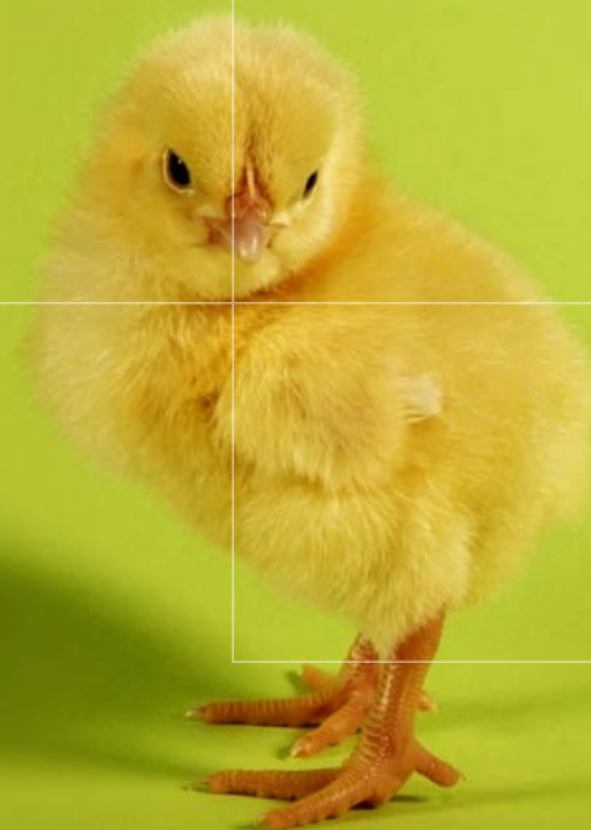


June 2007

A Molecular Safety Approach for Campylobacter (CampyFood)

- Novel molecular techniques in food safety
- Accurate detection and quantification of virulent *Campylobacter* spp. in food samples
- Food safety and hazard characterization with regard to quantitative risk assessment



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Title: A Molecular Safety Approach for Campylobacter (CampyFood)		
Nordic Innovation Centre project number: 04008		
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Abstract: The scope of the joint Nordic project was to harmonize ongoing research among the five participating Nordic laboratories through exchange of research personnel, internet homepage, integrated research projects, newsletters and workshops in order to prevent duplication of work and to ensure synergies. The overall objectives of the project have been (i) to implement novel molecular techniques in food safety, (ii) to accurately detect and quantify virulent Campylobacter spp. in food samples and (iii) to generate a better understanding of the virulence-determinant expression of food-borne pathogens in food matrices as a step towards formulating new strategies for food safety and hazard characterization with regard to quantitative risk assessment. Furthermore, implementation of effective control measures in primary production and further processing requires knowledge about the quantity of campylobacter's at various stages throughout the entire production chain. The network activities have generated: an internet homepage (www.campyfood.org), newsletters, workshops, and the mobility programme has so far involved eight exchanges, contributing to a closer collaboration between the CampyFood members. The mobility programme will end August 2007. The workshops have been open for attendance outside the CampyFood project for representatives from the food industry in the Nordic countries, private and governmental laboratories, food control authorities and companies marketing technologies for nucleic based analysis. The workshops have summarized the state-of-the-art for virulent Campylobacter spp., disseminated results obtained within the project, presented the developed molecular methods, and discussed the implementation of results.		
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Executive summary

The overall objectives of the project were:

- To harmonize ongoing research among the five participating Nordic laboratories through exchange of research personnel, internet homepage, integrated research projects, newsletters and workshops in order to prevent duplication of work and to ensure synergies.
- To implement novel molecular techniques in food safety
- To accurately detect and quantify virulent *Campylobacter* spp. in food samples
- To generate a better understanding of the virulence-determinant expression of food-borne pathogens in food matrices as a step towards formulating new strategies for food safety and hazard characterization with regard to quantitative risk assessment

These objectives have been met by:

- Generating coordinated projects within the network of CampyFood
- Generating two international spin off projects, namely (i) the Nordic NKJ project “Towards a standard for microarray-based characterisation of Salmonella isolates: a novel tool in risk assessments”, involving partners from National Food Institute, Denmark, MATFORSK, Norway and Lund University, Sweden and (ii) the European Integrated Research Programme BIOTRACER consisting of 47 partners with Jeffrey Hoorfar as coordinator.
- Demonstrating the usefulness of nucleic acid based techniques, and in particular diagnostic PCR, in food safety
- Improving the precision of quantifying food-borne pathogens using novel sample preparation methods prior to molecular techniques
- Improving the knowledge about microbial virulence expression in food matrices allowing us to formulate new molecular-based strategies for: (i) food formulation, (ii) food preservation and (iii) quantitative risk assessment.
- Using mathematical methods to improve molecular data interpretation

Method/implementation:

Information has been exchanged throughout the entire project by the use of an internet homepage, project meetings, progress reports and newsletters. Through harmonization and inter-Nordic validation of the methodology, the knowledge obtained during the project has been spread to industrial partners. This has been done through hands-on demonstrations, mobility research programmes and three workshops.

The workshops have been open for attendance outside the project for representatives from the food industry in the Nordic countries, private and governmental laboratories, food control authorities and companies marketing technologies for nucleic based analysis. The workshops have summarized the state-of-the-art for virulent *Campylobacter* spp., disseminate results obtained within the project, demonstrate the developed methods, and discuss the implementation of results. Yearly reports and a final report have been disseminated. The workshops have also strengthen the Nordic

network already established for PCR (www.pcr.dk), and extend it further by focusing on *Campylobacter* spp. in the MedVetNet Network of Excellence as well as the European Research Area Net “PROFORSAGE”.

The vehicle for know-how and knowledge transfer has been the exchange of research personnel; post-doctoral fellows, PhD students, MSc students as well as senior staff members. The research personnel performing work at the laboratories of DFVF, MATFORSK and/or Applied Microbiology have completed part of their research work during their stay in the host laboratories. Furthermore, transfer of the results to NMKL, NordVal and other Nordic standardization organs etc. have been organised by Prof. Jeffrey Hoorfar. He has extensive experience of exploitation and dissemination activities, and has been responsible for the internet homepage and the yearly newsletter.

Concrete results:

The network activities involving the following organisations Danish Meat Research Institute, Danpo, Icelandic Fisheries Laboratories, Lund University, Matforsk, National Food Administration (Sweden), National Food Institute (Denmark), National Veterinary Institute (Sweden), Norwegian Institute of Public Health, Poultry Processing (Finland), Prior (Norway), Procordia Food (Sweden), SIK (Sweden), Telemark University, University of Helsinki, University of Gothenburg, University of Umeå, VTT Biotech, have generated: an internet homepage (www.campyfood.org), five newsletters, and three workshops. The research mobility programme has so far involved eight exchanges, contributing to a closer collaboration between the CampyFood members. The mobility programme will end August 2007. Specific projects within the mobility programme have been (i) to develop new concepts for microarrays, (ii) to study microbial virulence expression, (iii) to investigate biofilm formation, (iv) to perform advanced trainee in real-time PCR, (v) to measure microbial exopolysaccharide formation, (vi) to study primer and probe-technologies on chicken meat and swine faeces and (vii) to investigate the colonisation levels of *Campylobacter jejuni* in chicken cecum samples.

Conclusions and recommendations:

CampyFood will continue the ongoing network activities in order to prevent duplication of work and ensure synergies. We see a need to improve and adjust molecular techniques for formulating new strategies for food safety and potentially for the development of novel control strategies. In addition, we see a need to improve our knowledge regarding virulence characteristics, and for a rapid detection/quantification of virulent strains/species in foods, as a novel approaches for risk assessment studies.

Furthermore, we will continue our efforts to generate spin off projects such as the ongoing NKJ project “Towards a standard for microarray-based characterisation of *Salmonella* isolates: a novel tool in risk assessments”, involving partners from National Food Institute, Denmark, MATFORSK, Norway and Lund University, Sweden. Recently a European Integrated Research Programme was initiated with Jeffery Hoorfar as coordinator. The project named BIOTRACER consists of 47

partners including several of the CampyFood organisations. The scope of the project is to improved bio-traceability of unintended microorganisms including *Campylobacter* and their substances in food and feed chains. In addition, BIOTRACER will integrate global microbial expression and survival data from food systems into existing empirical mathematical models, which will allow a holistic approach of these biological systems. The use of physiological data with information on virulence, gene expression and metabolite composition experienced in food-relevant conditions will generate data to model the behaviour of microorganisms introduced into the food chain. This will result in model systems in which the influence of the matrix on physiology and detection will be investigated.

Finally, obtained results will be of considerable interest to the scientific community, food manufacturers and regulators and so will be widely disseminated. This will include: refereed scientific publications, presentations at international conferences and articles in national/international magazines. For example members of the CampyFood network will be involved in the organisation of two upcoming international scientific conferences, FEMS Congress 2009 and Food Micro 2010. Both conferences will highlight microbial food safety/virulence.

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A Molecular Safety Approach for *Campylobacter*

Preface

Preventing the contamination of food by microbiological agents that can cause acute illness in humans and lead to considerable cost for society is a fundamental task of the highest priority. The scope of this three-year Nordic bridging project was to develop molecular methods for improved detection, quantification and virulence characteristics of food-borne pathogens, and in particular of *Campylobacter*. *Campylobacteriosis* is currently a commonly reported bacterial cause of acute gastroenteritis in the Nordic human population. Implementation of effective control measures in primary production and further processing requires knowledge about the presence and quantity of *Campylobacter*s at various stages in the entire production chain. Sensitive, efficient and quantitative detection methods will thus be necessary. Furthermore, knowledge on the nature, regulation, and mechanism of action of virulence determinants is indispensable for formulating new strategies for food safety and potentially for the development of novel control strategies.

A major concern is that new food-processing technologies are constantly being developed as human consumption patterns change. In particular, there is great concern regarding emerging pathogens often associated with convenience foods, such as fresh-cut vegetables, prepared sandwiches and ready-to-eat meals. The research proposed in this project will provide a better scientific understanding of how environmental conditions affect microbial virulence expression. Furthermore, areas such as the design of new food preservation methods, risk identification, risk assessment and risk management will all benefit from improved knowledge in this field. More natural ways of preserving food by adjusting abiotic factors so that, for example, virulence expression is repressed genetically, would not only render a decrease in the use of chemical additives to food possible, but also increase the quality of food. This is also a means of improving the health and well-being of consumers.

Background

Campylobacter is now recognized as a major enteric pathogen. Before 1972, when methods were developed for its isolation from faeces, it was believed to be primarily an animal pathogen causing abortion and enteritis in sheep and cattle. Today, food-borne enteropathogenic *Campylobacter* species *C. coli*, *C. lari*, and in particular *C. jejuni* are the leading cause of bacterial diarrhoea in many developed countries, including the Nordic countries. In several countries they cause more disease than *Salmonella* ssp. The incidence of *Campylobacteriosis* has risen continuously from 1992 to 2001 in the Nordic region. In Norway, the incidence increased by 145% from 1997 to 2001. The Nordic statistics of 2002 suggests that this increase may now have stopped due to improved routine analysis of broiler flocks etc. However, the actual number of human *Campylobacter* infections is presumably 10-20 times higher than the reported. The number of cases in Europe can be estimated to be as high as 5.7 million cases. The mechanisms by which *Campylobacter*s cause diarrhoea are poorly understood. The duration of illness is about 10 days, and most infections are self-limiting. Recently, it has been recognised that the paralytic condition, Guillian-Barré syndrome, can consist of a serious complication of *Campylobacter* infections.

*Campylobacter*s are widely distributed in the environment, and they are a natural part of the intestinal flora in both domesticated animals and wildlife. Sporadic infections in humans are mainly associated with contaminated food, water and pets: drinking

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unpasteurized milk, drinking undisinfected water, eating chicken, eating pork, barbecuing and living or working on a farm were identified as risk factors for indigenous campylobacter infection in the Nordic region. In Denmark 2002, the prevalence of thermotolerant *Campylobacter* spp. in raw chicken, turkey products, minced pork and minced beef was 42%, 21%, 0.2% and 0.1%, respectively. Consequently, contamination of meat may readily occur during the slaughter process. Consumption of poultry and poultry products is believed to be the primary source of human campylobacteriosis.

Main Objectives

The overall objectives of the project CampyFood are (i) to implement novel molecular techniques in food safety, (ii) to accurately detect and quantify virulent *Campylobacter* spp. in food samples and (iii) to generate a better understanding of the virulence-determinant expression of food-borne pathogens in food matrices as a step towards formulating new strategies for food safety and hazard characterization with regard to quantitative risk assessment. All these objectives have been met, and will be thoroughly discussed.

Furthermore, implementation of effective control measures in primary production and further processing requires knowledge about the quantity of campylobacter's at various stages throughout the entire production chain. However, the traditional detection of *Campylobacter* spp. using culture-based methods is time consuming, laborious, and difficult to adapt for quantitative analyses. In addition, viable but non-culturable cells are not detected. Nonetheless, it is still the most widely used tool for assessing the contamination within chicken flocks, animal herds, and in foods. Campylobacter-free chicken production relies today upon information about the status in flocks based on samples taken several days before slaughter. In addition, enumeration of *Campylobacter* spp. would be useful in monitoring the effect of intervention procedures ultimately aimed at reducing or eliminating the agent from foods.

Furthermore, knowledge on the nature, regulation, and mechanism of action of virulence determinants is indispensable in formulating new strategies for food safety and potentially for the development of novel control strategies. The Nordic participants, Nordic industry, food administrations and others will, based on the informal building of a contact network for industry and science – strengthening the links between industries, between industry and science, and between scientists – harmonize ongoing research and ensure synergistic effects.

Aim

The main purpose is to strengthen the bridge between the Nordic food industry, research institutes and universities in the field of microbiological food safety with special focus on campylobacters. We aim to use nucleic acid-based tools for accurate enumeration of virulent *Campylobacter* spp. in foods as well as to develop novel molecular tools for improved understanding of virulence characteristics of campylobacter. Such data would create the basis for effective control of virulent *Campylobacter* spp. in order to reduce the incidence of campylobacteriosis

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A Molecular Safety Approach for Campylobacter

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Mikael Skurnik, University of Helsinki
Maija-Liisa Suihko, VTT Biotech
Lea Lastikka, Poultry Processing

Homepage

The project has established a homepage with info about partners, meetings, publications, important links, guidelines etc. on www.campyfood.org. Stefan Jensen at the Department of Microbiology and Risk Assessment, National Food Institute, DTU, Denmark, is the webmaster (SJE@food.dtu.dk).

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Newsletter

Five newsletters have been published and distributed to the members of the cluster in order to inform the members about the development of the project. The newsletters are also available at the homepage in PowerPoint format.

Mobility Programme

The mobility programme has so far involved eight exchanges, contributing to a closer collaboration between the CampyFood members. The mobility programme will end August 2007.

1. Eyjolfur Reynisson, PhD student from Iceland has during a 3 months period 2005 been working at the Department of Microbiology and Risk Assessment, National Food Institute, DTU, Denmark with a special part of a Salmonella project. Eyjolfur did try some of the newest primer and probe-technologies on chicken meat and swine faeces as matrices and he did achieve some very good results, which will be of great value to this project and the CampyFood project.
2. Bettina Knudsen, Ph.D. student from KVL, Denmark, has during a three months stay 2005 been working at the Department of Applied Microbiology, Lund University, on alcohol-induced surface attachment of *Listeria monocytogenes*. Bettina has isolated and characterised extracellular polysaccharides thought to be involved in adhesion and biofilm formation, and used qRT-PCR in order to study the expression of the flagella gene, *flaA*, during growth in the presence and absence of 2,5% isopropanol.
3. Nina Wallin, Ph.D. student from Lund University has for a two month period 2005 been working within a campylobacter project at MATFORSK. Nina has investigated the colonisation levels of *Campylobacter jejuni* in chicken cecum samples. The quantity of *C. jejuni* relative to the normal flora was studied using real-time PCR together with TaqMan probes. The results were very encouraging, following the same trends as seen previously in the project. A manuscript is in progress including the results obtained through the collaboration.
4. Hugo Ahlm Grønlund, Ph.D. student from the Department of Microbiology and Risk Assessment, National Food Institute, DTU, Denmark, who by this time has worked six months at MATFORSK A/S in Norway 2006 developing a new concept in microarray to be used on *Salmonella*. Hugo will later in his Ph.D. be working four months at Lund University with the gene expression profile of *Salmonella* virulence in different environments.
5. Bettina Knudsen, Ph.D. student from KVL, Denmark, has been working in the Department of Applied Microbiology at Lund University for one month, starting October 2006, on alcohol-induced surface attachment of *Listeria monocytogenes*. Bettina will mainly be determining the expression of a flagella gene, *flaA*, in response to alcohol. The flagella genes are thought to be involved in adhesion and biofilm formation. She will be using qRT-PCR to measure the gene expression.
6. Terese Jensen, master student from KVL, Denmark, has during a one month period, October 2006, been working in the Department of Applied Microbiology at Lund University. Terese is working on her master thesis on Extracellular Polysaccharide (EPS) production by *Listeria monocytogenes*. EPS is a sticky substance thought to be involved in adhesion and biofilm formation. Terese will mainly be isolating EPS using alcohol precipitation for later characterisation using NMR.
7. Kerttu Valtanen, PhD student from the Department of Food and Environmental Hygiene at Helsinki University has during October 16-20, 2006 attended an advanced

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real-time PCR course in the Department of Applied Microbiology at Lund University. The course involved laboratory exercises in connection with lectures and seminars. The laboratory exercises cover different aspects of polymerase chain reaction such as (i) absolute and relative quantification, (ii) conventional and real-time PCR, (iii) optimisation of PCR, (iv) primer/probe design, and (v) sample preparation and strategies to generate PCR-compatible samples.

8. Hugo Ahlm Grønlund, Ph.D. student from the Department of Microbiology and Risk Assessment, National Food Institute, DTU, Denmark, has initiated his studies on virulence gene expression at the Department of Applied Microbiology, Lund University June 2007, see 4 above.

The First CampyFood Workshop

Forty-four scientists and industrial partners attended the first CampyFood Meeting at Tune Landboskole, Copenhagen, November 22-23, 2004. The Campylobacter situation was reviewed by four invited international experts, namely Professor Tom Humphrey (University of Bristol, UK), Dr. Gilles Salvat (Director of AFSSA, France), Dr Johan Lindblad (Swedish Poultry) and Professor Diane Newell (Veterinary Laboratory Agencies, UK). At the end of the meeting the most urgent questions for future research were highlighted and a writing group was established for the preparation of a grant application to be submitted to NKJ before June 2005.

Participants list

Denmark

Participant	Room	Dinner	2 x Lunch
Jeffrey Hoorfar	Yes	Yes	Yes
Stefan Jensen	Yes	Yes	Yes
Peter Saadbye	Yes	Yes	Yes
Michael Krause	Yes	Yes	Yes
Birgit Norrung	No	No	Yes
Bjarke Christensen	Yes	Yes	Yes
Hanne Rosenqvist	No	No	Yes
Soren Aabo	No	No	Yes
Berit Siemer	Yes	Yes	Yes
Anders Sorensen	Yes	Yes	Yes
Flemming Hansen	No	Yes	Yes
Lise Nersting	Yes	Yes	Yes
Niels Tornøe	Yes	Yes	Yes
Tom Humphrey	Yes	Yes	Yes
Gilles Salvat	Yes	Yes	Only one
Diane Newell	Yes	No	Only one

Sweden

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Peter Rådström	Yes	Yes	Yes
Patrik Ellström	Yes	Yes	Yes
Halfdan Grage	Yes	Yes	Yes
Elisabeth Holst	Yes	Yes	Yes
Torkel Wadström	Yes	Yes	Yes
Jörgen Nilsson	Yes	Yes	Yes

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Eva Olsson Engvall	Yes	Yes	Yes
Marika Hjertqvist	Yes	Yes	Yes
Elisabeth Borch	Yes	Yes	Yes
Marie-Louise Danielsson	Yes	Yes	Yes
Anna-Clara Rönner	Yes	Yes	Yes
Diana A Olsson	Yes	Yes	Yes
Börje Norling	Yes	Yes	Yes
Johan Lindblad	Yes	Yes	Yes

Norway

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Knut Rudi	Yes	Yes	Yes
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George Kapperud	Yes	Yes	Yes
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Finland

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Hilpi Rautelin	Yes	Yes	Yes
Rauni Karenlampi	Yes	Yes	Yes

Iceland

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Sigrun Gudmundsdottir	Yes	Yes	Yes
Franklin Georgsson	Yes	Yes	Yes

Poland

Participant	Room	Dinner	2 x Lunch
Kinga Wieczorek	Yes	Yes	Yes

Total number of participants: 44

The Second CampyFood Workshop

The second annual CampyFood meeting was arranged as a joined two-day seminar/workshop December 7-8, 2005 in Ski (Oslo), Norway. The name of the symposia was "Food Safety in a European Perspective". Plenary lectures were given by Dr. Herman BWM Koeter, European Food Safety Authority (EFSA), Dr. Maija Hatakka, European Commission, Health and Consumer Protection Directorate-General, Unit of Biological Risks, M. Sc. Annika Larsson, Arla Foods and Dr. Willem M de Vos, Wageningen Center for Food Sciences. The following issues were discussed within the CampyFood network:

1. Dr. Merete Hofshagen. Some information regarding the Norwegian Campylobacter surveillance program:
 - Norwegian broiler industry: Approx 500 farms with an average flock size of 12000.
 - An action plan started in May 2001 has produced a decrees of all pos. flocks.

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- No decrease in human cases.
- www.zoonose.no

2. Dr. Sigrun Gudmundsdottir. Information about Campylobacter has been showed on TV in order to make people think about work in the kitchen with food, chickens. Start 2001 all broiler flocks was and are tested for Campylobacter and action plans was started:

- Freezing of Campylobacter pos. broilers seems to have an effect.
- No decrease in Campylobacter positive flocks, despite the action.

3. Eva Møller Nielsen. Epidemiology of sporadic Campylobacter infections in Denmark:

- *C. jejuni* causes 95% of the campylobacter infections.
- Campylobacter are highly diverse – many different types.
- Different methods has been used to type,
- MLST, multi-locus sequence typing, has recently been developed for Campylobacter
- Indication of host-specific sequence types.
- Possible exchange of housekeeping genes between *C. coli* and *C. jejuni*

4. Even Heir. Some information on their work with typing:

- Strain-specific identification based on:
- Phenotyping characteristics
- Genetic characteristics
- Many of the current methods in use are not ok, so what kind of tool is needed?
- Indirect vs. direct typing – moving towards direct.
- MLST (see Eva Møller Nielsen) on Campylobacter ((Dingle et al 2001), (Miller et al. 2005), (Champion et al. 2005)) – www.genomesonline.org

5. Luca Cocoli. Some information on a larger project: “PathogenCombat”

- Control and prevention of emerging and future pathogens at cellular and molecular level.
- Objective; Provide new essential info and methods
- Implementation at industrial level; establish strategies and actions for dissemination
- www.pathogencombat.com

6. Nina Wallin. Detection of *Campylobacter jejuni* on colonization levels in chicken cecum samples using Real-time PCR

- The Biomek 2000 workstation was used for DNA isolation/purification
- ABI Prism 7900 platform is used for the PCR

7. Bettina Knudsen. Surface attachment and biofilm formation by *Listeria monocytogenes*.

- *L. monocytogenes* is very tough; 1 - 44°C, pH: 4,6 – 9,6, high salt tolerance, can grow in a refrigerator
- It was showed that isopropanol at low temperature gives a better production of extra-cellular polysaccharides (ESP).

8. Eyjolfur Reynisson. Salmonella, PCR, probes and chemistry in Real-time PCR

- Comparison of different dye/quencher combinations in a Taqman, Rotorgene and MX3000 (MX3005) assay.

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- Scorpions – The normal is a 2-step probe where the scorpions are a 3-step probe.
- LNA probes give a better CT than the other probes, based on MX3000.

The Third CampyFood Workshop

The final annual CampyFood meeting will be arranged, as part of a one-day seminar on the most up-to-date knowledge on food safety for industrial innovation. The meeting will be held at Danish Technological University in Lyngby, Denmark, August 29, 2007, entitled Food Safety for Industrial Innovation. The meeting is organised by Nordic Innovation Centre (NICE). Dr Marta Hugas from EFSA, Dr. Harmen Hofstra from the Safe Consortium, Prof Peter Raspor from the University of Ljubljana etc. will discuss the most current developments and focus areas within Food Safety in Europe, both from the perspectives of the EU government, the European research communities and the European food industry.

Detailed Scientific Report, Lund University, Sweden

Abstract

Real-time PCR is fast, sensitive, specific, and can deliver quantitative data; however, two disadvantages are that this technology is sensitive to inhibition by food and that it does not distinguish between DNA originating from viable, viable nonculturable (VNC), and dead cells. For this reason, real-time PCR has been combined with a novel discontinuous buoyant density gradient method, called flotation, in order to allow detection of only viable and VNC cells of thermotolerant campylobacters in chicken rinse samples. Studying the buoyant densities of different *Campylobacter* spp. showed that densities changed at different time points during growth; however, all varied between 1.065 and 1.109 g/ml. These data were then used to develop a flotation assay. Results showed that after flotation and real-time PCR, cell concentrations as low as 8.6×10^2 CFU/ml could be detected without culture enrichment and amounts as low as 2.6×10^3 CFU/ml could be quantified. Furthermore, subjecting viable cells and dead cells to flotation showed that viable cells were recovered after flotation treatment but that dead cell and/or their DNA was not detected. Also, when samples containing VNC cells mixed with dead cells were treated with flotation after storage at 4 or 20 degrees C for 21 days, a similar percentage resembling the VNC cell fraction was detected using real-time PCR and 5-cyano-2,3-ditolyl tetrazolium chloride-4',6'-diamidino-2-phenylindole staining (20% +/- 9% and 23% +/- 4%, respectively, at 4 degrees C; 11% +/- 4% and 10% +/- 2%, respectively, at 20 degrees C). This indicated that viable and VNC *Campylobacter* cells could be positively selected and quantified using the flotation method.

Executive summary (main objectives)

Campylobacter jejuni, *Campylobacter coli*, and *Campylobacter lari* have been recognized worldwide to be among the most frequently reported food-borne pathogens in humans. The traditional detection of *Campylobacter* spp. by culture-based methods is time-consuming, laborious, and difficult to adapt for quantitative analysis. Additionally, it has been reported that *Campylobacter* may go into a viable nonculturable (VNC) state, which is not detected by culture-based methods. The significance of this state for *Campylobacter* diagnostics is still controversial, as some studies have shown that organisms in the VNC state cannot colonize baby chicks, and for that reason the VNC state has been considered a degenerative form. Other studies have shown that VNC cells of this organism can in fact colonize baby chicks and

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suggest that the VNC state is a dormant form of the cell. Since no conclusive evidence seems yet available, there is a possibility that VNC cells may become infective and therefore should be detected by diagnostic methods for food-borne pathogens.

Recently, a novel sample treatment method called flotation, which is based on traditional buoyant density centrifugation, was developed. Using *Yersinia enterocolitica* as a model system, it was shown that this method can separate the target organism from environmental sample matrices and background flora (BGF) based on differences in buoyant densities. Further studies showed that this method could be successfully employed to reduce the risk of false-negative results due to detection of DNA originating from dead cells. The aim of the present study was to develop a flotation real-time PCR method which can be used to rapidly detect and quantify viable and VNC cells of *C. coli*, *C. jejuni*, and *C. lari* without false-positive results due to detection of DNA originating from dead cells. Also, to ensure correct detection of naturally contaminated samples, the buoyant density behaviour of different *Campylobacter* spp. strains was investigated at different time points during growth. Finally, this method was tested on spiked and naturally contaminated samples.

Method/implementation

For the spiked and naturally contaminated samples, chicken samples were spiked or analyzed directly by two different methods: direct plating using Preston agar and real-time PCR. To obtain quantitative real-time PCR measurements, a standard curve created for whole cells was used. For the second experiment, i.e., the VNC/dead cell experiment, cells of *C. jejuni* ATCC 33291, *C. jejuni* ATCC 29428, and *C. jejuni* CCUG 10937 were kept in Bolton broth for a period of 21 days at three different storage temperatures, i.e., 4°C, 20°C, or 42°C, under a microaerobic atmosphere and then stained with CTC (5-cyano-2,3-ditoly tetrazolium chloride) and DAPI (4'-6-diamino-2-phenylindole, dihydrochloride) as described by Cappelletti et al. Briefly, 0.5 ml brain heart infusion medium (Oxoid) was mixed with 0.1 ml of a 0.05 g/liter solution of pyruvic acid (Sigma) and 0.5 ml cell suspension. CTC was added to a final concentration of 5 mM and incubated for 4 h at 37°C. Cells were then harvested by filtration on black isopore polycarbonate membrane filters (0.2-µm pore size and 25-mm diameter; Millipore, Watford, England) and covered with 5 mg/liter DAPI (Sigma) solution for 5 min for counterstaining. Counts were obtained randomly by counting 2 times 20 fields per filter and, for each sample, two filters were counted.

The number of metabolically active cells (VNC) was divided by the total number of cells as determined by DAPI staining in order to acquire a relative amount of VNC cells in the mixture. To ensure the absence of culturable cells, plate counts were performed. After this, 1 ml of each mixture was subjected to flotation. After flotation, 1-ml samples were withdrawn from the *Campylobacter* recovery location and, as a control, from the top of the lower layer and the top of the upper layer. Afterwards the cells were added to 2-ml Eppendorf tubes, diluted with physiological saline to 2 ml (to obtain a density of the solution that allowed pelleting of cells), and centrifuged at $13,000 \times g_{\max}$ in a benchtop Eppendorf centrifuge for 5 min. Afterwards, 1.75 ml of the supernatant was removed and the cells were resuspended in the remaining 0.25 ml, after which they were heated for 5 min at 95°C to obtain cell lysis. These final samples were analyzed by quantitative PCR. Standard curves for absolute quantification were made with overnight cultures of known CFU/ml diluted in

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physiological saline. This implied that data acquired from both layers, even when originating from dead and VNC cells, were expressed in terms of CFU/ml.

Concrete results

Sample preparation prior to quantitative PCR (qPCR). The work on a novel discontinuous buoyant density gradient method, called floatation, in order to allow detection of only viable and viable nonculturable cells of thermotolerant campylobacters in chicken rinse samples has been finished and generated three publications.

Quantification of Campylobacter spp. from chicken rinse using floatation prior to real-time PCR. Real-time PCR is fast, sensitive, and specific and can be used to obtain quantitative data. However, two disadvantages are that this technology is sensitive to inhibition by food samples and that the method does not distinguish between DNA originating from viable, VNC and dead cells. For this reason, real-time PCR has been combined with a novel discontinuous buoyant density gradient method, called floatation in order to minimize PCR inhibition and to allow detection of only viable and VNC cells of *Campylobacter coli*, *C. jejuni* and *C. lari* in chicken rinse samples. Studying the buoyant densities of different Campylobacter strains showed that densities changed in the different growth phases, however all varied between 1.065 and 1.109 g/ml. These data were then used to develop a floatation assay. Results showed that after floatation and real-time PCR, cell concentrations as low as 8.6×10^2 CFU/ml could be detected without culture enrichment and amounts as low as 2.6×10^3 CFU/ml could be quantified using real-time PCR. Furthermore, subjecting viable cells and dead cells to floatation, showed that viable cells were recovered after floatation treatment but that dead cells and/or its DNA were not detected. Also, when samples containing VNC cells mixed with dead cells were treated with floatation, fractions ($20\% \pm 9\%$; $11\% \pm 4\%$) resembling the VNC cell fraction as determined by CTC-DAPI staining ($23\% \pm 4\%$; $10\% \pm 2\%$) were recovered. This indicated that VNC *Campylobacter* like viable *Campylobacter* cells could be positively selected and quantified.

Risk Assessment of False-Positive Quantitative Real-Time PCR Results in Food, due to Detection of DNA Originating from Dead Cells. Real-time PCR technology is increasingly used for detection and quantification of pathogens in food samples. A main disadvantage of nucleic acid detection is the inability to distinguish between signals originating from viable cells and DNA released from dead cells. In order to gain knowledge concerning risks of false-positive results due to detection of DNA originating from dead cells, quantitative PCR (qPCR) was used to investigate the degradation kinetics of free DNA in four types of meat samples. Results showed that the fastest degradation rate was observed (1 log unit per 0.5 h) in chicken homogenate, whereas the slowest rate was observed in pork rinse (1 log unit per 120.5 h). Overall results indicated that degradation occurred faster in chicken samples than in pork samples and faster at higher temperatures. Based on these results, it was concluded that, especially in pork samples, there is a risk of false-positive PCR results. This was confirmed in a quantitative study on cell death and signal persistence over a period of 28 days, employing three different methods, namely viable counts, direct qPCR, and finally floatation, a recently developed discontinuous density centrifugation method, followed by qPCR. Results showed that direct qPCR resulted

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in an overestimation of up to 10 times of the amount of cells in the samples compared with viable counts, due to detection of DNA from dead cells. However, after using floatation prior to qPCR, results resembled the viable count data. This indicates that by using of floatation as a sample treatment step prior to qPCR, the risk of false-positive PCR results due to detection of dead cells, can be minimized.

Food-borne microbial virulence, *Staphylococcus aureus*. A new project involving *Staphylococcus aureus* virulence expression was initiated 2005. It is known that certain *S. aureus* strains are able to induce staphylococcal food poisoning. It is not the bacteria themselves causing the disease, but toxins, so called enterotoxins, that are the causative agents. Today, the focus when producing safe food is eliminating and/or inhibiting the bacteria, the presence of toxins in the food is not examined. There is consequently a great need for characterizing the factors involved in enterotoxin production, to develop new control strategies. This can only be done through genetic studies of the bacteria. In this study a new real-time PCR method and an ELISA were developed and optimized to rapidly and specifically detect the genes and gene products of enterotoxins A and D in *S. aureus*, toxins closely associated with staphylococcal food poisoning. So far, we have developed a PCR method for the detection of one common *S. aureus* heat stable enterotoxin, namely enterotoxin A (SEA). A PCR assay of high specificity and sensitivity against SEA has been designed. The PCR assay was tested on nine different organisms, see Fig. 1, and 21 different *S. aureus* strains, data not shown. The assay showed only specificity against *S. aureus* enterotoxin A. The sensitivity was tested by running stepwise dilutions of DNA in the assay to determine the detection limit, see fig. 2. The detection limit was found to be at 10^{-9} g/ml of chromosomal DNA. The best amplification efficiency obtained was 0.92.

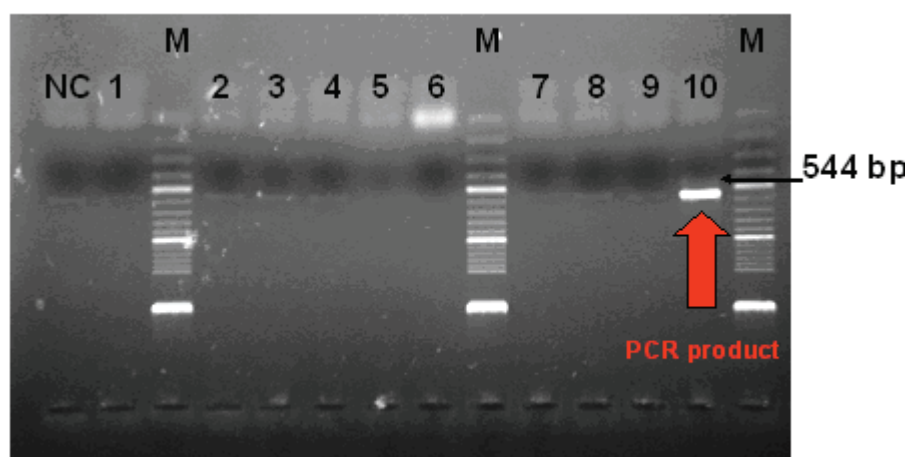
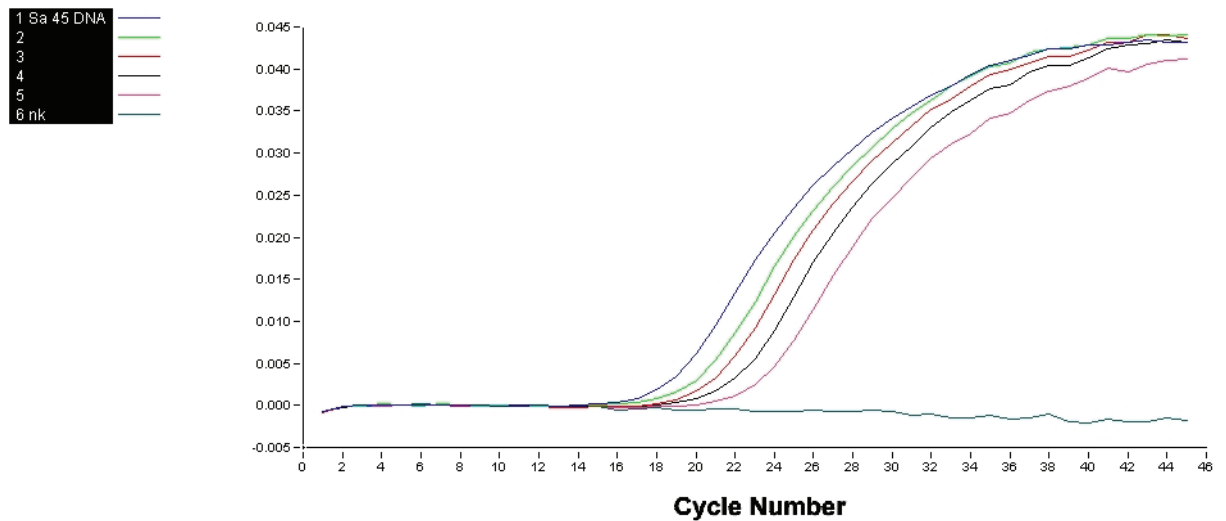


Figure 1. Agarose-gel picture after testing the specificity of the PCR assay. NC= negative control, M= molecular weight marker, lane 1: *S. aureus* NCTC 7428 (not producing SEA), 2: *E. coli* ATCC 25922, 3: *Bacteroides thetaiotaomicron* ATCC 29741, 4: *Stenotrophomonas maltophilia* CCUG 5866, 5: *Bacillus subtilis* 1A1, 6: *Proteus vulgaris* CCUG 6327, 7: *Proteus mirabilis* ATCC 43071, 8: *Serratia marcescens* ATCC 13880, 9: *Enterococcus faecalis* ATCC 29212, 10: *S. aureus* MU 50 (known to produce SEA)

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A



B

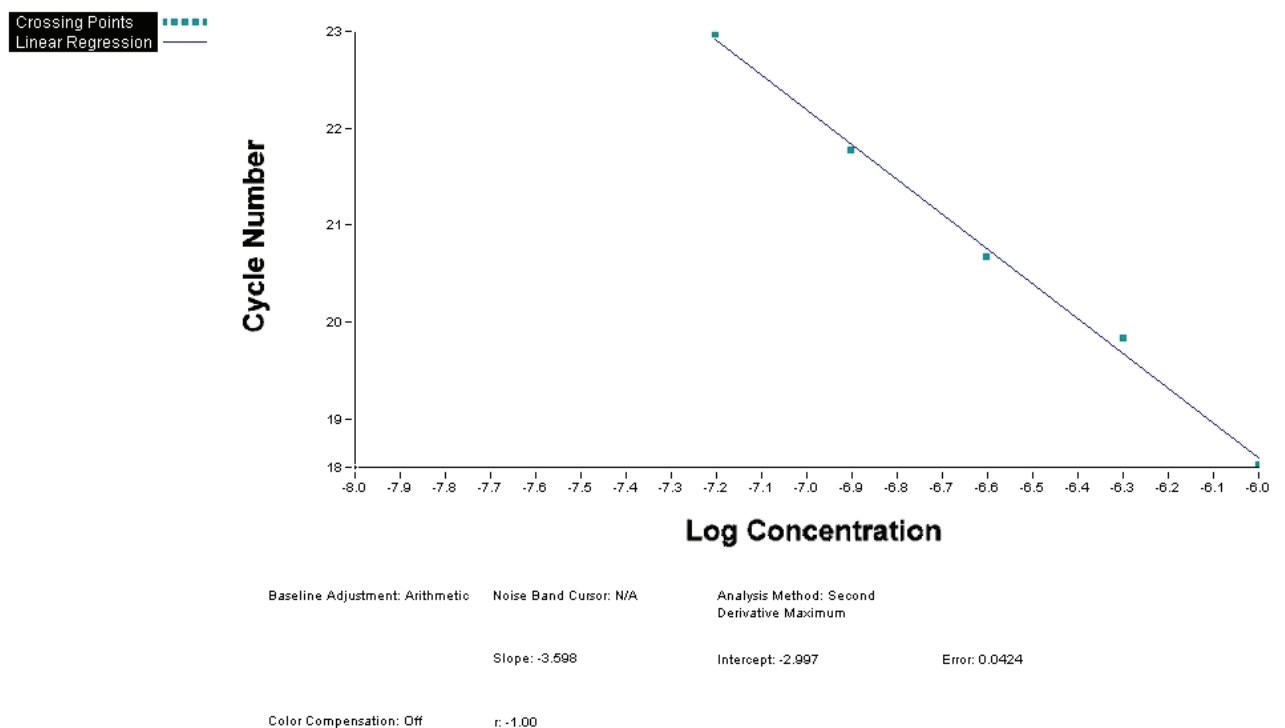


Figure 2. The graphs show one of the results of the sensitivity tests. Graph A shows the amplification of the DNA in the assay. Graph B shows the standard curve obtained by plotting the logarithms of initial DNA concentrations versus the cycle numbers.

Furthermore, we have designed a quantitative reverse transcription real-time PCR method (qRT-PCR) for the quantification of staphylococcal enterotoxin gene expression. *S. aureus* enterotoxin A was chosen for the method. If the assay wasn't functioning well the *S. aureus* strain would be exchanged or the PCR protocol modified. Our results demonstrate the correlation between the virulence expression levels (*sea* mRNA) to the levels of heat stable enterotoxin (SEA) using statistical

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models, see Fig. 1. The *sea* mRNA level was highest in late exponential phase while SEA was accumulating over time.

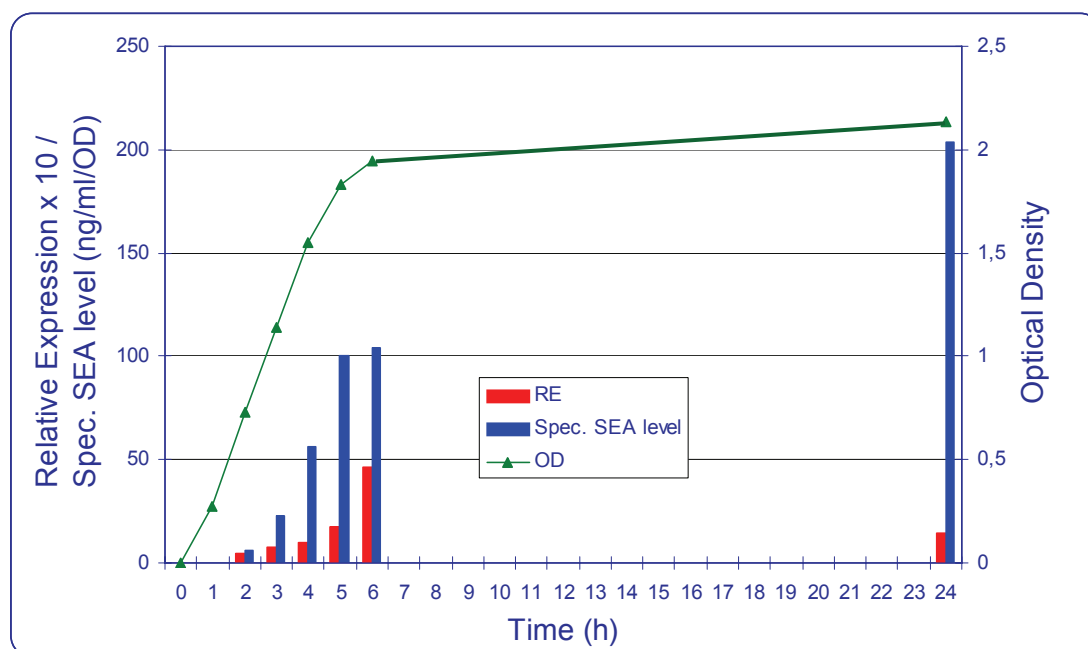


Figure 1. The growth curve, specific SEA level and relative expression of *S. aureus*. The expression of *sea* increased in the exponential phase and was highest in late exponential phase. The amount of SEA accumulated over time.

Food-borne microbial virulence, *Clostridium botulinum*. In recent years, non-proteolytic *Clostridium botulinum* has become a subject for concern due to the development of novel food products with alternative formulations and changing processing techniques, e.g. refrigerated processed foods of extended durability. One method that is used to extend shelf-life is modified atmosphere packaging. Previous studies on *C. botulinum* have mainly focused on the germination and outgrowth of spores in both microbiological growth medium and different foods. However, little is known about how environmental factors effect the expression of the botulinum neurotoxin gene. In the present study, we have followed the expression of neurotoxin using enzyme-linked immunosorbent assay, microarray, mouse bioassay and qRT-PCR. The effects of carbon dioxide (10%, 35%, and 70%) on type A, B and E botulinum neurotoxin gene expression were investigated during different growth phases. Significant differences were found between the different types, especially in the response to CO₂. For proteolytic *C. botulinum* type A and non-proteolytic *C. botulinum* type E, expression was the highest in late exponential phase, while it peaked in early stationary for *C. botulinum* type B. For non-proteolytic *C. botulinum* type B, neurotoxin expression increased with an elevated CO₂ concentration, with fivefold higher levels of mRNA and toxin being detected at 70% CO₂. The strongest induction of both growth and toxin expression for *C. botulinum* type E was observed at 35% CO₂. For *C. botulinum* type A maximum OD increased with increasing levels of CO₂, but no clear trend could be seen with regard to expression. The influence of the positive regulator gene, *cntR/A*, was also investigated and the overall expression profile studied using microarrays of *C. botulinum* strain Hall A.

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Statistical analysis and modelling. Before rapid methods such as conventional and real-time PCR or microarray analysis can be used on a regular basis, it has to be investigated to what extent these methods are influenced by each of the steps in the chain from sampling, through sample preparation and chemical analysis, to data analysis. The sampling strategy, sample preparation, and method of chemical analysis will determine what statistical methods and models will be appropriate, and the statistical analysis and the ensuing model will govern the design of the following experiments set up to optimise the analysis system as a whole. This has to be seen as an iterative process where the models entertained are successively refined through the accumulation and analysis of new data which has been obtained through carefully designed experiments based on the most adequate model in the previous step, applying the statistical principles for optimal design of experiments.

Statistical modelling of quantitative PCR: Statistical methods have been developed and evaluated as a step towards establishing a consensus on the correct way to analyse quantitative real-time PCR data in order to be able to compare the performance of different assays. Questions concerning detection limits, linear range of amplification, and amplification efficiency have been treated within a statistical framework, including repeated experiments, to account for the inherent variability in biological samples as well as in measurements. For this purpose, quantitative statistical methods, which embrace all the above mentioned aspects, have been developed and evaluated on real data. When an adequate model has been found and validated, it has been used as a platform for a rational, systematic, and efficient optimisation strategy, which will consist of a series of experiments carefully designed with the aim of optimising the PCR-system. The model-based statistical design of experiments employed at each stage in this procedure will ensure the most efficient use of the resources.

Statistical design and analysis of microarray experiments: Although the microarray technology certainly is a promising tool for comparative whole-genome expression analysis, the technology is still quite new and, consequently, there is a number of questions which still have not been answered in a conclusive manner. These questions range from the “low level” concerning the image analysis methods used to identify and distinguish the spots from the background on the microarrays and how to normalise or calibrate the obtained signals, to the “high level” of analysis where it has to be decided on how to interpret such a large number of gene expression levels and to find the adequate statistical methods to support exploratory analyses as well as establishing models for the testing of specific scientific hypotheses. The questions of modelling and analysis are closely linked to the issue of experimental design, e.g. for the development of appropriate methods for investigating gene regulation *in vivo*, comparing gene expression and transcription profiles under different environmental conditions. When using DNA-arrays for fingerprinting, the overall performance of the method for discrimination, classification, and identification may depend on the layout of the array and the micro-organism concerned, as well as on the statistical method applied. Thus, the methods under study have been evaluated and compared using well established statistical measures such as classification error rates. An additional question of importance is the robustness of the methods with respect to e.g. growth conditions and sample preparation. After proper validation, the model(s) chosen may be used for design of optimisation experiments, encompassing the choice of strains or species to include in the database on which the final model will be

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constructed, to ensure sufficient generality, and the number of replicate experiments needed to ensure sufficiently low error rates.

Conclusions

In conclusion, we have developed a new flotation technique for isolation of *C. coli*, *C. jejuni*, and *C. lari* in chicken rinse to be used prior to quantitative PCR, and the combined methods could detect *Campylobacter* spp. as low as 8.6×10^2 CFU/ml without culture enrichment. The method reduced PCR inhibition by BGF and chicken rinse samples and was able to minimize the risk of false-positive results due to detection of DNA from dead cells. Also, results indicated that VNC cells were recovered with viable cells and, therefore, false-negative results were avoided. Finally, the results emphasized the importance of studies concerning changes in buoyant density in different growth phases, especially when buoyant density is used for separation of target cells in sample treatment.

Recommendations

Due to the aforementioned problems with *Campylobacter* detection, PCR and real-time PCR have been increasingly used for detection. While this technology has tremendous potential for sensitive and simple identification, further studies addressing the quantification aspect of this technology are required before it can be widely accepted. There are limitations to the application of PCR technology. First of all, detection from food samples such as poultry or poultry rinses may be limited due to the presence of PCR inhibitors. Secondly, nucleic acid-based detection methods do not differentiate between nucleic acids originating from dead or viable/VNC cells, introducing a risk for false-positive results. This has resulted in a great demand for sample treatment methods that can overcome PCR inhibition and are capable of differentiating between viable and dead cells.

Publications and manuscripts acknowledging the Nordic Innovation Centre:

Wolffs P, Norling B, Hoorfar J, Griffiths M, Rådström P (2005) Quantification of *Campylobacter* spp. in chicken rinse samples by using flotation prior to real-time PCR. *Appl Environ Microbiol* 71:5759-5764

Wolffs P, Norling B, Rådström P (2005) Risk assessment of false-positive quantitative real-time PCR results in food, due to detection of DNA originating from dead cells. *J Microbiol Methods* 60:315-323

Wolffs P, Rådström P (2006) Real-time PCR for the detection of pathogens in meat. In LML Nollet and F Toldrá (ed.), *Advanced Technologies for Meat Processing*. CRC press Tylor & Francis Group, New York. p. 131-153

Van Niel EWJ, Velasco S, Grage H, Dueñas MT, Rådström P. A mathematical investigation on the influence of lactate on manganese homeostasis and exopolysaccharide production by *Pediococcus damnosus* 2.6. Manuscript

Årsköld E, Rådström P, van Niel EWJ. Modelling sucrose metabolism and reuteran formation by *Lactobacillus reuteri* ATCC 55730. Manuscript

Detailed Scientific Report, DTU, Denmark

Note: The Danish Institute for Food and Veterinary Research (DFVR) has been split in two institutes and a commercial unit and are now part of the Technical University of Denmark (DTU): The National Food Institute, the National Veterinary Institute and Dianova. Professor Jeffrey Hoorfar belongs to the Department of Microbiology and Risk Assessment within The National Food Institute from January 1, 2007.

Abstract

The production of certified, fresh *Campylobacter*-free poultry products has taken place in Denmark since 2002, and is the first example of fresh (unprocessed and non-frozen) chickens labelled as “*Campylobacter*-free”. In this study, a faster and automated real-time PCR was validated in comparative and collaborative trials according to international recommendations. The comparative real time PCR trial was performed against two reference culture protocols on 402 naturally-contaminated samples from: 99 shoe covers, 101 cloacae swabs, 102 neck skins from abattoirs, and 100 neck skin samples from retailers. The culturing included enrichment in both Bolton and Preston broths followed by the isolation on Preston agar and mCCDA. The comparative trial determined the relative accuracy, sensitivity and specificity of the real-time PCR method to be 98, 95 and 97 %, respectively. The collaborative trial included nine laboratories and consisted of testing neck skin, cloacae swab and shoe cover samples, spiked in three levels with *Campylobacter jejuni*. Analyzing the samples spiked with high levels of *Campylobacter*, an accuracy of 100 % was obtained for neck skin and cloacae swab samples. For the low levels of spiking, the accuracy was determined to be 100 % for neck skin and 92 % for cloacae swab samples. Thus, the method fulfilled the NordVal validation criteria on chicken neck skin and cloacae swab samples. Finally, on-site validation at the abattoir on a flock-basis was performed on 400 samples. The real-time PCR correctly identified all 10 out of 20 flocks as positive. The real-time PCR method has since been implemented at a major abattoir.

Executive summary (main objectives)

A Danish case-control study has identified consumption of undercooked poultry as one of the main causes of human infection. In 2003 approx. 35 % of Danish broiler flocks were infected with *C. jejuni*. Rapid detection methods are an important part of any *Campylobacter* intervention strategy, such as separated slaughtering of positive and negative flocks. The production of certified, fresh *Campylobacter*-free poultry products has been going on in Denmark since 2002, and is the world's first example of fresh chickens labelled for sale as “*Campylobacter*-free”. This certification has been based on a conventional gel electrophoresis PCR. In general, the superiority of PCR testing lies in its ability to detect the pathogen in cloacae swab samples within the same working shift, which due to its fast result makes it possible to slaughter chicken flocks with known *Campylobacter*-status, prior to suspected positive ones. Thus, PCR provides a rapid alternative to culture methods. The purpose of the present study was to validate a newly developed 16S rRNA gene based, *TagMan* PCR method in comparative and collaborative trials according to the recommendations of the Nordic validation organization, NordVal.

In order to increase the possibility of confirming PCR-positive results, the comparative validation was performed against two microbiological reference methods, applying different combinations of selective enrichment media and selective

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plating media. Most of the existing reports concerning the detection of thermotolerant *Campylobacter* have used only one microbiological reference method, whereas the present study included a combination of several protocols. In addition, to our knowledge, none of the published methods have been evaluated through a collaborative trial. It is essential for routine application that an analytical method is reliable and consistent. In order to establish these features, extensive testing of the robustness and performance characteristics of the method is required, preferably through a collaborative trial including end-use laboratories. Furthermore the PCR method used includes an internal amplification control (IAC), reinforcing its value as a diagnostic tool.

Method/implementation

The test reports and the real-time PCR analysis from the participating laboratories were carefully evaluated at return to the expert laboratory, and the results were approved for the inclusion in the statistical analysis, unless they fell into one of the following two categories: (i) Obvious performance deviation from the protocol, and/or (ii) Presence of target amplicons in the negative control results, indicating cross contamination at the participating laboratory.

The results obtained in the collaborative trial were analyzed according the recommendations from NordVal. The relative specificity was calculated for the un-spiked samples by the equation: $(1 - (FP / N-)) \times 100 \%$, where N- refers to the total number of un-spiked samples. The relative sensitivity was calculated for each level of spiking by the equation: $(TP / N+) \times 100 \%$, where N+ refers to the number of spiked samples. The relative accuracy was calculated for all levels of spiking by the equation: $((PA + NA + FP) / N) \times 100 \%$, where N is the number of samples tested.

On-site validation on flock-basis at abattoir:

At a major abattoir the real-time PCR method was validated against a routinely used gel-based PCR method approved previously by the Danish authorities to monitor the production. The two methods were performed in parallel on 400 pooled cloacae swab samples collected from 20 chicken flocks. Each cloacae swab sample was a pool of 25 swabs. Thus, 20 pooled samples represented 500 chickens per flock.

Concrete results

We developed a 12-hour *Salmonella* detection method, based on 8 hours of pre-enrichment followed by automated DNA extraction, and sensitive real-time PCR. The method was optimized to obtain the highest possible yield of cells and DNA. The growth of different *Salmonella* strains in various pre-enrichment media, and the effects of adding growth-promoting and selective reagents were explored, taking into account their PCR compatibility. The effects of 1) analysing larger volumes (1 to 5 ml) from pre-enriched samples, and introducing wash steps prior to DNA-extraction, 2) regulating the amount of paramagnetic particles (60 to 90 μ l) in the DNA-extraction, 3) eluting the DNA in reduced volumes (100 to 25 μ l), and 4) increasing the PCR template volume (5 to 20 μ l) were investigated. After 8 hours of pre-enrichment, buffered peptone water (BPW) yielded the highest number of *Salmonella*. Analysing minced meat samples, positive effects of increasing the initial sampling volume from 1 to 5 ml, and increasing the amount of paramagnetic particles to 90 μ l were observed. However, washing the pellet, and eluting the DNA in reduced volumes (25 and 50 μ l) had no positive effects and resulted in decreased

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reproducibility. Increasing the amount of PCR template DNA from 5 to 20 µl resulted in approx. 2 Ct-values improvement. The improved 12-hour PCR method was successfully compared to a reference culture method on 100 minced meat and poultry samples, with a relative accuracy of 99 %, a relative sensitivity of 98 % and a relative specificity of 100 %.

Conclusions

The Nordic poultry industry has benefited from this work. The PCR method has been implemented at Danpo A/S and AnalyCen A/S for the production of *Campylobacter*-free chickens. The former partner is the official testing laboratory of the Swedish Poultry producers. The real time PCR was validated and implemented at a large Danpo abattoir in Aars, Jutland, Denmark. The real-time PCR method correctly identified 10 out of 10 *Campylobacter*-positive, and 10 out of 10 *Campylobacter*-negative chicken flocks.

Recommendations

There is a substantial need for harmonization of DNA chip technologies from different Nordic countries for rapid and in-depth characterization and detection of *Campylobacter* isolates. This would create a novel tool to produce input for risk assessments.

Publications and manuscripts acknowledging the Nordic Innovation Centre

Josefsen MH, Krause M, Hansen F, Moos M, Hoorfar J (2007) Optimization of a 12-hour *TaqMan* PCR-based method for detection of *Salmonella* in meat. *Appl Environ Microbiol* 73:3040-3048

Detailed Scientific Report, MATFORSK, Norway

Abstract

Our main research focus during the period has been to develop high-throughput technologies for screening faecal samples for *Campylobacter jejuni* and other bacteria. We have addressed this aim developing an automated approach for DNA purification from chicken faecal samples. The quantification of *C. jejuni* was done by real-time PCR. We quantified *C. jejuni* relative to the total microflora. The total microflora was quantified by real-time PCR using the generally conserved gene 16S rDNA as target. We analysed triplicate samples of 253 randomly collected chicken caecal samples (sampled in 2001 and 2003) from Norwegian chicken flocks by real-time quantitative PCR. Our results are comparable to what we have previously reported for *C. jejuni* quantification. The difference is that we now do the quantification relative to the total flora. Normalisation to the total flora gives an increased reproducibility of the assay, and it allows us to determine the actual population fraction of the bacteria analysed. Furthermore, the DNA purification is universal, with the potential of detecting any bacteria in the samples analysed. We did a further evaluation and application of the high-throughput quantitative screening approach through the CampyFood exchange program (separate progress report by Nina Vallin). Matforsk was also responsible for the second annual CampyFood meeting held at Ski December 7 to 8 2005. During 2006 we have mainly focused on developing standardized approaches for using DNA microarrays in typing (Berget et al. 2007). The main challenge addressed by us is the combination of experimental

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error, typeability and discriminatory power. These challenges are very important when adapting DNA microarrays to routine diagnostics.

Executive summary (main objectives)

A bottleneck process in microbial community analysis is the nucleic acid purification. The current DNA purification procedures are generally manual and include steps that cannot easily be automated. Automation of the sample preparation process, however, is a requirement for high-throughput analyses. We have therefore developed an automated DNA purification system for high-throughput screenings of microbial diversity in gastrointestinal (GI) tract material. The automation was done by magnetic bead-based DNA purification using a Biomek2000 Workstation (Beckman Coulter, Fullerton, CA).

The method was evaluated, quantifying *C. jejuni* relative to the total microflora in chicken caecal samples.

The objective with the DNA microarray typing was to develop an approach for accurate and standardized typing of *C. jejuni*.

Method/implementation

Bacterial strains used for optimisation and validation of lysis treatment and DNA purification. *Lactobacillus reuteri* DSM 20016 (type strain; Deutsche Sammlung von Mikroorganismen) was used for the initial optimisation of the mechanical cell lysis step. This bacterium is known to have a rigid cell wall and is thus difficult to lyse. *Lactobacillus* spp. is also an important inhabitant in chicken GI tracts. We used 25 different strains in the evaluation of the optimized method. Strains were grown aerobically, microaerobically or anaerobically in MRS- (CM359, Oxoid), BHI- (CM225, Oxoid), Cooked Meat- (Difco), or Mueller-Hinton-broth (CM405, Oxoid) overnight at 30, 37, or 42°C (culture and atmospheric conditions dependent on species).

Optimisation of lysis treatment and DNA purification. Overnight cultures were concentrated by centrifugation in a microcentrifuge at 7,500 rpm for 10 minutes; supernatant was removed and pellets resuspended in 4M guanidinium thiocyanate (GTC). For enzymatic lysis, pellets were resuspended in an enzymatic lysisbuffer (lysozyme, mutanolysine and Solution 1 [50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0]) and incubated for 30 minutes at 37°C. 4 M GTC in ratio 2:1 was then added to the treated sample. Five hundred µl from each dilution were transferred to a sterile FastPrep-tube (Qbiogene Inc., Carlsbad, CA) containing 250 mg glass beads, 106 microns and finer (Sigma-Aldrich, Steinheim, Germany), and samples were homogenised for 40-80 seconds in FastPrep Instrument (Qbiogene). Wells in a 96-wells Greiner U-plate (Greiner bio-one, Frickenhausen, Germany) were filled with 170 µl sample and 10 µl paramagnetic Silica particles (Merck, Darmstadt, Germany) and transferred to a Biomek® 2000 Workstation (Beckman Coulter). One percent Sarkosyl was added after mechanical lysis, and the plate was incubated at 65°C for 10 minutes and another 10 minutes at room temperature. The supernatant was removed after lysis, and the paramagnetic beads were washed twice with ethanol (50-70 %). DNA was eluted from the silica particles by suspension of the particles in

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100 µl Buffer C (1 mM EDTA pH 8.0, 10 mM Tris-HCl pH 8.0) at 65°C for 15-30 minutes.

Optimisation and evaluation of automated DNA purification. Defined cultures were used for the initial optimisation of the automated DNA purification system. Parameters were optimised with respect to DNA yield, reproducibility and absence of PCR inhibitors. Parameters tested included a combination of enzymatic and mechanical lysis, ethanol concentration used for washing and elution time. Basically, the lysozyme treatment gave DNA that was more degraded compared to the use of a mechanical/chemical lysis (results not shown). A lysis procedure with an extended mechanical treatment, 80 seconds vs. 40 seconds of homogenisation, did not seem to result in any higher DNA yield. We found that a lower volume concentration (50 %) of ethanol gave a slightly better DNA yield than higher concentrations (70 %). The reason for this could be that ethanol increases the binding of DNA to the paramagnetic beads, making the DNA difficult to elute. We also found that elution time had an impact on the DNA yield. Doubling of the elution time from 15 to 30 minutes gave an improved yield. A mixing step before removing the paramagnetic beads after elution also had a positive effect on the yield. The final optimised protocol involves use of mechanical lysis for 40 seconds in the FastPrep® instrument, 50 % ethanol for washing and 30 minutes with elution of DNA from the silica particles.

The optimised protocol was evaluated using a selection of different bacterial strains (in total 25 isolates), both Gram negative and Gram positive bacteria. Purified DNA from these isolates was tested by real-time PCR using the universal 16S rDNA primer- and probe set. The different bacterial isolates gave CT-values in the range of 16-19, and there were no systematic differences in DNA yield between the G-positive and G-negative bacteria. The differences in CT-values could both be due to culture cell densities and differences in 16S rDNA gene copy numbers for the bacteria analysed. Further confirmation of lysis was done for all the strains by DAPI staining. DAPI stains nucleic acids, and all the samples had a more diffuse appearance after cell lysis due to the release of DNA compared to the culture stained before cell lysis (results not shown).

Important parameters when evaluating the automated DNA purification are the potential presence of PCR inhibitory compounds, cross contamination, DNA yield and reproducibility. These parameters were evaluated for chicken caecal material. After the DNA purification described here, DNA from more than 1 mg could be used in a 25 µl PCR reaction without detectable inhibition. Previous results have shown that chicken caecal material contains very high levels of PCR inhibitors, with less than 0.05 mg caecal material in 25 µl reaction being inhibitory. This indicates that our DNA purification removes most of the inhibitory substances present in these samples.

The potential for cross-contamination is a concern with automated DNA purification [23]. We tested cross-contamination in our automated DNA purification by filling every second well in a microtiter plate (n = 96) with caecum sample and sterile water (negative DNA isolation control), before running the isolation protocol. The amount of DNA in each well was subsequently tested using the universal 16S rDNA real-time PCR. The average CT-values for both the negative DNA isolation controls and the no template controls (NTC) were 33.1 (standard deviation of 0.5 and 0.4, respectively). The DNA detected in these samples is probably the DNA contamination of the DNA

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polymerase used. In contrast, the positive controls had a high DNA content with an average CT-value of 19.4 (standard deviation of 1.0). This indicates a very low DNA cross-contamination level of less than 1:10,000.

The reproducibility of the automated DNA purification was evaluated by DNA purification from 253 chicken caecal samples (with independent replicates per caecum sample, resulting in a total of 753 DNA purifications). The reproducibility was very high, as determined by the universal 16S rDNA real-time PCR. The mean of the obtained CT-values was 18.9, with a standard deviation of 1.1. DNA from all the 253 caecal samples could be amplified, and inhibition was observed in only 7 (0.9 %) of the individual DNA purifications. The biological results from the chicken caecal samples are presented in the main article.

We compared our magnetic bead-based DNA purification with the widely used column-based DNA isolation method, DNeasy, from Qiagen. We analysed a total of 16 caecal samples with both methods, and DNA yields were determined by universal 16S rDNA real-time PCR and gel electrophoresis. Both real-time PCR and gel electrophoresis showed that DNeasy gave slightly higher, but more uneven, DNA yields than the corresponding samples from the automated procedure. The purified caecal samples were also analysed by t-RFLP. The two methods gave equal band patterns for parallel samples, but the banding pattern was more even for the automated approach than for DNeasy (results not shown).

***C. jejuni* typing.** A total of 28 isolates of *C. jejuni* were included. The initial pilot experiment (termed pilot array) included 12 isolates. Eight of these, as well as an additional 16 isolates, were subjected to the subsequent experiments. These 24 isolates included eight human isolates from five outbreaks and a single sporadic isolate from a dog. The remaining 15 isolates included 10 isolates from human sporadic cases and 5 reference isolates (NCTC13255, NCTC 13259, NCTC 13261, NCTC 13263, NCTC 13264) described by (Wareing et al., 2003).

Array design, characteristics and experiments

Selected oligonucleotide probes were printed on Corning Ultragaps slides using 50% DMSO as a printing buffer with a primer concentration of 40 μ M, and were immobilized by UV-crosslinking (500 mJ). Each slide contained 8x3 subgrids. On each subgrid, three replicates of 66 different primers were printed. Samples for hybridization were applied individually to each subgrid. A total of 24 samples could be applied and simultaneously hybridized on each slide. Due to observed cross hybridization in preliminary experiments, only 26 (supplementary Table 2) of the 66 different primers were included in the labeling reaction. Including the pilot array, there are data from six different arrays and 28 isolates. Two replicates of 12 isolates were hybridized to the pilot slide, whereas five replicates of 24 isolates were hybridized to the five other slides. The pilot slide showed some evidence of systematic column variation; therefore, isolates were randomly assigned to the five experimental slides in order to eliminate possible column or array effects in the data.

Preprocessing of array data

The ImaGeneTM software (BioDiscovery Inc, Marina Del Ray, California, USA) was used for quantification of signal intensities. The background intensity was subtracted from the data, and the median of the three spot replicates was used for further

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analyses. All data analyses were performed with MATLAB version 7, service pack 2 (Mathworks, Inc.).

Microarray gene expression data contain systematic errors usually corrected for by data normalization (Cui et al., 2002). The normalization methods known from gene expression studies, however, are not well suited for the MLST arrays because the MLST array data are bimodal in nature, and there are no suitable control spots on the arrays. Some strategies for normalization were tested, but these did not improve the classification of the SNPs. Therefore, the data were not normalized. Because of random assignment on the location of isolates on the five experimental arrays, the possible column effect on the arrays should have minimal impact on the results.

Calculating score for classification of binary genotypes

All sequence types present in the MLST data base (or any other candidate list) can be represented by a vector of zeros and ones (the binary genotype), where ones represent polymorphic sites with the target SNP, and zeros represent sites with non-target SNPs. The binary genotype of any isolate can be predicted by array hybridization by classifying each spot as target/non-target SNP. After predicting the binary genotype from the array data, a bacterial isolate can be assigned to a sequence type in several ways. Here we tested two approaches. In the simplest approach, a sample was assigned to the candidates (sequence types) with the highest number of matches to the predicted genotype. With perfect classification at the SNP level, this will be equal to the number of SNPs used if the true genotype is present in the candidate list. In the other approach, a score was calculated for each candidate. In an experiment with K SNPs, N isolates and a list of M different candidates, the score of isolate i to candidate j was calculated according to

$$score_{ij} = \sum_{k=1}^K w_{ik} \cdot I(P_{ik} = G_{jk}) - \sum_{k=1}^K w_j \cdot I(P_{ik} \neq G_{jk}) \quad i = 1, \dots, N \quad j = 1, \dots, M$$

The $I(\)$ is the indicator function, which is one if the argument is true and zero otherwise, P_{ik} and G_{jk} is the value for SNP k in the predicted and the candidate genotype, respectively. If site k is predicted as a target site in the i'th sample P_{ik} equals one, likewise G_{jk} equals one if SNP k is a target site in the j'th candidate. Otherwise they are zero. The w_{ik} is the weight given to SNP k for sample i, and is set as $\frac{1}{2}$, where p_{ik} is the posterior probability of isolate i for having a target SNP in site k. A posterior probability of 0.9, for instance, means that the estimated change of x_{ik} coming from a spot with a target SNP is 90%. This means that w_{ik} equals 0 if the posterior is $\frac{1}{2}$ (poor classification), and approaches one if the posterior is close to 0 or 1 (good classification).

Basically, the score is a weighted sum of matches minus a weighted sum of mismatches, where the weights are set according to how much trust we have in the classification at the SNP level. An unknown isolate is assigned to the sequence type(s) with the highest score.

For methods distinguishing all four nucleotides, the same formula for calculating the scores can be applied, but the weights must be set differently since the posterior probability for “bad” classification will be $\frac{1}{4}$ instead of $\frac{1}{2}$.

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Generation of simulated data

We first simulated data by re-sampling from the observed data to test the ability of the MLST arrays to correctly identify sequence types registered in the MLST database using SNPs not included in the experiment. Next we used parametric distributions to simulate data from the two classes to investigate how the error rate at the SNP level affects the final classification of bacterial isolates.

A data point is denoted $x=[x_1, x_2]$ where x_1 and x_2 measures the Cy5 and Tamra intensities, respectively. Let X_t consist of experimental data from spots where the corresponding SNP is a target SNP, whereas X contains the rest of the data. In the resampling studies, artificial data were generated by re-sampling from X_t for target SNPs and from X for the non-target SNPs.

In the parametric simulations it is assumed that x_i has probability distribution p_1 if the corresponding SNP is a target SNP, and p_2 if it is a non-target SNP. Data for new genotypes were generated by sampling from p_1 and p_2 for target and non-target SNPs, respectively. Here we used bivariate normal distributions for p_1 and p_2 , with means and covariance matrices:

$$\mu_1 = [4 \quad 12] \quad \mu_2 = [4 \quad 4]$$
$$\Sigma_1 = \alpha \begin{bmatrix} 2 & 0 \\ 0 & 2 \end{bmatrix} \quad \Sigma_2 = \alpha \begin{bmatrix} 6 & 0.1 \\ 0.1 & 3 \end{bmatrix}$$

The alpha was varied between 1 and 14 to obtain error rates between 0% and 25% at the SNP level. In both types of simulations the average proportion of correct classifications at the strain level are reported, along with the mean reproducibility and the corresponding standard deviations. The average was calculated from 50 simulations.

Selection of SNPs from the MLST database

Full sequences were downloaded from the *C. jejuni* MLST website (<http://pubmlst.org/campylobacter/>). The sequences were reduced to binary genotypes by replacing all residues with the target SNP with 1 and all other bases with 0. In the following, SNP will refer to a polymorphic site in the binary or full sequence interchangeably. The binary genotypes were analyzed to find the most discriminating subsets of SNPs, using Simpson's diversity index (D). The best subsets were found for up to 40 SNPs. Note that the set of genotypes identified for a set of SNPs does not always correspond to a single sequence type, since several sequence types may have the same genotype for the selected SNPs. When we refer to genotype or binary genotype in the following section, we therefore refer to the unique classes identified for a set of SNPs in the MLST database.

Concrete results and conclusions

We evaluated the quantitative aspects of the automated DNA purification method by a 10-fold dilution of *C. jejuni* and *Cl. perfringens* cells into a background of *C. jejuni*- and *Cl. perfringens*- free caecum. Purified DNA from these samples was analysed by real-time PCR using universal 16S rDNA and *C. jejuni*-specific primer- and probe sets. Both the *C. jejuni*- and the *Cl. perfringens*- signal were normalised to the signal of the total flora. The normalised result shows a linear correlation between CT-values

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for *C. jejuni* and log input of *C. jejuni* cells relative to the total flora over a > 4 log range. minimum detectable fractions of target bacteria relative to the total flora were of approximately -7 log.

Our results showed that there were relatively large differences in the colonisation level of *C. jejuni* between chickens within the same flocks (Figure 1A). We also identified both *C. jejuni* positive and negative (below our detection limit of approximately -6 log relative to the total flora) chickens in some of the flocks. Our results are comparable to what we have previously reported for *C. jejuni* quantification. The difference is that we now do the quantification relative to the total flora. Normalisation to the total flora gives an increased reproducibility of the assay, and it allows us to determine the actual population fraction of the bacteria analysed. Furthermore, the DNA purification is now universal, with the potential of detecting any bacteria in the samples. The *C. jejuni* quantification illustrates the reproducibility and accuracy of our automated DNA purification.

New and more extensive knowledge about microbial communities can be obtained with the automated DNA purification from microbial communities in place. We have now removed a bottleneck process in the analyses of microbial communities. The next step is the combination with high-throughput detection approaches such as t-RFLP, QEXT (Quencher extension) or Restriction fragment melting curve analyses (RFMCA).

DNA microarray typing of *C. jejuni*. We estimated the ability to classify different genotypes and the uncertainty of these classifications by combining experimental data with simulations. This was not possible from the experimental data alone. From the parametric simulations we were able to study how the reproducibility and sensitivity changed as the error rate at the SNP level increased. The evaluation based on these simulations indicates that any method which can classify binary data (in our case SNPs) with a less than 2.5% error rate is able to obtain good classification at the typing level with 80-95% correct classification at the genotype level. When removing the SNPs with the poorest classification, the classification error for the experimental data was 3.2%. Hence, the accuracy of our SNE microarrays is close to the requirements for good genotype assignments, and thus promising for future high-throughput MLST typing.

Future success of SNE microarray MLST typing is also dependent on proving the advantages of this method over existing technologies. The limitation with the current assay is the reproducibility. A major part of the lack of reproducibility is due to printing and slide problems. We foresee that most of the technical problems with microarrays will be solved in the future as the technology matures. With respect to the SNE labeling, this technology is relatively well developed and tested. There are several commercial companies selling kits based on SNE labeling, for instance the Snap shot kit from Applied Biosystems and MASS array from Sequenom.

DNA sequencing is by far the most widely used approach for generating MLST data. The obvious limitation with DNA sequencing, however, is that multiple targets cannot be analyzed simultaneously. By using SNE microarrays this problem can be overcome. We have demonstrated four-gene detection, but this is certainly not the limit of SNE microarrays. In our approach, we have analyzed 24 arrays on a single

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slide. This can also easily be extended to 48 arrays. The major cost of our assay is the labeling reaction, which in principle is the same as for DNA sequencing. Thus, with fine-tuning and multiplexing, SNE microarray MLST typing could be a cost effective alternative to DNA sequencing.

Recommendations

Our recommendation for the future is to use high-throughput screening approaches to understand factors affecting the colonisation level of *C. jejuni*. When these factors are determined then we really have the possibility to control this bacterium.

When developing new typing approaches, it is very important to develop a statistical framework in parallel with the methods development. Accurate typing is very important in preventing *C. jejuni* entering the human food chain.

Publications and manuscripts acknowledging the Nordic Innovation Centre

Beate Skånseng, Magne Kaldhusdal and Knut Rudi (2006). Comparison of chicken gut colonisation by the pathogens *Campylobacter jejuni* and *Clostridium perfringens* by real-time quantitative PCR. *Molecular and Cellular Probes* 20: 269-279

Knut Rudi, and Kjetill S. Jakobsen (2006). Overview of DNA purification for nucleic acid-based diagnostics from environmental and clinical samples. *Methods in Molecular Biology* 345:23-35

Ingunn Berget, Even Heir, Jelena Petcovic and Knut Rudi (2007). Discriminatory power, typability and accuracy of Single Nucleotide Extension (SNE) microarrays. *AOAC International* (in press).

Trosvik P, Skånseng B, Jakobsen KS, Stenseth NC, Näs T, Rudi K. Multivariate analysis of complex DNA-sequence electropherograms for high-throughput quantitative analysis of mixed microbial populations. Accepted in *Applied and Environmental Microbiology*.

Detailed Scientific Report, Matís, Iceland

Note: IFL has the name Matís from 01.01.07. Eyjólfur Reynisson is the new country coordinator from Iceland after Sigrún Guðmundsdóttir.

Abstract

The main research area that IFL has been focusing on in the past year where molecular biological methodology has been used can be divided in two parts. First, the development of quantification and characterisation of total bacterial flora using real-time PCR and t-RFLP and second, quantification of the specific spoilage organisms (SSO) in fish using real time PCR technology. After the work conducted in 2005 we concluded that when LNA probe is used instead of regular TaqMan probe in a real time PCR reaction better sensitivity and higher fluorescence signals can be expected. Therefore, development of new real time PCR protocols at IFL aim to use the LNA technology whenever possible.

Total bacterial flora. Several primers sets targeting the 16S rRNA gene in combination with SYBR green or specific TaqMan probes were compared. The results from this comparison shows the limitations of SYBR green when compared to

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specific probes. In control reactions where no template was added a positive signal was generated with even as low as 25 Ct values, depending on the primer set used and the thermal program. After the PCR, a melting curve analysis was carried out. It revealed that the positive answer was not due to a contamination in the polymerase like has been suggested but from the formation of primer-dimers. Therefore, using SYBR green for the detection of total bacterial flora makes it impossible to gain sufficient detection sensitivity of the assay. SYBR green can be used when the total bacteria count is high (10^4 CFU/ml or more) However, when Taqman probe was used the sensitivity of the assay was increased from 2-3 orders of magnitude and the no template control (NTC) did not respond.

This quantification method was compared with conventional culturing in an experiment where the resistance of a naturally formed biofilm to different washing protocols was evaluated. In this project, ways to explore the possibilities of using more dilute detergents than recommended in fish processing surfaces were performed. Less concentrated detergents can lead to economical advantage for producers and more importantly, more environmental safe work practises. The study showed a high correlation between colony counting and real time PCR quantification. However, the PCR method detected about two log higher loads than colony counting. Many reasons could cause these results. First of all, with PCR, dead cells can contribute to the measurement since it is based on the presence of specific nucleic acids but not viable cells. The quantitative analysis is also based on number of 16S rRNA copies present in a sample and each bacteria species can contain from 2-11 copies of this gene in their genome. These factors are currently used for revision of the real-time PCR method and the standards used for quantification.

Another output of this project was the analysis of the bacterial flora of the biofilm. Two independent approaches were compared for this analysis: First, characterisation of cultured isolates with 16S rRNA sequencing and second, sequencing of clones which were obtained by molecular amplification and cloning of the 16S rRNA gene directly from the samples. Sequences were then BLASTed and a closest relative was matched for each sequence. Both of these methods showed a reasonable correlation. Manuscript for publication is being prepared.

SSO real time PCR development. The aim of this method is to have at hand a rapid objective test to evaluate the freshness of fish products. The main SSO have been described before and consist of *Shewanella putrefaciens*, *Photobacterium phosphoreum* and *Pseudomonas* spp. The goal is to have a multiplex PCR at hand to quantify these groups in one reaction.

Executive summary (main objectives)

Primer comparison for total count quantification with real time PCR. Primers to be tested were chosen from the literature. Those primers had either been used with SYBR green or a TaqMan probe. We tested four primer sets with SYBR green and two sets with TaqMan probe on a serial diluted template DNA isolated from *E.coli*. Using SYBR green, the detection limit was around 10^4 copies /reaction with most of the primer sets but when more diluted, primer-dimer was amplified preferably. When using a TaqMan probe the detection limit was lowered to 10^1 copies/reaction although a linear range (with 110% amplification efficiency) was reached between

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10^7 and 10^3 copies/reaction. The disadvantage of this assay however, is the low fluorescence signal obtained by the probe (5000 dR units on Mx3000P) in comparison to the *Salmonella* protocol reported last year (50.000 dR units on Mx3005) where LNA probe was used. The next step will be to exchange the TaqMan probe with LNA probe in an attempt to increase the fluorescence signal and the sensitivity of the assay.

In combination with these experiments we assessed a semi-automated nucleic acid purification instrument (Kingfisher) and compared the DNA quality and yield to the widespread Chelex boiling and another commercially available DNA purification kit (ChargeSwitch gDNA Mini Bacteria kit, Invitrogen). The automated method with Kingfisher proved to be more timesaving and gave DNA with better quality when analysed by spectrometry (Nanodrop), gel electrophoresis of gDNA and a real time PCR assay.

SSO real time PCR development. Search of candidate target genes quantification was carried out using literature and database search (NCBI) for the three organisms, *Shewanella putrefaciens* (S.p), *Photobacterium phosphoreum* (P.p) and *Pseudomonas* spp (P.spp). Specific identification of single species is a requirement for S.p and P.p. but a taxonomic identification for P.spp.

Primer pairs for the SSOs were tested and evaluated with SYBR green to assess secondary products and primer-dimer formation in the PCR. The primers for S.p. proved to be successful with SYBR green and thus the method was tested with a specific LNA probe. Using the LNA probe, a specific response and high fluorescence signal give rise to a promising method development although further testing and optimization needs to be carried out. The primers for P.p and P.spp need to be re-evaluated since the Ct values using SYBR green were unsatisfactory.

Method/implementation

1. Real time PCR development for the specific spoilage organisms in fish is in progress.
2. Terminal restriction fragment length polymorphism (t-RFLP) of a bacterial community in a biofilm. The t-RFLP profile was correlated against a 16S rRNA clone library and 16S rRNA sequences from isolates which were derived from the same biofilm samples.

Concrete results and conclusions

1. Real time PCR primers and an LNA probe targeting the species *Shewanella putrefaciens* and *Shewanella baltica* were established and verified for their specificity by testing them on other unrelated bacteria. Further optimization and validation will be carried out in 2007 along with a development of a protocol for the other SSOs.
2. t-RFLP, 16S analysis and bacterial count were the method of choice when analysing the effect of different washing parameters to remove a naturally formed biofilm from steel and plastic surfaces. Colony counting revealed better prevalence of bacteria on plastic surfaces than stainless steel surfaces. As expected, higher concentration of detergent and higher water temperature removed viable cells in the biofilm more effectively. A good correlation was achieved with 16S rRNA clone library and t-RFLP analysis when analysing the diversity of the bacterial community of an

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untreated biofilm. The major bacterial groups detected were *Aeromonas salmonicida*, *Pseudomonas* sp., *Shewanella baltica*, *Shewanella putrefaciens*, *Acinetobacteria* sp., *Serratia* sp. among with other less abundant groups.

Publications and manuscripts acknowledging the Nordic Innovation Centre

Reynisson, E., M.H. Josefsen, M. Krause and J. Hoorfar, *Evaluation of probe chemistries and platforms to improve the detection limit of real-time PCR*. J Microbiol Methods, 2006. **66**(2): p. 206-16.

Detailed Scientific Report, SIK, Sweden

Projects

1. The project “*Campylobacter*- karakterisering, smittkällor och spridningsvägar i Sverige” is a national project funded by KK-stiftelsen and Svensk Fågel. The project started 2001-08-12, and was ended 2006-10-20 by the presentation of a PhD thesis. The effective project duration is four years. The work is performed at SIK and at the department of Clinical Microbiology at the University of Göteborg. Professor Bertil Kaijser is the main supervisor and ass. prof. Elisabeth Borch is co-supervisor. A co-operation with the Swedish Food Administration (Livsmedelsverket) through Hans Lindmark was performed during 2006 on the quantification of *C. jejuni* on chicken meat by real time PCR.

2. “Development and optimization of a model system for biofilm and of PCR-DGGE for *Listeria* spp and *L. monocytogenes*” is a master thesis by Maria Lindeblom, department of Biotechnology, Chalmers University of Technology, performed at SIK, Göteborg during the supervision by Maria Lövenklev, SIK.

3. “Development of a real-time PCR for quantification of *Listeria* spp. in biofilm” is a master thesis by Elonore Falk, department of Cell and Molecular Biology, Göteborg university, performed at SIK, Göteborg and supervised by Maria Lövenklev, SIK.

4. “Real –time PCR for the detection of viable *Listeria monocytogenes*” is a master thesis by Karolina Josefsson, department of Applied Microbiology, Lund University, performed at SIK, Göteborg and supervised by Maria Lövenklev, SIK and Elisabeth Borch, SIK.

1a. Detection and quantification of *Campylobacter jejuni* on fresh chicken carcasses by real-time PCR.

Campylobacter jejuni is a Gram-negative, non-spore forming bacterium, which is currently the most common pathogen causing reported cases of food poisoning in Sweden, as well as in most other industrialised countries. Eating or drinking something containing these bacteria is often determined to be the source of infection and as few as 500 bacteria can be enough to cause illness. The cases are often sporadic and hard to trace. Chicken is a well-documented source for *Campylobacter* infections in humans, and the birds do not get sick by colonization of these bacteria. The focus of the thesis was on the characterisation of *C. jejuni* and *C. coli* from Swedish patients and chickens and to evaluate antibiotic resistance, genomic diversity, and to facilitate detection in food. The frequency of antibiotic resistance in *C. jejuni/C. coli*, in patients with campylobacteriosis, from 1996-2005, to the most commonly used antibiotics for treatment of *Campylobacter* infection in Sweden was

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studied. The antibiotic resistance among isolates from Swedish chickens at slaughter was also evaluated. Furthermore, the relatedness between strains originating from chickens and humans in Sweden was determined, and a comparison was made with strains originating from other parts of the world. Finally, a method was developed to find *C. jejuni* on chicken carcasses faster and more specifically. Resistance to norfloxacin increased (7 to 30 %), among the strains tested from patients acquiring the infection in Sweden. Among domestic isolates, the resistance to tetracycline varied between 4 % and 13 % during the decade analysed. The antibiotic resistance to erythromycin was consistently low among the domestic isolates tested (up to 4 %). It was concluded that antibiotic resistance among *C. jejuni/coli* strains is relatively uncommon in humans infected in Sweden (numbers in % earlier mentioned), as well as in Swedish chicken (only 3 %) for the most commonly used antibiotics for human treatment. Erythromycin was concluded to remain as the current drug of choice for the treatment of most patients infected abroad with *C. jejuni/coli*, since resistance to this drug was uncommon among all tested isolates. It was further concluded that a large increase in antibiotic resistance has developed for the quinolones (norfloxacin and ciprofloxacin) among domestic human *C. jejuni/coli* strains. A map of the antibiotic resistance over the time period of this study showed an ongoing emergence of resistance worldwide to norfloxacin for *C. jejuni/coli*. When PCR/REA was used, 100 % of the human *Campylobacter* strains and 98 % of the chicken isolates were identified as *C. jejuni*. Five percent of the human domestic isolates and 10 % of the chicken isolates were “falsely negative” by hippurate, using the PCR/REA assay as a reference. *C. jejuni* was observed to be a diverse taxon, based on PFGE profiling and cluster analysis. Over 50 % of the clones from Swedish patients and healthy Swedish chickens were not similar with respect to PFGE groups, which suggests that human infections can occur from sources other than chickens. Only 33 % of domestic isolates from human infection and Swedish chicken strains belonged to the same PFGE groups, indicating that other sources than chicken can be of importance for human campylobacteriosis. More isolates are needed to prove this indication. Similarities were observed between Swedish and Thai strains, suggesting global contamination pathways. In the quantification study, the number of bacteria on contaminated chicken meat was concluded to vary and that easily performed quantification methods, real-time quantitative PCR (q-PCR) and plate counting on selective media, with a highly standardized procedure, has the potential to sort out highly-contaminated meat (for direct heat treatment) from less- or non-contaminated meat. The results from the q-PCR was somewhat more stable than results from plate counting, indicating that small differences in the shape of the bacteria can influence the results when working with living bacteria grown on plates.

1b. Characterization of *Campylobacter jejuni* and *C. coli* from Swedish patients and chickens- antibiotic resistance, genomic diversity and detection

The risk of receiving a *Campylobacter* infection is largely dependent on the concentration of *Campylobacter*. Data on the prevalence of *Campylobacter* must, therefore, be complemented with quantitative data before the risk with poultry can be assessed satisfactory. Currently, quantitative detection is commonly performed by direct plating of chicken rinse on mCCDA plates. This medium can sometimes be overgrown by other bacterial species and non-*Campylobacter*. The objective of the present study was to investigate the possibility of quantitative detection of *C. jejuni* in chicken rinse by the use of real-time PCR, without any cultivation step. The detection limit was comparable with direct plating on mCCDA. The results from real-time q-

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PCR correlated well with those from direct plating when chicken rinse spiked with a tenfold dilution series was analysed five times. A correlation that was not as good between the methods was obtained when natural samples of chicken meat from retail was analysed. The concentrations obtained by the real-time q-PCR were generally higher than those obtained with plate counts, in contrast to when spiked samples were used. The presence of dead and/or viable but non culturable forms of *Campylobacter* in the natural samples may explain this discrepancy.

2. Development and optimization of a model system for biofilm and of PCR-DGGE for *Listeria* spp and *L. monocytogenes*

The model system should be reproducible as well as easy to use. It should also be easy to change the environmental parameters. Developing a PCR-DGGE protocol also included finding primers specific for *Listeria* that resulted in a PCR product where strains of *Listeria* could be differentiated from each other. The latter should then be used to investigate bacterial growth in the model system.

Two model systems were tested, of which the 6 well plate was found to be the best. It showed good results in reproducibility and was user-friendly. One disadvantage was the possible occurrence of contamination due to vaporization.

The *iap* gene was chosen target for PCR primers. It is both unique for *Listeria* and differs in the middle sequence between *Listeria* species. Protocols for PCR and DGGE were elaborated resulting in a separation not only among different species but also among different strains of *L. monocytogenes*.

L. monocytogenes was found to be as good a biofilm maker as other *Listeria* spp. in monoculture, but when mixed the presence of *L. monocytogenes* could not be indicated by PCR-DGGE analysis of the biofilm.

3. Development of a real-time PCR for quantification of *Listeria* spp. in biofilm

The food industry spends today millions dollar to create products with high safety. Knowledge about microorganisms and their lifestyles can be used to optimize processes and reduce the risk of contamination and in that way increase the safety of products.

The aim of this master thesis was to study and quantify *Listeria* spp grown in biofilm under various conditions, such as different surfaces materials and growth temperatures.

Both mono- and mixed cultures were examined regarding relative amount of produced biofilm and composition. Furthermore *L. monocytogenes* was quantified using real-time PCR. Two real-time PCR assays were optimized regarding running times, annealing temperatures by using gradient real-time PCR and PCR reagents. Target gene for *L. monocytogenes* was *hly*, which encodes virulence factor, called Listeriolysin O and is specific for all *L. monocytogenes* strains. The target gene for *L. innocua* was *lin02483*, this gene is located on the chromosome and is a hypothetical protein, only found in *L. innocua*. The optimized PCR assays for *hly* and *lin02483* was found to be very efficient when applied on genomic DNA and cell suspensions. Both assays are specific, reproducible and can detect low amounts of the target bacteria. Both assays were found to have a detection level of 15 cells with a detection probability of 87.5%. When quantifying cells in a biofilm, a more reliable method to remove cells from the surface would lead to more accurate quantification.

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4. Real –time PCR for the detection of viable *Listeria monocytogenes*

The aim of this project is to evaluate different methods with real-time PCR, to differentiate between viable and dead cells of *Listeria monocytogenes*.

The different methods are:

- Amplification of all the DNA, which will give a measurement of both living and dead cells, since the DNA from both viable and dead cells are detected
- Amplification of DNA in combination with ethidium monoazide (EMA) / propidium monoazide (PMA). EMA and PMA are DNA intercalating dyes which hopefully will lead to that the DNA from dead cells get inhibited so the amplification in the real-time PCR is only for the viable cells
- Extraction of the mRNA which is a measurement of viability since it is degraded very fast. The mRNA is translated to cDNA which in turn is amplified in the real-time PCR. The report is under finalization.

Publications

Falk, Eleonore. 2006. Development of a real-time PCR for quantification of *Listeria* spp. in biofilm. Master Thesis in Microbiology. Department of Cell and Molecular Biology, Göteborg University.

Lindeblom, M. 2006. Development and optimization of a model system for biofilm and of PCR-DGGE for *Listeria* spp and *L. monocytogenes*. Master thesis in Biotechnology, Chalmers University of Technology.

Rönner, A.-C. 2006. Detection and quantification of *Campylobacter jejuni* on fresh chicken carcasses by real-time PCR. PhD thesis. Department of Infectious Diseases, Göteborg University. ISBN: 91-628-6971-7.

Rönner, A.-C. and Lindmark, H. 2007. Detection and quantification of *Campylobacter jejuni* on fresh chicken carcasses by real-time PCR. In manuscript.

Detailed Scientific Report, Helsinki University, Finland

Abstract

Campylobacter project at the DEFH has the major aim to increase knowledge on epidemiology, ecology and transmission sources of *Campylobacter* in Finland. Activities in this field have continued in 2006 concentrating on epidemiology of *Campylobacter* infections in Finland by analysing population genetics of Finnish *C. jejuni* strains originating from poultry, cattle, water and human patients using MLST technique. Studies on antimicrobial resistance mechanisms and resistance acquisition kinetics in *C. jejuni* and *C. coli* have continued.

Executive summary (main objectives)

1. To continue studies on the epidemiology of human *Campylobacter* infections in Finland and the role of various sources in infections by collecting and analysing during one year period *C. jejuni* strains from human patients who have domestically acquired infections as well as strains from chicken meat from local retail shops in a small community in western Finland. A long-lasting family-based epidemic associated with drinking contaminated unpasteurised milk was studied (Schildt et al. 2006)

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2. To continue studies on the contamination level of Campylobacter in Estonian poultry (cooperation with Agricultural University, Tartu)
3. To study population genetics of Finnish *C. jejuni* strains from human domestic infections and from various animal sources collected during ten years period
4. Antimicrobial resistance mechanisms and kinetics of fluoroquinolone resistance in Campylobacter

Method/implementation

- For detection of campylobacter from various matrices (either food or fecal material) both conventional as well as PCR methods have been applied. Molecular tools are specific useful for detection of specific mutations associated with resistance (efflux pumps, fluoroquinolones, tetracycline etc) and for analysing presence/absence specific potential host-associated genes (1, 2, 3).

- Molecular typing of *C. jejuni* strains has been of primary importance in our project to

support epidemiological studies in comparison of PFGE genotypes occurring in animals and humans. PFGE typing has also been applied for studies on the risk factors associated contamination sources of turkey flocks with campylobacters during a rearing period (cooperation with Agricultural Institute, University of Helsinki)

- Epidemiological studies on risk factors associated with human domestically acquired Campylobacter infections have continued in cooperation with the Haartman institute (MD H. Rautelin)

- Population genetic analysis using Multi Locus Sequence Typing (MLST) technique for studies on the population structure (diversity, clonal groups and changes in time in population) of more than 400 *C.jejuni* strains. Both PCR methods used for multi-locus sequencing as well as statistical methods used for analysis of populations and sequence types (STs) have in under development

Concrete results and conclusions

1. The relative importance of various significant risk factors ($p < 0.05$) identified in our previous studies from 2002 were further analysed. The important finding of demographic characteristics of the patients was, confirming the results of our previous epidemiologic study that a significant proportion of the patients were > 60 years old (30.7%) and they were significantly ($p < 0.0001$) older than the general population in the three study areas (urban area/2 rural areas). Serotyping of the isolates did not show any association of the predominant serotypes (4-complex, Pen 6,7 and Pen 21) to any demographic characteristic, except geographic differences in common serotypes were evident. These results revealed that risk factors for acquisition of Campylobacter infection may differ in various geographic regions. Swimming water exposure was associated with Pen 6,7. In opposite, two risk factors, eating undercooked or raw meat (including poultry) and drinking water were not associated with any serotype (Schönberg et al. 2006). MLST analysis of these particular strains revealed that MLST complex 48 was significantly ($p < 0.005$) associated with tasting or eating raw meat. ST-45 was associated with contact with pet animals (Kärenlampi et al. 2006).

2. Population genetic analysis of 361 strains revealed that three MLST complexes, ST-21, ST-45 and ST-677 predominated because they covered 21, 45 and 11% of all strains. The presence of major ST types (ST-50, ST-677) common in Finland suggests that local adaptation is important in the epidemiology of *C. jejuni* populations. ST-45 was significantly associated with poultry. We further analysed these strains by typing

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with PFGE and analysing by PCR the presence/absence of six genes within the O-linked glycosylation locus proposed to be suitable markers for source (Champion et al. 2006). In our studies we not able to confirm that these genes could be suitable markers for livestock associated strains. Further studies are continued to increase understanding of population genetics of *C. jejuni* and its association with the epidemiology of campylobacter infections in Finland.

3. A cooperative project between our institute and the Estonian Agricultural University has been continuing for three years. In this project we have started first time to analyse *Campylobacter* contamination in Estonian poultry. In the study we found that in the beginning of 2000 when amount of imported poultry was high the contamination of imported meat was much higher than that of produced in Estonia. Antimicrobial resistance among *C. jejuni* isolates from 2005 was high (79,4%). The common single resistance was against enrofloxacin (73% resistant). Also multiresistant isolates were common (26.7%).

4. Therapeutic use of enrofloxacin (FQ) in poultry has been shown to induce persistent resistance in the *C. jejuni* population colonising in a flock. The resistance is 100% and persistent. Increasing number of multiresistant strains have been isolated from humans as well as from poultry in some countries. Our studies revealed that low-level resistance to FQs develops stepwise and the critical level for acquisition of high, clinically relevant level of resistance is approximately MIC 0.5 ug/ml. This MIC level is rather easily acquired by unspecific mechanism associated with use of some unrelated antimicrobials (tetracyclines, amoxicillin) which activate same efflux mechanism as FQs use. Strict control of use of antimicrobials in food animal production is required. To prevent increased resistance to single antimicrobial as well as multiresistance which is easily selected by a FQ controlled use of antimicrobials is required.

Transfer of the results to end users:

Oral presentations in several meetings directed to: 1. Food authorities, 2. Scientific community, 3. Food industry and 4. Media

Recommendations

- Targeted epidemiological studies to understand various epidemiological aspects in *C. jejuni*-host relationship are required to develop better prevention tools and improved diagnostics
- More studies on poultry food chain are required to develop efficient (cost-benefit) intervention strategies to prevent contamination and decrease human exposure and mathematical models for preventive control.
- Controlled use of antimicrobials in animal production is required to prevent evolvement of high level of resistance.
- More data is required to response on increasing antimicrobial resistance detected also among *Campylobacter*

Publications and manuscripts associated with the project:

Schönberg-Norio, D, Sarna, S., Hänninen, M.-L., Katila, M.-L., Kaukoranta, S.-S. and Rautelin, H. Strain and host characteristics in domestic *Campylobacter jejuni* infections in Finland. Clin Microbiol Infection 2006, 12, 754-760.

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Schönberg-Norio, D., Hänninen, M.-L., Katila, M.-L., Kaukoranta, S-S, Koskela, M., Uksila, J., Pajarre, S. ja Rautelin, H. Activity of telithromycin erythromycin, fluoroquinolones and doxycycline against *Campylobacter* strains isolated from Finnish subjects. *Antimicrobiol. Agents Chemother.* 2006 50, 1086-1088

Praakle-Amin, K., Roasto, M., Korkeala, H. and Hänninen, M.-L. Genetic characterization and antimicrobial susceptibility of *Campylobacter* in retail meat in Estonia. *Int J Food Microbiol.* (in press 2006)

Kärenlampi, R., Rautelin, H. Schönberg-Norio D, L. Paulin and Hänninen, M.-L. Longitudinal study of Finnish human *Campylobacter jejuni* and *C. coli* isolates using multilocus sequence typing, including comparison with epidemiological data, and poultry and cattle isolates *Appl. Environm. Microbiol.* (accepted 2006)

Kärenlampi, R., Rautelin, H: Schönberg-Norio, D. and Hänninen, M.-L. Evaluation of genetic markers and molecular typing methods for prediction of source of *Campylobacter jejuni* and *C. coli* infections. *Appl. Environ. Microbiol.* (accepted 2006)

Roasto, M. K. Juhkam, T. Tamme, A. Hörman, L. Häkkinen, M. Reinik, A. Karus and M.-L. Hanninen. Emergence of antimicrobial resistance in *Campylobacter jejuni* isolated from broiler chickens in Estonia (submitted 2006)



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