolitica* biotype 1 or *Y. intermedia*.

Table 3. *Comparison of a most probable number (MPN) method and a direct method for enumeration of coliforms in pasteurised milk*

	Number of samples	
Coliforms/ml	MPN	Direct
<10	138	172
10–10²	41	27
10²–10³	23	32
>10³	53	24
Total	255	255

Table 4. *Comparison of lactose-fermenting coliform counts and Enterobacteriaceae counts obtained from 164 samples of pasteurized cows milk*

	Number of samples	
MPN/ml	Coliforms	Enterobac-teriaceae
<3*	90	96
3–9	13	16
10–99	31	23
10²–10³	8	7
>10³	22	22
Total	164	164

* Level of detection = 3/ml.

Table 5. *Characterization of strains of* Yersinia *spp. isolated from cows milk*

No. of samples	Biotype	Serotype
6	*enterocolitica* 1	NT
5	*enterocolitica* 1	5, 27
3	*enterocolitica* 1	6, 30
5	*intermedia*	NT
3	*frederiksenii*	NT
3	Not biotyped	—

NT, not typable.

DISCUSSION

The methylene blue test was introduced in 1935 as a test of keeping quality for raw and pasteurized milk. It remains a statutory test for consumer milk samples. The modified test for cream samples is known to produce a significant number of anomalous results (Public Health Laboratory Service, 1971) and was not therefore made a statutory test. Anomalous results have been defined (Jenkins & Henderson, 1969) as decolourization of methylene blue immediately after overnight incubation by cream samples having a viable count of less than 10^4/ml, and failure to decolourize methylene blue in 4 h by samples with a viable count exceeding 10^5/ml. If this definition is also applied to milk, 10% of pasteurized samples and 34% of untreated samples also gave anomalous results. Seventy-nine per cent of pasteurized milk and 75% of untreated milk samples with viable counts exceeding

10^5/ml satisfied the methylene blue test. These anomalies have been explained by the low dehydrogenase activity of the psychrotrophic organisms found in refrigerated milks and creams (Lück, 1981). The high proportion of anomalous results from milk samples suggests that the status of the methylene blue test as a statutory test should be reviewed.

Pre-incubation conditions used in the statutory methylene blue test for milks are supposed to relate to conditions found at the relevant time of the year. From 1 May to 31 October, samples of milk are stored in a shadebox overnight. During the rest of the year, pre-incubation takes place in a water-bath at 18·3 °C. Considerable diurnal variation was found in shade temperatures, and despite specifications for the siting of the shadebox, inter-laboratory variations were noted in the number of samples which were not tested because shadebox temperature had exceeded 21 °C. As each laboratory taking part was within 30 miles of each other, it might be assumed that ambient temperatures were similar. The possibility therefore arises that samples of the same milk might give different results in different laboratories. Information pertaining to the keeping quality of milk is of most importance during the hotter summer months, and yet if the shadebox temperature exceeds 21 °C, the methylene blue test is not carried out, and no such information is obtained. Results obtained in this study showed that this problem was overcome by pre-incubation in the waterbath.

Whilst pre-incubation of the samples increases the number and metabolic activity of the bacteria present, this increase is not consistently related to the initial bacterial count (Lück, 1981). To obtain information about the effectiveness of dairy hygiene and the keeping quality of the product, it may be preferable to perform a total count and examine for specific groups of organisms. Spoilage in milk and cream is caused by the activity of bacterial lipase and proteases (Frank et al. 1985). Off-flavours developed as a result of enzymic activity can occur when the bacterial load is as low as 10^7/ml in milk samples (Lück, 1981). Viable counts from 6·5 % of pasteurized milk and 18 % of raw milk samples exceeded 10^6/ml. Counts as high as this at the point of retail sale suggest that the shelf-life will be unacceptably short to the consumer. The target for milks as this point might therefore be a total count below 10^5/ml. Samples with counts of 10^5–10^6/ml should be viewed with suspicion. Counts exceeding 10^6/ml suggest the need for improvements in stock rotation and control of storage temperature either in the distribution chain or at the retailer's premises.

Coliforms were detected in a large number of pasteurized milks on retail sale, reflecting the frequency with which post-pasteurization contamination takes place. As there were only 14 methylene blue failures found in pasteurized milks, there was obviously no relationship between the occurrence of coliforms and methylene blue test results. The absence of a linear relationship between the number of coliforms and total viable count means that the level of coliform contamination cannot be used to indicate the total number of bacteria present.

The occurrence of Yersinia spp. in 22 pasteurized milk samples is also thought to indicate post-pasteurization contamination (Greenwood & Hooper, 1985). Two outbreaks of yersiniosis in the USA have been attributed to the consumption of pasteurized chocolate milk (Black et al. 1978) and pasteurized milk (Tacket et al. 1984). Pasteurized milk contaminated with Yersinia spp. has also been

incriminated as the source of infection in this country (Greenwood & Hooper, in preparation).

Use of brilliant green bile lactose broth in a most probable number (MPN) method for detection of coliforms appeared to be more sensitive than a direct enumeration method using VRBA, but false positive results may occur unless presumptive positive tubes are subjected to a confirmatory test. Whilst this extra sensitivity may be desirable for examining samples immediately after pasteurization, it may be outweighed by the convenience of a direct enumeration method when examining retail samples. No additional information was obtained by examining for the presence of Enterobacteriaceae.

Results obtained in this survey indicate the need for reappraisal of the methylene blue test as a statutory method. Post-pasteurization contamination is known to be the most significant factor in limiting the keeping quality of refrigerated milk (Lück, 1981). The frequent occurrence of coliforms in this survey highlights the problem of post-pasteurization contamination. A viable count and coliform test may provide better information regarding dairy hygiene and the quality of the product at the point of retail sale.

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REFERENCES

BLACK, R. E., JACKSON, R. J., TSAI, T., MEDVESKY, M., SHAYEGANI, M., FEELEY, J. C., MACLEOD, K. I. E. & WAKELEE, A. M. (1978). Epidemic *Yersinia enterocolitica* infection due to contaminated chocolate milk. *New England Journal of Medicine* **298**, 76–79.

COUNCIL OF THE EUROPEAN COMMUNITIES (1985). Council directive on health and animal-health problems affecting intra-Community trade in heat treated milk (85/397/EEC). *Official Journal of the European Communities*. No. L 226/13.

FRANK, J. F., HANKIN, L., KOBURGER, J. A., & MARTH, E. H. (1985). Tests for groups of microorganisms. In *Standard Methods for the Examination of Dairy Products*, 15th edition (ed. G. H. Richardson), pp. 189–201. Washington D.C.: American Public Health Association.

GILCHRIST, J. E., CAMPBELL, J. E., DONNELLY, C. B., PEELER, J. T., & DELANEY, J. M. (1973). Spiral plate method for bacterial determination. *Applied Microbiology* **25**, 244–252.

GREAT BRITAIN (1977). Food and Drugs, Milk and Dairies (Special Designation) Regulations 1977. (Statutory Instrument No. 1033). London: HMSO.

GREAT BRITAIN (1983). The Milk-based Drinks (Hygiene and Heat Treatment) Regulations 1983. (Statutory Instrument No. 1508). London: HMSO.

GREAT BRITAIN (1983). The Milk and Dairies (Heat Treatment of Cream) Regulations 1983. (Statutory Instrument No. 1509). London: HMSO.

GREAT BRITAIN (1986). The Milk and Dairies (Semi-skimmed and Skimmed Milk) (Heat Treatment and Labelling) Regulations, 1986. (Statutory Instrument No. 722). London: HMSO.

GREENWOOD, M. H., COETZEE, E. F. C., FORD, B. M., GILL P., HOOPER, W. L., MATTHEWS, S. C. W., PATRICK, S., PETHER, J. V. S. & SCOTT, R. J. D. (1984). The microbiology of selected retail food products with an evaluation of viable counting methods *Journal of Hygiene* **92**, 67–77.

GREENWOOD, M. H. & HOOPER, W. L. (1985). *Yersina* spp. in foods and related environments. *Food Microbiology* **2**, 263–269.

Hartmann, P. A. & Lagrange, W. S. (1985). Coliform bacteria. In *Standard Methods for the Examination of Dairy Products* 15th edition (ed. G. H. Richardson), pp. 173–187. Washington, D.C.: American Public Health Association.

Jacobs, M. J. & Gerstein, M. J. (1960). *Handbook of Microbiology*. Princeton N.J.: Van Nostrand Company, Inc.

Jenkins, H. R. & Henderson, R. J. (1969). The source of bacteria in fresh cream, and the methylene blue reduction test as a guide to hygiene quality. *Journal of Hygiene* **67**, 401–408.

Lück, H. (1981). Quality control in the dairy industry. In *Dairy Microbiology*, vol. 2, *The Microbiology of Milk Products*. (ed. R. K. Robinson), pp. 279–324. London: Applied Science Publishers.

Miles, A. A. & Misra, S. S. (1938). The estimation of the bactericidal power of the blood. *Journal of Hygiene* **38**, 732–749.

Nelson, F. E. (1981). The microbiology of market milk. In *Dairy Microbiology*, vol. 1, *The Microbiology of Milk*. (ed. R. K. Robinson), pp. 165–207. London: Applied Science Publishers.

Public Health Laboratory Service (1958). The bacteriological examination and grading of fresh cream: report of a working party of the Public Health Laboratory Service. *Monthly Bulletin of the Ministry of Health* **17**, 77–87.

Public Health Laboratory Service (1971). The hygiene and marketing of fresh cream as assessed by the methylene blue test: report of a working party of the Public Health Laboratory Service, *Journal of Hygiene* **69**, 155–170.

Tacket, C. I., Narain, J. P., Sattin, R., Lofgren, J. P., Konigsberg, G., Rendtorff, R. C., Rausa, A., Davis, B. R. & Cohen, M. L. (1984). A multistate outbreak of infections caused by *Yersinia enterocolitica* transmitted by pasteurised milk. *Journal of the American Medical Association* **251**, 483–486.

Thatcher, F. S. & Clark, D. S. (1968). *Microorganisms in Foods. 1. Their Significance and Methods of Enumeration*. Toronto: University of Toronto Press.

Wauters, G. (1970). Contributions a l'étude de *Yersinia enterocolitica*. Thèse Vander Louvain, Belgium.

Wilson, G. S., Twigg, R. S., Wright, R. C., Hendry, C. B., Cowell, M. P. Maier, I. (1935). The bacteriological grading of milk. Special Report Series of the Medical Research Council, No. 206. London: HMSO.