

Program of the 1st meeting of WG4 of COST920
“Behaviour of zoonotic bacteria along the foodchain”
 (7-8. October, 2003, Budapest, Hotel Rege)

7th Oct. Tue., 8:30 am (loading of “.ppt” presentations from 7³⁰ – 8³⁰)

Introductions :

Nagy, B (l.o.) and Diana Bánáti (Hungarian COST representative) : Welcome 8³⁰ – 8⁴⁰

Williams, J. (Scientific officer, EU-COST): COST updates for MV members 8⁴⁰ – 9⁰⁰

Thorns, Ch. (Chairman of COST 920): COST920 as it stands today 9⁰⁰ – 9²⁰

*Colin, P. (Chairman of WG4): Introduction to the working plan of WG4:
 “Survival of zoonotic bacteria along the foodchain”* 9²⁰ – 9⁴⁰

Coffee Break 9⁴⁰ – 10¹⁰

Session 1: Epidemiology of zoonotic microorganisms through the food chain

Chair: *P. Colin*

*Humphrey, T.(invited lecture): The European Salmonella epidemic: what is so special
 about S.Enteritidis PT4 ?* 10¹⁰ – 10⁴⁰

*Mulder, R.W.A.W. (invited speaker): Microbial control, microbial survival
 in the poultry food chain* 10⁴⁰ – 11¹⁰

Methner, U.: Aspects on epidemiology of Salmonella in broiler flocks 11¹⁰ – 11³⁰

*Davies, R., Liebana, E., Breslin, M., (presented by: Pleydell, E.): Investigation
 of the distribution and control of Salmonella enterica serovar Enteritidis PT6
 in a layer breeding and linked commercial operation* 11³⁰ – 11⁵⁰

*Imberechts, H., De Baets, L., Saegerman, C., Jouret, M., De Greve, H.,
 Hernalsteens, J.-P.: Monitoring enterohaemorrhagic Escherichia coli
 in the abattoir and in cattle farms: a collaborative study* 11⁵⁰ – 12¹⁰

*Kakoyiannis, Ch.: Contamination of foods of animal origin with Salmonella,
 Campylobacter, L. monocytogenes and E coli O157 in Cyprus* 12¹⁰ – 12³⁰

Poster visits (see Abstracts p. 29-35) 12³⁰ – 13⁰⁰

Lunch (loading of “.ppt” presentations) 13⁰⁰ – 14⁰⁰

Session 2: Growth and survival along the food chain

Chair: *J. Olsen*

<i>Labadie, J.</i> (invited lecture): Biofilms of <i>Listeria monocytogenes</i>	14 ⁰⁰ – 14 ³⁰
<i>Aabo, S., Boel, J., Pedersen, J.R.</i> : Quantitative investigation of <i>Salmonella</i> Dublin on beef carcasses and the decay during cooling and vacuum packed storage	14 ³⁰ – 14 ⁵⁰
<i>Jordan, K.</i> : In situ localization of <i>E. coli</i> O157:H7 in food	14 ⁵⁰ – 15 ¹⁰
<i>Awad-Masalmeh, M.</i> : Verotoxigenic <i>E. coli</i> (VTEC) in milk and meat samples: possible origin and survival	15 ¹⁰ – 15 ³⁰
Coffee Break	15 ³⁰ – 16 ⁰⁰
<i>Colin, P., Abiven, A., Leleu, G., Malle, P.</i> : Epidemiology of <i>Listeria monocytogenes</i> along a rainbow trout food chain production	16 ⁰⁰ – 16 ²⁰
<i>Walsh, C., Duffy, G.</i> : Relationship between antibiotic resistance and thermotolerance in foodborne pathogens	16 ²⁰ – 16 ⁴⁰
Special lecture:	
<i>Hugas, M.</i> (invited): The Bio Hazard (BIOHAZ) Panel and it's activity	16 ⁴⁰ – 17 ⁰⁰
Dinner	19 ³⁰ –
We plan to go out to a Scandinavian dinner, on an individual basis.	

8th Oct. Wednesday, 9:00 am. (loading of “.ppt” presentations from 8⁰⁰ – 9⁰⁰)

Session 3: Bacterial changes along the foodchain

Chair: *H. Kruse*

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| Frees, D., Varmanen, P., Savijoki, K., Thorup, M., Lone Brøndsted, A.,
<i>Ingmer, H.</i> (invited lecture): Stress tolerance mechanisms influencing
virulence and survival of foodborne pathogens | 9 ⁰⁰ – 9 ³⁰ |
| Thomsen, L.E., <i>Olsen, J.E.</i> , Chadfield, M. S., Ingmer, H.: Links between stress
adaptation and virulence of <i>Salmonella</i> Dublin and <i>S. Typhimurium</i> | 9 ³⁰ – 9 ⁵⁰ |
| <i>Guerzoni, M. E.</i> , Vannini, L., De Cesare, A., Manfreda, G.: Stress response of
<i>S. Typhimurium</i> and <i>S. Enteritidis</i> isolates: relationship of cell fatty acids
and phagotypes | 9 ⁵⁰ – 10 ¹⁰ |
| <i>Newell, D. G.</i> , Ridley, A. M., Toszeghy, M. J., Wassenaar, T. M.: Genetic instability
as a mechanism for <i>Campylobacter jejuni</i> for environmental survival and
enhanced colonisation potential | 10 ¹⁰ – 10 ³⁰ |
| <i>Humphrey, T.</i> : Responses of <i>Campylobacter</i> to heat and cold | 10 ³⁰ – 10 ⁵⁰ |
| <i>Wagenaar, J.</i> , Jacobs-Reitsma, W., de Boer, P., Jan van der Wal, F.:
Practical aspects of <i>Campylobacter</i> typing along the food chain | 10 ⁵⁰ – 11 ¹⁰ |
| <i>Morabito, S.</i> , Tozzoli, R., Caprioli, A.: Whole genome comparison of
enterohaemorrhagic <i>E.coli</i> (EHEC) O157:
Definition of the minimum genomic core for pathogenicity. | 11 ¹⁰ – 11 ³⁰ |
| Coffee Break | 11 ³⁰ – 12 ⁰⁰ |

Session 4: Concluding session

Chair: *C. Thorns* and *P. Colin*

Finalization of the working plan of WG4: identification of areas of future research.

Possibilities to form proposals for FP6 from within WG4 (with possible links to other WGs).

Suggestions for topics, places and organization strategies for the 2nd workshop of WG4.

Conclusions by the Chair of WG4 (P. Colin) and by the Chair of COST920 Ch. Thorns

13.00 : Lunch (based on previous requests)

13⁰⁰ – 14⁰⁰

POSTERS (abstracts: pages 29-35)

Herpay, M.: Screening for VTEC in Hungary

Tóth I., Lancz, Zs., Nagy, B.: Sources and characteristics of bovine *Escherichia coli*: O157 and verotoxic *E. coli* (VTEC) in Hungary

Kostyák, Á. Veres, Z., Adrian, E.: *Salmonella* sero- and phage types in Hungarian laying and broiler flocks and products

Damjanova, I., Pászti, J., Jakab, M., Sréter-Lancz, Zs.: Epidemiological data on *Campylobacter* infections in Hungary

Kaszanyitzky-Juhász, É., Jánosi, Sz.: Hungarian veterinary antibiotic resistance monitoring system and the results on *Campylobacter* strains

Pieskus J., Milius, J., Stankevicius, A., Michalskiene, I.: Prevalence of *Salmonella* in poultry carcasses and their products in Lithuania

B. Sesma, C. Latasa, B. García, C. Solano, M. Alvarez, I. Lasa and C. Gamazo
Cellulose production by *Salmonella enteritidis* affects thermoresistance and adherence to egg shells

The survival of zoonotic bacteria along the foodchain

Pierre Colin

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Laboratoire d'études et de recherches en pathologie des poissons, Technopôle Brest-Iroise,
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The food chain includes all the operations which undergo foods since their production until their consumption.

In many cases, the modern food technology concentrate sites of production (breedings, slaughterhouses and industrial factories of transformation) and systems of distribution use more and more the cold chain, because of the spatio-temporal length of distribution networks. In addition, there is actually important changes in the consumer's habits influencing the food processing : requests of "organic" and "exotic" productions, products more and more elaborated, consumption of raw or undercooked products (meats, fishes and seafoods, milk and dairy products).

The microorganisms can be present in the various steps of the food chain and, during the operations of production and transformation of foods, the pathogenic bacteria are subjected to environmental factors modifying their capacities to survive or to multiply. During these operations foodstuffs undergo voluntary ,or not, modifications of their properties; these physical (heat, cold, mechanics), chemical (acidification, fermentation) treatments, modifying the intrinsic properties of products (temperature, a_w , pH, potential redox), disrupt naturally not only the bacterial flora, but also the capacities of these microorganisms to adapt themselves to these new environmental conditions.

Modifications can go towards an increase or a decrease to the expression of certain intrinsic capacities of bacteria. These capacities are translated by the expression of different phenotypic and molecular characters. These changes can affect the capability to link with other cells (biofilm, attachment...), to fit new environmental conditions including new molecules such as antibiotics, biocides, organic acids, to modify the expression of virulence.

So a better knowledge of the mechanisms of survival of these microorganisms throughout the food chain allows to bring an element of understanding in the procedure of evaluation of the risks. As examples : expression of the virulence according to the physiological state of the bacteria; characterization of the genes of virulence according to the environmental conditions; evolution of strains during the operations of transformation.

These analyses of the behavior of strains, their capacities to survive, to colonize or on the contrary to disappear will be of use as base to a better knowledge of the identification of the hazard (do all the strains of the same species present an identical hazard ?), of the the exposure assessment (what are the probability of obstinacy of such microorganisms since the production until the consumption?).

Session 1.

Epidemiology of zoonotic microorganisms through the food chain

Chairman : *P. Colin*

The European *Salmonella* epidemic: what is so special about *S. Enteritidis* PT4?

Tom Humphrey, Professor of Food Safety

Department of Clinical Veterinary Science
University of Bristol, UK

The last 20 years has seen a marked upsurge in the incidence of *Salmonella* infection in all parts of Europe. This has principally been associated with one serovar, *Salmonella* Enteritidis and mainly with one phage type (PT), PT4. It is not yet fully understood why this serovar and to a lesser extent, *S. Typhimurium*, is so successful as a human pathogen but it is likely to be associated with an invasive behaviour in food animals, which leads to the contamination of food interiors. Particularly important in the international *Salmonella* pandemic is the ability to infect chicken reproductive tissues and, from there, contaminate egg contents *in vivo*. It is believed that a few serovars including Typhimurium and Infantis are also able to do this but *S. Enteritidis* is overwhelmingly the most important. Persistence in chicken reproductive tissues is associated with *rpoS* expression and LPS structure, which may be linked. LPS structure is also important in governing survival in egg albumen, at hen body temperature and the differences between Enteritidis and Typhimurium in that respect are the reason for the different survival profiles of the two serovars. *Salmonella* Enteritidis survives significantly better than the other serovar. This probably explains the observation from an American group that although the isolation of the two serovars from reproductive tissues was essentially the same, *S. Enteritidis* was more frequently recovered from forming eggs. Work is in progress at Bristol to try and determine the reasons behind apparent differences between European and US strains of PT4. This may well be due to variations in LPS structure, particularly associated with ethanolamine and this and other matters will be discussed.

Microbial control in the poultry food chain

R.W.A.W. Mulder

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On global basis there is a trend toward increasing consumption of poultry meats and pork. In some countries also small animals as rabbits are becoming important meat sources.

Poultry meat is after pork-meat, the world's most popular animal food product. Its nutritional value, especially with respect to the healthier diets, which are demanded for the young but also for the ageing population, the diversity of available products, its convenience of preparation combined with an attractive price, have given poultry products a ranking above beef and pork products. Additional to these positive aspects poultry meat seems to be most widely accepted by different cultures or religion.

Despite these very positive points, the poultry industry as a whole, faces challenges with respect to animal welfare, environmental and food safety issues.

Of course these challenges are not new, as the industry always has been aware of these aspects. The recent Avian Influenza epidemic, has again given much public attention to food safety issues. Discussions on food safety issues include automatically environmental and animal welfare aspects, even leading to (emotional) opinions on drastic changes of future production.

Poultry products have always been considered as a microbiologically safe food, but due to general increased concerns regarding production methods in the food animal industry also poultry production is influenced.

As these aspects nowadays belong to the "quality of life" feelings of the modern consumer one cannot neglect them when discussing the topic of poultry and public health. As consumer's demands become more important the production chain has to react. This process of chain reversal started some years ago and due to recent events the implementation was already introduced.

The safety of poultry products and of all food of animal origin, depends upon the application of effective control measures at all stages of the production chain, literally from farm to fork. Eggs and poultry meat may be contaminated with a variety of micro-organisms. In eggs the food-borne pathogen *Salmonella* seems to be the most important organisms of concern. Also raw poultry meat may be contaminated with a variety of micro-organisms and although some are more relevant to the shelf life of the product, most concerns are with controlling agents of food-borne human disease.

The two principal food-borne pathogens associated with live poultry therefore are the salmonellas and *Campylobacter jejuni*. These rarely cause illness in the flocks and go unrecognised unless monitoring of flocks is carried out. However, the recent cases with *Salmonella paratyphi B* var *java* seem to contradict this statement.

This paper will concentrate on the potential human health risks resulting from poultry production and from consumption of poultry meat. The discussion will follow the production chain and some examples of intervention strategies will be given. Ideas on how research can contribute to control microbiological quality of industrial and more "organic" produced poultry meat will be presented.

Aspects on epidemiology of *Salmonella* in broiler flocks

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Comprehensive information on epidemiology of *Salmonella* infections in broiler farms is a prerequisite to introduce effective control measures. The need to reduce the occurrence of salmonellae in broilers is given by fact that poultry meat and raw meat products are now as ever an important source for *Salmonella* infection in humans.

The aim of the study was i) to gain general information on *Salmonella* epidemiology in broilers and ii) to study whether the serovar *Salmonella* Paratyphi B d-Tartrat+ has in comparison to other serovars of *Salmonella* the ability to spread more efficient within a flock and perhaps to exclude other *Salmonella* strains from intestinal colonisation. The need to study this question is due to the observation that the serovar *Salmonella* Paratyphi B d-Tartrat+ showed a considerable increase in recent years especially in the Netherlands but also in Germany.

In several farms with and without history of *Salmonella* Paratyphi B d-Tartrat+ infections follow-up examinations were undertaken to study the spread of *Salmonella* infection in broiler flocks under consideration of different factors e.g. *Salmonella* status of the broiler houses after cleaning and disinfection, *Salmonella* status of newly-hatched chicks and the use of antibiotics.

Molecular studies, especially plasmid profile typing and pulsed-field gel electrophoresis represent valuable tools for subtyping *Salmonella* Paratyphi B d-Tartrat+ isolates and, therefore, are appropriate to detect the contamination-infection cycle at both herd and farm level.

It could be shown that *Salmonella*-free day-old chicks are an essential prerequisite for the production of *Salmonella*-free broilers. Furthermore, it was evident that the *Salmonella*-status of broiler houses before introducing day-old chicks represents a substantial risk factor. Especially the contaminated feeding system within the broiler houses is of great importance as very young chicks are highly susceptible to *Salmonella* infection. Insects, e.g. darkling beetles or mealworms also seem to be a vector for *Salmonella* spreading between birds, however, further studies are needed to verify whether the beetles are involved in spreading between already infected birds only or whether they are also responsible as source of infection for very young birds. Independently effective measures to control insects in the houses have to be taken.

It could also be shown that the administration of antibiotics during the first days of life of the birds did not result in complete prevention or eradication of a *Salmonella* infection but only in a suppression for a limited time. A permanent use of antibiotics at the farm will result in the development of resistant intestinal and environmental bacteria and, over the time in a diminished efficacy of the antibiotics against the bacterial population of interest, and should therefore be avoided.

In experimental studies it was found that *Salmonella* Paratyphi B d-Tartrat+ isolates are not able to inhibit effectively the intestinal colonisation of *Salmonella* strains of other serovars. Therefore, other factors than colonisation inhibition must be responsible for rapid spreading of *Salmonella* Paratyphi B d-Tartrat+ in special areas or farms.

Investigation of the distribution and control of *Salmonella enterica* serovar Enteritidis PT6 in layer breeding and linked commercial operation

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Salmonella enterica serovar Enteritidis (*S. Enteritidis*), particularly phage type 4, has been responsible for an international epidemic of food poisoning during the last two decades. It is thought that the original spread of this organism may have been related to national and international trade in hatching eggs and breeding chicks but currently, in most developed countries, infection in breeding flocks is rare. Because of the enhanced capacity of *S. Enteritidis* to colonise ovarian and oviducal glandular tissue for long periods it is particularly important that infection in elite, grandparent and parent chicken breeding flocks should be prevented, since further spread of the organism in the hatchery may lead to infection in a large number of commercial laying flocks. If infection does occur then it is vital that it is identified early and that effective measures are taken to limit its further spread. This paper describes a bacteriological and molecular genetic investigation of an incident in which *S. Enteritidis* PT6 occurred in a layer-breeder company.

Intensive sampling was carried out in a layer breeder hatchery, a layer parent rearing farm, a layer parent farm and in a commercial pullet rearing and cage layer farm where *S. Enteritidis* PT6 had become established. PT6 was initially found in focal points in the hatchery, such as hatcher ventilation ducting, tray wash areas and waste areas, but improved disinfection following the installation of more accurate metering devices was followed by a rapid disappearance of contamination. Several different phage types of *S. Enteritidis* were found in the hatchery but most of these proved to be genotypically identical with PT6. Investigations of contaminated layer breeder and rearing sites within the company showed that the terminal disinfection programmes in place were effective in that no carry-over of infection occurred and the organism was rapidly eliminated from the organisation. Infection with PT6 originating from chicks despatched from the hatchery was investigated on a commercial pullet rearing farm. After vaccination for *S. Enteritidis* and several rounds of treatment with a fluoroquinolone antibiotic and competitive exclusion, no *Salmonella* was found in faeces or cloacal swabs but PT6 was present in dust in one of six houses. Sampling carried out after cleaning and disinfection and repopulation confirmed clearance of the organism from the site. The birds originating from the rearing farm were moved to a commercial cage laying farm and again treated with fluoroquinolone and competitive exclusion. Only small numbers of *S. Enteritidis* PT4 were found early in the life of the flock but PT6 predominated in late lay and persisted through cleaning and disinfection to infect the next flock of birds which was placed in the house at a higher frequency still. Molecular typing of isolates from the study, using Pst1/Sph1 ribotyping, PFGE and plasmid profile analysis suggested that some diversification of strains of PT6 had occurred during the time that infection had been present within the breeding company. One strain was however found on the contract breeding farm which was thought to be the source of the infection, in the hatchery and company breeding and rearing farms and in the commercial pullet rearing and laying farms.

The results of this study suggest that apparent differences in phage types and genotypes may reflect short-term diversification and sufficient samples should be tested to exclude this. The study also demonstrates the differences in standards of cleaning and disinfection and the success of control measures for *S. Enteritidis* at the different stages of the egg production pyramid. Despite vaccination persistent infection appears to be a danger on commercial cage egg production farms and improved terminal hygiene and biosecurity measures are needed to address this.

Monitoring of enterohemorrhagic *Escherichia coli* in the abattoir and in cattle farms: a collaborative study

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Abstract

Enterohemorrhagic *Escherichia coli* (EHEC) are a subgroup of verotoxin (VT) producing *E. coli* that, in addition to toxin, express other virulence factors, i.e. the attachment factor intimin (Eae), and enterohemolysin. The best-known EHEC is *E. coli* O157, which has been involved in many outbreaks and sporadic cases of food borne infections, but also other serotypes may be implicated in human disease. It is well known that ruminants, and especially cattle, are a reservoir for EHEC and that they may excrete the bacteria with their faeces. Through contaminated meat, milk or vegetables, through direct or indirect contact with infected animals and through direct human-to-human contact, *E. coli* O157 may infect man. In addition, it is known that the infectious dose for humans may be as low as a few hundred bacteria. For the moment, only hygienic measures at the farm, in the slaughterhouse and cutting plants and in the kitchen are essential in preventing human EHEC infections and clinical disease.

One of the important data in the assessment of the risk for humans to be infected with *E. coli* O157 or other EHEC is the prevalence of this bacterium in the bovine reservoir. In 2001, the Federal Agency for the Safety of the Food Chain set up a monitoring programme in slaughterhouses representative of the Belgian meat production. The results of this monitoring showed that less than 1% of the 1388 cattle carcasses tested were found positive for *E. coli* O157 EHEC. These cattle herds where *E. coli* O157 circulate represent a risk for public health when delivering animals to slaughter.

In case of the isolation of an *E. coli* O157 EHEC (with virulence factors VT, Eae and enterohemolysin) from a carcass at the slaughterhouse, the Federal Agency for the Safety of the Food Chain traced back the herds of origin. Subsequently, 10% of all bovines between 6 months and 2 years of age were sampled and analysed for *E. coli* O157. Bacteriological analysis was by selective enrichment, immunomagnetic separation, plating on selective medium Cefixime-Tellurite-SMAC, and agglutination of suspected colonies with O157 specific serum. Finally, the virulence factors VT and Eae, as well as the genetic basis for the O157 antigen were explored by PCR for confirmation.

The results after one year monitoring are presented. Obviously, sampling of animals from farms that have delivered EHEC to slaughter is significantly more sensitive than random sampling of cattle herds of which no history of *E. coli* O157 is known. In addition, our results confirmed that *E. coli* O157 could be isolated from feed and from dust, and that on a single farm various types of *E. coli* O157 can be detected.

**Contamination of Foods of Animal Origin with *Salmonella*, *Campylobacter*,
L. monocytogenes and *E coli* 0157 in Cyprus**

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Foods of animal origin worldwide are often contaminated with food-borne microorganisms. *Salmonella* and *Campylobacter* are the most common bacteria agents causing food-borne diseases. Although *Listeria* is not a very common food-borne microorganism, Listeriosis has a serious manifestation in the very young and the very old, pregnant women and in particular in the immunosuppressed people. *E.coli* 0157 is also a very dangerous food-borne microorganism for humans.

In Cyprus, results from our Laboratory in the last 15 years, show that poultry and quail carcasses on the average are contaminated at the rate of 54% and 77% with *Salmonella* and 49% and 81% with *Campylobacter*, respectively. Eighty three percent (83%) of poultry and 31% of quail isolates were *C. jejuni*. The remaining were *E.coli*. Additionally, 31% of chicken carcasses were contaminated with *L.monocytogenes*. Foods of animal origin such as beef, minced meat, meat products and live snails, were also found to be contaminated with *Salmonella* and *Listeria*. On the contrary, raw milk and milk products appeared to have a low contamination rate of 4.1% and 0.04% with *Salmonella* and 0.5% and 0.5% with *L. monocytogenes*, respectively. Poultry eggs (contents) were found to have a negligible contamination rate (0.01%) of *Salmonella*, while egg shells had a very low contamination rate (0.9%) with these microorganisms. In a more detailed investigation about *Campylobacter*, examining two poultry flocks, one known to be infected with *C. jejuni* and the other with *C.coli*, it was found after their slaughter, that the mean contamination levels of poultry carcasses with *C. jejuni* and *C. coli* were 1.2×10^6 and 5.7×10^5 , respectively. In one case of subclinical mastitis with *L. monocytogenes* from a cow, it was found that 10^6 cfu of this microorganisms were excreted per ml of milk from the infected teat. Out of 138 samples of various origin non was found to be contaminated with *E.coli* 0157.

Between 50 and 150 *Salmonella* food-borne cases are reported in humans (Laboratory diagnosed) every year in Cyprus. On the contrary there is no detailed information regarding *Campylobacter* and *Listeria* food- borne infections. No *E. coli* 0157 case has ever been reported in Cyprus. It is believed that poultry carcasses and other foods of animal origin may play an important role in food-borne diseases in Cyprus.

Session 2.

Growth and survival along the food chain

Chairman : *J. Olsen*

Biofilms of *Listeria monocytogenes*

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It has long been recognised that in natural environments, bacteria are found predominantly as biofilms. These biofilms generally are defined as microbial cells attached to a surface and encased in an extracellular polysaccharide matrix. It is also widely accepted that biofilms exhibit a specific phenotype which is characterised by a greater resistance to antimicrobial agents and biocides. This property is important notably in the food industry because established biofilms on food plant facilities are very often responsible of contamination during the product processing. *Listeria monocytogenes* is a Gram-positive bacterium widely distributed in the food-processing environment and frequently involved in food-borne disease outbreaks. This micro-organism is often isolated from the surfaces of processing lines in food plants, where it is able to survive for several days or months. This pathogenic bacterium is also able to set up specific survival strategy by up or down regulating genes involved in the colonisation of surfaces. As already indicated, one of the most striking properties of biofilm communities is their increased resistance to antimicrobial agents. This property is certainly the result of additive effects including several specific regulatory mechanisms inside the biofilms leading to long-term survival. Some of the proteins involved in long term survival of *L. monocytogenes* biofilms were identified by a proteomic approach. In the present work, mature biofilms of *L. monocytogenes* and planktonic culture of the same age were studied by a proteomic analysis of their protein content in order to investigate the induction or repression of individual proteins which could characterise the biofilm phenotype. Although no specific biofilm genes or proteins were identified, it is likely that those identified are necessary for long term survival of bacterial cells.

Quantitative investigation of *Salmonella* Dublin from cattle at slaughter and decay during cooling and vacuum-packed storage of beef.

S. Aabo¹, J. Boel¹ & J.R. Pedersen²

Salmonella Dublin is host adapted to cattle and is commonly associated with clinical salmonellosis. It is sporadically isolated from other animal species. In humans, however, causes severe generalised infections. *S. Dublin* is 10-20 fold more invasive than any other zoonotic *Salmonella* serotype with a human mortality rate of approximately 30%. In Denmark, the number of human cases has varied between 10-40 per year in recent years. Therefore, *S. Dublin* from beef is considered a serious food safety problem in Denmark.

Herds, which are specialised in raising calves for slaughter, has a particular risk of being *S. Dublin* infected compared to calves from diary herds. The excretion of *S. Dublin* at the time of slaughter has been studied by examination of 1708 calves from 35 herds, which only raised calves for slaughter. 1668 animals (97.5%) were tested negative while 40 animals (2.5%) excreted between 0.02-0.1 cfu/g feces and 100-1000 cfu/g feces. In another study, contamination of carcasses from herds with a history of clinical *S. Dublin* infection, have been investigated. One hundred and four animals from eight herds were examined at slaughter. Faecal shedding was quantified by examination of rectal content and carcass contamination were tested by swabbing of 2500 cm² carcass. Three animals (2.9 %) excreted *S. Dublin* at levels between 0.04 and 10 cfu/g faeces and three carcasses were contaminated at levels between 1 and 10 cfu *S. Dublin* per 2500 cm².

In order to determine any consumer protective effect from cooling or vacuum packaging, the decay of *S. Dublin* and *S. Typhimurium* DT104 was studied experimentally during prolonged cooling for 11 days or vacuum packaging for 43 days. In a seeding experiment with approximately 10⁴ cfu/ 100 cm², significant reductions of 3 fold for *S. Typhimurium* DT104 (one strain) and 200 fold for *S. Dublin* (one strain) were found during eleven days storage at 2-3°C. When spiked meat cuts were vacuum-packed for 43 days and stored at 2-3°C four different strains of *S. Dublin* showed reductions between 2-15 fold and four strains of *S. Typhimurium* DT104 showed reductions of 2-5 fold.

In conclusion, *S. Dublin* was found equally prevalent in calves from herds only raising calves compared to calves from herds with a history of clinical salmonellosis. Also, vacuum packaging of beef did not seem to provide any significant consumer protective effect towards either *S. Dublin* or *S. Typhimurium* DT104, while cooling may have some consumer protective effect towards *S. Dublin*. Presently, the effect of cooling with and without forced ventilation, are under investigation with a number of *S. Dublin* strains.

***In situ* localisation of *E. coli* O157:H7 on food surfaces.**

Kieran Jordan

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E. coli O157:H7 is among the food pathogens that are currently of most concern to the food industry. Its low infective dose and high risk of complications or even death are cause for concern. An integral part of the cycle of infection of *E. coli* O157:H7 is attachment to food surfaces. Because they are based on homogenisation, conventional microbiological methods, such as plate counts, do not allow a study of the attachment or localisation of bacteria in food. These factors are important as they contribute to our understanding of the importance of food surfaces as a micro-environment for pathogens.

Confocal scanning laser microscopy is a method that can be used to study *in situ* localisation of bacteria in food. Samples can be studied directly in the fully hydrated form and without any sample preparation such as homogenisation or extraction. The use of fluorescein isothiocyanate-labelled antibodies makes it possible to study specific bacteria, like *E. coli* O157:H7.

Using this method the striated muscle fibres and connective tissue (elastin fibres) of meat could be seen, with differential staining. *E. coli* O157:H7 cells were located primarily between muscle fibres and around the elastin fibres. Cells were also located within the connective tissue at a depth of 25 µm. In carrot, *E. coli* O157:H7 cells could be seen at cell junctions and in intercellular spaces at a depth up to 50 µm. In cheese, the cells appeared primarily in small clumps of about 10 cells, although a few single cells were also seen.

These results show the how *E. coli* O157:H7 cells attach firmly to food surfaces and how intracellular surfaces can be important micro-environments for this food pathogen.

Verotoxigenic E. coli (VTEC) in milk and meat samples: possible origin and survival.

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In studies done on the common of VTEC strains in intestinal flora of ruminants as well as in milk and in meat and meat products 1997 and in 2002 in Austria, the at most detected VTEC strains belonged to non- O157 H7 E. coli serotypes and they are widely distributed among cattle and sheep herds. The incidence of the O157 serotypes was 1-3 % and of those of the non-O157H7 serogroupe more than 2-25 % for individual animals and herds respectively.

In connection to this similar observations were also obtained in cases of milk, meat and meat products, when the samples tested for the incidence of all VTEC serogroupes. Isolated VTECS of non-O157 H7 of faeces (n = 200) samples belonged to 16, those of milk (40) to 6 and those of meat and meat products (80) to 11 O serogroupes. The VTEC serogroupes O101, O111, O114, O115, O118, O126, O128 and O137 were found among those of intestinal flora and meat isolates and the serogroupes O55, O78, O114, O125, O126 and O145 among those of milk and faecal flora, while the serogroupe O25, O26 and O114 among those of the three mentioned origins. With PCR on the common of virulence genes tested VTEC strains of the non-O157 and those of O157 on comparison to O157 H 7 of human origin have showed the following picture. *Stx₁* gene alone or in combination with *ehecA*, *eaA* or with both were found by 10% of those of intestinal flora, by 0% of milk and by 20% of meat products. Similarly *Stx₂* alone or with the before mentioned genes were observed by 18 of intestinal flora and by 2,5% of milk and by 60% of meat. *Stx_{1,2}* carrier were detected by those of intestinal flora in 72%, by milk in 97,5% and by meat and meat products in 20%. In the common and distribution of *catP*, *etpD*, *espP*, *col V* no correlation neither between O serogroupe nor between sources were seen. One VTEC strain of the serogroupe O114 were found to carry the same virulence gene spectrum isolated from intestinal, from milk and from meat.

Concerning virulence gene spectrum, a better correlation were seen in cases of VTEC of the O serotype O157 isolated from Human (n= 6), meat (n= 8) and intestinal flora (n=2) of cattle, inspire of the low number of detected and tested strains. Further a low number of VTEC strains belonged to the O serogroupe O26, O101, O111, O114, O115 and O118 of intestinal flora have carried the same virulence gene spectrum as the O157. E. coli severe disease of Human, are worldwide at most caused by VTEC of the Serogroupe O157 H7, inspite of their common in intestinal flora of ruminants in comparison to that of non-O157 very low. VTECS of Serogroupe O157 survive, according to primereyly study at low pH-value (2-3) longer than other strains careering the same virulence genes. They remained viable in milk(2-3 weeks) and on the surface of meat (2-3 weeks) and on the surface of eggs (2 days) at C°. However there are in this connection differences between strains, serotype and their source. In this regard more studies are needed to elucidate the secret of the dominance VTEC of the O157.

Epidemiology of *Listeria monocytogenes* along a rainbow trout food chain production

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The aim of this study was to identify the origin and to characterise *Listeria monocytogenes* strains isolated from different sites located along a river.

Three sites (A, B and C) were investigated during winter 2002. One hundred and two samples were collected by swabbing different places of the fish farms (A and B) and materials in the slaughter plant C, and by sampling live (mucus, fillets, gills...) and eviscerated fishes. One litre of water from upper and down river was also samples at each site. All samples were analysed for *Listeria monocytogenes* detection using an enrichment (Fraser broth) and isolation (ALOA medium) procedures, then typical colonies were identified using Microgen-*Listeria*-ID test. *Listeria monocytogenes* strains were characterised by Pulsed Field Gel Electrophoresis (PFGE) technique using *ApaI* and *AscI* enzymes.

Thirty-eight samples were contaminated by *Listeria monocytogenes* (table 1). A high number of positive samples (14/30) were found at the farm level situated up the river (site A). Concerning farms A and B, *Listeria monocytogenes* was present not only in the water upstream but also in the environment and mainly on the surface (mucus) of live fishes. In the slaughter plant, *Listeria monocytogenes* was found in the environment, especially during the first investigation, and again, on the surface of fishes. Nevertheless, the occurrence was lower during the second (3/18) and the third (2/21) series, due to the improvement of the cleaning and disinfecting procedures and of the hygienic conditions during slaughtering.

Table 1: Results of *L. monocytogenes* from different sites and samples.

Sites	A		B		C		
Series	1	2	3	4	5	6	7
Fishes	8/17	1/4	1/4	0/4	2/7	2/3	0/10
*	(4/4)	(1/1)	(1/1)	(0/1)	(1/2)	(1/1)	(0/3)
Environment	5/9	2/2	1/1	1/2	9/12	1/15	2/11
Water	1/4	1/2	0/1	1/4	-	-	-
Total	14/30	4/8	2/6	2/10	11/19	3/18	2/21

* () : Number of positive fishes out of number of analysed.

Concerning the mode of sampling, it seems that applying swabs (towels drunk with the enrichment broth) on the surface of fishes or on materials, is a very interesting methodology. In that way the mucus sampled on the surface of the fish is very useful.

Combination of *ApaI* and *AscI* results allows classifying isolates into PFGE types. Seven types were identified among 44 strains tested. Two types are predominant: the type 18 seems to be a common type, but was present only in the processing plant. These results confirm that strains present during the primary production do not get over the other during the processing step but it is difficult to explain these results because, in this study, the farm B and the processing plant are adjacent. Moreover the presence of the same type in the processing plant after several weeks and whether the improvement of hygienic procedures, confirms the ability of different resident strains to survive and colonise surfaces of equipment. The second one (type15) corresponds to the serotype 4b. These strains were present in the 2 farms, not only on the surface and intestines of fishes, but also in the environment, i.e. the net, and in the water. The other types are less common but one of them (19) isolates once on the surface of one fish belongs to the "yellow clone" known to be associated with foodborne outbreaks and sporadic cases.

Relationship between antibiotic resistance and thermotolerance in verocitotoxigenic *E.coli*.

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While the use of antibiotics has proven to be an effective means for the prevention and control of bacterial infection, their indiscriminate use has adverse consequences, by promoting the selection and prevalence of drug resistant microbial populations. *E.coli* O157:H7 and various other serotypes of enterohemorrhagic *E.coli* were first isolated in 1980. Since then they have been implicated in food poisoning cases around the world, many of which have been high profile outbreaks. Increasing levels of resistance in *E.coli* has been noted since 1956. There is a concern that antibiotic-resistant strains may react differently to antibiotic sensitive strains, during food processing. In this study, 5 strains of VTEC were studied. These included wild type and antibiotic resistant mutants (resistant to Nalidixic Acid and Streptomycin) of *E.coli* O157:H7 and *E.coli* O26 and a multi-antibiotic resistant strain of *E.coli* O157:H7 (resistant to 11 antibiotics). These were inoculated into mincemeat and were then heated to 55°C, with or without a prior heat shock treatment (48°C for 30 min). Samples were examined over a 50 min period and D-values (time in minutes to receive a 1 log reduction) were then calculated for all five strains. The possession of antibiotic resistant genes was found to affect the thermotolerance of VTEC in mincemeat. The wild-type strains of *E.coli* O157:H7 and *E.coli* O26 were found to be more thermotolerant than their antibiotic resistant counterparts. Interestingly, the D-value for the multi-resistant strain of *E.coli* O157:H7 were significantly lower (1.71 min) than the D-value for the wild *E.coli* O157:H7 (11.70 min) and wild type O26 (9.73 min) on non-selective recovery. These results suggest that antibiotic resistance induces thermal sensitivity in VTEC.

Session 3.

Bacterial changes along the foodchain

Chair: *H. Kruse*

Stress tolerance mechanisms influencing virulence and survival of food borne pathogens

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Bacterial cells respond to stress by expressing a variety of proteins. When the stress conditions lead to misfolding or denaturation of proteins the heat shock response is elicited. Many heat shock proteins belong to one of two categories, namely the chaperones or the proteases. While the chaperones have the ability to prevent misfolding of proteins and may promote refolding of unfolded proteins, the proteases are required for removing non-native proteins from the cells. To tightly control these processes bacterial cells have employed various regulatory strategies including positive regulation by alternative heat shock sigma factors or negative regulation by heat sensitive repressor proteins such as CtsR, HrcA and HspR.

For some years our studies have focused on the importance of proteases for tolerating conditions of environmental stress. We have found that in Gram-positive bacteria the Clp proteolytic complex is directly responsible for removing misfolded protein accumulating during stress and thus, cells lacking the Clp protease are stress-sensitive. In addition we have shown that in *Staphylococcus aureus* the Clp proteolytic mutants have a dramatic reduction in virulence compared to wild type cells. While we initially hypothesized that this was a consequence of the stress sensitivity, our data suggest that the Clp complex rather plays a specific role in controlling virulence gene expression and consequently affects virulence. Interestingly, the Clp proteolytic complex also controls the stress response itself as the repressor activity of the heat shock regulator, CtsR is modulated by Clp, and the Clp proteolytic activity is crucial for activating the novel heat and DNA damage response regulator, HdiR.

In Gram-negative bacteria the heat shock response is often controlled by alternative heat shock sigma factors. However, when the genome of *Campylobacter jejuni* was sequenced it was apparent that *C. jejuni* lacks such sigma factors but encodes homologues of the heat shock regulatory proteins, HrcA and HspR, normally found in Gram-positive bacteria. With the aim of understanding what governs the choice of regulatory control strategies we have undertaken an investigation of the HspR regulon in *C. jejuni* and the signals that controls its activity. In conclusion our studies underscores the extreme complexity of the bacterial response to stress and that additional knowledge is required to be able to predict the behaviour of food borne pathogens.

Link between stress adaptation and virulence of *Salmonella* Dublin and *S. Typhimurium*.

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Zoonotic *Salmonella* perform cycles of infections in food animals and infect humans via contaminated food. They encounter stressful conditions while surviving outside the animals, either in the farm environment or while being transported along the food chain to the consumer. Consequently, they maintain an apparatus, which enables adaptation to less favourable conditions. In the current study, we have investigated to which extent this apparatus is also needed to perform an infection.

An in-frame deletion was introduced into the gene *clpP*, the proteolytic subunit of the ATP-dependent Clp-protease, ClpXP. The mutant grew as well as the wild type strain at 37°C in rich medium, while it was impaired in growth at 45°C, in medium with 5% NaCl and when growing at pH 4.5. Transduction with an intact *clpP*-gene abolished the effect. The mutant invaded J774 macrophage-like cells to the same level as the wild type strain but contrary to this, it did not increase significantly in numbers over a 48-hour period, and it was significantly less cytotoxic to the cells. The mutation resulted in a delayed oxidative burst of the J774 cells. The mutant was completely attenuated in a mouse virulence assay. Like the growth effects, transduction with an intact gene complemented this. In summary, an intact *clpP* gene is required for stress adaptation and virulence in *S. Typhimurium*. The reason for this remains to be investigated.

In *S. Dublin* we investigated a library of signature tagged transposon mutants that were pre-selected for mutations that lead to attenuation in a mouse model. These were subjected to heat stress (45°C), and a mutant in the LPS-associated gene, *manC* (*rfbM*) was identified as impaired in growth. It was also impaired in growth when subjected to salt, pH and oxygen stresses. The mutant expressed the natural LPS profile but produced less LPS than the wild type strain. This is the first report that LPS may be important for the natural ability of *Salmonella* to adapt to stressful conditions. The mutant and the wild type strain invaded and survived inside J774 cell at the same level, but the mutant showed a reduced oxidative burst of the macrophage-like cells. It was also attenuated in intra-gastric challenge of mice. The exact mechanism underlying the influence on stress adaptation and virulence has to be investigated.

**STRESS RESPONSE OF *SALMONELLA* TYPHIMURIUM AND *SALMONELLA* ENTERITIDIS ISOLATES:
RELATIONSHIP BETWEEN CELL FATTY ACIDS AND PHAGE TYPES**

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INTRODUCTION

Cyclopropane fatty acid (CFA) formation is a post-synthetic modification of the lipid bilayer (PL) that generally occurs abruptly as cultures of *Escherichia coli* and many other bacteria enter into the stationary phase (Chang and Cronan, 1999). CFAs are present in the cell membranes of several pathogenic species or genera including *Helicobacter*, *Salmonella*, *Campylobacter*, *Vibrio* and *Yersinia* (Guckert *et al.*, 1986; Haque *et al.*, 1996); however, the physiological rationale for cyclopropane ring formation in bacterial membranes remains obscure (Grogan and Cronan, 1997).

Various culture parameters have been observed to determine the extent of cyclopropanation of the membrane PL: in exponential phase cells of *E. coli* the acid habituation was associated with a significant conversion of unsaturated fatty acids (UFAs) to cyclopropane FAs (Brown *et al.*, 1997). However, the conversion of UFAs to CFAs, as a sudden response to stress exposure, and its relation with survival has not been investigated before neither in *E. coli* nor in other microbial species.

The aim of this work was to establish whether cyclopropanation of membrane UFAs is a stress response mechanism in pathogenic and non-pathogenic phage types of *Salmonella* Typhimurium and *Salmonella* Enteritidis. In particular membrane phospholipid CFA changes of 12 food related strains of *Salmonella* Enteritidis and *Salmonella* Typhimurium, when exposed to abrupt oxidative, cold and heat stresses, were evaluated. The 12 strains isolated from poultry meat and poultry meat based foods were chosen because of their different genetic and phenotypic traits (antibiotic resistance profiles and phage types) and membrane fatty acid composition under standard conditions.

RESULTS AND DISCUSSION

The ability of bacteria to act as foodborne pathogens depends in part on their capability to maintain the integrity of their cytoplasmic membrane throughout the food chain as well as host gastric stomach and colonize the intestinal traits of humans.

The comparison of the viability and membrane fatty acid adaptation patterns to the various environmental changes showed that each strain of *Salmonella* Enteritidis and *Salmonella* Thyphimurium considered in this work exhibited its unique stress response although some general remarks can be outlined:

- 1) the differences in CFA's proportion of the various strains under standard conditions were considerable and they were not related to the serotype, genetic traits nor antibiotic resistance profiles of the strains.
- 2) The survival of the majority of the strains considered during the exposure to various stresses was not dependant on the initial CFA level, but appeared to be associated to the ability to rapidly or instantaneously convert unsaturated C18:1 and C16:1 fatty acids to their corresponding CFAs. This response pattern, which was associated with a major stress tolerance, was observed only in the isolates belonging to the phage types PT4, PT6, DT12, DT104 regarded as those frequently isolated from livestock and cases of human diseases.
- 3) Only few strains, characterised by a minor heat and oxidative tolerance regardless of their CFAs initial level, did not modify the proportion of CFAs during stress exposure. These strains belonged to the phage type DT193 that is regarded as a non-pathogenic one for humans.

The function of the rapid or instantaneous conversion of UFA to CFA is not obvious, but appeared to be linked to a major thermotolerance during the heat treatment or oxidative stress, although this FA modification is clearly not the only mechanism ensuring a more efficient survival.

The exposure to acid, high osmolarity, starvation, heat and oxidative stresses "*in se*" can be regarded as a putative virulence factor. In fact, as suggested by Bodnarek and Olden (1996), some virulent *Yersinia enterocolitica* strains have the potential for maintaining virulence when grown under adverse conditions due to the occurrence of some key fatty acids including CFAs. Moreover, much remains to be investigated about the physiological functions of these membrane components or better of their more or less instantaneous conversion on the regulation of cyclase gene expression during stress exposure and ultimately on its role in the host adaptation.

Genetic instability as a mechanism for *Campylobacter jejuni* for environmental survival and enhanced colonisation potential

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The plasticity of the *Campylobacter* genome is well recognized and has been observed both over the total chromosome and in hot spots such as the flagellin genes. These effects are frequently reflected in the instability of genotypes by techniques such as PFGE and Fla typing. A hypothesis has been proposed (Wassenaar and Newell, 2001) that mosaic rearrangement provides a mechanism whereby *Campylobacter jejuni* can increase its genetic potential to survive environmentally stressful conditions. This hypothesis has been investigated using sets of clonally-related strains derived from geographically and/or temporally related sources. Strain sets were exposed to heat and cold stress consistent with poultry meat processing. These results indicated that mosaic rearrangement can be induced by such stresses. In order to determine whether such rearrangements enabled certain variants colonisation advantages, strains from a single batch of poultry meat were investigated using an in vivo model of chick colonisation. This group of isolates were previously shown to possess the capacity for genomic rearrangement as demonstrated by minor differences in PFGE profiles whilst their genotypes detected by other methods remained unchanged (Wassenaar et al , 1998). Isolates were selected for study that had undergone changes in PFGE genotype, but not Fla type, following transport and storage, and isolates that displayed different genotypes from a single sampling swab. Isolates representative of the different PFGE genotypes derived from the same swab were passaged, in groups of 2 to 3, through the one-day old chicks, and any predominance in this competitive environment determined. Overall, one of the PFGE types was predominant after 2 and 4 weeks passage, suggesting that this type preferentially colonized the chick gut. However, for some strain combinations new PFGE types were observed in one or two, but not all, birds in that group. This observation suggests that at least in some birds genomic rearrangement, as observed by the formation of new genotypes, was occurring in *C. jejuni* colonizing the chick intestine. An infected seeder bird model was used to compare prevalence of genomic instability in *C. jejuni* colonizing the chick intestine by a horizontal route. Preferential selection of a single PFGE type, with evidence of genomic rearrangement in some birds, was once again the outcome. Thus genetic instability as a result of environmental stress can enhance colonization potential.

Responses of *Campylobacter jejuni* to heat and cold

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Campylobacter jejuni is a supremely important human pathogen and the World Health Organisation (WHO) estimates that ~ 1% of the population of developed countries will be infected each year. This represents a huge health and economic burden. *Campylobacter jejuni* is an interesting organism and there is a general view in the international scientific community that it is highly sensitive to the extra-intestinal environment, which does not fit easily with its high success rate as a zoonotic pathogen and the fact that many vehicles have been implicated in human infection. It now becoming clearer, particularly when appropriate recovery methods are used, that *Campylobacter* spp. are much more robust than previously thought and can be recovered, for example, from dry surfaces 24 hours after the initial contamination event, albeit in low numbers. Recent work in Bristol is also helping to provide other possible explanations for the success of *C. jejuni* as a human pathogen. Three areas of study are being pursued. The first has shown that *C. jejuni*, in marked contrast to *Salmonella* spp. and *Escherichia coli*, does not become heat-sensitive when held at refrigeration temperatures. In fact, heat tolerance shows a small but significant increase (Hughes, unpublished). The second area of work is examining the effects of attachment on heat tolerance and has found that cells of *C. jejuni* attached to chicken muscle are much heat-tolerant than free cells. The final work programme is looking at *C. jejuni* gene expression at 4-6°C. The cells are quite metabolically active at these temperatures and it has just be shown (Cogan *et al*, unpublished) that 144 genes shown a significant increase in activity at low temperature. The gene, which shows the greatest increase in expression is a novel two-component regulator. Fifty genes also show significant down-regulation. These and other matters will be discussed.

Practical aspects of *Campylobacter* typing along the food chain

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Considerable efforts have been made to develop molecular typing methods for *Campylobacter*. A series of appropriate methods is available and has been tested for reproducibility and robustness, and for the possibility to exchange results between laboratories. These techniques have been used in studies on the epidemiology of *Campylobacter*. Some of these studies conclude that some specific genotypes are restricted to certain hosts whereas other studies conclude that the lack of shared genotypes in animal reservoirs and humans exclude these animals as source for human infection. We think such studies should be interpreted with great care, as discussed below.

From *in vitro* studies it is known that some *Campylobacter* strains are natural transformable. In *in vivo* experiments we demonstrated that there is a rapid and considerable exchange of genetic material between *Campylobacter* strains in the chicken gut. In broilers and on poultry meat samples, the presence of mixed cultures of *Campylobacter* are common. However, in epidemiological studies in which faecal or product samples are cultured to isolate/enrich *Campylobacter*, usually only one or a few colonies per sample are typed. The non-dominant *Campylobacter* strains will almost never be detected.

The combination of the rapid exchange of genetic material between *Campylobacter* strains, and the fact that in mixed cultures the lower coloniser will not be detected, makes that with the current practice of isolating strains on plates and subsequent genotyping of isolated colonies, conclusions have to be drawn carefully.

Whole genome comparison of Enterohemorrhagic *E. coli* (EHEC): Definition of the minimum genomic core for pathogenicity

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Sequencing of *E. coli* O157 genome has recently shown the presence of a large amount of DNA horizontally acquired in the chromosome of this micro-organism. Besides the LEE and the Stx-converting phages, in this DNA are recognisable numerous putative pathogenicity islands (PAIs) that could be involved in the pathogenesis of EHEC infections.

To assess if these PAIs belonged to EHEC O157 only or if they were instead more diffuse in the different EHEC and EPEC clones, we used a whole genome approach based on the use of commercially available microarray slides containing 5500 genes from EHEC strains EDL 933 and Sakai.

As a pilot experiment, we tested these slides twice against two O45 LEE-positive *E. coli* strains isolated from pigeons believed to be of reduced pathogenicity but still able to induce attaching and effacing lesions on Hep2 cells and producing a typical Stx2 toxin (*Stx2f*).

Using this approach, we identified 80 ORFs absent from both the O45 isolates. Sixty-nine of these are common to *E. coli* K12, but the remaining 11 are instead specific to EHEC O157 and are part of putative PAIs O#52, O#57, O#93 and O#166 in the EDL 933 strain.

The absence of these PAIs from strains with reduced pathogenicity may indicate that these mobile genetic elements could be involved in the pathogenetic process, therefore their presence among the EHEC and EPEC clones must be evaluated. To this purpose, the presence of these genes is now under extensive testing by PCR against a large panel of EHEC and EPEC strains representative of the different pathogenic clones.

Posters

Screening for VTEC in Hungary

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Shiga toxin (verocytotoxin) - producing *Escherichia coli* strains (STEC/VTEC) are not frequently detected in Hungary. Regional variations in VTEC infection rates occur for several reasons including the occurrence of outbreaks, variations in screening and testing policies, and real variations in incidence. Apart from the predominant serotype O157, sporadic non - O157 strains have also been isolated. Sixty eight VTEC were detected: O7:HNT (1), O18ab:NM (2), O19:HNT(1), O26:H11(3), O39:H48(1), O76:HNT (4), O76:NM (2), O86:NT (1), O91:HNT (1), O96:HNT (1), O98: NM (2), 109:NT (1), O138:NM (1), O146:H28 (2), O146:NT (2), O157:H7 (9), O157:H33 (1), O157:NT (4), O157:NM (27), ONT:HNT (2). Twelve cases were associated with small family outbreaks and 56 VTEC strains were isolated from sporadic infections. Two non - O157 VTEC strains (O39:H48 and O138:NM) originated from animals. Three O157 VTEC strains fermented D-sorbitol. Twenty three were Stx1, eight Stx2 and thirty five Stx 1+2 producer. The yearly isolation rate of the main without significant evolution time. Although some variation in the yearly isolation rate of the main serogroups was observed, no significant trend appeared. Clinical data were available in 40 patients: 26 (%) presented with uncomplicated diarrhoea, often not bloody, 12 with hemorrhagic colitis (%) and two with HUS (%). In conclusion , VTEC infection in our country was typically sporadic and the isolation rate remained stable in time.

Sources and characteristics of bovine *Escherichia coli* O157 and verotoxic *E. coli* (VTEC) in Hungary

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The presence of *E. coli* O157 and Verotoxin-producing *E. coli* (VTEC) in beef and in dairy cattle was investigated by using O157 specific immunomagnetic separation (IMS) and VTEC-Screen kits, and the isolated *E. coli* strains were characterised. Altogether 3190 *E. coli* colonies - originated from different sources including from the colon of slaughtered beef (n=245), from the faces of healthy dairy cattle (n=60) and from fresh milk samples (n=114) - were screened. Total of 8.9 % (22/245) of colon and 7.4 % (8/114) of milk samples contained *E. coli* O157 strains. No *E. coli* O157 was isolated from the faces, but 8.3 % (5/60) of these samples proved to be Verotoxin-producing out of which two non-O157 VTEC strains were identified by PCR. Four out of the 23 (17.4 %) colon strains and 1 out of 8 (12.5 %) milk origin *E. coli* O157 strains harboured *stx* gene. A previously isolated milk origin *E. coli* strain did not contain *stx* gene either. Three Verotoxin-producing colon origin *E. coli* O157 strains carried *stx1*, *stx2* genes whereas *stx* gene of the non-O157 colon strain was untypable by *stx1* and *stx2* primers. The only VT⁺ *E. coli* O157 strain of milk origin harboured *stx2* gene, which was proven by *stx2*-specific agglutination. All the *E. coli* O157 strains of colon origin had *eae* gene encoding for intimin. Five of these strains had γ type *eae*, one strain had β type *eae* and α *eae* was found in any of the O157 strains. Eighteen (86 %) *E. coli* O157 strains of colon origin had the *E. coli* O157:H7 serotype-specific plasmid (pO157) -encoded *entHly* gene and 15-15 (68%) strains had *espD* and *paa* genes. None of the strains had *cnf* or *cdt* genes. One of the VTEC strains isolated from the faces had *eae* gene, as well. *E. coli* O157 strains did not ferment sorbitol, were motile and did not produce colicin.

In conclusion, by using the above preselection methods we could isolate *E. coli* O157 and non-O157 VTEC strains from bovine sources. The *stx* gene occurred with low frequency (4/31) in these *E. coli* O157 strains, although the strains had LEE pathogenicity island (*eae*⁺, *espD*⁺), and pO157 (*entHly*⁺). On the basis of these results we can assume that the majority of the *E. coli* O157 strains isolated and characterised in this study can not be regarded highly pathogenic enterohaemorrhagic *E. coli* (EHEC), but they seem to have potential to become EHEC as a result of *stx* gene transfer as was demonstrated elsewhere.

***Salmonella* sero- and phage types in Hungarian layers, broilers and products**

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The *Salmonella* Reference Laboratory of the National Food Investigation Institute examines the serotypes of *Salmonellae* isolated by the units of the animal health & food control service (animal health institutes, animal health and food control stations). It also investigates the phage type and antimicrobial resistance of *Salmonella Enteritidis* and *S. Typhimurium* strains of animal origin.

The Hungarian and international *Salmonella* outbreaks encourage the interest against the *Salmonella* contamination of egg, egg products and poultry meat.

The EU Directive on zoonoses admitted by the WHO urged the Hungarian scientific societies and the Ministry of Agriculture to elaborate and conduct a *Salmonella* eradication programme. This programme has been existing from the middle 90's. The results of its operation are the following: *Salmonella Enteritidis* **infection**, which was the most common salmonellosis of *Gallus gallus*, decreased from 79% to 26%. *Salmonella Typhimurium* infection also decreased, from 4,3% to 2,8%. Unfortunately during this period serotype *Infantis* became the most popular serotype, and the level of infection is growing (38%).

Data of *Salmonella* **contamination** are the following:

S. Enteritidis: decreased from 58,8% to 7,6%

S. Typhimurium: increased from 1,8% to 3,7%

S. Infantis increased from 15,6% to 83%.

Data collected from the results of eradication programme prescribed by the 49. / 2002. (V. 24.) FVM Order:

S. Infantis: 74%

S. Enteritidis: 14%

S. Typhimurium: 1,6%

Concerning serotype *Enteritidis*, 6 and 6b are the most common phage types; in most cases all kinds of isolates belong to these two types, except for the strains of turkey origin, in which 15 is the most frequently isolated variant. Among *S. Typhimurium* strains 2 and 2c phage types can be isolated most frequently. 2 is much more characteristic for pork and cattle than for other species. 2c is common in poultry. 2 and 2c phage types of the Felix-Callow system used by our laboratory refer to the multiresistant definitive type (DT) 104 of the phage scheme of Anderson.

Epidemiological data on *Campylobacter* infections in Hungary

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As in the most of developed countries, the annual incidence of diseases caused by thermotolerant campylobacters in Hungary shows a significant increase in the last decade. To understand the mode of transmission of the pathogen in sporadic cases a cooperative work was initiated with the participation of the National Center for Epidemiology and the National Food investigation Institute.

The aim of the study was to evaluate the contamination level of food and to implement the characterisation of the isolates of food and human origin by the same methods. 92 % of chicken meat, 50% duck and goose, 16 % pork and 1.8% of the raw milk samples were found to be contaminated with thermotolerant *Campylobacter* spp.

Because of the problems with the identification to species level by culture based techniques performed by different laboratories (e.g. hippurate-negative *C. jejuni* isolates) a multiplex PCR system worked out by Cloak and Fratamico (2001) was introduced. It seems that 15 % of the strains had previously been misidentified by the routine labs.

Our data on antimicrobial susceptibility show a high level of resistance to fluoroquinolones (76% at chicken, 37 % at the water fowl and 52 % at the human isolates). There is a very low level of resistance to erythromycin at present (some human isolates has also been found, but not included in this examinations).

A commercially available serotyping system based on the heat- stable antigens was evaluated for the typing of the strains, however more than 40% of them were non-typeable. Therefore, molecular typing methods, as ERIC-PCR and PFGE (*Sma*I, *Kpn*I) were introduced for the further characterisation of 50 sporadic human (sent to the National Center for Epidemiology by the routine labs) and 30 isolates of food origin (all isolated in a month period in Budapest). ERIC-PCR can be a useful tool for the first grouping of the isolates as a relatively cheap and easy method in case of a high number of samples to be compared (sporadic cases, comparison of strains or different origin as food and human isolates), however the reproducibility is not always satisfactory. Based on the PFGE pattern, the 80 examined strains could be ranged into 24 major *Sma*I genotypes or into 44 complex (*Sma*I and *Kpn*I) genotype). We could observe the presence of the same complex genotypes from epidemiologically previously non-related samples from different districts of Budapest and/or from samples of food origin from big retailers. The genetic diversity of *C. coli* isolates in this study were much lower than that of *C. jejuni*. Raw poultry meat seems to be highly contaminated with thermotolerant campylobacters in Hungary as well, contributing to the increasing incidence of human infections. Therefore the need at least to reduce the frequency and the level of contamination in the farms and during meat processing also focusing on the meat handling by the end consumer would play an important role in the infection control.

Hungarian veterinary antibiotic resistance monitoring system and the results on *Campylobacter* strains

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Because of the rapid development and spread of antimicrobial resistance it is important to monitor antimicrobial resistance of pathogenic zoonotic and commensal bacteria. Susceptibility testing of bacteria from different routine samples of animal origin has been carried out in veterinary institutes for a long time but by an inconsistent methodology. The disc diffusion method proposed by the NCCLS was introduced in all institutes in 1997.

In order to obtain a coherent view of the antimicrobial resistance of bacteria a computer system was established, consisting of a central computer to store all data and as many local computers as necessary attached to it through the network. Local computers are connected to video cameras, which displays the picture of agar plates on their monitors. The inhibition zone diameters of bacteria can be drawn with the mouse. The software measures the diameters, determines if the bacteria are susceptible or not. The evaluation is based on the data of the NCCLS.

This integrated system is suitable for monitoring trends in antimicrobial resistance of bacteria from animals, food and humans, facilitating comparison of the occurrence of resistance for each circumstance in the chain. It depends on the examiners which antibiotics they want to examine. Thirty-two different antibiotic panels were compiled, taking into account the active ingredients of drugs permitted for veterinary use in Hungary, intrinsic resistance and cross-resistance, the mechanism of resistance and the special requirements of the various animal species, and the recommendations of the OIE Expert Group on Antimicrobial Resistance. We can change the members of the panels any time, even during the measuring process.

Since January 2001 each of the 19 counties of Hungary has submitted three tied colon samples from a stock of cows, pigs and broiler chickens to the Central Veterinary Institute every month. We culture *E. coli*, *Salmonella*, *Campylobacter* and *Enterococcus* strains from the samples.

Within the last 2 years 176 *Campylobacter* strains were isolated from slaughter chicken, 204 from slaughter pigs and 142 from slaughter cows. Cephalotin resistant strains were examined for hippurate hydrolysis. Hippurate positive strains were identified as *C. jejuni*, and the others are *C. coli* or *C. lari*. The resistance of the strains is tested by disk diffusion and E-test. The results of the 2 tests correlate with each other.

Nalidixic acid resistance was the highest and erythromycin resistance was the lowest among the strains from all of the 3 animal species.

Prevalence of *Salmonella* in poultry carcasses and their products in Lithuania

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In this study, a survey was carried out to determine the prevalence of *Salmonella* in Lithuania in poultry products for the period of time 2000-2002. The samples included chicken carcasses, chicken parts (wings, legs, and breast) as well as carrions, outwash from slaughterhouse and excrements. A total number of 3379 chicken samples were analysed by bacteriological, biochemical, serological and PCR methods. *Salmonella* was isolated from 19 (1.28%) out of 1480 samples in year 2000, from 7 (4.7%) out of 883 samples in 2001 and from 12 (4.1%) out of 1066 samples in year 2002. According to our results, only three (0.76%) excrement samples for *Salmonella* from 393 day-to-day chicken were positive, meanwhile the excrement from 4-16 week age chicken were positive for 20 (1.22%) of 1634 investigated samples. The results obtained using conventional identification methods showed that poultry products were contaminated with *Salmonella* belonged to 11 different serotypes. *Salmonella enteritidis* and *Salmonella typhimurium* were the most prevalent serotypes. *Salmonella typhimurium* were the most frequently isolated from chicken meat samples, meanwhile *Salmonella enteritidis* was isolated from chicken carcasses as well as from carrions, outwash and excrements. The other serotypes were *S. isangi*, *S. gallinarum*, *S. bovis* and *S. spp* with only one isolate of each. The results of our investigations indicate that *Salmonella typhimurium* and *Salmonella enteritidis* are the dominant serological type in Lithuanian chicken products, as they are in many other countries.

Cellulose production by *Salmonella enteritidis* affects thermoresistance and adherence to egg shells

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Summary

Most of the environmental *Salmonella* strains are able to form biofilms on materials of different nature and under different growth conditions. It is recognised that bacterial biofilms exhibit enhanced resistance to adverse conditions such as desiccation, extreme temperatures and the effect of antibiotics or sanitizers.

Different studies have shown that cellulose is an essential component of the biofilm matrix produced by *salmonella* and other *enterobacteriaceae* like *Escherichia coli* or *Klebsiella pneumoniae*(1,2) . It has also been demonstrated that, although the ability to produce cellulose does not affect to the virulence of the bacteria, it protects them from the action of chemical agents such as NaClO (2). In the present study we have compared wild type strains of *Salmonella enteritidis* that show different abilities to produce cellulose and also wild type strains *versus* their isogenic cellulose deficient mutants for the ability to adhere to the external surface of hen egg shells and also for the sensitivity against heat stress in different growth conditions. Therefore, polar effects could not be excluded because this work has been performed using wild and isogenic mutants specifically required for the biofilm development.

Experimental procedures and Results

Salmonella strains were divided in four groups, in function of the media where they were able to produce the extracellular matrix. Adherence studies were performed using 1 cm² shell pieces from one day old eggs and thermoresistance assays were carried out starting from 10⁶ ufc/ ml exponential phase bacteria suspensions.

Egg shells inoculated with strain 3934bcs-E-Km mutant showed 10 fold less adherence after two hours at 37°C than wild strain 3934. Furthermore no differences were detected in the attachment of wild type and mutant strains to shells after 14 hours

Heating at 55°C during 120 minutes there was a significative difference (p<0.05) of survival between the cellulose producing and cellulose non producing strains.

Conclusions

1-As the growth rate of the cellulose minus mutants is not decreased, the production of this exopolysaccharide is not involved in the bacterial survival.

2-The cellulose producing strains have higher thermoresistance than the cellulose minus mutant strains

3- The ability to produce extracellular cellulose seems to be a mechanism to enhance survival of *Salmonella enteritidis* in surfaces.