Application of New Methods to Regular Water Hygiene Control

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1. Introduction

Many years of experience have shown that monitoring hygienic water quality by classical microbiological tests is an important means for the supply of safe drinking water. Nevertheless, well-known disadvantages of conventional methods are that they are time-consuming, labour-intensive, and results are often taxonomically inaccurate. Additionally, in the last decade there has been increased sensibility towards so-called new pathogens, for instance viruses, giardia, and cryptosporidium, which are not or only partially covered by standard indicator systems. Recent developments, particularly concerning molecular biological methods, could help to overcome these problems. Thus, evaluation studies and standardization of procedures are necessary to force the approval of new methods by the health authorities, this being the basic requirement for routine application.

In the following, some new methods are presented in brief and the experiences gained by use of those methods in regular water hygiene control are summarized. In the microbiological laboratory of the public utilities of the city of Mainz several new biochemical, immunological and molecular biological methods are employed, partly by application in parallel to standard techniques. Some techniques were developed or are still under development in our institute Water Research Mainz (WFM Wasserforschung Mainz GmbH), others are commercial products.

2. Detection of E. coli and Coliform Bacteria

As alternative to the conventional detection of E. coli/Coliform Bacteria, an immunological test was developed which recognises members of the family Enterobacteriaceae including indicators and pathogens [1] (basic methodology in co-operation with Bitter-Suermann and co-workers, Medical University of Hannover). The test is based on a monoclonal antibody against the genus-specific antigen ECA (Enterobacterial common antigen) and is performed as Sandwich-ELISA in microtiter plates (fig. 1). A short microbiological enrichment step guarantees that the detected bacteria belong mostly to the E. coli/Coliform group. Following internal and external evaluation studies with about 2800 water samples, the test was approved as German Pre-Standard [2] and is now commercially available (ELISA Systems Enterobacteriaceae, Riedel-de Haën). Including 16-20 h pre-enrichment, results are available within 24 h.
Figure 1: Working scheme of the ECA-ELISA for the detection of Enterobacteriaceae
The rapid test Colilert (IDEXX) [3,4], which meanwhile has been noted in the National Primary Drinking Water Regulations in the United States [5], was evaluated in our laboratory with about 350 water samples [6]. Samples are prepared with readily packed reagents and incubated for 18 h. After that, specific enzymatic activities result in an emerging of colour or colour and fluorescence indicating the presence of E. coli and Coliform Bacteria.

Another helpful means for E. coli and Coliform analysis are recently developed chromogenic solid media, based on the same biochemical principles as Colilert, e.g. Chromocult Coliformen Agar (Merck). After a conventional enrichment step, subculture on this medium allows the distinction of E. coli and Coliform Bacteria by coloured colonies.

The methods mentioned before are regularly applied in our routine laboratory, which enables us to get the results much earlier then by using only standard techniques. Because of an intensive information and co-operation policy, the local health authorities accept results obtained with new methods - provided that the water samples are prepared with the standard methods in parallel. Thus, e.g. sanitation or construction work at the pipes can go on with only short delay, which already has saved a lot of money and trouble to the water works.

3. Detection of Legionella

On the national level there has been no standardization of methods for the detection of Legionella yet, and on the international level the standardization activities are still going on [7]. Conventional testing usually includes 3 to 7 days of preculture followed by selective subculture and eventually identification by biochemical serotyping, which makes up to 10 days altogether. In our institute a colony blot assay was developed [8] based on a monoclonal antibody against a genus-specific heat-shock protein (basic methodology in co-operation with Bitter-Suermann and co-workers, Medical University of Hannover). Legionella colonies from conventional pre-cultivation are blotted onto a membrane and treated as shown in figure 2. They may be enumerated very easy as blue coloured dots. Other identification steps are not necessary, results are available after 2 hours. The method has been evaluated by testing about 300 water samples in 6 different laboratories in Germany and one in the United States [9]. Results do harmonise very well with those obtained by conventional identification [10]. The test is now commercially available (ELISA Systems Legionella, Riedel-de Haen) and is one of the methods recommended for Legionella identification in the actual ISO draft for the detection of Legionella [7].
Figure 2: Working scheme of the colony blot assay for the detection of Legionella
Further, a gene probe test kit (EnviroAmp™ Legionella Kits, Applied Biosystems) has been tested [10]. Hereby members of the genus Legionella are detected and additionally the presence of Legionella pneumophila is indicated by hybridization of amplified sequences to probes specific for the Legionella 5SrRNA gene and the mip gene, respectively. Sample preparation by filtration and DNA-extraction, PCR, hybridization and colour development take about 4 h altogether. As results, blue dots appear on a membrane strip where the probes have been immobilized. The detection of Legionella is possible down to $10^1$-$10^2$ CFU in the sample and the concentration can be estimated by the intensity of the colour. Our investigations confirmed that the test was highly specific and sensitive, but in consequence is at risk of false-positive results. First, inhibition of PCR was a problem with several water samples, but occurred only very seldom with an amended version of the testkit [12]. In comparison to cultivation techniques, much more water samples appeared positive for Legionella. This is caused by the fact that cultivation techniques are optimized for Legionella pneumophila, whereas the gene probe test kit detects non-pneumophila species with nearly equal sensitivity. As a consequence, comparison of results obtained by conventional culture and by using the gene probe test kit is not practicable. However, the lack of standard methods (in Germany) enables the use one of the new methods for regular water samples which certainly saves a lot of time and might possibly enhance the accuracy of the results.

4. Detection of Enterococci

Recently we developed a method for the detection of enterococci in water samples by use of 23S rDNA targeted oligonucleotide probes in cooperation with Schleifer and coworkers from the Technical University of Munich [13]. Other results of this research project are presented by H. Meier elsewhere in the proceedings, especially dealing with in situ hybridization. In our institute a test scheme using PCR and specific hybridization was developed [14]. A short microbiological enrichment step preceding PCR appeared to be necessary to keep a low detection limit. The next step is a reverse hybridization in microtiter plates coated with immobilized probes followed by a colour development reaction (fig. 3). Enterococci may be identified as a taxonomic group as well as single species or group of species. The method yields results one day after reception of the water samples.

Ongoing investigations are aimed at an improvement of the technique, especially concerning the enrichment step. To improve enrichment and extraction of target DNA and to exclude possible PCR-inhibitors, the application of magnetic beads coated with rDNA probes is tested. Furthermore, the range of target bacteria to be detected by this test scheme will be widened including e.g. E. coli and Pseudomonas aeruginosa by use of specific probes, respectively. Therefore the test might in future be very helpful concerning the fast and specific detection of important indicator bacteria.
Detection of Enterococci by 23S rDNA-targeted oligonucleotides
(in development)

**Microbiological enrichment**
- Water sample → Enrichment broth → Incubation at 37 °C overnight

**PCR with incorporation of Digoxigenin**
- PCR-mix
- Microtiter plate coated with immobilized probes → Color development

**Hybridization**

**Detection**
- Photometer
- Total 24 to 26 hours

**Figure 3:** Working scheme of the detection of Enterococci by PCR and reverse hybridization
5. Examination of biofilms

In the last years it became notorious, that many problems with bacterial regrowth depend on biofilm formation in water conditioning and distribution systems. Research work is going on by use of different staining techniques and in situ hybridization of bacteria (fig. 4). Our intention was to get material for the investigations from biofilms growing in the distribution system directly, not from artificial systems. Therefore the biofilms were grown on different materials in modified flow devices (‘Robbin’s devices’) installed in the plumbing.

First results showed that bacterial diversity and activity is significantly depending from the raw water quality and the pipe materials [15]. Additionally, the presence of exemplary hygienically relevant bacteria is investigated.

6. Conclusions

As conclusion, we do believe that improvement of analytical methods in hygienic water control is beneficial. Even water works practising the state of technique have to be aware of dysfunctions in nearly every part of their systems. Therefore it has always been the policy of our institute to enforce the development and application of new and promising methods and to make the results of evaluation studies public. A little success has been the acceptance of rapid alternative methods for the detection of E. coli and Coliform Bacteria by the local health authorities, if standard methods are applied in parallel. Therefore, not only a lot of time and costs has already been saved to the public utilities, but also for the customers the safety of the distributed drinking water has been markedly enhanced.

However, new methods have not yet been considered by the Drinking Water Commission, and this lack of official approval strongly limits their application to regular water hygiene control. Of course, broad-range evaluation studies, performance of round robin, and standardization of the procedures are inevitable requirements to be fulfilled before a new technique may be applied in routine water hygiene control. An international regulation of the validation proceedings certainly would be helpful regarding the approval of methods by national or international Drinking Water Commissions. Regarding the advantages, the necessity to think and work in new categories should not be a hindrance for the application of new methods.
Examination of Biofilms

**Total cell count**
- DAPI-staining
  - (fluorescence change from yellow to blue when bound to DNA)

**Respiratory activity**
- CTC-staining
  - (reduction to fluorescent, insoluble formazan)

- biofilm
- Bacterium
- DNA
- enzymes
- specific gene probes
- extraction of DNA
- amplification of specific DNA sequences (PCR)
- in situ-hybridization
- in vitro-hybridization

Molecular biological identification of bacteria from biofilms

Figure 4: Working scheme of the examination of biofilms
References


