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Methods of detection and molecular characterisation of pathogenic E. coli

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Executive Summary

The subject of the first official PEN conference was methods of detection and molecular characterisation of pathogenic *E. coli*. The aim was to focus on non-O157 Shiga toxin producing *E. coli* (STEC) and other pathogenic *E. coli*, though most of the information discussed was relevant to STEC. Although many aspects of methodology were discussed, there still appears to be no consensus on the best way for approaching the detection and isolation of non-O157 STEC from foods, environmental samples, animals and clinical specimens. However, two common approaches have been adopted by most researchers working in this area depending on the focus of the study. Firstly there is testing for specific serotypes of STEC, this approach is used to target the most common and clinically significant STEC. This is particularly relevant for workers investigating outbreaks and sources of clinical infection where the serotype of STEC is known and for investigating the ecology and epidemiology of relevant STEC serotypes. The second approach targets the broad group of STEC and generally uses methods focussed on the detection of either Shiga toxins or the genes which encode them (*stx*).

ISO/CEN has a working group looking into a standard method for STEC detection, the advantages of having such a method would be the ability to compare data between different areas and laboratories and as a benchmark for other methods to be developed. It was suggested that an open method may be the best approach, eg. any method could be used providing it gave a certain sensitivity, specificity, false positive and false negative rate. However, the problems associated with defining STEC (eg. does the method need to target only certain STEC or does it need to target all STEC etc) will mean this approach is still a long way from being incorporated into an ISO standard.

Some other interesting information coming out of the meeting was the push from USDA to have a regulatory system in place for non-O157 STEC. Peter Feng (USDA) was a keynote speaker and suggested that the US will eventually have to test for non-O157 STEC, particularly the STEC of serotypes O26:H-, O103:H2, O111:H-, O121:H7 and O145:H- as well as O157:H7. However there is the dilemma of having no clear definition of non-O157 STEC and which of the STEC should be targeted. Peter Feng is keen on using the seropathotype groupings of (Karmali et al. 2003) where STEC are divided into 5 seropathotypes (seropathotypes A to E) based on their ability to cause outbreaks and their association with severe human disease. However, European researchers are pushing for a combination of targeting specific serotypes and looking at Shiga toxin genotyping. The latter is of interest as there is increasing evidence that the type of Shiga toxin is clinically relevant. More information about the virulence aspects of STEC will be presented in the next PEN meeting to be held on the 6-7th March, 2008. This second meeting may provide further insight into what makes an STEC potentially virulent, then an appropriate STEC definition can be developed and the methods for detection will follow.

General Summary of Meeting

The first official PEN meeting was held at the Chipping Campden Food Research Association (CCFRA) in Chipping Campden (Figure 1). The title of the meeting was Methods of detection and molecular characterisation of pathogenic *E. coli*. The meeting consisted of 10 invited presentations and 16 posters. There were 59 registrants for the meeting.

Figure 1. PEN delegates arriving at the CCFRA. The meeting was held on the top floor of the bulding on the left, the bottom floor of this building had been flooded 5 days earlier and it was a credit to Chris Baylis and the staff of CCFRA for having the venue ready for the meeting.

The next meeting will be held in Rome in March 6-7th 2008 and will focus on pathogenicity, virulence and emerging pathogenic *E. coli* (and the anticipated July meeting will be shifted back into September and will be located in Sweden).

General summary:

- there is no clear definition of STEC that covers all clinically significant strains
- the complexity of nomenclature around STEC, Shiga toxins and other pathogenic *E. coli* needs to be simplified to assist in a way forward for defining STEC and hence developing detection methods
- diarrhoeagenic *E. coli* (DEC) have a high incidence in the human population, particularly ETEC in Belgium (thought to be a result of the changing food supply with more produce entering the EU from countries such as Israel)
- other types of *E. coli* such as attaching and effacing *E. coli* (A/EEC) are difficult to find due to the problems of defining these groups and the lack of reporting clinical cases
- there appears to be a low incidence of typical EHEC (eg. *eae* positive) in foods while atypical EHEC are often more common (eg. *eae* negative)

- different methods may need to be applied to different foods particularly when investigating an outbreak therefore standard methods may need to be flexible and must maintain the ability to be modified to suit new situations
- several research groups are undertaking large evaluations to develop media for isolation of non-O157 STEC (particularly the serotypes O111, O26, O103, O145)
- array technology may allow rapid (but costly) identification of STEC and other pathogenic *E. coli* though simultaneous screening of multiple virulence genes
- currently no regulatory position in the US for non-O157 STEC, but this is likely to change in the future
- no regulatory position in the EU for *E. coli* O157 or non-O157 STEC

General Ecology

Analysis of publications on STEC has shown that different serotypes have the potential for different ecology. There were 4,111 reports of STEC with the most common sources of isolates identified as sick humans, healthy animals (cattle, sheep) and sick animals. Reports of *E. coli* O26:H11 found the most common sources of these isolates in decreasing order were sick humans, sick cattle, healthy cattle and healthy sheep. For *E. coli* O111 the most common sources were sick humans and sick cattle, the prevalence in healthy cattle was very low. *E. coli* O91:H- was isolated from sick humans and healthy sheep which was different to *E. coli* O91:H21. There may be host specificity for specific serotypes. Analysis of the serology of patients from the Australian outbreak associated with mettwurst found that the severity of disease and the number of complications increased with exposure to multiple serotypes of STEC. There was consensus that there are often double infections in humans with two or more STEC serotypes present. Isolates without *stx* are often found in patients potentially indicating that these isolates may be the survivors after phage induction has taken place and resulted in release of the toxin.

A survey of diarrhoeagenic *E. coli* (DEC) in Denmark has been conducted over the past 10 years. The incidence of enterotoxigenic *E. coli* (ETEC) was found to increase, as did the incidence of enteropathogenic *E. coli* (EPEC). The STEC incidence in Denmark is 2nd only to that found in Scotland. The incidence of various DEC fluctuates but attaching and effacing *E. coli* (A/EEC) were frequently isolated. A/EEC contain *eae*, do not carry *stx* and do not belong to known EPEC serotypes. The cause of food borne disease as a result of imported foods was highlighted by an outbreak of ETEC resulting from basil imported into Denmark from Israel. This raised the issue of change in global food production and how diseases once thought of as travel specific, may become more common due to imported foods.

Pathogenic E. coli in Foods

Studies from Germany investigating the incidence of STEC in foods were conducted between 2002 and 2005. STEC were isolated from 8-16% of raw milk cheese and from 7 to 12% of meats (including game but not poultry). The methods used involved a 6h enrichment followed by an *stx* ELISA. Colony blots targeting *stx* were used to isolate the organisms. Of the 219 STEC isolates detected, only 43% carried the *stx* genes (stx_{2a} and stx_{2d}) associated with greater virulence in humans. Only 5% of the isolates carried *eae* and 45% *ehx*, indicating that typical EHEC are rare in foods. Atypical EHEC, considered as those isolates which carried stx_2, but did not carry *eae* were

more frequent and belonged to serotypes O8, O22, O91, O113, O174 and O178 and were mostly H21.

In addition to EHEC and STEC, there was a presentation on Enteroaggregative *E. coli* (EAEC). But are these *E. coli* important in developed countries? Cooked meats, fresh produce and water and ice have been identified as vehicles of EAEC infection. Like EHEC, there has been difficulty in defining this group of *E. coli*. Some of the virulence genes associated with these bacteria are not exclusive to EAEC and many of the genes are carried on a large plasmid hence stability of the genes can be an issue. There are various potential gene targets which include fimbiral genes, toxin genes and plasmid encoded genes. There is no diagnostic marker for these organisms as they are a heterogenous group of *E. coli*. The targets chosen by the group at the Health Protection Agency included the fimbrial genes *aaT*, *aaP* and *aaiA*, as well as the *uid* gene specific for most strains of *E. coli*. Different enrichment broths and incubation temperatures were tested with the most appropriate of these for PCR screening found to be TSB at 41.5°C for 24 hours. Screening with PCR worked well for foods including lettuce, cooked ham, milk, bean sprouts and raw beef. Detection in foods which may contain other *E. coli* (such as raw beef) continues to be challenging as PCR screening tests may be positive but it is difficult to find the EAEC once the sample has been plated and colonies picked for testing for the presence of virulence genes. It was estimated that for every 1 EAEC colony tested, there were at least 40 naturally occurring *E. coli* making it necessary to screen a large number of colonies.

Detection

In Europe the major non-O157 STEC serotypes are considered to be O26, O103, O111 and O45, but also O121 and O91 to a lesser extent. The first of these serotypes have been targeted by using a PCR specific for the O antigens. In addition, an extensive study on the phenotypic properties (such as carbohydrate fermentation) and resistance to inhibitory and selective compounds of these organisms has been conducted. The aim was to develop a selective and differential medium to assist in the isolation of these organisms and there is currently a patent under application for the production of this medium. The most appropriate enrichment was determined to be a 2 step process involving 6h at 37°C with moderate concentrations of inhibitory compounds (such as bile salts) followed by an 18h enrichment at 42°C with higher concentrations of inhibitory compounds. This enrichment process followed by plating onto the selective and differential medium developed had reasonable success in isolating target STEC from cheese, pasteurised milk and ground beef. However, the target organisms were more difficult to detect in faeces and cheese made from unpasteurised milk. The use of immunomagnetic separation (IMS) after enrichment was not tested and is recommended to assist in the recovery of these organisms. An IMS and multiplex PCR validation study using ring trials will be considered towards the end of this year. No naturally contaminated samples have been processed using this method.

Molecular detection tools have been produced to detect various serotypes of STEC. A group working in France have developed a real time PCR for detection of *stx* genes, seropathotype A (eg. *E. coli* O157:H7) and seropathotype B (*E. coli* of serotypes O145, O103, O26 and O111) isolates. The PCR targets included the *wzx* and *wbd* genes (O-antigen related) of O26, O111 and O103, the *galE* of O103 and the small inserted island (*isl*) of O145. The master mix consists of 10

primers and 5 TaqMan probes for the serotypes. It only works on isolated colonies in pure culture, but there are studies underway to investigate the potential for use on enriched foods. The problem always associated with PCR on foods is that you can not be sure that all gene targets exist within a single isolate. The proposal for foods is to screen with the *stx* PCR, if negative, then no further action is required. If positive, then the enrichment can be screened with the serotype PCR, if the sample tests negative, it can be assumed that the sample does not contain important or clinically significant STEC and no further action is required. If the serotype PCR is positive, then isolation of the STEC is required. The format of the PCR system is a Genedisc. These are ready to use disposable discs that can be used in a Genedisc cycler. There is one for *E. coli* O157 (which targets the rfb and fliC genes, also *stx* and *eae* as well as positive and negative controls) and another that targets the seropathogroups (*E. coli* O26, O111, O145, O103 and O157 as well as positive and negative controls). The major disadvantage of this system is that it requires a dedicated instrument. A small survey conducted on raw milk resulted in 21% of 300 samples testing positive for *stx*, but of these only 4.8% contained one of the seropathogroups (eg. 8 samples with O145, and one each of O157 and O103). A similar investigation of minced meat found 15% of 205 samples were positive for *stx*, while 2.6% of these were positive for one or more of the seropathotypes (4 samples positive for O145 & O157, 2 for O145 and O103, and one sample each was positive for both O145 and O26 and another for both O157 and O103). No attempt was made to isolate the STEC from this work.

Characterisation

Protocols for the typing of non-O157 STEC are currently under development for PulseNet. The protocols have been designed for pulsed field gel electrophoresis of the STEC serotypes considered most important in the USA, *E. coli* O26, O111, O103, O121, O45 and O145 and should be available at the end of 2007 or early 2008. The method will be different to that currently used for *E. coli* O157 and will probably more closely follow the PFGE conditions used for *Campylobacter*. Multi-locus variable number tandem repeat analysis (MLVA) and Single Nucleotide Polymorphism (SNP) methods are also being investigated by CDC as possible typing methods. MLVA sometimes groups isolates differently to PFGE, and vice versa. There is a need to look at multiple loci for SNPs to be useful. It is eventually hoped that a microchip will be developed which will include both the ability to determine the presence of specific virulence genes and also allow the subtyping of isolates.

Various microarray technology platforms were considered to be of benefit in the future for determining the virulence potential of STEC isolates. Arrays have been developed which can test for multiple virulence traits for different pathogenic *E. coli* as well as the development of those which can also determine the presence of genes encoding for different serotypes of *E. coli*.

Work from the European Committee for Standardization on the possibility of developing a standardized horizontal method for the detection of STEC (CEN/TC 275 WG6) was presented. There is EU legislation (2003/99/EC) which states that member countries need to monitor the food chain for the presence of STEC. The method recommended by the ad'hoc working group is based on the detection of Shiga toxin genes using PCR. The group is currently developing a method proposal but are struggling to develop a clear definition of STEC and are therefore currently basing the approach on detecting all STEC. The method relies on screening enrichment

broths with PCR targeting the Shiga toxin genes (or possibly other genes such as the *eae* or O serotype specific genes). The PCR product needs to be detected and verified. Isolation would consist of streaking onto TBX (ChromAgar TBX from Merck), incubating at 41.5°C for 18 – 24h. Up to 50 colonies are picked and point inoculated onto nutrient agar. Pools of colonies are combined in water and subjected to another PCR for detection of the target genes. If the pools are positive, the individual colonies are picked and tested. This method is still under development with many questions still to be answered, eg. should novobiocin be included in the enrichment, should faecal samples be enriched in BPW, what primers/probe should be used, will it be a real time PCR test, what controls are needed, what type of DNA preparation is required etc. There is a large amount of robust validation work to be performed before such a method can be used. Some of the participants of the meeting were concerned about the labour involved in testing so many colonies. The group is currently discussing defining certain aspects about the method, such as using a performance based approach rather than prescribing a particular protocol (eg. target organism, requirements for specificity and sensitivity, limit of detection etc).

Regulatory Issues

The US currently has no regulatory policy in relation to non-O157 STEC although there is a push from the USDA to introduce such regulations. The difficulty with producing regulations is knowing what to target as not all STEC are likely to be pathogenic to humans and therefore of no health significance. There is expected to be a new definition of EHEC coming out soon which may provide regulators with guidance on how to define these organisms (eg. should it be serotype, seropathotype or virulence marker based and there are both advantages and disadvantages with each of these approaches). The major non-O157 STEC of clinical significance in the US are O26:H-, O103:H2, O111:H-, O121:H7 and O145:H-. The US are wary of introducing regulations as once in, they are hard to change. The example of the regulations for enteropathogenic *E. coli* in cheese and cheese products was provided. The wording in the regulation refers to enteropathogenic *E. coli* but is actually meant to apply only to enterotoxigenic *E. coli* (ETEC). The regulations state that no more than 1,000 ETEC/g of product be present, which is based on the infectious dose of ETEC infections. ETEC analysis is only performed if the *E. coli* count in the product is >10,000 cfu/g, but if this limit was reduced to 100 cfu/g, then there would be little need for ETEC testing as they are difficult to detect and this level would provide a better limit for ensuring a reduced risk of infection. Currently the USDA rely on the FDA-BAM method and MLG.5A methods for testing for *E. coli* O157, but there has been flexibility to change the methods used to meet the requirements of the investigation (eg. analysis of produce and juice where the isolation of *E. coli* O157 is not always possible using the current standard and recommended methods). The general consensus is that molecular methods will have a major role to play in any methods aimed at the detection of non-O157 STEC.

A proposal was put forward to create a working group on risk assessment of STEC. This group would gather published epidemiological data on human infections, compile a list of serotypes and the virulence markers they carry from different sources including animals, environmental samples and food, and investigate methods for rapid identification of clinically significant STEC. Their goal would be to have a current list of STEC which are highly pathogenic to humans and those that may cause diarrhoea but not haemorrhagic uraemic syndrome (HUS) or haemorrhagic colitis (HC) and those that frequently occur in nature but have not been implicated with disease in

humans. There is already a group working out of the European Food Safety Authority (EFSA) which are involved in a similar activity titled Monitoring of verotoxigenic *Escherichia coli* (VTEC) and identification of human pathogenic VTEC types. A report is due from this group by the end of the year. The suggestion of forming another group within the PEN project for investigating this issue was put on hold until after the release of the EFSA report.

Meeting Summary

Chris Baylis summed up the meeting by highlighting the importance of methods and listing the following points:

- there is complexity in nomenclature and defining STEC
- the USA is likely to develop a regulatory position for non-O157 STEC
- different methods may need to be applied to different foods, sample types
- methods must be flexible and able to be modified to meet emerging issues
- there is complexity in developing isolation media for improving isolation of STEC
- diarrhoeagenic *E. coli* have a high incidence in foods (particularly atypical EHEC)
- typical EHEC found in low incidence in foods
- there are difficulties in defining A/EEC and EAEC which hampers detections and reporting
- PCR (including real time PCR) likely to be used for screening (including new technologies such as the Genedisc)
- current differences in methods make comparisons between studies and different countries very difficult
- reducing time for identification is important, maybe microarray and typing technologies such as MLVA will help
- the ecology of *E. coli* is complex.

Poster Presentations:

There were 24 poster abstracts submitted for the PEN meeting, but only 16 posters were present (Figure 2). A brief summary of the main points from the most relevant posters presented at the PEN meeting appears below:

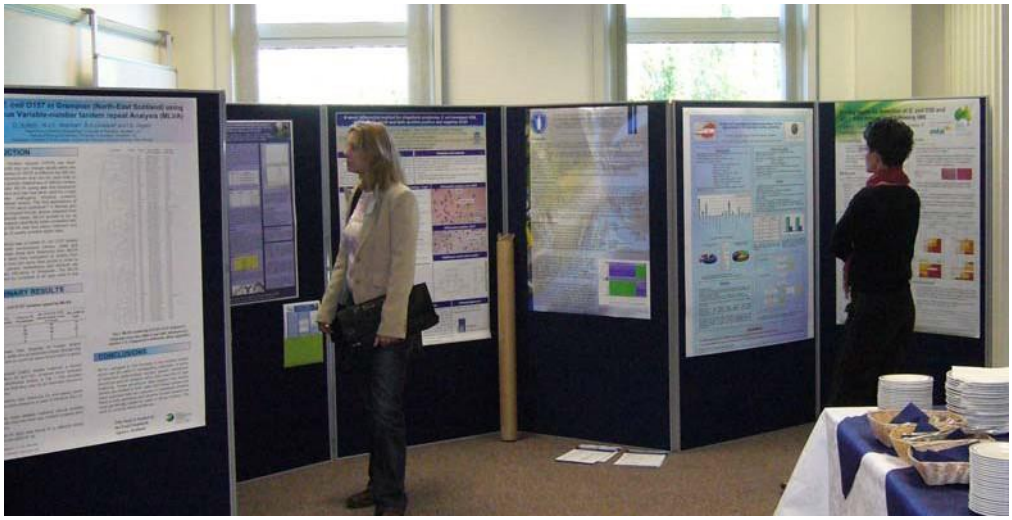

Figure 2. Poster viewing at the PEN meeting in CCFRA.

- increase in incidence in STEC in the Republic of Ireland thought to be due to increased surveillance and increase awareness
- non-O157 STEC is a rising issue in Ireland
- MLVA has been recommended as a tool for studying *E. coli* O157 dissemination in feedlots and for identifying the source of human infections
- preliminary results of a pan-genome array (based on 24 sequenced *E. coli* and 8 *Shigella* strains) investigating the relationship between *E. coli* O103 isolates (causing a high level of HUS cases in Norway) showed strains possessed many genes which were identical to those of *E. coli* O157:H7 (EDL933) but many of the specific O157 genes and some phage genes were missing.
- the Shiga toxin genotype is more important than the amount of toxin produced in progression to HUS with stx_2 and stx_{2c} more often associated with HUS than other *stx* and may be a useful indicator of the potential to cause severe human disease
- Shiga toxin genes types are often specific to animal groups (eg. stx_{1c} in lamb and sheep, stx_{2e} associated with pigs)
- there was no association between the virulence genes found in avian *E. coli* isolates (including those isolated from birds with colibacillosis) and those found in extra-intestinal pathogenic *E. coli* associated with clinical disease in humans
- real time PCR screening followed by plating onto Chromocult media containing cefixime, cefsulodin and vancomycin and subsequent screening of 5 colonies from each plate was successfully used for detection of *E. coli* serotypes O111, O26, O103 and O145 from beef carcases
- Sequencing data has been obtained for the O antigens of the following *E. coli* serotypes, O2, O63, O123, O148, O149 and O174 and will be used to develop specific PCR for targeting these serotypes
- some non-O157 strains of *E. coli* are sensitive to novobiocin
- commercially available kits can be used for detection of Shiga toxins in food samples

Potential Collaborations:

Teagasc: Dr Geraldine Duffy from Teagasc approached FSA about joining the ProSafeBeef EU program (www.prosafebeef.eu), the title of the project is Advancing Beef Safety and Quality through Research and Innovation. Currently John Thomspon at UNE is involved in this program. FSA will be not be funding work for such a project and will therefore not be involved in this activity. However, continued links with Teagasc through future PEN meetings may help ascertain information from the ProSafeBeef program.

Prof Christine Vernozy-Rozand of the National Veterinary Laboratory in Lyon, France was keen to collaborate on a study of *E. coli* O26 isolates. France has recently had an outbreak of *E. coli* O26 associated with consumption of cheese made from unpastuerised milk. The ecology of *E. coli* O26 appears to be similar in both France and Australia with this organism associated more with dairy cattle and calves than cattle used for beef production. It is proposed that *E. coli* O26 strains are exchanged between FSA and the NVL of France to enable a study of virulence genes and survival characteristics to occur. The FSA component of this work will involve an honours student and is dependant on attracting such a student. A visit to the NVL is proposed in conjunction with the next PEN meeting in Rome, in 2008.

Karmali, M.A., Mascarenhas, M., Shen, S., Ziebell, K., Johnson, S., Reid-Smith, R., Isaac-Renton, J., Clark, C., Rahn, K. and Kaper, J.B. (2003) Association of genomic O island 122 of *Escherichia coli* EDL 933 with Verocytotoxin-producing *Escherichia coli* seropathotypes that are linked to epidemic and/or serious disease. *J Clin Microbiol* **41**, 4930-4940.