

Final Programme of the 2nd session of WG1 in COST 920

Quantification and detection of microbes by novel molecular tools

4. - 5. November 2004, Vienna, University for Veterinary Medicine,
Lecture Hall BOO, Institute for Milk Hygiene, Building GA.



and
University of Veterinary Medicine, Vienna



Oral presentations

Thursday, 4. 11. 2004

13 00 - 13 30 Introductions:

von Fircks, Wolf-Dietrich (Rector) Welcome

Thorns, Chris: COST920: a model of success

Threlfall, John: The current and the future of WG1/ COST 920 "Harmonisation of diagnostic and typing methods"

13 30 – 15 30 Session A. Development and harmonisation in microarray technology today

13 30 – 14 00

A.1 Key note lecture:

*Hoorfar, Jeffrey (invited speaker, Coordinator of FOOD PCR 1&2, DFVF, Denmark, confirmed)
The urgent need for harmonized molecular diagnostic tools throughout Europe*

14 00 – 15 30

A.2 Lectures (20 min):

*Bodrossy et al. (AT, Austrian Research Centers):
Microbial diagnostic microarrays - current potential and future perspectives*

*Malorny et al. (GE, Bundesinstitut für Risikobewertung):
Characterization and typing of Salmonella by DNA microarrays*

*Giammarinaro et al. (FR, INRA):
"Staph. Array" a new tool to identify staphylococcal strains*

*Schmidt et al. (AT, VBC Genomics):
Solid phase PCR on Microarrays for the detection of pathogens (not confirmed)*

15 30-16 00 Break

16 00-18 00 Session B: Risk Assessment needs qualitative data: Solutions to the problems of data generation by real-time PCR

16 00– 16 30

B.1 Key note lecture (30 min):

*Stüger, Peter (invited lecture, Joanneum Research Graz, Austria, confirmed):
Sampling for reliable data generation*

16 30– 17 30

B.2 Lectures (20 min):

*Jurgelevicius et al., (LT, National Veterinary Laboratory)
Application of Real-Time PCR Assay for the detection of Salmonella enterica in poultry carcasses and their products in Lithuania*

*Ripabelli et al., (IT, University of Molise)
Comparison of isolation and identification methods für Campylobacter isolated from meat samples collected in Italy*

*Grant et al., (UK, Health Protection Agency)
Real time PCR for detection of bacterial toxin genes*

17 30– 18 00

B.3 Special lecture (30 min):

*Fach, Patrick (invited lecture, AFSSA, France, confirmed)
Real-time PCR for detecting Shiga-toxin producing E.coli (STEC) associated with the world's most frequent clinical cases*

Evening reception (optional): Concert by the "Wiener Geigenquartett" (Wiener Philharmoniker) , Ceremony Hall of the UVM

Friday, 5. 11. 2004

09 00-11 30 Session C. Quantification of pathogens by traditional and molecular tools

09 00 – 09 30

C. 1 Key note lecture (30 min)

*Bruns, Ute (Institute for Animal Breeding and Genetics, Austria, confirmed)
Quantification of DNA/RNA from complex matrices at trace levels*

09 30 – 11 00

C.2 Lectures (20 min)

*Hein et al., (AT, University for Veterinary Medicine)
Difficulties to overcome in quantitative real-time PCR analysis*

*Rodríguez-Lázaro et al., (UK, Bristol University)
Application of real-time PCR for detection and quantification of *Listeria monocytogenes* in food*

*China et al., (BE, University of Liège)
Quantification of *Salmonella* in meat by real time PCR using a genetically modified strain*

*Flekna et al., (AT, University for Veterinary Medicine)
Differentiation of viable from non-viable *Campylobacter* by EMA-real-time PCR*

11 00 – 11 30

C.3 Special lecture (30 min)

*Beumer, Rijkelt (invited speaker, Wageningen University, The Netherlands, confirmed)
Enumeration and identification of pathogenic bacteria in food: traditional versus modern methods*

11 30 – 12 30 Lunch Break

12 30 – 15 00 Session D. Solutions to problems regarding the detection of pathogens in microbial communities

12 30 – 13 00

D.1 Key note lecture (30 min):

*Wagner, Michael (invited lecture, Institute of Microbial Ecology, Austria, confirmed)
Detection and quantification of unculturable bacteria*

13 00 – 14 30

D.2 Lectures (20 min):

*McLaughlin et al., (UK, Health Protection Agency)
Molecular tests for detection of gastrointestinal pathogens directly in faeces*

*Rossmannith et al., (AT, University of Veterinary Medicine)
Real Time PCR-inhibitory compounds in commonly used enrichment media and different food matrices*

*Possè et al., (BE, Department for Animal Product Quality):
Development of a detection method for non-O157 VTEC in food*

*Monika Ehling-Schulz et al., (GE, Technical University of Munich)
Molecular Assays for detection of emetic toxin producing *Bacillus cereus**

14 30 – 15 00

D.3 Special lecture (30min):

*Loessner, Martin (invited speaker, ETH Zürich, Switzerland, confirmed):
CBDs and tail spikes: novel approaches for isolation of bacterial cells*

15 00-15 10 Concluding remarks and Farewell

Poster Presentations:

*Bergsjø et al., (NO, National Veterinary Institute)
Molecular epidemiology of *Salmonella* spp. from gulls, factories, humans and animals in Norway*

*Wasył et al. (PL, National Veterinary Research Institute)
Characterisation of *Salmonella* Agona swine isolates*

*Suess et al. (AT, University for Veterinary Medicine)
Performance testing of three different real-time PCR assays for the quantification of *Salmonella* spp.*

*Kiem Mac et al. (GE, Bundesinstitut für Risikobewertung)
Practicability of a scheme for the simultaneous detection and isolation of *Yersinia* spp. in broilers*

*Martínez-Blanch et al., (ES, Institute of Agrochemistry and Food Technology, Valencia)
Detection of Toxigenic members of the *Bacillus cereus* group by quantitative Real-Time PCR*

The urgent need for harmonized molecular diagnostics tools throughout Europe

Jeffrey Hoorfar (Danish Institute for Food and Veterinary Research, Dept. of Microbiological Food Safety, Copenhagen)

Recognizing the need of standardization, in 1999 the European Commission approved the research project of 35 European institutes (FOOD-PCR www.PCR.dk), which aims to validate and standardize the use of diagnostic PCR for detection of bacterial pathogens in foods. Standard PCRs were devised for 5 pathogens: *S. enterica*, thermotolerant *Campylobacter spp.*, enterohemorrhagic *E. coli*, *L. monocytogenes* and *Y. enterocolitica*. The methodology was focused on 3 sample types from primary food production: chicken-rinse, pig-swab, and milk. The 3-year project comprised 6 work packages and 20 tasks. The tasks resulted in certified DNA material, preparation of a thermocycler proficiency testing, strategies for sample preparation and performance of ring trials. The work on automated detection resulted in several real-time PCRs. The validation proceeded in three phases. First, expert laboratories prepared DNA material, promising PCRs, and tested them for efficiency and specificity against comprehensive collections of reference strains. The final selected PCRs were optimized, and taken forward into Phase 2 to conduct inter-laboratory trials to confirm their specificity. This was conducted in two steps: in the first all reagents were supplied by the originating laboratory, while in the second the participants used their own. This provided a thorough evaluation of the efficiency and robustness of the PCRs. The sample pretreatment methods developed were based on current ISO pre-enrichment procedures. These procedures were adopted to allow PCR to replace conventional detection. Finally, the entire methods were subjected to inter-laboratory trials. The most important outcome of the project has been the production of standardized PCR publications, a biochemical kit for validation of different types and brands of thermocyclers and Certified Reference DNA material. The outcome of FOOD-PCR will facilitate the implementation of sensitive and cost-effective methods for detecting food borne pathogens by diagnostic laboratories. The Food-PCR project (www.foodpcr.com) will continue under the 6th RTD Programme, as part of the Network of Excellence MedVetNet (www.medvetnet.net).

Microbial diagnostic microarrays – current potential and future perspectives

Levente Bodrossy (Austrian Research Center, Seibersdorf)

Microbial diagnostic microarrays (MDMs) are a very promising tool in the detection, identification and community analysis of microbes. Their application potential spans most areas of microbiology. We have developed a set of techniques for microbial diagnostic microarrays, developed and validated a microarray and applied this in full scale studies. We are currently developing further arrays aiming at pathogen detection from different samples. Besides the above, future perspectives in MDMs will be discussed.

Characterization and typing of *Salmonella* by DNA microarrays

Burkhard Malorny, Beatriz Guerra and Reiner Helmuth (National Salmonella Reference Laboratory, Germany, Federal Institute for Risk Assessment, Berlin)

The development and validation of a DNA microarray for the characterization and typing of *Salmonella* isolates will be presented. The aim of the approach is the screening of routinely received *Salmonella* isolates for the presence of genes encoding serogroup specific antigens, fimbrial antigens, pathogenicity and virulence markers, antibiotic resistance determinants, phagetype specific markers, etc. The current version of the array contains 112 oligonucleotide probes with a length of mainly 40mers. Cy5-labelled genomic *Salmonella* DNA is hybridized to the oligonucleotide probes overnight, unbound DNA molecules are removed by washing and finally Cy5- signals are detected with a GenePix 4000B scanner (Axon Instruments, CA, USA). The validity of the results are confirmed by gene specific PCRs or phenotypic methods (O-antigens, H1- and H2- antigens).

The microarray could be a straightforward approach for the identification of new outbreak strains and to study the epidemiology of *Salmonella* on the genotypic level. An extension of the target probes is in progress.

"Staph. Array" a new tool to identify staphylococcal strains

Phillippe Giammarinaro, S. Leroy, J.P. Chacornac and R. Talon (INRA, Clermont-Ferrand)

Identification of Staphylococci is essential for clinicians or food microbiologists. As conventional methods shown their limits, we developed a rapid and accurate tool for identification of staphylococcal strains at species level by using oligonucleotide array technology. From variable regions of the *sodA* gene, encoding for a manganese dependent superoxide dismutase, 35 oligonucleotidic probes were selected. The *sodA* gene amplification products of 35 different species reference strains were hybridised to the array. In the condition of hybridisation we used, we observed that some probes could hybridised with targets obtained from closely related species. However a characteristic pattern of hybridisation can be relied to each species. We used these characteristic patterns to identify 80 strains from clinical, food or food environmental samples. Results of the array were confirmed by sequencing. In conclusion the oligonucleotide array we designed offers an accurate and rapid method for the identification of staphylococcal strains, isolated from clinical, environmental or food samples, at species level.

Solid-phase PCR on microarrays for the detection of pathogens

Wolfgang M. Schmidt (VBC-GENOMICS Bioscience Research GmbH, 1030 Vienna)

We present a DNA microarray based solid-phase PCR genotyping method for the direct analysis of a natural DNA sample with no requirement for extra sample preparation and purification steps. The method, which we call "on-chip PCR", is suitable for the amplification and simultaneous sequence characterization of DNA. In contrast to conventional PCR, the reaction is performed directly on the flat surface of a glass chip and reaction products are visualized by fluorescence scanning of the chip. On-chip PCR combines a liquid phase PCR using two sequence-specific primers with a solid-phase PCR using nested, allele-specific PCR primers covalently bound to the chip surface. The allele-specific oligonucleotide primer microarray is designed such that positive signals reveal the presence and nature of the target DNA of interest. The described method utilizes simultaneous probing of sense and antisense strand information in a multiple, highly replicated testing format allowing computer automated, unsupervised assignment of sequence information with high accuracy. We present an application of the described procedure for the identification of bacteria in clinical samples by amplifying either ribosomal rDNA or species-specific gene sequences. Our results show how on-chip PCR can be employed for the detection and identification of pathogens and demonstrate the beneficial combination of conventional PCR amplification with microarray technology for the development of simple and rapid DNA diagnostic systems.

Sampling for reliable data generation

Hans Peter Stüger (Institut für Angewandte Statistik und Systemanalyse, Joanneum Research, Graz)

The talk will cover principles of random sampling, data analysis and risk analysis. Sampling is one instrument to reduce uncertainty about real world phenomenons. Practical sampling requires determination of sample size, sample structure, frequency, population definition and measurement methods. It depends heavily on the purpose of the investigation and the subsequent decisions. The results of sampling based analysis are also connected with uncertainty, which can be quantified by confidence intervals. These depend on the accepted significance level and accuracy. Methods for construction of confidence intervals are classical statistics and bootstrap. There will be given a short introduction to a different approach, Bayesian statistics, which incorporates prior knowledge.

Application of Real-Time PCR Assay for the detection of *Salmonella enterica* in poultry carcasses and their products in Lithuania

Vaclovas Jurgelevicius, Jonas Milius, Juozas Pieskus, Irena Michalskiene (Lithuanian National Veterinary Laboratory, Institute of Immunology, Vilnius)

The objective of this study was to develop rapid, reproducible, and robust method for detecting *Salmonella enterica* in poultry carcasses and their products.

For the extraction and purification of DNA from the preenrichment culture, five methods (boiling, alkaline lysis, Chelex, Nucleospin, automated platform for nucleic acid extraction ABI PRISM 6700) were compared. The results of comparisons among the five DNA extraction methods showed significant differences except for the results from the boiling and Chelex methods (the two methods that produced the lowest threshold cycles). Boiling was selected as the preferred extraction method because it is the simplest and most rapid. The results of this study demonstrate that the 5' nuclease (TaqMan) PCR assay with the ABI Prism 7900HT system constitutes an effective and easy-to-perform method for detecting serotype Enteritidis in poultry carcasses and their products samples. Real-Time PCR is suitable for the rapid detection of salmonellae in chicken feces and, thus, to diagnose *Salmonella*-infected flocks.

Comparison of isolation and identification methods for *Campylobacter* isolated from meat samples collected in Italy

Ripabelli G., Fanelli I., Sammarco. M. L., Luzzi I.* (Department of Health Science, University of Molise, Via De Sanctis, 86100 Campobasso, Italy, *Department of Infectious, Parasitic and Immunomediated Disease, Istituto Superiore di Sanità, Rome).

Introduction: *Campylobacter* spp., are recognized as significant human bacterial pathogens, being responsible for increasing numbers of gastroenteritis cases worldwide.

Aim: To compare: 1) CCDA agar and membrane filtration techniques for the isolation of *Campylobacter* from meat samples; 2) to compare phenotypic and genotypic methods for the identification of *Campylobacter*.

Methods: 352 meat samples were examined for *Campylobacter* using selective enrichment. Two methods were used and these comprised Preston enrichment broth followed by cultivation onto selective charcoal cefoperazone desoxycholate agar (CCDA), and filtration through 0.45 µm cellulose triacetate membranes that were placed on non-selective blood agar. Suspected *Campylobacter* colonies were identified to genus and species level using phenotypic reactions and PCR for a fragment of the 16S rRNA gene (*Campylobacter* spp.), and of *mapA* (*C. jejuni*) and *ceuE* (*C. coli*) genes.

Results: Isolates phenotypically identified as *Campylobacter* spp. were recovered from 80 samples: 38 by both microbiological methods, 31 by selective agar only and 11 by membrane filtration only. Seventy-four of the isolates were confirmed as *Campylobacter* by 16S rDNA PCR and the six 'unconfirmed' were all isolated using CCDA agar. Using phenotypic reactions, *C. jejuni* was recovered from 43 samples, *C. coli* from 36, and both *C. jejuni* and *C. coli* from the remaining sample. The results of the phenotypic species identification were confirmed by PCR for all cultures, except for two *C. coli* identified by PCR as *C. jejuni*.

Discussion: although CCDA agar gave higher isolation rate than membrane filtration, all six unconfirmed cultures came from the selective agar plates. The physical selection performed by membranes provides more specific results, although the sensitivity was too much low to identify all positive samples. The low biochemical activity and frequent variability in results provides difficulty in the identification of *Campylobacter* spp.. Moreover, even kits, such as the API Campy system, cannot clearly differentiate among some species (On 1996). This could be responsible of the cultures unconfirmed by PCR. The Similar finding were described by Diergaardt and colleagues (2004) where *Campylobacter* spp. were confirmed in only 3 out of 22 isolates when analysed by 16S rRNA gene sequencing. Molecular methods, particularly PCR, have marked an important step forward in bacterial diagnostics. In species identification, PCR and gene sequences for 16S rRNA and 23 rRNA are used very frequently. Problems in biochemical species definition can be in the differentiation between *C. jejuni* and *C. coli*. The main phenotype discrimination test is the hippurate hydrolysis that is positive only for *C. jejuni*. Hence, a reliable identification of *Campylobacter* spp. should be supplemented with a molecular method.

Bibliography

- On SL. Identification methods for *Campylobacters*, *Helicobacters*, and related organisms. Clin Microbiol Rev. 1996;9(3):405-22.
- Diergaardt SM, Venter SN, Spreeth A, Theron J, Brozel VS. The occurrence of *Campylobacters* in water sources in South Africa. Water Res. 2004;38(10):2589-95.

Real time PCR for detection of bacterial toxin genes

Kathie Grant (Health Protection Agency, Colindale)

No abstract available

Real-time PCR for detecting Shiga-toxin producing *E. coli* (STEC) associated with the world's most frequent clinical cases

Fach Patrick (AFSSA-LERQAP, Laboratoire d'études et de recherches sur la qualité des aliments et sur les procédés agro-alimentaires, 23 Av du Général De Gaulle, 94706 Maisons-Alfort)

STEC O157 has emerged as an important cause of food-borne infections, but it has been recognized that STEC strains causing gastrointestinal diseases may belong to a wide range of serogroups. PCR assays have been described for detecting STEC and identifying their genes of virulence, but in STEC outbreaks it is important to determine the STEC serogroup rapidly to identify clusters of related cases. The conventional serotyping determination is a time-consuming procedure and is now being gradually replaced by PCR. We described 5'-nuclease PCR assays for detecting eight O-serogroups, H7 flagellar antigen and *stx* genes from the STEC associated with the world's most frequent clinical cases. A single set of primers was used to detect the genes *stx1* and *stx2* in the same reaction by 5'-nuclease PCR. Serotyping by 5'-nuclease PCR of STEC was based on the selection of primers and probes targeting the O-antigen gene clusters of *E. coli* O26, O55, O91, O111, O113, O157, the *eae* gene of *E. coli* O103, the O-island 29 of *E. coli* O145, and the flagellar H7 antigen gene. This work provides sensitive and specific tests for the rapid, reliable detection of the main pathogenic *E. coli* O-serogroups of major public health concern.

Quantification of DNA/RNA from complex matrices at trace levels

U. Bruns, S. Müller, R. Steinborn, B. Strobl, M. Karaghiosoff and M. Müller (Institute for Animal Breeding and Genetics, University for Veterinary Medicine, Vienna)

Real-time PCR is a potentially powerful tool for molecular-biological analytics due to its high sensitivity and specificity. This enforces its application in forensic analyses and studies of gene expression patterns at basal levels.

In this contribution, we will present a study dealing with species-specific meat and bone meal quantification in feedstuff. As light microscopy is still the only accepted method for EU legislative investigations, real-time PCR might become a routine use alternative once validated, for control of the EU wide MBM ban in feed intended for ruminants. We will line out the intrinsic problems of this application and the current state of potential.

In the second part we will consider aspects of Q-RT-PCR used for basal gene expression studies at low levels and point out some problems arising there regarding chemistry and source of RNA to quantify. Considerable differences were observed among cell-types as well as among cell-lines. New up-coming methods for high-throughput applications include e.g. custom-designed TaqMan array experiments (fluidic cards) for relative gene expression studies. However, these still need to be evaluated carefully despite their highly on reproducibility orientated design.

Difficulties to overcome in quantitative real-time PCR analysis

Ingeborg Hein¹, Gabriele Flekna², Peter Rossmann¹ and Martin Wagner¹ (Department of Veterinary Public Health and Food Science, ¹ Institute of Milk Hygiene, Milk Technology, and Food Science, ² Institute of Meat Hygiene, Meat Technology, and Food Science, University of Veterinary Medicine, Vienna)

Real-time PCR is an accurate method for quantification of DNA. The method has the potential to be applied for the direct quantification of microorganisms in food. However, there are some critical steps which will influence the quantitative out-put and thus have to be considered very carefully.

During DNA isolation a certain amount of target may be lost. Therefore the efficiency of the complete isolation procedure should be taken into account when analysing the results and an effort should be made to correct for loss of target during the DNA isolation.

Pathogenic bacteria are quantified in food in relation to a certain amount of this food. Therefore they have to be quantified absolutely and the result will depend on the correctness of the method which was chosen for preparation of the standard. This topic is also very important when it comes to the characterization of a real-time PCR assay with respect to the detection limit. The pros and cons of different approaches to create a standard as well as different methods for DNA measurement will be discussed.

PCR efficiency can vary between different real-time PCR assays but even between samples run on the same plate as well. Current methods to calculate the PCR efficiency within each PCR tube and correct the results for these differences will be presented.

Application of real-time PCR for detection and quantification of *Listeria monocytogenes* in food

David Rodríguez-Lázaro (Veterinary Molecular Microbiology, University of Bristol, Bristol)

Listeria monocytogenes is a human bacterial pathogen that is frequently found in food products. Ingestion of foods contaminated with this bacterium can result in listeriosis. The spread and persistence of *Listeria monocytogenes* in food products and food-processing factories are big concerns. Thus, the quality assurance programmes must include adequate microbiological control measures. The standard method for the identification of *L. monocytogenes* involves growth in pre-enrichment and selective media, followed by a battery of confirmatory biochemical and serological tests. However, it can take up to 10 days to obtain definitive data. The disadvantages of conventional culturing methods have prompted the development of alternative rapid molecular-based methods such as the real-time PCR (RTiPCR).

In this work different pre-PCR sample processing strategies to be coupled with a previously developed real-time PCR assay for the quantitative detection of *L. monocytogenes* in food products are evaluated. With the appropriate pre-PCR strategy, it was possible to detect and quantify very low load of *L. monocytogenes* in different products with excellent accuracy compared to the standard plate count method. In addition, a duplex RTiPCR system was developed to a simultaneous detection of *L. monocytogenes* and *Listeria* spp. Thus, the described strategies could be a promising alternative for the quantitative detection of *L. monocytogenes* in food products and processing factories.

Quantification of Salmonella in meat by real time PCR using a genetically modified strain

B. China (University of Liège, Liège)

Salmonella, one of the most frequent foodborne pathogen, is mainly responsible for gastrointestinal diseases. The detection of salmonella in food by classical methods involves a pre-enrichment step since the amount of Salmonella present can be low in comparison with the normal flora. Therefore, the quantification of the initial Salmonella content involves several dilutions meaning a lot of tubes and a lot of plates. Alternatively, it is possible to detect Salmonella by real time PCR. Nevertheless due to the pre-enrichment step the quantification is not directly possible. Nevertheless, a genetically modified Salmonella strain was constructed. Before the pre-enrichment step, a known amount of the mutant strain was added to the initial suspension. After pre-enrichment, a real time PCR was performed using a specific probe for wild type Salmonella and a specific probe for mutant Salmonella. If the growth rate of both bacteria has been the same, the ratio between wild type and mutant salmonella will be the same before and after pre-enrichment. Therefore, it is possible to estimate this ratio by real time PCR after pre-enrichment and from that to estimate the amount of wild type Salmonella present before pre-enrichment.

Differentiation of viable from non-viable *Campylobacter* spp. by EMA – real-time PCR

Gabriele Flekna¹, M. Wagner², F.J.Smulders¹, and Ingeborg Hein²

(Department of Veterinary Public Health and Food Science,¹ Institute of Meat Hygiene, Meat Technology, and Food Science, ² Institute of Milk Hygiene, Milk Technology, and Food Science, University of Veterinary Medicine, Vienna)

The permanent improvement of PCR detection systems for food-borne pathogens makes a differentiation of viable and dead bacteria indispensable. In order to improve a *Campylobacter* spp. quantification system we combined a real-time PCR detection system with ethidium bromide monoazide (EMA). EMA selectively enters dead bacteria and is irreversibly linked to DNA after light exposure thus inhibiting PCR amplification. It has been shown that the addition of this dye reduces the signal derived from dead bacteria, however no direct comparison between the results of the plate count method and real-time PCR has been presented so far. This comparison was the focus of the present work.

As a model we used a mixture of heat-treated (= non viable) and viable *C. jejuni* to optimize incubation and exposure time. We compared real-time PCR results with plate count derived colony counts. The effect of the treatment, especially conditions of incubation and exposure to light, on the results was studied with and without the addition of EMA.

Detection and Quantification of *Campylobacter jejuni* from chicken carcasses by real-time PCR.

Hans Lindmark and Paula Ågren (National Food Administration, Oslo)

DNA extraction was done directly on chicken carcass rinses and concentrated eluate was used as template. The 5´-nuclease PCR method described by Nogva et al. 2000 Appl. Environ. Microbiol 66:4029-4036 was used with minor modifications. The developed method was tested in parallel with the NMKL 119 method and direct plating on CBFS-plates. Twenty-two of 46 tested chicken carcasses were positive with the PCR-method. One PCR-positive sample was not detected with the culture methods and three culture positive samples was not detected by the PCR method. These three isolates were later identified as *Campylobacter coli*, which is not discovered by the PCR-method. Direct plating recorded *Campylobacter* concentrations between 0.5 and 88 cfu/g. The PCR, but not the DNA extraction, was done in duplicate for each sample. For the lower concentrations there was a considerable variation in the number of campylobacter between the two runs and also against the number from direct plating. The variation decreased with increased bacteria concentration. In conclusion; the sensitivity of the described method is equal with traditional culture methods. Concentrations above 50 cfu/g is likely to be required in order to do reliable quantifications.

Enumeration and identification of pathogenic bacteria in food: traditional versus modern methods.

R.R. Beumer and W.C. Hazeleger (Wageningen University, Wageningen)

Three basic protocols for the detection of pathogens exist: direct plating on selective media, direct selective enrichment and pre-enrichment on non-selective media. Dependent on the number of cells to be expected in a sample and/or the standards described (by law or otherwise), one or more of these procedures may be used for detection. The success of all three basic protocols depends on (1) the number and the state of the micro-organisms in the sample, (2) the selectivity of the media (a balance between inhibition of competitors and inhibition of the target organism), (3) conditions of incubation (time, temperature, presence of oxygen) and (4) the electivity of the isolation media (the ease of distinction between the target organism and competitive microflora).

Since traditional methods for the recovery and identification of pathogens in food are both time consuming and labour intensive, rapid and/or automated detection methods have been developed which achieve comparable results in less time. Food producers and distributors as well as public health authorities have great interest in rapid methods. In principle, these methods permit a more efficient control of raw materials, processes and products and may play an important role in food trade and product liability. However, improvements of traditional methods, in particular enrichment procedures for foodborne pathogens, are necessary to have full profit of molecular methods.

Detection and quantification of unculturable bacteria

Michael Wagner (Institute of Microbial Ecology, Austria, confirmed)

No abstract available

Molecular Tests for the detection of gastrointestinal pathogens directly in faeces.

Jim McLaughlin (Health Protection Agency, Colindale)

Enteric pathogens comprise a diverse group of viral, bacterial and eukaryotic parasites. Current methodologies to detect these pathogens are similarly diverse and include direct detection by light and electron microscopy, growth *in vitro* and subsequent characterisation, detection of specific antibody responses in the host, cell culture, and detection of pathogen-specific antigens by immunoassays. These methods, although reliable, can be labour intensive, time consuming, subjective and require a high skill base.

The purpose of this presentation will be to outline experience in the development and application of molecular tests for the tests for detection of pathogen-specific nucleic acid through amplification by PCR. Generic extraction of nucleic acid from faeces were adapted from the method of Boom utilised guanidinium isothiocyanate and activated silica. This extraction technique was combined with: reverse transcriptase with random priming to generate complementary DNA from viral RNA; and mechanical disruption with zirconium beads together precipitation with polyvinyl pyrrolidone for bacterial and parasite DNA. PCR based methods were developed for a range of pathogens based on nested and un-nested reactions together with block based and real-time formats. Examples of assay design and application, including the scavenging of DNA from conventionally stained faecal smears of glass microscope slides will be given.

Real Time PCR-inhibitory compounds in commonly used enrichment media and different food matrices

Peter Rossmannith, Ingeborg Hein and Martin Wagner (Institute for Milk Hygiene, University for Veterinary Medicine, Vienna)

Real-time PCR possibly provides a new tool for direct detection of food-pathogens out of food matrices and enrichment media. The ability to detect and quantify very small amounts of DNA with real-time PCR leads to new necessities regarding sample preparation and enrichment procedures.

We have tested the influence of various enrichment media used in detection of food-borne pathogens on real-time PCR with respect to inhibition of the reaction and effects on detection caused by fluorescence. The influence of food matrices on real-time PCR was also investigated. A real-time PCR assay with an artificial template and various labeled probes was performed, plate reads with the Stratagene Mx3000p thermocycler have been carried out and fluorescence within the samples was measured with a fluorescence-spectrometer. Regarding the enrichment media we found a stable real-time PCR assay crucial. Inhibitory effects on real-time PCR result from an interaction between media compounds as well as sub optimal performance of the assay itself. Fifteen representative food matrices have been investigated and three showed inhibitory effects on PCR, which were caused by compounds of the matrices. Interference of fluorescent compounds of the food matrices during data collection carried out by the Mx3000p thermocycler could not be shown.

Development of detection method for non-O157 VTEC in food

Björn Possé, L. De Zutter, L. Herman, M. Heyndrickx (Department for Animal Product Quality, Ghent)

A research team was assembled in close cooperation between the University of Ghent, represented by prof. L. De Zutter, and CLO-DVK (Department of Animal Product Quality and Transformation Technology), represented by dr. L. Herman, dr. M. Heyndrickx and lic. B. Possé to improve detection methods for non-O157 VTEC.

The verocytotoxin producing *Escherichia coli* strains (VTEC) are forming an important group of zoonotic human pathogenic *E. coli*. A collection of strains from serotypes often associated with human infections (ranging from abdominal pains to HUS) and a collection of non-pathogenic *E. coli* was fully characterised by PCR (virulence typing), RAPD (genetic fingerprinting) and specific culture media and broths.

Carbohydrate fermentation patterns, antibiotic susceptibility, acid tolerance and other characteristics were analysed to develop selective detection and isolation procedures for several non-O157 VTEC and for sorbitol positive *E. coli* O157. Several carbohydrates were identified as selective substances in differentiating VTEC serotypes and acid treatment together with antibiotic resistance analysis allows development of selective or elective culture media.

These procedures will be implemented in detection and isolation of non-O157 VTEC in food products and farm related samples and will provide preliminary information on non-O157 VTEC prevalence in Belgium.

Molecular Assays for Detection of Emetic Toxin Producing *Bacillus cereus*.

Molecular assays for detection of emetic toxin producing *B. cereus*

Monika Ehling-Schulz, Martina Fricker, Monica Grinell, and Siegfried Scherer (Technical University, Munich)

Bacillus cereus is a ubiquitous gram positive, endospore-forming, motile rod that is a commonly isolated from food. It can cause two types of food poisoning syndromes: emesis and diarrhoea. The emetic type of the disease is attributed to the heat-stable depsipeptide cereulide and symptoms resemble *Staphylococcus aureus* intoxication, but there were no rapid methods available to detect *B. cereus* strains causing this type of disease. Based on a PCR screening technique, a PCR fragment of unknown function was identified, which turned out to be specific for emetic toxin producing strains of *B. cereus*. The sequence of the fragment was determined and on this basis, a specific DNA probe was designed and a PCR assay was developed. Recently, a peptide synthetase gene was identified that was shown to be responsible for the non-ribosomal production of cereulide [1]. PCR primers, derived from this sequence, were used to develop a specific primer system for the cereulide synthetase gene. We will present the first molecular assays that allow a rapid and conclusive detection of emetic *B. cereus* strains in foods within a day and that provide the basis for the development of quantitative PCR systems.

[1] Ehling-Schulz *et al.*, 2005, AEM, in press.

CBDs and tail spikes: novel approaches for isolation of bacterial cells

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Listeria bacteriophages encode several different endolysins, which are cell wall lytic enzymes responsible for release of progeny from infected cells. The enzymes are composed of distinct functional domains: the N-termini harbour the catalytic activity, and the C-terminal cell wall binding domains (CBD) direct the enzymes to their substrate. They mediate specific activity of the enzymes towards *Listeria* cell walls, based upon their ability to bind to specific carbohydrate ligands with high affinity.

CBD polypeptides and fusions with GFP found a number of interesting applications. Besides specific labeling of target cells, they form the basis for a novel magnetic separation assay (termed CBD-MS), designed for rapid recovery and detection of *Listeria* cells from foods. The method is based on immobilization of target cells onto the surface of paramagnetic beads coated with recombinant CBD polypeptides. CBD-Beads are very effective and provide recovery rates in excess of 90% of *Listeria* cells from mixed bacterial communities. They enable rapid and sensitive detection from selective enrichment cultures. An alternative approach, suitable for Gram-negative bacteria such as *E. coli* or *Salmonella*, is the use of phage short tail spike proteins. They recognize and bind to components of the outer membrane, and are also able to immobilize target cells on solid surfaces.

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Molecular epidemiology of *Salmonella* spp. isolates from gulls, factories, humans and animals in Norway

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The study investigated the possible epidemiological connection between thirty one different isolates of the serovars *Salmonella* Agona, *S. Montevideo* and *S. Senftenberg* from sea gulls along the Norwegian coast and sixty seven corresponding isolates from other sources in Norway within the same period (2000-2001.) The PFGE patterns of the isolates indicate the presence of identical salmonella strains in sea gulls, fish meal factories and fish feed factories. The results suggest a risk of transmission between these sources and domestic animals or humans.

Characterisation of *Salmonella agona* swine isolates

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Salmonella Agona is one of the prevalent serovars found in symptomless infections in pigs in Poland. A retrospective study was performed to answer the question of *Salmonella Agona* clonal spread within the herds as well as to evaluate typing techniques. Biochemical characterisation revealed several biotypes within 27 tested isolates. Six resistance profiles and four plasmid profiles were observed in the tested isolates. Most of the strains harboured single, 92 kb plasmid. PFGE revealed 10 XbaI profiles. Most of the strains were unrelated showing ca. 50% of genome similarity. Several strains isolated in a short period of time (1998/1999) showed high genome similarity pointing out probable clonal spread with minor genetic rearrangement identified with PFGE analysis. Contaminated feed could also be a common source of *Salmonella Agona* infection. However as observed in other serovars, usually the infection is self-limiting. Typeability of the applied methods ranged from 0.52 to 1.00 while discrimination power did not exceed 0.89. *Salmonella Agona* due to self-limiting infections and low antibiotic resistance is not considered a primary swine pathogen in Poland. However the attention should be drawn on symptomless infections in swine herds because of public health concerns.

Performance testing of three different real-time PCR assays for the quantification of *Salmonella* spp.

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The need to rapidly assess possible foodborne pathogen contamination of raw materials and finished products, as well as the microbiological contamination risk during manufacturing procedures, demands for highly sensible, fast and reliable analytical tools. This has led to the development and refinement of alternative methods of analysis that are quicker and/or easier to perform than classical microbiological protocols. Real-time PCR is such a promising technique, able to quantify the burden of microbial pathogens by amplification of pathogen-specific target sequences.

In this study, we present a comparison of three different real-time PCR primer and probe sets for detection of *Salmonella* spp in over night enrichment cultures. Two of the assays target the *ttra* gene, encoding a regulatory factor for tetrathionate respiration, and the third one is located in the invasion protein A gene (*invA*).

Each assay was thoroughly assessed in terms of specificity of the target region and folding characteristics of the primer/probe combinations, specificity (exclusive detection of 101 *Salmonella* strains and failure of detection of 45 non-*Salmonella* strains), and sensitivity (limit of detection and limit of quantification).

It could be shown that each assay is highly specific for detecting *Salmonella* spp. in enrichment cultures. However, there are differences among the assays regarding PCR efficiency and hence sensitivity as well as robustness in the linear range of quantification indicating restricted power in routine use.

Practicability of a scheme for the simultaneous detection and isolation of *Yersinia* spp. in broilers

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Yersinia (Y.) enterocolitica is considered to be a foodborne pathogen. Many selective isolation and enrichment methods of *Yersinia* spp. in foods have been described. However, no single isolation procedure has focused simultaneously on the fast recovery and determination of *Yersinia* spp. in broilers. This study to determine the practicability of such a procedure was part of a research project dealing with the effect of *Yersinia enterocolitica* on broilers during a 36-days period. The procedure combined a minimum of biochemical tests, the PCR and the skills of the technical assistants involved. PCR was used to detect and confirm the genus *Yersinia*, the species *Yersinia enterocolitica*, the chromosomal *ail* and the plasmid-borne *virF* and *yopT* genes. It was the method of choice to cope with the accumulating amount of isolated strains during the research period. The PCR and isolation scheme presented here proved its applicability for this project despite rare occurrence of obstacles.

Detection of toxigenic members of the *Bacillus cereus* group by quantitative Real-Time PCR

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The *Bacillus cereus* group is a subdivision of the genus *Bacillus* that includes the closely related species *B. anthracis*, *B. cereus*, *B. thuringiensis* and *B. mycoides*. The species *B. pseudomycoides* and *B. weihenstephanensis*, more recently described, are also phylogenetically related and to a lesser extent considered within the group. These Gram-positive, spore-forming organisms are typically soil saprophytes that spread easily on many types of foods (Turnbull et al. 2002). *B. cereus* is widely recognized as a food poisoning organism. It produces an emetic toxin which causes vomiting and different enterotoxins causing diarrhea. Several *B. thuringiensis*, *B. mycoides* and *B. weihenstephanensis* strains also possess genes and produce enterotoxins similar to those of *B. cereus* (Hansen et al. 2001). These organisms can survive mild heat treatments and cause food poisoning or spoilage which constitutes a potential problem in the food industry.

Detection of this pathogen is usually carried out by culture techniques that include the use of selective media, biochemical reactions and other parameters for bacterial identification. This traditional procedure is time consuming and usually leads to misidentifications due to the high phenotypic relatedness between the species in the *B. cereus* group.

In the present work we have applied molecular genetic techniques as an alternative for a rapid and unequivocal detection of *B. cereus* group which would be very valuable in food safety assessment. A total of 68 strains were phenotypically identified according to ISO 7932 procedure and API test, and genotypically characterized by ISR and RAPD-M13 analysis. They included reference strains and food isolates within the *B. cereus* group and related *Bacillus* species and were tested for the presence of different virulence genes (cereolysine O (*clo*), enterotoxin FM (*entFM*), phosphatidylcholine-specific phospholipase C (*pc-plc*) and sphingomyelinase (*Sph*)) by PCR amplification using specific primers, previously described. The *pc-plc* gene showed the highest prevalence among the strains tested and was chosen for the design of *B. cereus* specific primers. Sequences were analyzed with ARB software package (<http://www.arb-home.de>) and specific primer sets were designed using Primer Express software version 1.0 (ABI Prim, PE Biosystems) in order to develop a SYBR Green I real-time PCR procedure. Specificity of designed primers was tested by conventional and quantitative PCR (Realtime PCR) using purified DNA of the *Bacillus* strains included in the study.

Key words: *Bacillus cereus* group, detection, identification, PCR.

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