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THE ESTIMATION OF NUMBERS OF COLI BACTERIA IN FOODS BY MEANS OF A DEEP AGAR TUBE TECHNIQUE WITH A MODIFIED VIOLET RED BILE AGAR

By
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In bacteriological food control coliform tests have found a wide field of application especially in programmes for the testing of pasteurized or otherwise low-temperature heated products, in which gramnegative rods as a whole — in accordance with the low thermoresistance of this group — should be absent. In addition to this long established practice the possibility of selective testing of heattreated — especially non-hermetically packed products — as well as of non-heattreated foods for Escherichia coli I has aroused some interest (*Jepsen* 1959). The presence of E. coli I generally is considered indicative of a recent fecal contamination with concurring risk of introducing enteric pathogens such as Salmonella and Shigella. Hence a reliable selective quantitative cultural test for E. coli I in foods would seem desirable. A test which should combine speedy performance with a reasonable degree of accuracy so as to allow for immediate action on unsatisfactory results.

Coli media. A large selection of coli media, fluid as well as solid, is available. In fluid media formation of gas from lactose fermentation indicates growth of coliform bacteria, whereas in solid media the production of acid serves as indication. Fluid

media in common use are f. inst. Brillantgreen-Lactose-Peptone Broth, Eosin-Methyleneblue Broth, Desoxycholate Broth, Gentianviolet-Bile-Lactose-Pepton Broth and Mc. Conkey Broth. In this country Gentianviolet-Bile-Lactose-Peptone Broth in Durham tubes has been widely used in bacteriological testing of milk, a test dosis of 0.1 ml. being inoculated into three parallel tubes. However, fluid media may produce a certain number of false positive as well as false negative results because of a too narrow range between selective and inhibitory action of the specific components of the media (*E. Malling Olsen* 1952, *Egon Petersen* 1953).

The solid media in common use are f. inst. Endo-Agar, Eosin-Methyleneblue Agar, Desoxycholate Agar, McConkey Agar and Violet Red Bile Agar (VRB Agar). In Danish food control laboratories Eosin-Methyleneblue Agar has been widely used, recently also VRB Agar has become a common choice.

The agar plate technique for the enumeration of 44° E. coli I

When solid media are used for the enumeration of coliforms in foods, dilutions are plated in ordinary Petri dishes and incubated for 18—24 hours at 37°C, whereas the enumeration of E. coli I (fecal type) requires the plates to be incubated at 44°C. In view of the critical influence of the temperature of incubation upon the selectivity of the method ordinary airfilled incubators can hardly be considered satisfactory. Especially when large numbers of plates are piled in a small space, the delay in reaching equillibrium of temperature may result in germination and growth of low-temperature coliforms other than E. coli I. To overcome this objection *Hauge* (1959) has proposed to place the Petri dishes, wrapped in waterproof plastic bags, in a water filled incubator. The greatest problem in applying this technique lies in the waterproof wrapping because ordinary commercial grades of plastic bags quite often do not prove watertight even when selecting apparently heavy and good quality plastfoils. To secure a rapid equillibrium of temperature with large numbers of Petri dishes submerged in a comparatively small amount of water also may be difficult, unless the incubator tub is designed with a special heating unit circulating hot water through the tub (Cyclotherm).

For these reasons a simplification of the technique was sought for by substituting deep agar tubes for ordinary Petri dishes.

The deep agar tube technique for the enumeration of 44° E. coli I

Medium. In a series of comparative investigations, initiated by The Nordic Committee for Food Analysis Violet Red Bile Agar was found a most satisfactory medium. This also has been the experience of other authors. In a comparative examination of 8 solid coliform media (VRB Agar, Eosin-Methyleneblue Agar, Lactose-Bromthymolblue-Trypaflavin Agar, Desoxycholate-citrate Agar, Ammonium-Coli Agar, Ammonium-Coli Agar with 3 per-cent peptone, McConkey Agar and Chapman Agar) *S. Østerling* (1951) found VRB Agar superior in combining a high degree of selectivity with a minimum of inhibitory effect on coliforms. Similar results are reported by *Bartram & Black* (1937), *Miller & Prickett* (1938) in the testing of milk, and by *Babel & Parfitt* (1936) who tested icecream. Also *Malling Olsen* (1952) in his work on solid coliform media placed VRB Agar among the best.

VRB Agar used in our investigations was prepared according to the formula given by The Nordic Committee on Food Analysis (1960).

Basal medium:

Bacto peptone	g	8
Bacto Yeast extract	g	3
NaCl	g	4
Agar	g	15
Water	ml.	1000

pH 7.2—7.4

To 200 ml. of melted and cooled basal medium is added immediately prior to use:

Sodium desoxycholate Gurr Bact.	mg.	300
Neutral Red (Vital Stain) Gurr	mg.	6
Methyl Violet (Vital Stain) Gurr	mg.	0.4

Sterile 10 per cent solution of lactose ml. 20.

The dyes are kept in alcoholic solution while the bile salt is dissolved in the lactose solution by heating to 45°C. The molted medium is poured into the Petri dishes and mixed with the inoculum.

Bacteria belonging to the lactose fermenting coliform group produce after 18—24 hours of incubation red to red-violet stained colonies surrounded by a zone of precipitate showing the same

colour. This reddish precipitate consists of a bile acid/dye complex which has been precipitated by the lowering of the pH level following the formation of acid from lactose fermentation.

When cultivating coliforms in deep agar tubes of usual VRB Agar the problem arises of disruption of the agar column due to the formation of gas from lactose fermentation.

Investigations by *Pakes & Jollyman* (1901) have demonstrated all aerogenic glucose fermenting bacteria to be capable of decomposing formic acid into hydrogen and carbon dioxide whereas bacteria which do not attack formic acid were found to form acid only and no gas from glucose. From this observation the authors concluded that formic acid is the intermediate product from which gas developed in bacterial fermentation of carbohydrates originates. Later *Stephenson & Stickland* (1932) showed that the formation of hydrogen and carbon dioxide from formic acid is catalyzed by an enzyme for which they proposed the name formic acid hydrogenlyase.

Pakes & Jollyman (1901) also discovered the inhibitory effect of potassium nitrate (1 per cent) upon formation of gas from glucose or formic acid media inoculated with Coli-Aerogenes organisms. They supposed the reduction of nitrate to nitrite would consume the hydrogen atoms thus leaving no free hydrogen. *Tubiash* (1951) found nitrate and nitrite ions to prevent the production of gas by Escherichia-Aerobacter from glucose as well as from lactose and galactose, and he could demonstrate that concentrations of nitrate to the equivalent of 50—100 p.p.m. of nitrate-nitrogen will stimulate growth of E. coli in lactose broth. *Billen* (1951) in studying the inhibitory effect of nitrate upon hydrogenlyase puts forward the hypothesis that nitrate is promoting the formation of nitratase. By this process the medium becomes depleted of nitrogen which also is needed for the synthesis of hydrogenlyase. On the other hand *Gest* (1954) arrives at the conclusion that the inhibitory effect actually is caused by nitrite resulting from the action of nitratase enzym on nitrate. No explanation, however, is given as to the mechanism by which the nitrite ions exercise their inhibitory action.

OWN INVESTIGATIONS

Preliminary investigations proved that sodium nitrite could prevent formation of gas in deep agar cultures of E. coli. The minimum level of sodium nitrite that would prevent completely

formation of gas was determined as 0.5 ‰. At the same time, however, a certain growth inhibiting effect of sodium nitrite on E. coli was observed. Parallel counts in the same medium without sodium nitrite in Petri dishes showed higher results and larger and more developed colonies. In the following experiments therefore $NaNO_2$ was substituted for $NaNO_3$ 0.5 ‰ which proved just as effective and less inhibitory, but still the developing colonies of E. coli remained smaller than E. coli colonies in plate cultures without the addition of sodium nitrate. Attempts to improve conditions of growth in the deep agar tubes by addition of potassium permanganate to rise the redox potential of the medium failed and it was decided to run a series of comparative counts in VRB Agar plates without sodium nitrate and in the same medium with 0.5 ‰ sodium nitrate in deep agar tubes, using flat Miller-Prickett tubes with a white enamel glass plate inside instead of ordinary round tubes. The enamel glass plate divides the medium in the flat Miller-Prickett tubes into two thin layers whereby an exact counting of the colonies is greatly facilitated as compared to the difficult counting of the round tube colonies.

Comparative coli counts in deep agar tubes with V.R.B.-Nitrate Agar. The material consisted of samples of pasteurised market milk, kindly selected by dr. *O. Winther* of the Food Control Laboratory Division, City of Copenhagen Health Department. Before the samples were handed over to us they had been tested in the food control laboratories and found to contain suitable numbers of coliform bacteria.

From each milk sample two parallel series of saline dilutions were set up. One ml. inocula of the appropriate dilutions were transferred to Petri dishes and to Miller-Prickett tubes respectively. The plates were poured with 15 ml. of VRB Agar, and after the medium had solidified a cover layer of uninoculated medium was added to avoid the formation of surface colonies which due to an oxidative type of fermentation frequently do not produce typical red-coloured precipitation. The Miller-Prickett tubes were filled up with 15 ml. of VRB-Nitrate Agar while mixing the inoculum with the medium by turning the tubes twice. After the medium had solidified again a cover layer about two cm. in height was applied, partly for the same reason as mentioned above, and partly to provide for growth of any bacteria which might have been deposited on the glass lining above the agar column during the turning of the tubes. Very rarely, however, growth was observed in the cover layer.

After incubation for 18—20 hours at 37° C counts were made of the plates and tubes. As far as possible only plates or tubes with a colony count between 30 and 300 were used for the enumeration. Of a total of 43 samples examined by this method 8 samples yielded either too low or too high counts, and one sample was lost due to dilution errors, leaving altogether 34 samples valid for comparison. The optimal and typical appearance of red zones of precipitation around coliform colonies is seen only with well separated colonies i. e. when the colony count does not exceed about 150 per plate or tube, whereas more dense growth results in smaller colonies lacking the typical zones of precipitation. In a number of cases some lactosefermenting colonies in plates as well as in tubes remained surprisingly small in size within the first day of incubation. After an additional 18—20 hours of incubation these minute colonies as a rule would develop into typical normal size coli colonies; but some remained unaltered. A selection of such minute colonies was subcultivated on Triple Sugar Iron Agar slants by which all of the dysgonic strains reacted as typical coli (yellow/yellow gas). In a few cases the plate cultures showed a number of small greyish non-fermenting colonies which easely could be distinguished from coli colonies. Such strains were identified as members of the Alcaligenes group.

Statistical analysis

In accordance with the general observation that logarithms of bacterial counts show better approximation to the normal symmetrical curve of variation than do arithmetical numbers (*Robertson & Frayer,* 1930 and *Devereux,* 1937) log. counts have been used instead of arithmetical numbers in the calculations (Table 1). In this series of comparative counts the plate count method has resulted in a higher geometric mean than the deep agar tube count (142,000 versus 101,000). The difference, however, is found not to be statistically valid as the standard error of the difference exceeds the difference ($\frac{D}{m_D} = 0.88$).

Modification of the VRB-Nitrate Agar medium.

Although the deep agar tube method was found equal to the plate count method with respect to numerical results the pro-

blem still remained of the colonies being smaller and showing less typical development in the deep agar tubes when compared to the corresponding plate colonies. This is probably due to a lower oxygen tension during growth in deep agar tubes. Experiments were undertaken to overcome this deficiency by decreasing the agar contents of the deep agar medium. It was found that a medium with 0.9 per cent of agar resulted in a considerable increase in size of the coli colonies in deep agar tubes and also in the development of the typical appearance with surrounding zones of purple coloured precipitate. Further reduction of the agar strength was unsuitable because of confluent growth. Experiments showed that the change in agar strength did not influence the optimal nitrate concentration. So the final formula of the VRB-Nitrate medium for deep agar tubes was fixed to 0.9 per cent of agar and 0.5 ‰ of $NaNO_3$.

Comparative coli counts in deep agar tubes with a modified VRB-Nitrate Agar. With the medium described above another series of comparative counts was set up using 3 strains of E. coli I, 3 samples of cows feces and 3 samples of raw bulk milk. (The milk samples for this experiment were kindly supplied by dr. *Sven J. Olsen* of the laboratories of The Trifolium Dairy Company). From each sample dilutions were counted in 5 parallel plates and 5 parallel deep agar tubes with two series of tests incubated at 37° C and 44° C respectively for 18—20 hours. The 44° cultures were placed in a thermostatically controlled water bath with circulating waterflow. The tubes were placed in racks while the plates were submerged wrapped in plastic bags (double layer).

Statistical analysis. The results of each series of counts have been evaluated separately, and the consequently arithmetic means were used in place of geometric means.

In counting pure cultures of E. coli I (Tables 2 and 3) the deep agar tube technique on an average yielded higher counts than the plate technique both at 37° C and at 44° C. The difference was found to be statistically valid in all but two cases, the difference exceeding the standard error of the difference ($\frac{D}{m_D}$ 37° = 3.06, and $\frac{D}{m_D}$ 44° = 2.54).

The feces counts (Tables 4 and 5) and the bulk milk counts (Tables 6 and 7) showed smaller differences of which only a

Table 1.
Statistical analysis of comparative coli counts of milk and cream in V.R.B. Agar plates and V.R.B. Nitrate deep agar tubes.

Sample no.	Milk grade	Coli plate count	Log. coli plate count x_1	Coli tube count	Log. coli tube count x_2
3 a	Pasteurized skim milk	9,200	3.964	8,600	3.935
3 b		8,500	3.929	4,400	3.643
5 a	Pasteurized whipping cream	29,000,000	7.462	1,300,000	6.114
5 b		22,600,000	7.354	5,400,000	6.732
6 a	„ „ „	102,000	5.009	10,000	4.000
6 b		120,000	5.079	46,000	4.663
8 a	HTST pasteurized milk	1,500,000	6.176	2,600,000	6.415
8 b		11,200,000	7.049	7,200,000	6.857
9 a	Pasteurized cream I	1,770,000	6.248	260,000	5.415
9 b		2,350,000	6.371	920,000	5.964
10 a	Stassanized milk	390,000	5.591	520,000	5.716
10 b		240,000	5.380	180,000	5.255
11 a	„ „	4,100,000	6.613	3,500,000	6.544
11 b		6,900,000	6.839	3,200,000	6.505
12 a	HTST pasteurized milk	3,100,000	6.491	4,800,000	6.681
12 b		11,400,000	7.057	1,200,000	6.079
13 a	Stassanized milk	10,700	4.029	5,800	3.763
13 b		10,900	4.037	4,200	3.623
14 a	„ „	14,000	4.146	15,000	4.176
14 b		12,500	4.097	13,000	4.114
15 a	„ „	19,500	4.290	17,200	4.236
15 b		26,100	4.417	16,500	4.218
16 a	Pasteurized skim milk	5,700	3.760	2,400	3.380
16 b		5,200	3.716	3,100	3.491
17 a	Pasteurized whipping cream	3,100	3.491	3,300	3.519
17 b		4,500	3.653	1,000	3.000
18 a	Stassanized milk	27,000	4.431	21,000	4.322
18 b		23,000	4.362	24,000	4.380
21 a	HTST pasteurized milk	22,000	4.342	17,000	4.230
21 b		24,000	4.380	15,500	4.190
22 a	Pasteurized cream III	16,000	4.204	41,000	4.613
22 b		19,000	4.279	26,000	4.415
23 a	Pasteurized skim milk	7,300	3.863	5,700	3.756
23 b		8,100	3.909	6,700	3.826
24 a	Pasteurized cream I	7,000	3.845	7,300	3.863
24 b		7,700	3.887	6,500	3.813
25 a	Pasteurized whipping cream	62,000	4.792	41,000	4.613
25 b		102,000	5.009	69,000	4.839
26 a	HTST pasteurized milk	67,000	4.826	43,000	4.634
26 b		72,000	4.857	99,000	4.996
27 a	Pasteurized cream III	58,000	4.763	24,000	4.380
27 b		26,000	4.415	55,000	4.740

Table 1 (continued).

Sample no.	Milk grade	Coli plate count	Log. coli plate count x_1	Coli tube count	Log. coli tube count x_2
28 a	Pasteurized whipping cream	180,000	5.255	165,000	5.248
28 b		135,000	5.130	190,000	5.279
29 a	Stassanized milk	1,290,000	6.111	1,330,000	6.124
29 b		1,650,000	6.218	1,160,000	6.065
30 a	„ „	680,000	5.833	750,000	5.875
30 b		550,000	5.740	590,000	5.771
31 a	„ „	2,860,000	6.456	1,950,000	6.290
31 b		860,000	5.935	1,970,000	6.294
35 a	Pasteurized cream III	470,000	5.672	2,540,000	6.405
35 b		1,730,000	6.238	2,630,000	6.420
36 a	Stassanized milk	1,220,000	6.086	430,000	5.634
36 b		1,050,000	6.021	500,000	5.699
37 a	„ „	720,000	5.857	320,000	5.505
37 b		900,000	5.954	170,000	5.230
38 a	Pasteurized whipping cream	170,000	5.230	200,000	5.301
38 b		120,000	5.079	100,000	5.000
39 a	Pasteurized cream I	120,000	5.079	50,000	4.699
39 b		460,000	5.663	90,000	4.954
40 a	Stassanized milk	800,000	5.903	640,000	5.806
40 b		860,000	5.935	340,000	5.532
41 a	Pasteurized whipping cream	68,000	4.833	69,000	4.839
41 b		86,000	4.935	89,000	4.949
42 a	Stassanized milk	132,000	5.121	225,000	5.352
42 b		215,000	5.332	245,000	5.389
43 a	Pasteurized cream I	17,000	4.230	36,000	4.556
43 b		14,000	4.146	19,000	4.279

V. R. B. plates:

$n_1 = 68$

Σ_1 log. count $= 350.404$

$\bar{x}_1$ mean log. count $= \frac{350.404}{68} = 5.153$

Geometric mean = 142.000 coliform bacteria/ml. of milk

$s_1 = \pm \sqrt{\frac{70.56}{67}} = \pm 1.026$

$m_1 = \pm \frac{1.026}{\sqrt{68}} = \pm 0.1244$

V. R. B. deep agar tubes:

$n_2 = 68$

Σ_2 log. count $= 340.113$

$\bar{x}_2$ mean log. count $= \frac{340.113}{68} = 5.002$

Geometric mean = 101,000 coliform bacteria/ml. of milk

$s_2 = \pm \sqrt{\frac{64.82}{67}} = \pm 0.9835$

$m_2 = \pm \frac{0.9835}{\sqrt{68}} = \pm 0.1193$

$$D = 5.153 - 5.002 = 0.151$$

$$m_D = \pm \sqrt{0.1244^2 + 0.1193^2} = \pm 0.1724$$

$$\frac{D}{m_D} = 0.88$$

Table 2.

Statistical analysis of comparative counts of E. coli I cultures in V. R. B. Agar plates and V. R. B. deep agar tubes (0.9 per cent of agar, 0.5 ‰ of $NaNO_3$) at 37°C.

	Dilution factor	Method of cultivation	Number of colonies (x) a	b	c	d	e	Σx	n	$\bar{x}$	s	m	D	m_D	$\frac{D}{m_D}$
Strain 1.	10^{-7}	Plate	120	103	75	63	53	414	5	82.8	± 27.91	± 12.48	112.4	± 14.13	7.95
		Tube	205	202	201	199	169	976	5	195.2	± 14.80	± 6.62			
	10^{-8}	Plate	71	64	63	58	40	296	5	59.2	± 11.69	± 5.23	17.2	± 7.14	2.41
		Tube	94	79	72	71	66	382	5	76.4	± 10.89	± 4.87			
	10^{-9}	Plate	41	20	20	19	14	114	5	22.8	± 10.47	± 4.68	20.6	± 4.79	4.30
		Tube	47	44	43	42	41	217	5	43.4	± 2.30	± 1.03			
Strain 2.	10^{-8}	Plate	6	5	4	4	4	23	5	4.6	± 0.90	± 0.40	2.8	± 1.85	1.51
		Tube	13	10	6	5	3	37	5	7.4	± 4.04	± 1.81			
	10^{-9}	Plate	0	0	0	0	0	0	5	0	0	0	0.8	± 0.85	0.94
		Tube	2	2	0	0	0	4	5	0.8	± 1.90	± 0.85			
Strain 3.	10^{-7}	Plate	87	83	81	77	60	388	5	77.6	± 10.48	± 4.69	3.0	± 5.02	0.60
		Tube	78	77	76	74	68	373	5	74.6	± 3.98	± 1.78			
	10^{-8}	Plate	12	8	8	6	6	40	5	8.0	± 2.45	± 1.14	5.6	± 1.47	3.81
		Tube	17	14	13	12	12	68	5	13.6	± 2.07	± 0.93			
	10^{-9}	Plate	4	1	1	0	0	6	5	1.2	± 1.70	± 0.76	0.6	± 1.15	0.52
		Tube	4	2	1	1	1	9	5	1.8	± 1.93	± 0.86			

Table 3.

Statistical analysis of comparative counts of E. coli I cultures in V. R. B. Agar plates and V. R. B. deep agar tubes (0.9 per cent of agar, 0.5 ‰ of $NaNO_3$) at 44 °C.

	Dilution factor	Method of cultivation	Number of colonies (x) a	b	c	d	e	Σx	n	$\bar{x}$	s	m	D	m_D	$\frac{D}{m_D}$
Strain 1.	10^{-7}	Plate	72	72	56	54	28	282	5	56.4	± 18.02	± 8.06	98.8	± 9.69	10.20
		Tube	211	199	197	191	178	976	5	195.2	± 12.03	± 5.38			
	10^{-8}	Plate	72	60	50	49	26	257	5	51.4	± 16.97	± 7.59	11.4	± 14.69	0.78
		Tube	85	84	68	61	16	314	5	62.8	± 28.12	± 12.58			
	10^{-9}	Plate	40	33	16	15	13	117	5	23.4	± 12.26	± 5.48	22.4	± 7.58	2.96
		Tube	55	53	50	45	26	229	5	45.8	± 11.69	± 5.23			
Strain 2.	10^{-8}	Plate	8	5	4	4	3	24	5	4.8	± 1.92	± 0.94	2.8	± 1.29	2.18
		Tube	10	8	8	7	5	38	5	7.6	± 1.83	± 0.82			
	10^{-9}	Plate	1	1	0	0	0	2	5	0.4	± 0.55	± 0.25	0.4	± 0.46	0.88
		Tube	2	1	1	0	0	4	5	0.8	± 0.84	± 0.38			
Strain 3.	10^{-7}	Plate	81	71	70	64	54	340	5	68.0	± 9.93	± 4.44	1.6	± 4.73	0.34
		Tube	76	69	68	68	67	348	5	69.6	± 3.65	± 1.63			
	10^{-8}	Plate	10	8	8	7	5	38	5	7.6	± 1.83	± 0.82	0.6	± 1.67	0.36
		Tube	10	9	9	4	3	35	5	7.0	± 3.24	± 1.45			
	10^{-9}	Plate	2	1	0	0	0	3	5	0.6	± 0.90	± 0.40	0.2	± 0.45	0.45
		Tube	1	1	1	1	0	4	5	0.8	± 0.45	± 0.20			

Table 4.

Statistical analysis of comparative coli counts of cows feces in V. R. B. Agar plates and V. R. B. deep agar tubes (0.9 per cent of agar, 0.5 ‰ of $NaNO_3$) at 37°C.

	Dilution factor	Method of cultivation	Number of colonies (x) a	b	c	d	e	Σx	n	$\bar{x}$	s	m	D	m_D	$\frac{D}{m_D}$
Sample 1.	10^{-4}	Plate	43	38	36	34	27	178	5	35.6	± 5.86	± 2.62	19.8	± 2.93	6.76
		Tube	60	56	55	54	52	277	5	55.4	± 2.92	± 1.31			
	10^{-5}	Plate	8	8	3	3	3	25	5	5.0	± 2.74	± 1.23	1.2	± 1.40	0.86
		Tube	6	4	4	3	2	19	5	3.8	± 1.48	± 0.66			
	10^{-6}	Plate	3	1	1	1	0	6	5	1.2	± 0.92	± 0.41	0.2	± 0.52	0.38
		Tube	2	1	1	1	0	5	5	1.0	± 0.71	± 0.32			
Sample 2.	10^{-6}	Plate	23	16	15	13	13	80	5	16.0	± 1.31	± 0.59	3.8	± 2.25	1.69
		Tube	18	16	11	10	6	61	5	12.2	± 4.82	± 2.16			
	10^{-7}	Plate	4	3	1	1	0	9	5	1.8	± 1.64	± 0.73	0.8	± 0.89	0.90
		Tube	4	3	3	2	1	13	5	2.6	± 1.14	± 0.51			
	10^{-8}	Plate	1	0	0	0	0	1	5	0.2	± 0.45	± 0.20	0.2	± 0.32	0.62
		Tube	1	1	0	0	0	2	5	0.4	± 0.55	± 0.25			
Sample 3.	10^{-5}	Plate	9	6	4	3	1	23	5	4.6	± 3.05	± 1.36	1.2	± 1.48	0.81
		Tube	9	7	6	5	2	29	5	5.8	± 2.59	± 1.16			
	10^{-6}	Plate	2	1	1	1	0	5	5	1.0	± 0.71	± 0.32	0.2	± 0.71	0.28
		Tube	2	2	1	1	0	6	5	1.2	± 1.40	± 0.63			

Table 5.

Statistical analysis of comparative coli counts of cows feces in V. R. B. Agar plates and V. R. B. deep agar tubes (0.9 per cent of agar, 0.5 ‰ of $NaNO_3$) at 44°C.

	Dilution factor	Method of cultivation	Number of colonies (x) a	b	c	d	e	Σx	n	$\bar{x}$	s	m	D	m_D	$\frac{D}{m_D}$
Sample 1.	10^{-4}	Plate	33	29	23	22	7	114	5	22.8	± 9.91	± 4.43	16.0	± 5.76	2.78
		Tube	48	43	40	37	26	194	5	38.8	± 8.23	± 3.68			
	10^{-5}	Plate	5	5	4	4	2	20	5	4.0	± 1.23	± 0.55	2.0	± 1.19	1.69
		Tube	10	6	5	5	4	30	5	6.0	± 2.35	± 1.05			
	10^{-6}	Plate	2	2	0	0	0	4	5	0.8	± 1.10	± 0.49	0.4	± 0.55	0.73
		Tube	1	1	0	0	0	2	5	0.4	± 0.55	± 0.25			
Sample 2.	10^{-6}	Plate	2	1	0	0	0	3	5	0.6	± 0.90	± 0.40	4.2	± 1.89	2.22
		Tube	11	7	3	2	1	24	5	4.8	± 4.14	± 1.85			
	10^{-7}	Plate	3	2	2	1	0	8	5	1.6	± 1.14	± 0.51	0.8	± 0.64	1.26
		Tube	2	1	1	0	0	4	5	0.8	± 0.84	± 0.38			
Sample 3.	10^{-5}	Plate	2	1	0	0	0	3	5	0.6	± 0.90	± 0.40	2.0	± 0.72	2.77
		Tube	4	4	2	2	1	13	5	2.6	± 1.34	± 0.60			
	10^{-6}	Plate	0	0	0	0	0	0	5	0	0	0	0.6	± 0.40	1.50
		Tube	2	1	0	0	0	3	5	0.6	± 0.90	± 0.40			

Table 6.

Statistical analysis of comparative coli counts of raw bulk milk in V. R. B. Agar plates and V. R. B. deep agar tubes (0.9 per cent of agar, 0.5 ‰ of $NaNO_3$) at 37°C.

	Dilution factor	Method of cultivation	Number of colonies (x) a	b	c	d	e	Σx	n	$\bar{x}$	s	m	D	m_D	$\frac{D}{m_D}$
Sample 1.	10^{-3}	Plate	65	54	53	42	37	251	5	50.2	± 10.99	± 4.91	6.4	± 6.57	0.97
		Tube	67	67	52	51	46	283	5	56.6	± 9.77	± 4.37			
	10^{-4}	Plate	45	15	14	13	10	97	5	19.4	± 14.43	± 6.45	0.6	± 7.27	0.08
		Tube	31	22	21	13	13	100	5	20.0	± 7.48	± 3.35			
	10^{-5}	Plate	1	0	0	0	0	1	5	0.2	± 0.45	± 0.20	0.4	± 0.63	0.63
		Tube	3	0	0	0	0	3	5	0.6	± 1.34	± 0.60			
Sample 2.	10^{-5}	Plate	22	20	19	17	16	94	5	18.8	± 2.39	± 1.07	6.2	± 2.00	3.10
		Tube	18	15	11	10	9	63	5	12.6	± 3.78	± 1.69			
	10^{-6}	Plate	5	5	5	3	2	20	5	4.0	± 1.41	± 0.63	2.0	± 1.05	1.90
		Tube	5	2	2	1	0	10	5	2.0	± 1.87	± 0.84			
	10^{-7}	Plate	1	0	0	0	0	1	5	0.2	± 0.45	± 0.20	0	± 0.28	0
		Tube	1	0	0	0	0	1	5	0.2	± 0.45	± 0.20			
Sample 3.	10^{-4}	Plate	136	120	106	99	93	554	5	110.8	± 17.31	± 7.74	0	± 9.39	0
		Tube	125	121	108	103	97	554	5	110.8	± 11.88	± 5.31			
	10^{-5}	Plate	53	50	47	30	29	209	5	41.8	± 11.43	± 5.11	2.2	± 6.78	0.32
		Tube	51	49	37	32	29	198	5	39.6	± 9.94	± 4.45			
	10^{-6}	Plate	8	8	5	5	4	30	5	6.0	± 1.87	± 0.84	0.4	± 1.13	0.36
		Tube	7	7	6	5	3	28	5	5.6	± 1.67	± 0.75			

Table 7.

Statistical analysis of comparative coli counts of raw bulk milk in V. R. B. Agar plates and V. R. B. deep agar tubes (0.9 per cent of agar, 0.5 ‰ of $NaNO_3$) at 44°C.

	Dilution factor	Method of cultivation	Number of colonies (x) a	b	c	d	e	Σx	n	$\bar{x}$	s	m	D	m_D	$\frac{D}{m_D}$
Sample 1.	10^{-1}	Plate	[1])	69	67	66	51	253	4	63.25	± 8.26	± 4.13	1.35	± 5.65	0.24
		Tube	75	68	67	61	52	323	5	64.6	± 8.62	± 3.86			
	10^{-2}	Plate	12	9	9	6	5	41	5	8.2	± 2.78	± 1.24	0.8	± 1.39	0.58
		Tube	10	10	10	8	7	45	5	9.0	± 1.41	± 0.63			
	10^{-3}	Plate	5	3	2	2	1	13	5	2.6	± 1.52	± 0.68	0.8	± 1.27	0.63
		Tube	6	1	1	1	0	9	5	1.8	± 2.39	± 1.07			
Sample 2.	10^{-1}	Plate	93	92	88	87	76	436	5	87.2	± 6.76	± 3.02	6.0	± 6.21	0.97
		Tube	100	84	80	74	68	406	5	81.2	± 12.13	± 5.43			
	10^{-2}	Plate	19	18	14	14	13	78	5	15.6	± 2.70	± 1.21	3.0	± 2.05	1.46
		Tube	18	14	12	11	8	63	5	12.6	± 3.72	± 1.66			
	10^{-3}	Plate	4	3	2	1	0	10	5	2.0	± 1.58	± 0.71	0.6	± 1.40	0.43
		Tube	7	3	2	1	0	13	5	2.6	± 2.70	± 1.21			
Sample 3.	10^{-2}	Plate	18	17	14	11	11	71	5	14.2	± 3.27	± 1.46	2.0	± 2.07	0.97
		Tube	17	14	11	10	9	61	5	12.2	± 3.27	± 1.46			
	10^{-3}	Plate	3	3	2	1	0	9	5	1.8	± 1.30	± 0.58	0.6	± 0.61	0.98
		Tube	2	1	1	1	1	6	5	1.2	± 0.45	± 0.20			
	10^{-4}	Plate	1	0	0	0	0	1	5	0.2	± 0.45	± 0.20	0	± 0.28	0
		Tube	1	0	0	0	0	1	5	0.2	± 0.45	± 0.20			

[1]) Contaminated.

Table 8.

Statistical analysis of numbers of "atypical" colonies developing from raw bulk milk in V. R. B. Agar plates and V. R. B. deep agar tubes (0.9 per cent of agar, 0.5 ‰ of $NaNO_3$) at 37°C.

	Dilution factor	Method of cultivation	Number of colonies (x) a	b	c	d	e	Σx	n	$\bar{x}$	s	m	D	m_D	$\frac{D}{m_D}$
Sample 1.	10^{-3}	Plate	7	6	4	3	2	22	5	4.4	± 2.07	± 0.93	0.4	± 1.41	0.28
		Tube	8	8	5	2	1	24	5	4.8	± 3.27	± 1.46			
	10^{-4}	Plate	2	1	1	1	0	5	5	1.0	± 0.71	± 0.32	1.2	± 1.47	0.82
		Tube	6	3	1	1	0	11	5	2.2	± 2.39	± 1.07			
Sample 2.	10^{-5}	Plate	13	13	9	7	7	49	5	9.8	± 3.03	± 1.36	0.4	± 3.33	0.12
		Tube	17	13	12	5	0	47	5	9.4	± 6.80	± 3.04			
	10^{-6}	Plate	2	1	0	0	0	3	5	0.6	± 0.89	± 0.40	0.4	± 0.60	0.67
		Tube	2	2	1	0	0	5	5	1.0	± 1.00	± 0.45			
	10^{-7}	Plate	2	0	0	0	0	2	5	0.4	± 0.89	± 0.40	0.2	± 0.14	1.43
		Tube	1	0	0	0	0	1	5	0.2	± 0.45	± 0.20			
Sample 3.	10^{-4}	Plate	52	50	38	30	28	198	5	39.6	± 11.11	± 4.97	0	± 7.58	0
		Tube	55	51	34	33	25	198	5	39.6	± 12.80	± 5.72			
	10^{-5}	Plate	7	7	6	5	4	29	5	5.8	± 1.30	± 0.58	3.2	± 1.96	1.63
		Tube	15	11	8	7	4	45	5	9.0	± 4.18	± 1.87			
	10^{-6}	Plate	1	1	1	0	0	3	5	0.6	± 0.55	± 0.25	1.8	± 1.22	1.48
		Tube	5	4	2	1	0	12	5	2.4	± 2.07	± 0.93			

few were found to be statistically valid. Out of a total of 49 separate counts the plate method yielded higher results in 16 cases (32.7 per cent), but only one was statistically valid. In 30 cases (61.2 per cent) the deep agar tube method yielded higher results of which 11 were statistically valid. 3 cases showed no difference.

Small retarded colonies. In the feces cultures practically all colonies presented the typical appearance. Six small retarded colonies all could be identified as coliform strains (T. S. J. Agar slants: yellow/yellow, gas). In the raw bulk milk cultures several small atypical colonies were observed, mostly in 37° cultures. Such colonies were counted separately and not included in the statistical analysis. 14 atypical colonies from 44° cultures all were identified as E. coli I. Of 88 atypical colonies from 37° cultures 56 strains behaved as typical aerogenic coliforms, whereas 32 strains were either anaerogenic or slow gas producing coliforms (7 strains E. coli I, 4 strains E. coli II, 2 E. freundii, 16 Klebsiella and 2 strains of irregular intermediate type).

As no non-coliform bacteria have been encountered among the small atypical colonies it seems permissible to include such colonies in the coliform count.

In Table 8 a comparison is made of the numbers of atypical retarded colonies developing in 37° plate cultures and the corresponding tube cultures. Although there is a tendency for slightly higher numbers of atypical colonies in the tube cultures, the differences are not statistically valid.

The deep agar tube method has proved satisfactory also when applied in a number of routine examinations of icecream, confectionary, mayonnaise salads, meat paste and fish paste. The results were in good agreement with those obtained from parallel plate counts, numerically as well as regarding size and appearance of the colonies.

Selectivity of V. R. B. Agar. Pure cultures of various species of microorganisms commonly seen as contaminants in food products were plated in VRB Agar at 37° C. The results are tabulated below:

	Growth in VRB Agar 37° C.
Micrococcus pyogenes	No visible growth
Non-hemolytic micrococcus	„ „ „
Pyogenic streptococci	„ „ „
Fecalis streptococci	No visible growth after 24 hours. Some strains pin-point, red colonies after 48—72 hours.
Viridans streptococci	No visible growth
Streptococcus uberis	„ „ „
Lactobacilli	„ „ „
Corynebacterium bovis	„ „ „
Proteus	Pin-point, slightly reddish colonies after 24 hours. Complete decolouration of the colonies and the medium after an additional 24 hours (alkaline reaction).
Achromobacter	No visible growth
Alcaligenes	No visible growth after 24 hours. After 48—72 hours pin-point, slightly reddish colonies which on prolonged incubation developed decoloration of the medium.
Pseudomonas	After 24 hours small noncoloured or greenish colonies. On prolonged incubation the medium tended to become decolourized.
Bacillus	No visible growth
Yeast	„ „ „

CONCLUSION

These results together with the finding that retarded lactose fermenting colonies belong to the coliform group demonstrate the high degree of selectivity of the VRB medium and support the conclusion that in counting plate cultures as well as deep agar cultures, all distinctly red coloured colonies which after 24 hours of incubation have developed above pin-point size with a fair degree of accuracy can be considered as coliform bacteria.

Furthermore it is concluded that the deep agar tube technique with the modified VRB-Nitrate Agar described in this report can replace the ordinary plate techniques with solid coli media as a reliable method for estimating the number of coliform bacteria in foods, milk etc., that the deep agar tube method offers special practical advantages over the plate technique when aiming at selective counting of E. coli I in 44° cultures, because large number of tubes easely can be placed in ordinary water bath incubators while Petri dishes cannot, unless specially wrapped in water-tight plastic bags.

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SUMMARY

The author has investigated the possibilities of substituting the agar plate technique for a deep agar tube technique in estimating numbers of coliform bacteria in foods. Especially when water bath incubation at 44°C is used for selective counting of E. coli I a tube technique would seem to offer considerable practical advantages over the ordinary Petri dish plate technique. The medium selected for the purpose is Violet Red Bile Agar. However, in order to be able to cultivate coliform bacteria in VRB-deep agar tubes the formation of gas from fermentation of lactose must be suppressed. To achieve anaerogenic growth of coliform bacteria the author has utilized the existing knowledge of the inhibitory effect of nitrate- and nitrite ions upon the formic acid hydrogenlyase enzyme which catalyses the decomposition by coliforms of formic acid into hydrogen and carbon dioxide. Nitrate was found preferrable to nitrite because of less inhibitory effect. The optimal concentration of nitrate in VRB Agar was determined to 0.5 ‰ $NaNO_3$. Flat Miller-Prickett-tubes with an inlaid white enamel glass plate were found to facilitate counting. To obtain well developed colonies with a typical zone of precipitation the agar contents of the medium was reduced to 0.9 per cent. Two series of comparative coli counts with the modified VRB-Nitrate agar in deep agar tubes against ordinary VRB-Agar in Petri dishes at 37°C and 44°C showed on the whole no statistically valid differences.

The problem of small retarded colonies appearing in tube cultures and plate cultures as well, mostly when seeded with raw milk, was investigated. All of 108 such strains except two could be identified as members of the coliform group. The selectivity of the medium was tested by plating a variety of non-coliform organisms. Only a few strains of fecal streptococci, Proteus, Alcaligenes and Pseudomonas were capable of scanty growth, and in no instance the resulting colonies could be mistaken for colonies of coliform bacteria.

ZUSAMMENFASSUNG

Colibestimmung in Lebensmitteln mit Hilfe einer Hochagartechnik mit modifiziertem Rot-Violett-Galleagar.

Der Verfasser untersuchte die Möglichkeiten, die Agarplattenmethode durch eine Hochagartechnik zur Zählung coliformer Bakterien in Lebensmitteln zu ersetzen. Besonders bei einer Inkubation im Wasserbad bei 44°C scheint die Hochagartechnik praktische Vorteile gegenüber dem gewöhnlichen Gebrauch von Petrischalen zu bieten. Zu den Untersuchungen wurde Rot-Violett-Galleagar (R.V.G.-Agar) angewandt. Um eine Zählung coliformer Bakterien in Hochagarröhren mit R.V.G.-Agar vornehmen zu können, muss die bei der Vergärung von Laktose entstehende Gasentwicklung verhindert werden; es wird festgestellt, dass man — auf Grund der Kenntnis bei flüssigen Kulturen — auch in festem Substrat die hemmende Wirkung der Nitrat- und Nitritionen auf das Ameisensäure-Hydrogenlyaseenzym ausnutzen kann, das die Spaltung der coliformen Bakterien von Ameisensäure in Sauerstoff und Kohlendioxyd katalysiert. Es zeigte sich, dass Nitrationen den Nitritionen vorzuziehen waren, und als optimale Nitratkonzentration wurde 0,5 ‰ $NaNO_3$ bestimmt. Ferner erwies es sich, dass flache Miller-Prickett-Tuben mit eingelegter weisser Emailglasplatte die Koloniezählung wesentlich erleichterten. Zwecks Erzielung gutentwickelter Kolonien mit einer typischen Präzipitatzone wurde der Agarinhalt des Mediums auf 0,9 % reduziert. Zwei Aussaatreihen zur vergleichenden Colizählung in modifizierten R.V.G.-Nitrathochagar gegenüber gewöhnlichem R.V.G.-Agar in Petrischalen sowohl bei 37° als auch 44°C zeigten im grossen ganzen keinen statistisch sicheren Unterschied.

Bei der Züchtung besonders aus roher Milch wurde sowohl in den Hochagarröhren als auch im Plattenmedium eine Reihe sehr kleiner und scharf begrenzter Kolonien wahrgenommen. 108 von diesen Stämmen — mit Ausnahme von 2 Stämmen — liessen sich in die Gruppe coliformer Bakterien einreihen. Im Hinblick auf die Untersuchung der selektiven Eigenschaften des R.V.G.-Agars wurde die Aussaat einer Reihe von nichtcoliformen Mikroorganismen ausgeführt. Hierbei zeigte es sich, dass nur einige wenige Streptokokkenstämme (zur Faecalis-Gruppe gehörig) sowie Proteus, Alcaligenes und Pseudomonas imstande waren, in diesem Medium zu wachsen, jedoch in keinem Falle mit Kolonien, die mit Colikolonien verwechselt werden konnten.

RESUMÉ

Colibestemmelse i levnedsmidler ved hjælp af højagarteknik med modificeret Rød-violet-galde-agar.

Forfatteren har undersøgt mulighederne for at erstatte agarpladem etoden med en højagarteknik til tælling af coliforme bakterier i levnedsmidler. Specielt når inkubationen sker i vandbad ved 44°C må højagarteknikken antages at byde på praktiske fordele frem for anvendelse af almindelige petriskåle. Til undersøgelserne er anvendt Rød-violet-galdeagar (R.V.G.-agar). For at kunne foretage tælling af coli-

forme bakterier i højagarrør med R.V.G.-agar må luftudviklingen, fremkommet ved bakteriernes forgæring af laktose, hindres, og det konstateres, at man — lige som det er kendt for flydende kulturers vedkommende — også i fast substrat kan udnytte nitrat- og nitritioners hemmende virkning på myresyrehydrogenlyaseenzymet, som katalyserer de coliforme bakteriers spaltning af myresyre i brint og kuldioxyd. Det fandtes, at nitrationer var at foretrække frem for nitritioner, og den optimale nitratkoncentration blev bestemt til 0,5 ‰ $NaNO_3$. Endvidere er fundet, at flade Miller-Prickett-tubes med indlagt hvid emaljeglasplade letter tællingen af kolonier væsentligt. For at opnå veludviklede kolonier med en typisk præcipitatzone, blev mediets agarindhold reduceret til 0,9 %. To udsædsserier til sammenlignende colitælling i modificeret R.V.G.-nitrat-højagar kontra almindelig R.V.G.-agar i petriskåle ved såvel 37° som 44°C viste stort set ingen statistisk sikker forskel.

Ved dyrkning især fra rå mælk iagttoges i såvel højagarrørene som plademediet en del meget små og skarpt afgrænsede kolonier. 108 af disse stammer viste sig alle — med undtagelse af 2 — at kunne henføres til gruppen coliforme baktereir. Med henblik på undersøgelse af R.V.G.-agars selektive egenskaber er foretaget udsæd af en række non-coliforme mikroorganismer. Herved har kun nogle få streptokokstammer (tilhørende fæcalis-gruppen) samt Proteus, Alcaligenes og Pseudomonas vist sig i stand til at vokse i mediet, men i intet tilfælde med kolonier, der kunne forveksles med colikolonier.

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