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New Molecular Approaches to Determine the Viability of Pathogens

Quantifying infectious agents in environmental samples or in foodstuffs plays a major role in preventing epidemics or infections in agriculture, too. Traditional methods of determining pathogens are increasingly being replaced by molecular biology techniques. However, these methods can only distinguish dead from live or infectious cells, if a selective step causing metabolism activation is included upstream of the measurement. This paper introduces such molecular biological techniques.

Reliable infection protection is of central importance in human, animal and phytohygiene. It serves to protect humans, animals and plants against pathogens, which could be taken up or be introduced into a certain area.

Accordingly, routine investigations are prescribed for sensitive areas on so-called indicator germs for example by the EU hygiene regulation, fertilizer or biowaste regulations. A positive result indicates that pathogens can be present in the examined material. The charge of such material can then not be further used according to the original destination and has typically to be hygienized again, which can cause high costs.

For the monitoring of the hygienic status classical microbiological, cultivation dependent methods are used. These have the advantage that viable organisms able to reproduce, pathogens or indicators, are determined. However, many of these methods show unsatisfactory specificity, produce false positive or negative results, need a too long operating time, require particularly trained personnel and expensive equipment, are too expensive or have other disadvantages. In addition, some important pathogens such as cryptosporidia or noroviruses cannot be cultivated or are very difficult to cultivate.

For this reason it was tried in the recent past in many fields to replace established classical methods by new molecular biological quantification procedures or to develop alternative methods. Meanwhile particularly PCR (Polymerase Chain Reaction) based procedures were introduced in food quality control monitoring, but also in plant protection serological procedures, which usually involve antibodies, against distinct surface structures of the target organisms are used apart from different PCR methods in routine analysis. Although above all the specificity and speed of the serological and PCR based molecular biological methods are beyond dispute, there is nevertheless doubt whether positive proofs with these and other molecular biological approaches are based on the presence of viable target organisms. In particular after a hygienization step, the target molecules could remain determinable for a long time in the sample, despite dying of the target organisms or loss of their infectivity and thus cause false positive results. This can lead to unnecessary costs and insecurity.

The goal of the present article is therefore to give an overview of the molecular biological techniques used in agricultural applications for the determination of pathogens, and to outline their areas of application and their limitations. Emphasis is put on PCR based techniques. Special consideration is given to the question in how far they are suitable to assess viability of the target organisms.

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Keywords

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Literature

Literature references can be called up under LT 07114 via internet http://www.landwirtschaftsverlag.com/landtech/local/literatur.htm.

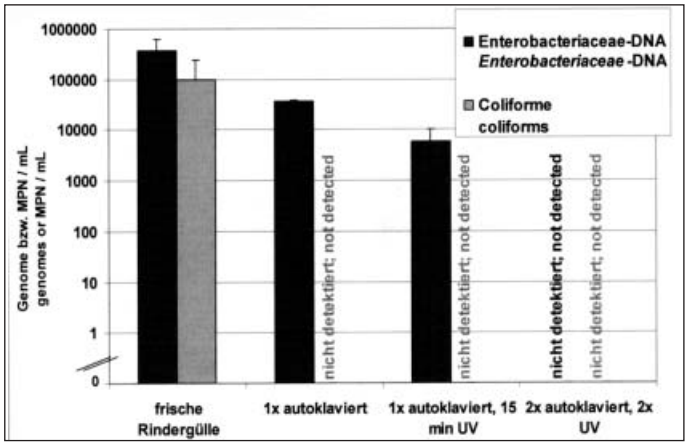

Fig. 1: Reduction by different physical hygienization treatments of DNA copies of Enterobacteriaceae and of viable counts (most probable number, MPN) of coliforms

Molecular biological methods and applications, an overview

The methods introduced in the following can be applied in principle for all agriculturally relevant media such as plants and their derivatives and/or processing products, samples of animals and animal products, soil and water. From these carrier media either the organisms/pathogens or the target molecules typically have to be extracted and be transferred into a pure or purer phase (not for „in-situ" methods) for quantification. This is mainly done to avoid bias of the measurement by disturbing compounds in the carrier media and for target concentration. Suitable routine methods were developed meanwhile, and extraction kits are commercially available. Mostly chemical/physical extraction routines are applied, occasionally in addition with a serological component [1, 2, 3]. The knowledge of the extraction efficiency is a substantial point for the determination of the method detection limit, which should be documented [4]. This limit must be as low as possible, the method thus have the best possible sensitivity from the sample to the measuring signal. This is important, because already traces of pathogens can cause diseases once they have settled in the host.

Mostly only small sample quantities are analyzable. This poses a problem, since they should be representative for usually large charge quantities. Accordingly sample selection for pooled samples must be well chosen, so that the data obtained can be extrapolated in a statistically sound way.

The methods can be subdivided according to the nature of the target molecules.

Surface structures

Serological methods use the ability of (marked) antibodies to bind specifically to distinct surface structures as from proteins or lipids, thereby rendering quantifiable the marked organism by a calibration curve. The determination can be made for example by an Enzyme Linked Immunosorbent Assay (ELISA) or by fluorescence microscopy and image analysis. Since surface structures are stable beyond death, their detection does not allow for assessing viability unless further steps are included upstream.

Deoxyribonucleic acid (DNA)

Different techniques have been derived in the meantime from the classical PCR with downstream gel electrophoresis verification of the formed products for the work on various questions. Specificity is controlled by the primer design and the temperature profile in the PCR. For reliable quantification only so-called Real-Time systems (qPCR, quantitative PCR) are suitable. Spiking of the sample with defined standards (standard spiking) allows for an absolute quantification [1, 4, 5].

The qPCR approach is highly specific, and with a suitable upstream extraction routine it can be applied for the very sensitive quantification in samples even from semi-liquid cattle manure. The determined DNA copies and the counts of viable organisms are virtually identical in systems with high biological activity like fresh manure. However, the values diverge partly drastically after killing measures or sublethal stress [6, 7] (*Fig. 1*). Because of the possibility for high throughput with rapid results and its high specificity and sensitivity, qPCR has outstanding suitability for the initial screening of samples. In such an „early warning system" the involvement of qPCR can be very economical and time-saving, since only the positive samples must be confirmed with physiology-based tests [1, 4, 5]. A still higher throughput might theoretically be achieved with DNA microarray systems. However, DNA amounts of the pathogens are needed here at a level as they usually do not occur in environmental samples.

Ribonucleic acids (RNA kinds)

Of the different RNA kinds mostly ribosomal RNA (rRNA) and messenger RNA (mRNA), but rarely transfer RNA (tRNA), are used as target molecules beside viral RNA (vRNA). With an upstream Reverse Transcription (RT, transcription of RNA in DNA) each RNA species can in principle be quantified by RTqPCR. The same applies to the NASBA (Nucleic Acid Sequence-Based Amplification), which specifically amplifies RNA in an isothermal reaction. The initial RNA concentration in the sample can be quantified by real-time product detection similarly as with qPCR. However dependent on the physiological state of the target organisms, variable net amounts of RNA molecules per cell or organism may be present. This makes an absolute quantification of organisms using RNA difficult and requires standardization.

In principle Fluorescence in-situ Hybridization (FISH) could be used for quantification of target organisms in environmental samples, however, microscopic image analysis is demanding and particularly with inhomogeneous sample material statistically hardly to be secured. Additional uncertainties arise particularly from the variable permeabilization of the target cells and the variable accessibility for the probes in complex environmental samples. The advantages of the FISH approach are more in the spatial representation of interactions between (micro) organisms and substrate.

Combined methods

If the methods listed above are applied without upstream methodological extension after a killing step, also dead organisms will be detected because of the stability of surface structures, DNA and RNA [5, 6, 7, 8]. They are therefore suitable for the documentation of hygienization or dying only with an upstream selection step which contains an activation of the metabolism. Because of the outstanding suitability of the molecular methods for specific, sensitive and rapid quantification, several combined procedures were established in the meantime which permit the assessment of viable organisms or infectious pathogens.

Including a selective enrichment step before the specific determination for instance by PCR (preferably qPCR) is a suitable option particularly for cultivable bacteria. Dead units do not propagate and are decisively thinned out. Quantification is done by Most Probable Number (MPN) analysis. Exemplarily this was shown for thermophilic campylobacters [7]. Although a cultivation step is included, this approach nevertheless saves time and money in relation to conventional procedures.

For pathogens which cannot be grown in artificial media, for example parasites such as cryptosporidia or certain viruses, cell culture systems (or animal hosts) can take over the step of the selective enrichment of living and the exclusion of dead and non-infectious units. Quantification can be done with a standard calibration curve, e.g. using (q)PCR or immunfluorescence (PCR, cell culture IFA) [7, 8]. However, cell cultures are extremely sensible, partly lengthy and require well trained personnel. For the routine practice attempts are made therefore to replace them by alternative procedures.

Here we could recently develop a procedure, which is based on quantification of the induceability of suitable genes (the ability to transcribe mRNA from DNA) [8]. The more specific mRNA is transcribed in a certain time after the induction (heat, substrate), the more activity is or active units are present. While after the killing treatment no mRNA production was induceable anymore, the untreated controls showed higher mRNA induceability and contents due to transcription during the induction time.