Comparison of Three Techniques for the Total Count of Anaerobes from Intestinal Contents of Pigs

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In the study of the gastrointestinal microflora of pigs, an exact qualitative and quantitative determination of facultative and obligate anaerobes is necessary. Indeed, it is well known that in the posterior part of the digestive tract (cecum, large intestine) the microflora consist principally of anaerobic populations (Bacteroides, lactobacilli, streptococci and probably many other genera) (4, 5, 9, 10). Hence, it is clear that for a study of these organisms a suitable anaerobic technique should be used.

In this note, we describe the results of a comparative study of three anaerobic techniques: the roll-tube method of Hungate (7, 8); the anaerobic ringed-plate technique of Van der Heyde, developed and currently used in our laboratory for some years (11, 12); and incubation of conventional petri dishes in anaerobic jars (GasPak anaerobic jar, Bioquest, Cockeysville, Md.). Cecum and colon samples were obtained from slaughtered pigs. The origins and feeding regimen of the animals were unknown. Immediately after slaughtering, cecum and colon were each pinched off and transported to the laboratory. From each organ, one sample of about 1 g was taken with a syringe and diluted anaerobically in a rubber-stoppered tube containing a mineral solution (1 volume of solution A plus 1 volume of solution B plus 2 volumes of water), and cysteine-HCl (0.03% w/v) (8). Dilution and inoculation of the roll tubes in duplicate was carried out as described earlier (8). The gas phase was 90% N₂, 10% CO₂, and the roll tubes were incubated at 37 C. For the three methods, reinforced clostridial medium agar (Oxoid) with 0.001% hemin was used (1, 3, 6, 13). For the roll-tube method, the agar content of the medium was increased to 2% (w/v).

Further samples in duplicate from the cecum and colon, respectively, were homogenized and diluted in 0.1% peptone (w/v) with 0.85% NaCl (w/v) and 0.04% agar (w/v). One drop of each dilution was inoculated in duplicate on ringed plates and incubated anaerobically under 90% N₂, 10% CO₂ in a water bath, according to the technique of Van der Heyde (11, 12). Finally, one drop of each dilution was inoculated in duplicate on conventional petri

| Table 1. Total counts* of anaerobes from cecum and colon, as obtained with three techniques |
|---------------------------------|--------|--------|--------|-------|-------|-------|
| Animal no. | Cecum | | | Colon | | |
| | RT* | RP* | AJ* | | | |
| | | | | RT | RP | AJ |
| 1 | 10.00e | 9.37e | 9.00e | 10.04 | 9.10 | 8.80 |
| 2 | 10.16 | 9.37 | 9.20 | 10.00 | 9.07 | 9.02 |
| 3 | 10.45 | 9.96 | 9.20 | 10.33 | 9.30 | 8.85 |
| 4 | 9.41 | 9.07 | 8.77 | 9.63 | 9.15 | 9.25 |
| 5 | 9.68 | 9.17 | 9.07 | 9.91 | 9.47 | 9.72 |
| 6 | 9.49 | 9.22 | 9.20 | | | |
| 7 | 9.76 | 8.55 | 8.62 | 9.78 | 9.30 | 9.20 |
| 8 | 10.04 | 9.32 | 9.45 | 9.91 | 9.80 | 10.27 |
| 9 | 9.33 | 8.82 | 9.02 | 9.61 | 9.32 | 9.30 |
| 10 | 9.10 | 8.25 | 8.30 | 9.40 | 8.70 | 8.70 |
| 11 | 9.25 | 8.50 | 8.60 | 9.60 | 9.05 | 9.20 |
| 12 | 9.78 | 9.30 | 9.35 | 9.60 | 9.60 | 9.85 |
| 13 | 8.75 | 8.10 | 8.25 | 9.08 | 8.90 | 8.90 |
| 14 | 8.81 | 8.05 | 8.10 | 8.93 | 9.20 | 9.20 |
| 15 | 9.29 | 8.77 | 8.50 | | | |
| Mean value | 9.55 | 8.85 | 8.84 | 9.68 | 9.22 | 9.24 |

*Log values per gram of contents.
*Abbreviations: RT, roll-tube method; RP, ringed-plate technique; AJ, anaerobic jar incubation.
*Mean value of counts on one sample, diluted and inoculated in duplicate.
*Mean value of counts on two samples, plated out in duplicate.
*Sample lost.
Table 2. Proportional composition of the dominant flora obtained with the roll-tube method and the ringed-plates technique

| Method               | Intestinal segment | No. of colonies examined | Group 1 (%) | Group 2 (%) | Group 3 (%) | Group 4 (%) |
|----------------------|--------------------|--------------------------|-------------|-------------|-------------|-------------|
| Roll-tube method     | Cecum              | 106                      | 76          | 15          | 9           | 0           |
|                      | Colon              | 105                      | 47          | 36          | 8           | 9           |
| Ringed-plates method | Cecum              | 183                      | 26          | 49          | 15          | 10          |
|                      | Colon              | 155                      | 21          | 69          | 7           | 3           |

* For both methods, from highest dilution, all the colonies were picked, gram-stained, and examined on morphology. Classification in groups was done as outlined in the text.

Table 3. Total counts and proportional composition of the dominant flora of anaerobes, obtained with the roll-tube method and ringed-plates technique, using the same subsamples

| Determinations | Cecum | Colon |
|----------------|-------|-------|
|                | RTa   | RP    | RT   | RP    |
| Animal no.     |       |       |      |       |
| 16             | 8.50b | 8.10b | 8.80 | 8.60  |
| 17             | 9.30  | 8.20  | 9.20 | 8.90  |
| 18             | 9.00  | 8.80  | 9.50 | 9.20  |
| 19             | 9.20  | 8.80  | 9.30 | 9.10  |
| 20             | 9.40  | 8.40  | 9.90 | 8.70  |
| Mean value     | 9.08  | 8.46  | 9.36 | 8.90  |
| Composition: dominant flora (%) |       |       |      |       |
| Group 1        | 42    | 4     | 33   | 0     |
| Group 2        | 32    | 56    | 37   | 74    |
| Group 3        | 26    | 40    | 30   | 26    |
| Group 4        | 0     | 0     | 0    | 0     |
| No. of colonies examined | 43    | 51    | 25   | 27    |

*a* Total counts: log value per gram of contents. Samples (about 1 g) were taken with a syringe and immediately diluted anaerobically in stoppered tubes. From these tubes, inoculation of roll tubes and further dilution and inoculation of the ringed plates were carried out.

*b* RT, roll-tube method; RP, ringed-plates technique.

*c* Mean value of counts on one sample, inoculated in duplicate.

dishes and incubated in anaerobic jars with the GasPak system (BBL).

After 72 hr, colonies were counted. The results are presented in Table 1. It is clear that for every Cecal sample highest counts were obtained with the roll-tube method. The same is true for most of the Colon samples. Mean values indicate that on the Cecum and Colon samples, respectively, about five (0.70 log values) and three (0.44 log values) times more bacteria were counted. Furthermore, for the first and second method, we investigated the composition of the dominant flora. Therefore, from the highest dilution, all of the colonies (samples 10-15) were picked and identified by Gram staining and morphological examination. On this basis, classification was done as follows: group 1, gram-negative, polymorphic rods (probably mainly Bacteroides group); group 2, gram-positive cocci; group 3, gram-positive rods; group 4, non-classified organisms (unclear Gram stain, irregular morphological form).

In Table 2, the proportional composition of the dominant flora is given. It is interesting to note that with the roll-tube technique not only were higher counts obtained, but the composition of the dominant flora was also different, compared to the ringed-plates technique. Indeed, a considerable shift towards group 1 was observed. This effect is more pronounced for the Cecum samples. Group 1 is probably composed mainly of members of the genus Bacteroides and similar groups.

Because the use of different subsamples in this comparison could have influenced the total count, a further experiment was carried out in which roll tubes and ringed plates were inoculated with samples from the same diluted sample, held in rubber-stoppered tubes. Further dilutions, from which the ringed plates are inoculated, were prepared this time in the same dilution fluid as used for the roll-tube method. From the results presented in Table 3, which are in agreement with the first results, it is evident that the higher counts obtained with the roll-tube method (Table 1) are not due to the use of different subsamples or dilution fluids. The difference in counts is probably due to different oxidation-reduction potentials of the media or the inoculation procedure. Indeed, with the ringed-plates technique, this manipulation is carried out aerobically.
From these results, it seems that the roll-tube method is the best of the three methods studied for examination and isolation of the predominant bacteria in the digestive tract of pigs. Investigating the microbial flora of stomach contents of primates, Bauchop also obtained highest counts with Hungate's technique (2). As the ringed-plate method is very well suited for routine work with a high number of samples, it could be important to modify and adapt the technique for counting strict anaerobes. Sampling, homogenization, dilution, and inoculation of the samples should be done under strictly anaerobic conditions.

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