Applicability of the ISO Enterobacteriaceae Test for Determining the Suitability of Pasteurized Milk for Shipment

Jun Sato*1,†, Haruka Ohno*2 and Chinatsu Matsui*2
(*1 Faculty of Food and Nutritional Sciences, Toyo University, Izumino, Itakura-machi, Ora-gun, Gunma 374-0193; † Corresponding author)
(*2 Faculty of Life Sciences, Toyo University, Izumino, Itakura-machi, Ora-gun, Gunma 374-0193)
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We compared the desoxycholate agar (DOA) media method to investigate the presence of 
coliforms at milk plants and its proposed substitute, the ISO Enterobacteriaceae test. In raw milk 
samples with only coliforms or Enterobacteriaceae, there was a good correlation (r≥0.98) between 
the number of bacteria on DOA and violet red bile glucose (VRBG) agar. In raw milk samples 
that contained Pseudomonas, which is a psychrotrophic bacteria, as well as coliforms or Enterobacteriaceae, the correlation between the number of bacteria on DOA at 30°C and VRBG agar at 
37°C was weak. It was assumed that this was because the capability of supporting the growth of 
psychrotrophic bacteria by VRBG agar is inferior to that demonstrated by DOA; the difference in 
incubation temperatures greatly influenced this effect. The ratio of the bacteria grown on VRBG/ 
DOA agar plates (VRBG/DOA) using the coliforms such as E. aerogenes NBRC 13534T and K. 
oxytoca JCM 1665 was 0.67–1.09 and 0.95–1.31, respectively. Because the bacterial count of Pseu-
domonas grown on VRBG agar was not suitably counted, the count differed depending on the 
strains used, incubation temperature, and layering conditions. For tests verifying the suitability 
of raw milk for shipment, if the purpose is to detect the presence of Enterobacteriaceae only, then 
the ISO Enterobacteriaceae test can be used. However, if psychrotrophic bacteria are also to be 
detected, then using the ISO Enterobacteriaceae test may not be suitable.

Key words: ISO, Enterobacteriaceae, coliform, pasteurized milk, VRBG

1. Introduction

Europe and North America have adopted International Organization for Standardization (ISO) 
and U.S. Food and Drug Administration’s Bacteriological Analytical Manual (FDA/BAM) as the 
method for detecting bacteria in food, and these have become the world standards (http://www. 
fda.gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/default. 
htm). However, Japan has its own conventional standard tests for food sanitation which are integrated 
into the standard component of the Food Sanitation Law, and the culture media, culture 
conditions, and other culturing procedures are different from those used in the Europe and U.S. Japan 
therefore stands alone in food safety tests; this may cause problems when importing and ex-
porting raw and processed food in today’s accelerated and globalized food market. Under such an 
environment, if ISO standards will be introduced in Japan in the near future, it is important to 
speculate, in advance, what type of problems would be encountered at the inspection place in 
food factories.

One well-established test that will be adopted in Japan in the future is the ISO Enterobacteriaceae 
test. Members of Family Enterobacteriaceae are oxidase-negative bacteria that ferment glucose 
and form pink, red and purple colonies on violet red bile glucose (VRBG) agar. Because their 
taxonomy has been well established, they are widely used as indicator microorganisms instead of 
coliforms in the European Union (EU). The Enterobacteriaceae-negative standard was first 
 promulgated in Japan for tests on raw meat in September 2011, presumably as a response to this 
trend as well as a way of harmonizing regu-
tions. This adoption represents the first appearance of a new set of indicator microorganisms, the *Enterobacteriaceae* in Japan. Although there are few reports on the effectiveness of the ISO *Enterobacteriaceae* test in Japan, Saito et al. compared a simple EB plate method with the ISO method on meat, sea food, and prepared foods. Teramura et al. tested 53 commercial meat samples to evaluate a simple ISO *Enterobacteriaceae* test on Compact Dry ETB media, by comparing it with VRBG agar and Petrifilm EB. Nakamura et al. compared the effectiveness of the official Japanese standard coliform test using deoxycholate agar (DOA) with VRB, VRBG agar and enzyme substrate containing medium, and they tried to detect and isolate coliforms (or *Enterobacteriaceae*) from food and the environment.

However, there are a number of factors that need to be considered if the conventional coliform test is substituted by the ISO *Enterobacteriaceae* test. For example, concerning the coliform test conducted in pasteurized milk plants, some tests detect the presence of the psychrotrophic bacteria *Pseudomonas* spp. In this case, the incubation temperature is 30°C. Further, DOA is incubated at 30°C and not only the dark red colonies are counted but all colonies that grow on this medium are counted for the control. Thus, products that need to be refrigerated, such as pasteurized milk (except for milk products storable at room temperature), where *Pseudomonas* species is one of the indicators of contamination, this is considered to be a very strict food sanitation test. Because the ISO *Enterobacteriaceae* test uses different media and incubation conditions, if this test is adopted in pasteurized milk plants, it is important to determine if it can detect *Pseudomonas* species, which can be detected by the current coliform test. In this study, we compared the DOA media method used as the coliform test in pasteurized milk plants with the proposed alternative, the ISO *Enterobacteriaceae* test (ISO21528-2: 2004), and we evaluated the usefulness of the proposed alternative test in determining the suitability of pasteurized milk for shipment.

2. Materials and Methods

2.1 Test strains

The following 4 Gram-negative strains were used.

*Enterobacter aerogenes* NBRC 13534

*Klebsiella oxytoca* JCM 1665

*Pseudomonas putida* NBRC 14164

*Pseudomonas aeruginosa* ATCC 9027

2.2 Counts of coliforms on DOA

Following the “Ministerial ordinance on milk and milk products concerning compositional standards, etc.”, samples were cultured using pouring method with DOA (Eiken Chemical Co., Ltd., Tokyo).

2.3 Counts of *Enterobacteriaceae* on VRBG agar

Following the ISO21528-2 test method, samples were cultured using the pouring method with VRBG agar (Oxoid). Representative colonies grown on VRBG agar were used for the oxidase test and glucose fermentation test.

2.4 Changes in bacterial contamination of raw milk after freezing

Raw milk was obtained from the manufacturer and frozen at −20°C for 0, 3, and 7 days. The change in the plate counts of coliforms (or *Enterobacteriaceae*) and non-coliforms (or non-*Enterobacteriaceae*) was measured on DOA, VRBG agar, and XM-G agar (Nissui Pharmaceutical Co., Ltd., Tokyo). They were identified by colors of colonies.

2.5 Comparison of bacterial counts from raw milk on DOA and VRBG agar

Raw milk was incubated for overnight at 30°C to allow bacteria to multiply at various concentrations, or raw milk samples were appropriately diluted with pasteurized milk, and each sample was tested on DOA and VRBG agar. All colonies grown on DOA were counted regardless of color. However, only the pink, red, and purple colonies on VRBG agar were counted.

2.6 Validating the coliform test for milk

Each milk sample was inoculated so that approximately 10^2–10^3 CFU/ml of the test strains were present in the milk. Each sample was plated on DOA and VRBG agar and the number of colonies was counted.

2.7 Identification of microbial isolate

Twenty-four representative isolates from DOA were identified using the API20E and AP-20NE identification system (SYSMEX bioMérieux).
3. Results

3.1 Changes in bacterial contamination of raw milk after freezing

In order to intentionally create samples of raw milk in which only the coliforms or *Enterobacteriaceae* are present as gram-negative bacteria, the milk was frozen at $-20^\circ$C for 0, 3, and 7 days. The changes in coliforms (or *Enterobacteriaceae*) or non-coliforms (or non-*Enterobacteriaceae*) numbers were measured for each time period on DOA, VRBG, and XM-G agar (Table 1). The condition for incubation was $30^\circ$C for 20 hr on each medium. On all media, the count of coliform (or *Enterobacteriaceae*) reached a magnitude of $10^7$ CFU/ml. This could have been because it took time to obtain the raw milk samples.

On DOA, the coliform count remained stable for all time periods. The non-coliform count also did not change at 3 days after freezing, but at 7 days after freezing, the count decreased to 0 CFU/ml. On VRBG agar, the *Enterobacteriaceae* count was stable at all time periods. The initial count of non-*Enterobacteriaceae* was $6.5 \times 10^5$ CFU/ml. The count decreased to $2.5 \times 10^5$ CFU/ml at 3 days after freezing and to 0 CFU/ml at 7 days after freezing. If these results are compared with the count of non-coliform on DOA, the count of non-*Enterobacteriaceae* at 0 and 3 days after freezing was an order of magnitude lower than the count of non-coliform on DOA. On XM-G, the coliform count decreased gradually with time, but there was no change in the magnitude until 7 days after freezing. The non-coliform count remained stable at 0 CFU/ml from the beginning.

These results show that on freezing raw milk for 7 or more days at $-20^\circ$C, the non-coliforms and non-*Enterobacteriaceae* were undetectable on all 3 media types; this indicates that freezing raw milk for 7 or more days either kills or damages these bacteria.

3.2 Comparing plate counts on DOA and VRBG agar

Samples of raw milk, that had been frozen for 7 or more days at $-20^\circ$C to ensure that only coliforms or *Enterobacteriaceae* were present, were plated onto DOA and VRBG agar, and the numbers of bacteria were counted. In order to detect psychrotrophic bacteria, DOA plates were incubated at $30^\circ$C and $37^\circ$C for 20 hr, and VRBG agar plates were layered and incubated at $37^\circ$C for 24 hr (Fig. 1). Results showed a strong correlation between the numbers of bacteria on DOA and VRBG agar regardless of the incubation temperature ($r \geq 0.99$ at $30^\circ$C and $r \geq 0.98$ at $37^\circ$C).

Next, the same tests were conducted on raw milk that had not been frozen, and therefore, contained non-coliforms or non-*Enterobacteriaceae* as well as coliforms or *Enterobacteriaceae*. In this case, differences between the media depended on the presence or absence of layering in VRBG agar (Fig. 2). The numbers of bacteria on VRBG agar plate at $37^\circ$C were lower than those on the DOA plate at $30^\circ$C ($r \leq 0.78$) regardless of the layering conditions. There was also a strong correlation

| Table 1. Effect of the freezing duration at $-20^\circ$C on Gram-negative bacterial counts in raw milk (log N [CFU/ml]) |
|-------------------------------------------------|---|---|---|
| Freezing duration (days)$^1$ | 0 | 3 | 7 |
| **Desoxycholate agar (DOA)** | | | |
| Coliform | 7.11 | 7.52 | 7.15 |
| Non-colo-form $^2$ | 6.36 | 6.18 | 0.00 |
| **Violet red bile glucose (VRBG) agar** | | | |
| *Enterobacteriaceae* | 7.34 | 7.15 | 7.11 |
| Non-*Enterobacteriaceae*$^3$ | 5.81 | 5.40 | 0.00 |
| **XM-G agar** | | | |
| Coliform | 7.56 | 7.30 | 7.11 |
| *E. coli* | 6.26 | 6.41 | 5.78 |
| Non-coliform | 0.00 | 0.00 | 0.00 |

Two raw milk samples were tested for each freezing duration and the average Gram-negative counts are shown in the table.

$^1$ Raw milk samples were frozen at $-20^\circ$C.

$^2$ All colonies which grew on DOA were counted.

$^3$ All colonies which grew on VRBG were counted.
between the number of bacteria grown on VRBG agar and DOA plates when both were incubated at 37°C, again regardless of the layering conditions ($r \geq 0.94$). When the numbers of bacteria grown on DOA plate at both incubation temperatures and on VRBG agar plate at 37°C with or without the layering conditions were counted, the difference between the mean of the numbers of bacteria was significant at 5%; there was no significant difference between the number of bacteria on DOA plate at 37°C and VRBG agar plate at 37°C without layering (data not shown).

Twenty-four representative isolates from DOA medium using raw milk samples (containing all bacterial types as described above) were identified. All the isolates taken from dark red colonies on DOA plate were oxidase negative and glucose fermentation positive. Eighteen strains were identified as 8 species from the Enterobacteriaceae genera Citrobacter, Enterobacter, Hafnia, Klebsiella, and Serratia. All the strains from colonies that were not dark red on DOA plate were oxidase positive and glucose fermentation negative. Four strains belonged to 3 Pseudomonas species and 2 isolates were identified as a species of Ralstonia (Table 2). Taking into account these findings, it was assumed that the non-coliforms or non-Enterobacteriaceae in raw milk were mainly psychrotrophic Pseudomonas species.

3.3 Validating the coliform test for pasteurized milk

The test strains were inoculated into raw milk, and the number of bacteria was counted on DOA and VRBG agar. DOA medium was incubated at 30°C for 20 hr. VRBG agar was incubated at 30°C and 37°C for 24 hr, with and without layering. Further, we confirmed the difference in the number and ratio (the number of bacteria on VRBG agar/the number of bacteria on DOA) of bacteria (Table 3). Both coliforms *E. aerogenes* NBRC 13534T and *K. oxytoca* JCM 1665 had approximately the same plate counts on VRBG agar and DOA regardless of incubation temperatures or layering conditions. The ratio of bacteria was 0.67–1.09 for *E. aerogenes* NBRC 13534T and 0.95–1.31 for *K. oxytoca* JCM 1665.

However, for *P. putida* NBRC 14164T incubated at 30°C on VRBG agar, regardless of layering, the plate count was less than half of that on DOA, and the ratio of bacteria was ≤0.85 with layering and ≤0.83 without layering. On the other hand, no *P. putida* colonies were found on VRBG agar at 37°C. For *P. aeruginosa* ATCC 9027 grown on VRBG agar at 30°C and 37°C with layering, the
plate count was drastically decreased compared with that on DOA. However, without layering, regardless of the temperature, the plate count was almost the same as that on DOA.

Accordingly, although VRBG agar has almost the same capability for detecting coliform as that of DOA, for *Pseudomonas* indicator species, the effectiveness differed depending on the species. In addition, the incubation temperature and the presence/absence of layering also caused a considerable difference.

### Table 2. Identification results of microorganisms isolated from raw milk samples which were stored at 5°C

<table>
<thead>
<tr>
<th>Enterobacteriaceae*¹</th>
<th>Number of isolates</th>
<th>Non-Enterobacteriaceae*²</th>
<th>Number of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Citrobacter freundii</em></td>
<td>1</td>
<td><em>Pseudomonas fluorescens</em></td>
<td>1</td>
</tr>
<tr>
<td><em>Citrobacter sp.</em></td>
<td>3</td>
<td><em>Pseudomonas luteola</em></td>
<td>1</td>
</tr>
<tr>
<td><em>Enterobacter cloacae</em></td>
<td>2</td>
<td><em>Pseudomonas sp.</em></td>
<td>2</td>
</tr>
<tr>
<td><em>Hafnia alvei</em></td>
<td>3</td>
<td><em>Ralstonia pickettii</em></td>
<td>2</td>
</tr>
<tr>
<td><em>Klebsiella oxytoca</em></td>
<td>3</td>
<td>Subtotal</td>
<td>6</td>
</tr>
<tr>
<td><em>Serratia liquefaciens</em></td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Serratia marcescens</em></td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Serratia odorifera</em></td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Subtotal</strong></td>
<td>18</td>
<td></td>
<td>24</td>
</tr>
</tbody>
</table>

*¹ Dark red colony on DOA, oxidase: —, glucose fermentation: +<br>
*² Not dark red colony on DOA, oxidase: +, glucose fermentation: —

### Table 3. Comparison of the colony counts of coliforms (Enterobacteriaceae) and *Pseudomonas* spp. on DOA and VRBG plates

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Order (CFU/ml)</th>
<th>log DOA (CFU/ml)</th>
<th>log VRBG (CFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>30°C w/o*¹</td>
<td>30°C w/t*²</td>
</tr>
<tr>
<td></td>
<td></td>
<td>w/t<em>² w/o</em>¹</td>
<td>w/t<em>³ w/o</em>¹</td>
</tr>
<tr>
<td><em>Enterobacter aerogenes</em></td>
<td>10²</td>
<td>2.43 (0.97)</td>
<td>2.35 (1.00)</td>
</tr>
<tr>
<td>NBRC13334³</td>
<td>10¹</td>
<td>1.94 (0.95)</td>
<td>1.86 (0.96)</td>
</tr>
<tr>
<td></td>
<td>10⁰</td>
<td>0.90 (0.78)</td>
<td>0.60 (0.67)</td>
</tr>
<tr>
<td><em>Klebsiella oxytoca</em></td>
<td>10²</td>
<td>2.80 (1.02)</td>
<td>2.78 (0.99)</td>
</tr>
<tr>
<td>JCM1665</td>
<td>10¹</td>
<td>1.85 (1.04)</td>
<td>1.76 (0.95)</td>
</tr>
<tr>
<td></td>
<td>10⁰</td>
<td>0.85 (1.31)</td>
<td>1.00 (1.18)</td>
</tr>
<tr>
<td><em>Pseudomonas putida</em></td>
<td>10²</td>
<td>2.55 (0.83)</td>
<td>1.86 (0.73)</td>
</tr>
<tr>
<td>NBRC14164³</td>
<td>10¹</td>
<td>2.33 (0.85)</td>
<td>1.94 (0.83)</td>
</tr>
<tr>
<td></td>
<td>10⁰</td>
<td>1.30 (0.69)</td>
<td>0.95 (0.73)</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>10²</td>
<td>2.72 (0.53)</td>
<td>2.73 (1.00)</td>
</tr>
<tr>
<td>ATCC9027</td>
<td>10¹</td>
<td>1.58 (0.00)</td>
<td>1.64 (1.04)</td>
</tr>
<tr>
<td></td>
<td>10⁰</td>
<td>0.60 (0.00)</td>
<td>0.30 (0.50)</td>
</tr>
</tbody>
</table>

*¹ Without layering of DOA
*² With layering of VRBG
*³ Without layering of VRBG
*⁴ Ratio of (log VRBG (CFU/ml))/log DOA (CFU/ml)

4. Discussion

To examine the change in the number of bacteria that contribute to the contamination of frozen raw milk samples, three different selective culture media for Gram-negative bacteria, i.e., DOA, VRBG, and XMG, were used. Selective agents in these media are sodium deoxycholate, bile salts No. 3, and sodium lauryl sulfate, respectively. Of these three selective agents, media including bile salts has been reported to be toxic against Gram-negative enteric bacteria².⁸. The
reason why XM-G cannot be used for non-coliform counts is believed to be related not only to the differences in nutrients present in this media but also because sodium laurel sulfate is very selective and inhibits the growth of psychrotrophic bacteria such as Pseudomonas spp.

When the same raw milk samples that had been frozen for 7 or more days at −20°C were tested on DOA and VRBG agar plates, there was a strong correlation between the bacterial counts on the two media regardless of the incubation temperature (Fig. 1). Table 1 shows that although the capability of VRBG for detecting Pseudomonas spp. is inferior to that of DOA, there was no difference between the two media to detect the coliform. This was because the non-coliforms or non-Enterobacteriaceae in raw milk samples frozen for 7 days at −20°C either died or were damaged due to freezing; the coliforms and Enterobacteriaceae selectively survived. No major difference could be detected between the number of bacteria grown on the two different media. The mechanism of this decline of non-coliforms or non-Enterobacteriaceae could have some relationships with the fact that death rates of microbes in the process of freezing are different among various kinds of microbes and they could also be affected by freezing speed and the production of a large number of minute ice particles in a cell[6]. If the raw milk sample was not frozen, the number of bacteria present on VRBG agar at 37°C was lower than that present on DOA at 30°C regardless of layering (Fig. 2). It was assumed that VRBG agar had inferior capability of supporting the growth of Pseudomonas species in comparison with DOA; the incubation temperature could have also influenced this effect. There was a strong correlation with respect to plate counts between DOA and VRBG agars at 37°C regardless of layering conditions. Further, the growth of Pseudomonas species was inhibited on DOA at 37°C, resulting in the predominant growth of coliforms.

These results show that DOA and VRBG agar have approximately the same bacterial plate count values when only coliforms or Enterobacteriaceae are present. When psychrotrophic bacteria such as Pseudomonas species as well as coliforms or Enterobacteriaceae are present, the correlation between DOA incubated at 30°C and VRBG agar was weak. Because almost no contaminated microorganisms are present in Japanese pasteurized milk subjected to UHT sterilization[7], in this study, we carried out tests using raw milk instead of processed milk. The tests to determine whether raw milk can be shipped are conducted using DOA incubated for 20+/−2 hr at 32–35°C. In milk plants that detect the presence of not only coliforms but also psychrotrophic bacteria, the culture is incubated at 30°C[8]. This means that when testing milk products to determine if they are suitable for shipment, the ISO Enterobacteriaceae test is perfectly suitable if detecting its presence is the main concern. However, if a more strict test that detects the presence of psychrotrophic bacteria is required, then the ISO Enterobacteriaceae test is not suitable.

In pasteurized milk samples in which coliforms were inoculated, for both coliform species, the bacterial plate count on DOA was approximately the same as that on VRBG, and the ratio of bacteria was 0.67–1.31 (Table 3). In pasteurized milk samples in which Pseudomonas was inoculated, the growth depended on the species used, incubation temperature, and layering conditions. The results showed a low correlation between the number of bacteria on DOA at 30°C and VRBG agar when psychrotropic bacteria consisted mainly of the genus Pseudomonas as well as coliforms or Enterobacteriaceae. The number of P. aeruginosa ATCC 9027 grown on DOA and VRBG agar without layering was almost the same. Because Pseudomonas bacteria are aerobic, there is a possibility that the presence/absence of layering conditions would have greatly influenced the bacterial growth.

Acknowledgments

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References

3) International Organization for Standardization, ISO 21528–1. Microbiology of food and animal feeding stuffs—Horizontal methods for the detection and enumeration of Enterobacteriaceae—Part 1: Detection and
腸内細菌科菌群試験法の牛乳出荷判定検査への適用
佐藤 順*1†・大野 陽華*2・松井 千夏*2
(*1 東洋大学食環境科学部、*2 東洋大学生命科学部)

牛乳工場で大腸菌群検査に用いられている「DOA培地法」と、その代替法として想定されるISO法である「腸内細菌科菌群試験法」を比較検討した。グラム陰性細菌として大腸菌群あるいは腸内細菌科菌群のみ存在する生乳では、DOA菌数とVRB菌数との間にはr=0.98以上の良好な相関が得られた。大腸菌群あるいは腸内細菌科菌群に加え、低温細菌であるPseudomonas属等が存在する生乳では、DOA菌数（30℃）とVRB菌数（37℃）との相関は低かった。大腸菌群であるEnterobacter aerogenes NBRC 13534及びKlebsiella oxytoca JCM 1665のVRB菌数/DOA菌数（対数値）の値は、前者で0.67～1.09、後者で0.95～1.31の範囲となった。一方、Pseudomonas属菌株の菌数はVRBで十分計測できず、菌株や培養温度の相違、重層の有無によって発育状況が異なった結果となった。牛乳の出荷判定検査において、腸内細菌科菌群のみを管理の対象とする場合は「腸内細菌科菌群試験法」を適用できるが、低温細菌を含めた管理を行う場合には適用は難しいことが明らかとなった。