Various taxo- and ecogroups of bacteria as index organisms for the enteric contamination of bottled waters: their significance and enumeration

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EPIDEMIOLOGICAL FUNDAMENTALS

Drinking water has repeatedly been observed to spread enteric diseases, even in recent times [1–7]. This may also apply to bottled waters, although there is very little convincing epidemiological evidence to suggest that this has already happened. Nevertheless it is wise for experts in public health not to neglect this aspect of the bottling of mineral waters.

Enteric diseases of various origin — salmonellosis, shigellosis and yersiniosis — can be transmitted by water that is only very sparsely contaminated with the causative bacteria, even in normoacidic persons [2]. This contrasts sharply with the elevated numbers of cells, *i. e.* the order 10^6, that seem to be required to trigger salmonellosis and most types of shigellosis when these bacteria are ingested with food [8]. It is, however, in accordance with the observation that chocolate can cause salmonellosis when only very sparsely contaminated, *i. e.* at the level of 1–10 cfu/g [9–10].

This apparent contradiction has prompted us to carry out some investigations on the underlying mechanism. In our experiments 99m Tc labeled drinking water was given to healthy volunteers and its fate followed with a gamma camera in line with a computer. This investigation revealed that amounts of water up to 50 ml when *taken between meals* passed the pyloric area with virtually no delay. Enteric pathogens contained therein are, hence, hardly exposed to the bactericidal effect of gastric juice. Thus they reach the duodenum in almost the same numbers as ingested and subsequently may trigger the corresponding disease [11]. The same phenomenon may

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be expected to occur when small amounts of chocolate are eaten between meals. However, when enteric pathogens are absorbed with meals, intragastric retention times are considerable, resulting in a dramatic reduction in numbers of colony forming units. In the latter instance the customarily accepted high numbers of cells have to be absorbed to allow at least one to penetrate the duodenal area. Thus the infecting dose of enteric pathogens may indeed be very low, provided they are absorbed between meals with relatively limited volumes of water.

This situation is aggravated in the case of bottled waters, because a considerable part is used by patients. In compromised hosts, obviously depending on the underlying disease, intragastric bactericidal mechanisms may be greatly impaired, to the extent that often every single organism, even if only potentially pathogenic, reaches the duodenal area.

In assessing the potential role of bottled waters in terms of provoking enteric disease the ecological properties of such beverages have to be taken in account. The presence of carbon dioxide is the parameter of paramount importance. It is quite certain that enteric bacteria will not grow under appreciable relative pressures of CO_2; they will, on the contrary, die off more or less rapidly, dependent on other attributes of the water under consideration, such as its pH [12–13]. In « still » waters the situation is different. Dependent on the types and concentrations of chemical compounds that can serve as nutrients for bacteria in such waters, organisms can remain viable for a rather long period of time [13]. And even waters, initially very poor in nutrients, may present a hazard. If, in such products, autotrophic, or only oligotrophic bacteria [14–15] develop to considerable numbers of cfu per ml [16–19], secondary (« cadaveric ») growth of strictly heterotrophic bacteria, such as enteric pathogens may occur. The same situation may be observed, following nitrogen fixation [20–22] in bottled waters. This occurrence is obviously limited to opened containers, which may, however, often be stored for quite a while before being completely consumed.

ESSENTIALS OF SANITARY QUALITY ASSURANCE

Contrary to the belief obstinately held in some circles, adequate bacteriological quality of bottled water cannot be assured by what is called the retrospective, or analytical, approach. This consists of drawing samples from consignments, examining these and comparing the results obtained with generally accepted microbiological standards for drinking water. Such an approach is, first of all, far too insensitive from the mathematical point of view. In addition, results are often obtained only after the sampled consignment has been shipped; thus this approach has the character of a *post mortem*,

And, even when alarming laboratory data are obtained in time, the effect is often nil because no one concerned is capable of correcting a situation in which poor results are obtained.

It is clear from these considerations that assurance of bacteriological safety of bottled water must be sought by the preventive approach. This is defined as the laying down of, and adherence to, strict Codes of Good Manufacturing Practice, often referred to as GMP. In the case of bottled waters this rests on carefully adjusting the mode of bottling to the nutritive properties of a given mineral water. Pertinent attributes in this context are, as we have seen in the previous paragraph: nutrient composition, pH and pCO_2.

An often posed question in this connection is: how good GMP's will have to be. For example, given the nutrient composition and pH of a given « still » mineral water, what measures should be taken to guarantee that an infinitesimally low initial level of enteric bacteria will have an insignificant probability of growth or even survival? The answer, as always in the field of Health Sciences, has to be based on an empirical approach, in this particular instance a suitably designed challenge test. In such assessments samples of the commodity are inoculated with reasonable numbers of enteric pathogens whose fate under practical conditions is subsequently followed. Based on the results of such studies GMP's for a given type of water can be developed, which, clearly have to be verified by intensive sampling and examination.

Once this preventive principle of sanitary quality assurance has been adhered to it is striking how fast previously marginal or even unacceptable quality improves [23–24]. In addition spreads of results between subsequent consignments will be considerably reduced [25–26]. The latter, in turn, enables efficient monitoring to be done by third parties. This applies particularly to examination for licensing importation, or by buyers who wish to compare various suppliers. For the statistical reasons outlined before, such monitoring work has an extremely low benefit/effort quotient when suppliers exert no preventive quality control, whereas it is most effective in cases where, subsequently to introduction of GMP, quality fluctuations between consignments have been reduced to acceptable levels.

PRINCIPLES OF METHODOLOGY

Choice of organisms

Direct tests for enteropathogenic bacteria of aquatic origin such as *V. cholerae*, *Salmonella typhi*, *Salmonella paratyphi*, the mostly food-borne, other salmonellae, shigellae, enteropathogenic serotypes of *E. coli* and *Yersinia* are mostly not carried out, because even under the worst conditions

they occur in rather low numbers. However, there is no essential experimental difficulty in this sense, because almost unlimited amounts of bottled waters can be filtered over suitable membranes, which can subsequently be cultured as is done in the examination of food samples [8]. Therefore, no deterrent exists from the methodological point of view to test water for pathogens where this may be required. Yet, for routine purposes testing for valid index organisms is the procedure of choice.

The coli–aerogenes group of bacteria, known also as « coliform» bacteria, are customarily used for the monitoring of water for safety, as their presence above certain levels is an indication of an unsafe initial condition, contamination upon bottling or combination of these defects. A significant improvement to this time–honoured practice was suggested about 1950, independently by Seeliger [27] and Habbs [28] in Germany and Hendriksen [29] in Norway. This involved the use of the entire group of the *Enterobacteriaceae* [30], to which the coli–aerogenes bacteria belong, as indicator organisms. This change results in the following advantages: *a*) as the definition of the *Enterobacteriaceae* is well established severe problems due to the illdefined taxonomy of the « coliforms » [31–32] are resolved; *b*) this substitution eliminates the possibility of obtaining false negative results due to the presence of lactose–negative strains — to which, *inter alia*, most pathogens belong! — or of « slow » lactose fermenters as the predominant enteric contaminants. Hence, both the reproducibility and the sensitivity of testing for *Enterobacteriaceae* is far better than that of the estimation of the coli–aerogenes group and so without increased effort or required skills.

There is a definite need to continue the good practice of testing water, in addition, for more robust index organisms, particularly the streptococci of the D–group of Lancefield [8]. These bacteria are certainly not exclusively of enteric origin — hence the need to avoid indicating them as « faecal » streptococci. Nevertheless their occurrence in water, and certainly in bottled waters which are often used for dietetic purposes (*vide supra*), points to contamination from a suspect source. In addition experience shows that their presence above given low levels can be easily avoided. Given their relatively high resistance to unfavourable external conditions they are, finally, reliable index organisms for viral contamination of water, although, obviously, by no means every water sample containing unacceptable levels of Lancefield group D streptococci will also be contaminated *e. g.* with hepatitis A virus, or one of the newly recognized, reoviruses [33–34].

Whether or not to examine water routinely for other organisms such as *E. coli* and *Pseudomonas aeruginosa* is a matter of opinion.

When bottled waters are found to be consistently « free » from *Enterobacteriaceae* there is little need to test them, additionally for *E. coli* as this species is included in the *Enterobacteriaceae* group. However, when occasio-

nally, low levels of *Enterobacteriaceae* are encountered, identification of the types may be appropriate. *E. coli* is then the first species to test for, while an examination for *Klebsiella* types is also highly recommended. Whether or not consignments of bottled water containing low numbers of innocuous types of *Enterobacteriaceae* only are acceptable, is debatable. Faithful supporters of the GMP–approach will certainly recommend systematic filtering of such waters, prior to bottling, thus avoiding any risk at all for the often compromised consumers.

P. aeruginosa can thrive well even in almost pure water [35]. Hence testing of still bottled waters for this organism is imperative. When sufficient numbers of repetitions of such examinations have given consistently negative results, the frequency of further testing can be considerably reduced, eventually even to perhaps once every month.

The need for recovery of injured cells

Bacteria of the *Enterobacteriaceae* and Lancefield D streptococci groups are known to be inhibited by selective agents currently employed in media used for their enumeration, if the cells carry sublethal lesions. Such injury has been demonstrated to result from heating [36–38], freezing [39–41] drying [42–44] and exposure to low pH [45], low temperature [46–48] and antimicrobial agents such as chlorine [49], in low concentrations. However, more and more evidence is obtained that bacteria occurring in ecospheres that, at first sight, do not seem to be very hostile, *e. g.* fresh meats [50–51] milk [52] and surface water [53–58], may also carry injury in the sense that they become abnormally sensitive to inhibitors occurring in the selective media customarily used for their detection. It is striking that even in suspensions of recently harvested bacteria, a considerable proportion of cells may show increased sensitivity to such selective agents. This has prompted the question as to whether lesions of this type have not to be considered the rule for microbial populations rather than the exception.

At any rate nothing will ever be lost, and quite an amount of underestimation will be avoided by systematically applying a treatment, prior to enumeration in selective media, referred to as «resuscitation». The conditions required for such a repair phase obviously depend on many factors: the treatment or agent having caused the lesions, the time of exposure to such damaging influences, the condition of the cells before stressing occurred, and particularly also the composition of the selective medium to be used, after the resuscitation treatment, to ultimately enumerate the repaired cells [59–62]. It is obvious that in such repair treatments the situation has to be avoided in which readily resuscitated cells and those that had not been damaged, start dividing, whereas considerable numbers of more tardily

restored cells still require more time to attain such a degree of recovery that they will grow in the customary selective media. It is striking that full recovery of most types of damaged bacteria can usually be achieved by exposing them to a non-selective restorative medium for no more than 2 hours at 20 °C, provided the selective medium used for their subsequent culturing is suitable [55, 63–66]; *vide infra*.

The need for adequately standardized procedures

Procedures to be applied in the microbiological monitoring of bottled waters have, as always, to be rigorously standardized. Where this is neglected, microbiological examination of any commodity in international trade becomes impractical if not unworkable. A special aspect of this imperative need for codification should be particularly stressed.

It has been demonstrated repeatedly that many preparations of selective media show inhibitory properties towards non-impaired cells of the bacterial types for whose detection they have been devised [67]. This phenomenon is all the more vexing as different commercially available media, marketed under exactly the same name, and even consecutive batches of the same brand may vary considerably in the degree of this intrinsic toxicity. We have found it quite possible to avoid such shortcomings of selective media by choosing the right types and concentrations of the most frequently involved medium components, particularly indicators, triphenyl methane dyes, bile, bile salts and other surface active compounds. This incidental suboptimal functioning of selective culture media is, clearly, to be distinguished from the essential shortcoming of most selective media *vis à vis* impaired cells.

Erroneously low results which would have been obtained by the use of media, deficient in the former sense should be avoided by routinely evaluating batches of selective media before allowing their use in practical monitoring work. For this purpose the following procedure has been found useful [68]. Trial plates or tubes are sparsely inoculated with at least two organisms, each of two types, *viz.* (a) one robust and the other more fastidious among the types to be detected; (b) one rather sensitive, the other more refractory, chosen from the most frequently encountered interfering organisms. The former two will both have to be recovered easily, whereas the last of group (b) should in principle not show too much growth and the first strain of group (b) not develop at all.

SUGGESTION FOR A ROUTINE ANALYTICAL PROCEDURE

In all monitoring work the principle is that the responsible bacteriologist takes into account the relation between analytical effort spent and information obtained therefrom. The two alternative approaches existing in the

field of the bacteriological examination of bottled waters are membrane filtration and the Presence–or–Absence (P–A) approach of Clark [69–71]. Both techniques have their *pros* and *cons*. The latter is rather voluminous, membrane filtration is still somewhat hampered by intrinsic problems, like the influence of the quality of the filter on the reliability and sensitivity of the enumeration procedure based on common, or special « M »–media [72–76].

In our hands the common stem P–A technique of Clark has given most satisfactory results. It comprises the following steps:

– a resuscitation treatment applied to a suitable portion, but at least 100 ml, *in the original bottle* [77], to be attained by the addition of equal volumes of double strength buffered glucose tryptone soya peptone (« tryptone soya ») broth, and incubation for 2 hours at approximately 20 °C;

– one single enrichment procedure of the resuscitated aliquots, *viz.* in MacConkey purple (MCP) broth, added as an equal volume of double strength broth, subsequently incubated at 30 °C;

– passing samples not leading to turbidity upon incubation for 18 to 24 hours;

– subculturing turbid enrichment cultures onto plates of (*a*) violet red bile glucose (VRBG) agar [30] for the isolation of *Enterobacteriaceae*; (*b*) kanamycin aesculin azide (KAA) – agar [78] for the isolation of Lancefield group D streptococci.

This basic procedure, though in itself quite adequate, can — just like the modern automobile — be extended to all degrees of sophistication. A few examples may illustrate this.

When *E. coli* is to be sought, positive enrichment cultures in MCP are streaked onto slants of McConkey nr. 3 agar and incubated at 44 $\pm$ 0.1 °C. If colonies with typical purple halos are obtained within 18 to 24 hours, this is quite reliable presumptive evidence for *E. coli*. Full identification can be achieved by testing colonies in MacKenzie, Taylor and Gilbert's modification of Eijkman's test (formation of indole and fermentation of lactose in the presence of bile salts and brilliant green at 44 °C), combined with assessment of absence of citrate assimilation on Simmons' agar and a negative oxidase reaction [79].

Where it may be required to differentiate between colonies of *Enterobacteriaceae* and those of *Aeromonadaceae*, vibrios and non fermentative Gram negative rod shaped bacteria, isolates obtained on VRBG showing a distinct purple halo should be examined in Gram negative diagnostic tubes. These consist of an approximately 2 cm bottom layer of VRBG, a barrier layer

of 2 cm water agar and a 2 cm layer of SIM–agar with 0.5 % NaCl added. Such tubes allow (*a*) determination of the mode of attack on glucose, to be assessed from the bottom layer; (*b*) the oxidase reaction to be carried out with the biomass obtained from the top layer; (*c*) reading of motility and H_2S formation and assessment of indole formation [80].

Rather easy detection of *P. aeruginosa* is possible by streaking positive MCP enrichment cultures onto slants of King *et al's* agar with 100 mcg/ml of nitrofurantoin added and incubating these at 42 °C [81]. Any growth obtained presents strong evidence for the presence of *P. aeruginosa*. Full identification requires (*a*) stabbing into Gram negative tubes (*vide supra*), allowing determination of motility, oxidase reaction and absence of anaerobic attack on glucose; (*b*) stabbing into plain VRBG tubes to assess oxidative attack on glucose; and (*c*) streaking onto glycerol mannitol acetamide cetrimide (GMAC) agar slants, which, when incubated at 42 °C, will turn cherry red in case of *P. aeruginosa* [81].

It goes without saying that these more complicated isolation and identification techniques are not required and quality assessment becomes very simple when consistently negative results are obtained upon enrichment in MCP. It is an ironic coincidence of microbiological monitoring of bottled waters that here, too, crimes against GMP do not pay, because of the expensive aftermaths when organisms, having no place there, are yet detected.

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Summary. — Bottled « still » waters may present bacterial and viral risks, particularly for compromised consumers and when stored for some length of time in opened containers. Therefore, bottling has to be supervised carefully and any sanitary deficiencies discovered, corrected immediately. When plants are thus controlled by a preventive system, bacteriological examination of samples will be warranted, whereas this is senseless, for mathematical reasons, in non–supervised bottling plants.

A direct search for enteric pathogens is discouraged. Instead, detection of the entire *Enterobacteriaceae* group, *P. aeruginosa* and Lancefield group D streptococci, is advocated. Deficiencies of customarily used methods of detection of the coli–aerogenes group of bacteria are outlined. The need for resuscitation of cells of bacterial groups used as index organisms, debilitated by storage in bottled waters, before their inoculation into currently employed selective media is demonstrated.

For routine purposes the following procedure is recommended:

– opening of the containers under aseptic conditions, leaving at least 100 ml of the contents in the original bottle or can;

– addition of an equal volume of double–strength tryptone soya peptone broth ;

– allowing this mixture to stand in the laboratory for two hours, to restore sublethally injured cells;

– addition of an equal volume of double–strength MacConkey purple broth;

– overnight incubation at 30 °C.

No growth of bacteria should occur in this test. When growth is nevertheless observed, the enrichment fluid should be examined for the presence of *Enterobacteriaceae* (and, if present, particularly for *E. coli*), *P. aeruginosa* and Lancefield's group D streptococci. Media and methods that have been found useful for these purposes are described.

Résumé (*La signification et la détection des taxogroupes et des écogroupes de bactéries, utilisés comme indicateurs de la contamination entérique des eaux plates présentées en bouteilles*). — Les eaux « plates » en bouteilles peuvent présenter des risques du point de vue bactériologique et virologique, en particulier pour les consommateurs sensibles et après stockage en flacon ouvert. C'est pourquoi leur mise en bouteille doit être soigneusement controlée et les éventuelles déficiences sanitaires doivent être corrigées sans délai.

Lorsque les entreprises sont ainsi controlées de manière préventive, l'examen bactériologique des échantillons est utile, alors que ceci est insensé si la mise en bouteille n'est pas surveillée, pour des raisons mathématiques.

La recherche directe des *Enterobacteriaceae* pathogènes n'est pas recommandée. Il est conseillé d'effectuer plutôt les contrôles bactériologiques suivants: *a*) le groupe des *Enterobacteriaceae*; *b*) *P. aeruginosa*; *c*) les streptocoques du groupe D de Lancefield. Les déficiences des méthodes usuelles pour la détection des « coliformes » sont démontrées. La nécessité impérieuse d'effectuer une revivification préalable des cellules lesées par un séjour dans les eaux minérales des organismes témoins, avant leur culture sur les milieux sélectifs usuels est soulignée.

Pour l'examen de routine le mode opératoire suivant est recommandé:

– ouvrir les bouteilles sous asepsie stricte, laissant un volume d'au moins 100 ml dans les flacons;

– ajouter un volume égale de milieu de revivification à double concentration, p. ex. le bouillon tryptone soya;

– laisser reposer pendant 2 heurs à la température ambiante, afin de restaurer les cellules lésées;

– ajouter un volume égal de « MacConkey purple broth » (bouillon lactosé aux sels biliaires et pourpre de bromocrésol) à double concentration;

– incubation à 30 °C pendant 18–24 heures.

Aucune croissance bactérienne ne doit être observée dans ces conditions. Dans le cas contraire, il est nécessaire de rechercher, dans les cultures d'enrichissement, la présence des groupes de bactéries suivants: *Enterobacteriaceae*, *E. coli*, *P. aeruginosa* et streptococques du groupe D. Les modes opératoires pour cette recherche sont récommandés.

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