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GENERAL PAPER

Development of reference materials for microbiological analysis

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Abstract The reliability of reference materials (RMs) depends on properties such as fitness, robustness, commutability, stability and homogeneity. The development of RMs for microbiological analysis is especially challenged through questions around the stabilisation and recovery of viable cells, the dispersion of precise numbers of cells, matrix effects and, when using molecular techniques, the presence of nucleic acids (e.g. DNA) of dead and live target organisms. However, RMs are indispensable tools for quality control in microbiological analysis. The Institute for Reference Materials and Measurements (IRMM), as part of the European Commission, concentrates its efforts on the development of RMs to support the development, implementation and monitoring of EU legislation. A special focus is given to highly precise RMs for presence/absence and enumeration tests in microbiological food and water analysis. Another group of new RMs certified by the IRMM comprise DNA-based materials to control the identity of microorganisms in qualitative assays. All of these activities serve to improve quality control in microbiological analysis.

Keywords Reference materials · Microbiological analysis · Quality control · Food microbiology · Water microbiology · Pathogens

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Introduction

The Institute for Reference Materials and Measurements' (IRMM) activities in the field of microbiological analysis are triggered by its mission, i.e. "to promote a common and reliable European measurement system in support of EU policies." This implies the development and "dissemination of internationally accepted quality assurance tools, including validated methods and reference materials." It is widely accepted that reference materials (RMs) are indispensable tools in analytical sciences and that RMs are at the edge of building "confidence in the comparability of measurements." RMs and reliable analytical methods are, generally, the cornerstones of any serious quality control methodology.

Several RM producers worldwide produce and distribute RMs for microbiological analysis. Prominent RM formats include spray-dried contaminated milk powder (from IRMM, EC; and RIVM, NL), dried plano-convex discs (from HPA, UK), freeze-dried "BioBalls" (from BTF, Australia) or dried semi-solid starch matrix materials (from Institut Pasteur Lille, F). This selection is, by far, not complete and does not represent a classification, but it shows that different formats of RMs for microbiological analysis can be used by analytical laboratories and method developers, which could also satisfy the demands of accreditation bodies and others. However, efforts are necessary to improve the properties of new RMs in order to overcome potential shortcomings of established formats. Such challenges could be the robustness of a material, attempts to minimise matrix interferences and to decrease inter-unit variations, further the development of commutable materials for application in several

methods and several areas of analysis, the development of more precise low colony-forming unit (CFU) materials for presence/absence tests or of precise high CFU materials for water microbiology, the development and certification of RMs for the rapid and reliable quantification of pathogens using nucleic-acids-based methods or to develop RMs for the control of the identity of regulated organisms in qualitative assays.

It is well understood that, especially, the stabilisation of live micro-organisms presents a challenge and, consequently, limits possibilities to develop highly reliable RMs based on viable cells. However, it is also evident that the proper use of reliable RMs in microbiological analysis is essential to support classical fields of application, such as method development, method validation and quality control. A thorough quality control in microbiological analysis and diagnostics helps to assure, e.g. a reliable control and sourcing of bacterial and viral load, and can, thereby, directly influence the quality of life in a general sense. Examples for the beneficial effects of a successful quality control, such as the identification and elimination of *Burkholderia cepacia* from a disinfectant used in a blood bank [1] or for the detection of micro-organisms in blood culture milieu [2], were recently described.

Apart from RMs based on viable cells, another challenge is the development of RMs for quantitative and qualitative molecular methods in microbiological analysis, comprising RMs based on nucleic acids, proteins or other molecular targets to identify selected organisms or parts thereof. Special demands here are certainly RMs for quantitative molecular analysis.

Legislation and microbiological criteria in the EU

Legislation related to the production/processing and placing on the market of foodstuffs in the EU was set out for decades in several Directives and Decisions as summarised in Table 1. In 2006, Regulation (EC) 852/2004 entered into force to ‘‘harmonise rules on the hygiene of foodstuffs, specific hygiene rules for food of animal origin, and specific rules for controls on products of animal origin intended for human consumption.’’ One of the goals is the introduction of relevant and meaningful microbiological criteria, based on a thorough risk assessment.

Another recent EC regulation (EC) 2073/2005 redefines limits for all major pathogens or their toxins/metabolites in different kinds of foodstuffs, and adapts limits to developments in food processing, food storage and consumer behaviour.

Table 1 Selected Council directives and Commission decisions regulating aspects of food production and marketing in the EU. Microbiological criteria were defined in several of these directives and decisions

Directive/decision	Short description
80/777/EEC	Exploitation and marketing of natural mineral waters
89/437/EEC	Marketing of egg products
91/492/EEC	Marketing of live bivalve molluscs
91/493 EEC	Marketing of fishery products
92/46 EEC	Marketing of raw milk, heat-treated milk and milk-based products
93/51 EEC	Criteria applicable to production of cooked crustaceans and molluscan shellfish
94/65 EC	Marketing of minced meat and meat preparations
2001/471 EC	Rules on general hygiene by operators
2073/2005 EC	Microbiological criteria for food

The IRMM's strategic activities and goals

Certified reference materials (CRMs) based on viable bacteria intended for quantitative analysis in microbiology

Existing CRMs with certified values for CFUs

Several pathogens such as *Salmonella* or *Listeria* shall not be present in foodstuffs. Therefore, the aim has to be to prove their presence/absence or allow only their presence below a certain limit of CFUs, depending on the matrix. Several internationally agreed standards (ISO or others) for microbiological methods exist and reliable microbiological RMs support the development, implementation and monitoring of methods described in these standards. Problematic for the development of reliable RMs for microbiological analysis is not only the commutability of the materials but also biological aspects, such as the stabilisation, survival and reactivation of viable bacteria.

The IRMM's portfolio of certified reference materials (CRMs) currently consists of six different micro-organisms (see Table 2), which were certified in the 1990s. These RMs were originally developed by RIVM (NL) using contaminated spray-dried milk powder as the matrix. All materials proved to be stable over the last decade (example given in Fig. 1), the materials are sufficiently homogeneous and are well accepted in many customer laboratories.

vOne major challenge, as extensively explained in the certification report for BCR-507R, is the fact that capsules with low CFU materials of, e.g. *Salmonella*, might not contain viable cells (see Table 3) and result in

Table 2 Certified reference materials (CRMs) for microbiology currently available at the IRMM

Organism	Unit	Certified value	Matrix	Application
Enterococcus faecium (BCR-506)	CFU	76[a], 72[a], 109[a]	Cells in milk powder	Cell count
Enterobacter cloacae (BCR-527)	CFU	34	Cells in milk powder	Cell count
Bacillus cereus (BCR528)	CFU	53.4–55.8[a]	Cells in milk powder	Cell count
Escherichia coli WR1 (BCR-594)	CFU	36[a], 40[a], 49[a], 56[a]	Cells in milk powder	Cell count
Listeria monocytogenes (BCR-595)	CFU	7.2	Cells in milk powder	Cell count
Salmonella typhimurium (BCR-507R)	CFU	5.0	Cells in milk powder	Cell count
E. coli O157 (IRMM-449)	–	Identity	Genomic DNA	Diagnostic PCR
L. monocytogenes (IRMM-447)	–	Identity	Genomic DNA	Diagnostic PCR

[a] Depending on media and/or method

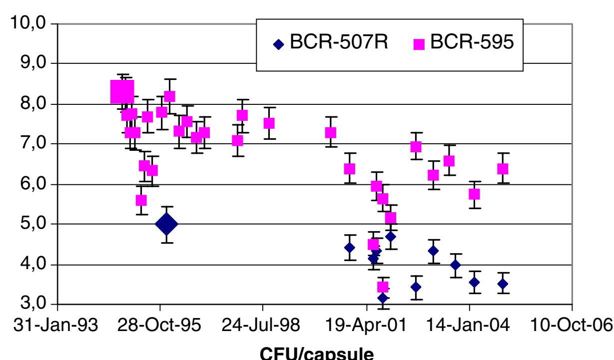

Fig. 1 Stability data of two low colony-forming unit (CFU) certified reference materials (CRMs) at the IRMM (BCR-507R *S. typhimurium*, BCR-595 *L. monocytogenes*). The drop in CFUs observed around 2001 for BCR-595 was most probably due to an unsatisfactory laboratory performance and not to a loss in stability

"zero counts" when used. In BCR-507R, this holds true for 1.1% of all measured capsules, i.e. 6 out of 554 samples. In practical terms, the CFU numbers per capsule follow a Poisson distribution and, consequently, all currently known "low CFU" materials always comprise samples with a large scatter around the mean of the respective batch and a fraction of "empty" samples. If this fraction is known, the probability to obtain zero counts can be calculated and the minimum number of capsules to be tested can easily be deduced. Based on this number, conclusions on the method and/or laboratory performance might be drawn when using such "low CFU" RMs. The sole possibility to overcome these practical and statistical inconveniences is the analysis of a certain (higher) number of samples, which, consequently, increases the price for quality control.

Therefore, the IRMM is engaged in the development of new "low CFU" materials that do not exhibit zero counts and which can be used in enumerations as well as in presence/absence tests.

CRMs under development and certification

The IRMM launched a worldwide call for the expression of interest in 2005 to acquire raw materials for the certification as microbiological RMs. The call focussed on 5 and 100 CFU RMs of *Escherichia coli* O157 and *S. enteritidis*. Amongst others, BTF Ltd., Sydney, Australia, responded to the call and we currently run the certification of 5 CFU BioBalls materials of *E. coli* O157 and of *S. enteritidis*. We intend to certify the numbers of CFUs for complete (ISO) methods. BTF Ltd. is known as the company that develops microbiological RMs as so-called "BioBalls," a product based on flow cytometry rather than on aliquoting methods [3]. This might be the reason for an increased precision of resulting RMs, a statement which is currently assessed on various RMs by the IRMM.

The *S. enteritidis* material showed in the homogeneity study a mean CFU value of 4.7 (SD 1.7) and single counts between 2 CFU and 7 CFU, and, hence, a more narrow distribution was observed with the BioBalls as compared to, e.g. BCR-507R. When testing the materials further on the selective RSV (Rappaport-Vassiliadis medium) and XLD (xylose lysine deoxycholate) media (see Fig. 2), the numbers decreased slightly, but no sample gave a zero count. The final CRM will be useful to cover the complete ISO standard 6579:2002 (Horizontal method for the detection of *Salmonella* spp.).

Table 3 The IRMM's low CFU CRMs and certified fractions of empty capsules

Organism	Certified value	95% confidence limits	Fraction (%) of capsules with no viable cells
S. typhimurium BCR-507R	5.0 CFU	4.5–5.4 CFU	1.1/1.6
L. monocytogenes BCR-595	7.2 CFU	6.8–7.6 CFU	0.075/1.2

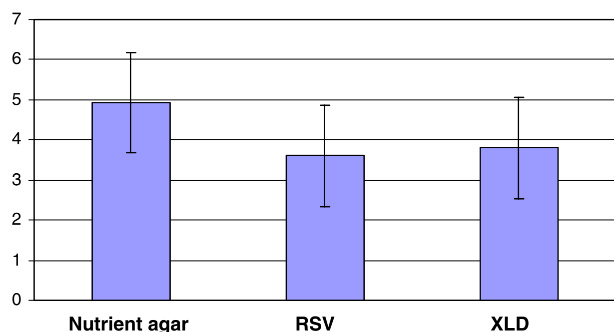

Fig. 2 Mean CFU (±SD) counts of IRMM-352, 5 CFU *S. enteritidis* BioBalls plated on three different media as defined in the relevant ISO method (ISO 6570:2002). RSV=selective Rappaport-Vassiliadis medium; XLD=xylose lysine deoxycholate agar

A number of additional BioBall RMs for water microbiology [4] are currently being assessed before entering the certification exercise. This includes *Pseudomonas aeruginosa*, *Candida albicans*, *Legionella pneumophilae*, *Citrobacter freundii* and several other important micro-organisms.

CRMs based on bacterial genomic DNA: CRMs for qualitative analysis to confirm the identity of selected bacteria

Precise quantitative analysis in microbiology is important for the enumeration of pathogens, e.g. in foodstuff, where certain organisms like salmonella shall not be present, or for clinically relevant micro-organisms. The development of PCR-based (polymerase chain reaction) methods has facilitated the precise identification of micro-organisms in all kinds of matrices. The IRMM has already released two CRMs and is currently certifying several additional RMs intended for qualitative analysis in microbiology. All materials are based on genomic DNA, which can be either mechanically sheared to lower sized molecules (up to ~20 kb) or can be intact DNA, allowing the analysis of complete bacterial genomes.

CRMs based on sheared genomic DNA

The IRMM's two CRMs [5, 6] based on sheared genomic DNA, IRMM-447 (genomic DNA of *L. monocytogenes*) and IRMM-449 (genomic DNA of *E. coli* O157) are listed in Table 2. Both materials are intended to be used as positive control in PCR reactions carried out for diagnostic purposes. The certification of these materials revealed several problems. One of the most important initial questions was how to stabilise the DNA and then how to recover it to a maximum after processing. Finally, the DNA was freeze-dried and filled under an argon atmosphere in specific plastic tubes, allowing a high degree of recovery. Both CRMs are certified for their identity and proved to be stable at –20°C to 18°C for at least 24 months. DNA stored at 40°C for 2 years could still be used in a PCR reaction, even if the DNA was no longer visible in an ethidiumbromide-stained agarose gel (see Fig. 3). This observation is in line with interesting results on the longevity of nucleic acids under favourable conditions, e.g. for the detection of pathogens in well conserved mummies [7].

The IRMM also started the certification of genomic DNA of *Campylobacter jejuni*, an important food pathogen, which is difficult to grow in culture.

CRMs based on intact genomic DNA

The European Council directive 70/524/EEC on the use of probiotic bacilli as additives in feeds and Directive 93/113/EC on the marketing of micro-organisms and their preparations in animal nutrition regulate the approval of specific genera of bacteria as feed additives. Initiated in a co-operation with the FEFANA (EU Feed Additives and Premixtures Association), the IRMM currently produces in a first step IRMM-311, an RM in the form of intact genomic DNA of *Bacillus licheniformis* DSM5749. This organism is officially approved by the EC as probiotic feed additive. The certified property of the RM will be the

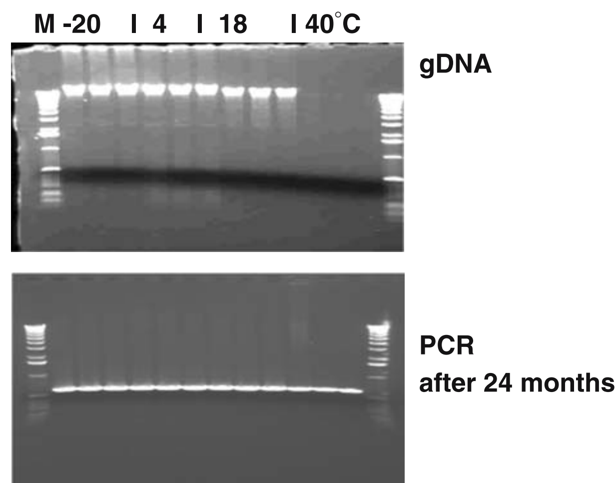

Fig. 3 Long-term stability of genomic DNA of IRMM-449 (*Escherichia coli* O157). No more DNA is visible after 24 months of storage at 40°C, but the material is still stable enough for its intended use: to serve as the template in polymerase chain reaction (PCR) reactions. A 693-bp-long fragment of the *fli* C gene was amplified in the PCR and correct products were obtained with all templates stored from –20 to 40°C for 24 months

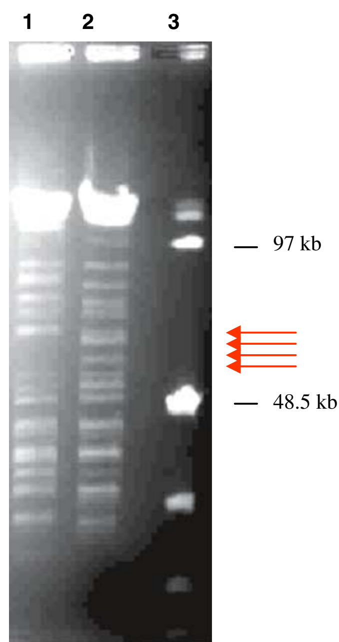

Fig. 4 Pulsed field gel electrophoresis (PFGE) of *Sfi*I digested genomic DNA containing agarose plugs of *Bacillus licheniformis* 7559 (*lane 1*) and IRMM-311 *B. licheniformis* DSM 5749 (*lane 2*). The *arrows* between 48.5 kb and 97 kb indicate an important region for the certified identity of IRMM-311. *Lane 3* is a DNA size marker of λ-DNA concatemers (BioRad, Belgium)

identity of this probiotic species. The plugs (agarose cubes) containing unsheared genomic DNA should be usable in pulsed field gel electrophoresis (PFGE) experiments in order to confirm the identity of the approved bacillus strain. Therefore, the DNA plugs will be subjected to digestion with the restriction enzyme *Sfi*I and the resulting DNA fragments will be separated by PFGE (see Fig. 4). The restriction fragments in a defined range, here between 60 kb and 100 kb, will allow the comparison of the isolated bacillus strains with the approved *B. licheniformis*.

The certification report will be accompanied by a method describing how to prepare intact genomic DNA of bacilli, which shall be controlled.

Perspectives

To summarise, the IRMM's main target is the development of RMs with the best possible accuracy, reliability and commutability. The materials shall allow either a quantification or an identification of a target organism or a target molecule.

One of the main aims is the development of robust CRMs that overcome matrix interferences in microbiological analysis. We search for RMs which are ideally applicable to different methods and cover a large range of fields, such as food, water, feed or environmental analysis. The best possible materials shall have a low interunit variation and, most importantly, must be fit for the intended purpose.

We, therefore, focus further on the development of new highly precise RMs for presence/absence tests (NO empty vials!) in quantitative microbiological analysis, put emphasis on research projects towards the development of rapid quantitative methods and develop RMs for the control and validation of such methods. Wherever information on quantities is not relevant, "identity RMs" for a qualitative analysis become attractive. The IRMM further pursues this path and extents its portfolio of microbiological RMs certified for their identity. Especially, the development of nucleic-acid-based analytical methods will be brought forward through new precise and reliable RMs. These challenges are to be tackled by the IRMM and its co-operation partners.

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