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SHIGA TOXIN PRODUCING *Escherichia coli*: GERMANY 2011 *Escherichia coli* O1O4:H4 OUTBREAK LINKED TO SPROUTED SEEDS

Introduction

The outbreak of Shiga Toxin producing *Escherichia coli* O104:H4 linked to bean sprouts led to over 3800 confirmed cases of illness that included more than 823 cases of Heamolytic Uremic Syndrome (HUS) and 44 deaths (Frank *et al*., 2011). Although it appeared that the O104:H4 serotype came from nowhere the pathogen had actually been isolated in Germany 10 years ago. The outbreak in Germany was more a consequence of two vulnerabilities (highly virulent pathogen and a high risk food) within the food chain coming together with disastrous consequences.

The purpose of this Scientific Information Bulletin (SIB) is to provide background into Shiga Toxin *Escherichia coli* and how the O104:H4 serotype has changed our understanding of pathogenicity of *E. coli*. The future challenges in controlling STEC and research needs will also be discussed.

Escherichia coli

E. coli is encountered in the gastrointestinal tract of all warm blooded animals including humans. The bacterium has a close relationship with mammals that was established some 140 million years ago when the first rodents appeared on the Earth. The vast majority of *E. coli* are non-pathogenic and actually are essential for health. For example, *E. coli* produces Vitamin K that we use as part of cell repair (blood clotting) and are far more effective probiotics than lactic acid bacteria in out-competing would be pathogens such as *Salmonella* and *Clostridium difficile*. *E. coli* has also found utility as an indicator for fecal contamination in foods and water. Its presence indicates the potential presence of virulent pathogens such as *Salmonella*, *E. coli* O157 and *Shigella*, amongst others. Finally, *E. coli* has been the workhorse of molecular biology and provided a wealth of information on the function of genes and how these can be manipulated to produce valuable products such as insulin used to treat diabetes. One of the main features that makes *E. coli* easy to manipulate in genetic engineering is the ease with which genetic material can be introduced and alter the physiological traits of the bacterium. Indeed, it is the acquisition of genes that gave rise to a sub-set of *E. coli* termed pathogenic *E. coli*. The pathogenic *E. coli* can be sub-grouped on the mode by which they cause illness and are classified as Enteropathogenic *E. coli* (EPEC), Entero-toxigenic *E. coli* (ETEC), Entero-Diffuse *E. coli* (EDEC) and Entero Invasive *E. coli* (EIEC). The additional two groups of relevance here are Entero Aggregative *E. coli* (EAggEC) and Shiga Toxin Producing *E. coli*.

Shiga Toxin Producing *Escherichia coli*

Escherichia coli harboring shiga toxin(s) genes collectively fall with the STEC group and encompasses over 200 different serotypes (Couturier *et al*.. 2011; Lindqvist *et al*., 2011; Mathusa *et al*., 2010). The different serotypes making up the STEC group were known since the 1980's but apart from O157 were

only considered significant from an academic interest in terms of bacterial evolution given that most showed little or no virulence (Pennington, 2010). Indeed, over 70% of STEC are considered low risk and rarely associated with illness. However, a sub-group of non-STEC have increasingly been associated with serious illness called Heamolytic Uremic Syndrome (kidney failure) that has hitherto being mainly restricted to the *E. coli* O157:H7 serotype.

How does STEC cause illness?

The ability of the STEC serotype to cause illness is dependent on the complement of virulence factors. When *E. coli* O157 is ingested it can survive the acid of the stomach and eventually reaches the colon where it binds to a receptor on the epithelial cells of the gastro-intestinal tract. The *E. coli* O157:H7 then produces a protein called intimin (encoded by *eae* gene) and Tir (encoded by *tir* gene) that facilitates attachment to the epithelial cell surface. *E. coli* O157:H7 also forms a tube (Type III secretion system) to enable proteins to be transferred from the bacteria into the host. Although there are 25 proteins transferred from *E. coli* O157:H7 into the epithelial cell the most significant is the Shiga like toxin (encoded by *stx* gene). The shiga toxin acts to cut an adenine unit from the ribosomal RNA (rRNA) of the host. Although this may appear a trivial alteration to RNA structure it is sufficient to stop protein synthesis thereby resulting in epithelial cell death. *E. coli* O157:H7 also produces a heamolysin that dissolves blood cells thereby contributing to the virulence of the pathogen.

When *E. coli* O157:H7 cells are ingested, the infected person will start developing the common characteristics of foodborne illness such as fever, nausea, stomach cramps and diarrhea. In healthy individuals the immune system will neutralize the *E. coli* O157:H7 thereby removing the infection from the body (Pennington, 2010). However, in susceptible individuals (young, old and immuno-compromised) the *E. coli* O157:H7 can continue to proliferate and the shiga-toxin eventually enters the blood stream and targets the kidneys causing Hemolytic Uremic Syndrome (HUS). Blood appears in the urine and diarrhea and eventually leads to kidney failure that can be fatal. Thrombotic thrombocytopenic purpura (TPP) can also occur whereby patients develop blood clots. The elderly are more prone to TPP and like HUS it can be potentially fatal (George, 2009; Karpman, 2008; Sanchez *et al*., 2010; Tarr, 2009).

It has long been considered that the virulence of O157 could be attributed to the presence of the shiga toxin (stx2), attachment protein (eae) and heamolysin. With non-O157 STEC the virulence factors are missing or non-functional. In addition the less potent form of the shiga toxin (stx1) is produced. Consequently, the majority of non-O157 STEC have low pathogenicity towards humans or give mild symptoms similar to 24 hour flu (viral gastroenteritis).

Emergence of virulent non-O157 STEC

Within the last 5 years there has been an increased incidence of HUS linked to non-O157 STEC although *E. coli* O157:H7 still remains the main pathogen of concern. In the US the incidence of O157 STEC is estimated at 0.9 cases per 100, 000 population that compares with 0.5 for non-O157 STEC (Kasper *et al*., 2010). This translates to an estimated 73,000 cases of *E. coli* O157 infections accounting for 61 deaths. In comparison, non-O157 STEC infections accounts for 36, 000 cases leading to 30 deaths. The incidence of HUS from non-O157 STEC infections is <2% that compares to 8% in the case of *E. coli* O157.

"Top 6" STEC

To address the increased food safety risk of non-O157 STEC it was proposed by the USDA Food Safety Inspection Service (FSIS) to classify all STEC as adulterants (i.e., zero tolerance as with *E. coli* O157). In effect this meant that meat processors would be required to screen for STEC and if positive issue a product recall or send the sample for re-processing. However, both regulators and industry acknowledged that not all STEC serotypes represented the same food safety risk and a large proportion of serotypes encountered in foods had no or low virulence. In addition, no reliable methods were available for differentiating the non-pathogenic STEC from those that can cause HUS. Therefore, a compromise was reached whereby focus would be placed on those STEC that had been previously implicated in cases of HUS. From the incidence of non-O157 STEC reported to date there are six serotypes that have been commonly linked to clinical infections. The "Top 6" serotypes (O26, O111, O103, O121, O45 and O145)

accounted for non-O157 STEC cases that resulted in HUS (Kasper *et al*., 2010). A common feature of the Top 6 non-O157 STEC is the expression of stx2 and eae thereby fitting the theory that virulent strains have the full complementary of factors required to cause the fatal HUS condition (Gill and Gill, 2010). Therefore, diagnostic tests for screening for the presence of the Top 6 STEC are based on presence of *stx2* and *eae* with absence of either being interpreted as a negative result. The method was AOAC approved and it is anticipated that the Top 6 will be classed as adulterants by the end of 2011.

Alternative virulence factors present in STEC

The general theory that STEC required stx2 and eae was accepted although failed to explain why certain serotypes could cause HUS in the absence of eae. For example, in 1993 there was a small cluster of three HUS cases linked to serotype O113:H21 that was stx2 positive but eae negative (Paton, 1994). A strain of *E. coli* O104 (stx2 positive, eae negative) that was related to the 2011 outbreak strain caused a single case of HUS in Germany in 2001. The ability to cause HUS in the absence of attachment protein was proposed to be through hyper production of shiga toxin and an alternative virulence factor called subtilase (a toxin that shuts down the protein factory of the host) (Biscola *et al*., 2011; Bosilevac *et al*., 2011; Lin *et al*., 2011; Schaffzin *et al*., 2011). The origins of subtilase remains unclear although it has genetic sequences related to the virulent pathogens *Bacillus anthracis* (causes anthrax), *Salmonella typhi* (typhoid causing) and *Yersinia pestis* (plague). The toxin is part of the AB_5 toxin family along with shiga toxin, cholera toxin and ricin (Paton *et al.,* 2004).

***Escherichia coli* O104:H4**

E. coli O104 is a non-O157 STEC that has been rarely implicated in foodborne illness outbreaks. There was an outbreak of O104:H21 centered in Helena, Montana, USA, linked to fecally contaminated pasteurized milk (Anon, 1995). The outbreak resulted in 17 cases 67% of which were female with a median age of 35. Four cases of HUS resulted from the outbreak with no deaths. Serotype O104:H4 was isolated in Africa in 2001 from patients with diarrhea and again from a woman in Korea who had contracted HUS. A single case of HUS within Germany in 2001 was attributed to a strain of O104 although it was not recovered again until the 2011 outbreak.

The *E. coli* O104:H4 implicated in the outbreak centered in Germany was fully genetically characterized within a week of being identified as the causative pathogen (Anon, 2011; Bielaszewska *et al*., 2011).

The description of *E. coli* O104:H21 implicated in the outbreak is as follows:-
Shiga toxin 1 negative
Shiga toxin 2 (*stx*2a) positive
Intimin (*eae* gene) negative
Enterohemolysin negative
EAEC (enteroaggregative *E. coli*) virulence plasmid:
*aat*A positive (ABC-transporter protein gene)
*agg*R positive (master regulator gene of Vir-plasmid genes)
aap positive (secreted protein dispersin gene)
agg positive (AAF/I-fimbral subunit-gene)
*agg*C positive (AAF/I-fimbral operon-gene)
Antimicrobial resistance profile: Resistant to ampicillin, amoxicillin/clavulanic acid, piperacillin/sulbactam, piperacillin/tazobactam, cefuroxime, cefuroxime-axetil, cefoxitin, cefotaxime, cetfazidime, cefpodoxime, streptomycin, nalidixic acid, tetracyclin, trimethoprim/sulfamethoxazol.

From the genes identified within the outbreak strain there was found to be 93% similarity to another type of pathogenic *E. coli* referred to as Entero Aggregative E. coli (EAggEC) with the presence of the stx2 linking the serotype to the EHEC group.

Entero Aggrative *Escherichia coli*

EAggEC is commonly responsible for infant diarrhea primarily in developing countries and also travelers to developing countries (i.e., Travelers Diarrhea) (Morabito *et al*., 1998). There have been outbreaks

within industrial countries where EAggEC has been responsible for sporadic diarrhea although is rare (Huang *et al.,* 2006). The symptoms of EAggEC infection comprises watery diarrhea, occasionally with blood and mucus lasting 7 – 14 days but the condition is non-lethal provided the patient remains hydrated.

EAggEC strains exhibit a spectrum of pathogenicity that is dependent on the presence of virulence factors required to cause illness. *E. coli* within the EAggEC group harbor a 60MDa plasmid (pAA) that encodes for surface appendages (fimbria AAF/I, AAF/II and AAF III) and genes encoding for cell-associated enterotoxins (EAST1 and serine protease) are also found on the pAA plasmid regulated by aggR (Zamboni *et al.,* 2004).

The fimbria on the surface of EAggEC act to attachment between cells and the host mucosa. The bacterium forms a "brick-like" structure that essentially forms a biofilm on the lining of the gastro-intestinal tract thereby providing firm attachment (Elias *et al*., 1999). The cell associated toxins induce dilation of crypt openings leading to fluid accumulation in the lumen manifested by the observed perfuse diarrhea. The enterotoxin encoded by EAST-1 is not restricted to EAggEC but is also present in Enterotoxigenic and Enteropathogenic *E. coli* (also associated with Travelers Diarrhea). Some EAggEC strains express heamolysin, common in *E. coli* that cause urinary tract infections.

Although EAggEC do not express shiga toxins the genes it has been noted that several strains harbor the genes or more correctly the lysogenised bacteriophages (Iyoda *et al*., 2000). *E. coli* O157:H7 acquired the shiga toxin phenotype via bacteriophages and it is likely that *E. coli* O104:H4 also acquired the toxin via the same mode. The "shiga toxin producing" bacteriophages are lysogenic that integrate stx into the chromosome of the *E. coli* host. When the *E. coli* is subjected to stress (for example, presence of antibiotics) the SOS response is induced that triggers the replication cycle of the phages, including expression of shiga toxin that is released when the cell lyses (Turner, 2011). This is the primary reason why antibiotics are not administered when treating HUS patients. The over use of antibiotics, ~~is~~ leading to an increase prevalence of stx carrying bacteria is considered a possibility.

Serotype O104:H4 was also found to express ESBL (Extended Spectrum Beta lactamase) which is an enzyme that degrades antibiotics such as penicillin. ESBL is commonly encountered in urinary tract infections caused by *E. coli*.

What was the likely source of *E. coli* O104:H4 in the 2011 German outbreak?

The outbreak of *E. coli* O104:H4 was eventually traced to bean spouts produced by an organic farm outside Hamburg (Frank *et al*., 2011) Sprouted seeds have been implicated in over 40 outbreaks of foodborne illness within the last decade (Warriner *et al*., 2009). The traditional source of contamination associated with sprouts is the seed although contamination introduced during the sprouting process has also been implicated in a small number of cases. The seed originally becomes contaminated in the field and can be exposed to a wide range of hazards derived from manure, contaminated irrigation water, in addition to wildlife. Pathogens can reside on seeds in a dormant state and rapidly multiply under the warm (20-25°C) and humid (>90% relatively humidity) conditions of the sprouting room. It has been demonstrated that pathogens such as *E. coli* can rapidly multiply from 1 cell to attain 100, 000, 000 cells per g within 48h of the sprouting period (Warriner *et al*., 2003). In addition, pathogens can readily become internalized within sprouting seeds and cannot be removed by washing.

E. coli O157:H7 outbreaks are commonly linked to meat (ground beef) and leafy greens such as spinach. In contrast, non-O157 STEC have a tendency to be linked to person-to-person contact or food service/community locations. Furthermore, whilst O157 STEC primarily affects the young and old, non-O157 STEC appeared to cause fatal HUS over a broader age range especially young adults. In the case of O104:H4, EAggEC are exclusively linked to human sources given that no animal reservoirs have been identified to date. If the contamination was introduced onto the seed during cultivation via a sewage spill or transfer to the sprouting seed bed during sprouting remains to be confirmed.

Conclusions

The *E. coli* O104:H4 outbreak linked to bean sprouts was one of the largest foodbo~~u~~rne illness outbreaks ever recorded. Over 3000 cases were confirmed with 38 deaths and an unusually high incidence of HUS

(30%) that primarily affected young adult females. The outbreak underlines the food safety issues linked to sprouted seeds that have been responsible for over 40 outbreaks over the last decade. In addition, there was the emergence of an apparent new EHEC and EAggEC hybrid in the form of *E. coli* O104:H4. However, there have been previous cases of HUS linked to serotypes lacking the *eae* attachment protein although these have been relatively rare. It is likely that in the German outbreak the O104:H4 serotype proliferated to high levels during the sprouting process thereby leading to those affected receiving a high dose of the pathogen. It could be that ordinarily the population is exposed to low doses of the pathogen and hence is rarely encountered. Still there are many features of O104:H4 that need to be known in order to enable control of the serotype. For example, what is the prevalence of the bacterium within the population and why are women more susceptible. Possible theories for the latter includes the administration of birth control pills increasing the receptors required for O104:H4 attachment and/or the involvement of the pathogen in urinary tract infections. Also there is a need to understand how the stx2 gene was transferred to O104:H4 and if there is a risk of the toxin becoming more widely distributed amongst *E. coli*. Current evidence would suggest that antibiotics may have a direct role in the mobility of the stx gene between strains although that needs to be confirmed.

The question of whether more outbreaks of O104:H4 will occur in the future remains open to speculation. The fact that the pathogen is more likely to reside in humans as opposed to animal reservoirs would suggest that the prevalence of O104:H4 would be lower than that of O157 that is frequently encountered in cattle. The infectious dose of the O104:H4 serotype will also need to be determined given that this would contribute to the risk associated with the pathogen. Regardless of this factor the question is posed whether a further subgroup of STEC should be established to account for non-O157 serotypes that belong to the EAggEC and that would be missed in standard assays based on detecting *stx*2 and *eae* genes.

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