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DNA sequencing and analysis of the virulence plasmid in E. coli O26:H11:K60 ED21

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1. INTRODUCTION

Escherichia coli is part of the normal microflora of the gastrointestinal tract of mammals and birds, but certain strains have been associated with gastrointestinal diseases in both humans and animals. These *E. coli* strains have been categorised into pathogenicity groups, based on their virulence properties. One of these groups is characterised by the production of potent cytotoxins that inhibit protein synthesis within eukaryotic cells. These toxins are either termed verocytotoxins (VT), because of their activity on Vero cells, or Shiga toxins (Stx), because of their similarity with the toxin produced by *Shigella dysenteriae*. Therefore, these strains are either termed Shiga toxin-producing *E. coli* (STEC) or verotoxigenic producing *E. coli* (VTEC).

STEC infections have been described in a wide range of both domestic and wild animal species, but their natural pathogenic role has been demonstrated only in young calves (diarrhoea or dysentery), weaning pigs (oedema disease), and dogs (cutaneous and renal vasculopathy in greyhounds). In humans, can be

asymptomatic or can cause illnesses such as diarrhea, bloody diarrhea, haemorrhagic colitis (HC), and haemolytic uremic syndrome (HUS), especially among children and the elderly. Enterohaemorrhagic *E. coli* (EHEC) constitute a subset of serotypes of STEC that has been firmly associated with bloody diarrhoea and HUS in industrialised countries. The majority of the cases of disease worldwide are caused by strains of serotype O157:H7, but infections sustained by EHEC strains belonging to serogoups other than O157, like O26, O111, O103, and O145 are increasingly being reported. These strains are now usually referred to as non–O157 EHEC. STEC/EHEC represent the only pathogenic group of *E. coli* that has a definite zoonotic origin, with cattle being recognised as the major reservoir for human infections. (25)

2. ANIMAL RESERVOIRS AND ECOLOGY

STEC can be found in the gut of numerous animal species, but ruminants have been identified as a major reservoir of STEC that are highly virulent to humans, in particular EHEC O157. Knowledge about the routes of transmission and the sources of human infections has increased during the past twenty years, as numerous epidemic events have been investigated. It appears evident that STEC may be transmitted from animal reservoirs to humans not only via the ingestion of contaminated foods or drinking water, but also by contact with STEC-positive animals or with their environment. (25)

2.1. Cattle

Cattle are considered to be the most important source of human infections with EHEC O157, being asymptomatic excretors of the organism, which is a transient member of their normal gut microflora. The presence of EHEC O157 in cattle excreta appears to be influenced by the age of the animals. Studies conducted in the

United States have shown that EHEC O157 could be isolated from the faeces of less than 1.5% of calves under two months of age (129) and from 1.8 to 5% of the calves aged between two and four months (210).Thereafter, the prevalence of EHEC O157 declines (78), suggesting that faecal shedding might be more intense and frequent immediately after weaning. Experimental infections in calves have confirmed that EHEC O157 is shed longer and more intensely in calves than in adult cattle (36) and that shedding largely increases after weaning (66). A higher rate of faecal shedding of STEC after weaning has also been observed in a Japanese study regardless of the serotype. *stx* genes were detected in faecal samples from 39.4% of calves less than 2 months of age, 78.9% of calves from 2 to 8 months of age, and 40.8% of adult cattle (172).

The prevalence of EHEC O157 in cattle also depends on the season, since increased rates of faecal shedding have been repeatedly reported in warmer months (17, 29, 75). However, a study conducted on the presence of STEC in grazing beef cattle regardless of their serotype showed a higher prevalence in the

winter than in the summer (182). Studies on the presence of STEC in cattle have been performed worldwide. In North America, STEC have been isolated both from beef cattle (169,181) and dairy cattle (182). Hancock and co-workers (74) reported the isolation of EHEC O157 from 0.28% of dairy cattle (8.3% of the tested herds) and from 0.71% of beef cattle (16% of the herds). In Brazil, *stx* genes were detected in the faeces of 82% of dairy cattle and 53% of beef cattle, but EHEC O157 was isolated from only 1.5% of the samples. In Australia, *stx* genes were present in 16.7% of faecal samples from dairy cattle (31) and EHEC O157 and EHEC O26:H11 were isolated from 1.9 and 1.7% of the samples respectively. In Japan, *stx* genes were detected in the faeces of 46% of tested calves, 66% of heifers and 69% of cows (102); EHEC O26, O111 and O157 were isolated in 9 of the 78 herds tested (11.5%). In Europe, studies on STEC faecal shedding have been performed in many countries. The rate of animals carrying STEC ranged from 2.8% of the tested animals in the UK (208) to 75% in Norway (190). When EHEC O157 was isolated, the rate of positive animals ranged from 0.2% in France

(154)to 16.6% in Italy(16). In Spain, STEC have been isolated from both calves and adult cattle, with prevalence rates ranging from 20.3 to 36.7%. In these studies, up to 83% of the tested herds were positive and the main STEC serogroups isolated were O8, O20, O22, O77, O113, O126 and O162. Most of the isolates were negative for the *eae* gene. In France, a study performed at the slaughterhouse indicated that 18% of faecal samples were positive for *stx* genes, and STEC were isolated from 7.9% of these samples (163). In another study, however, a much higher prevalence of *stx* genes (70%) was observed, and STEC were isolated from more than 30% of the samples.(154) STEC belonging to serogroups other than O157 can frequently be isolated from young calves with diarrhoea. STEC strains pathogenic to calves usually possess the LEE, (locus of enterocyte effacement), produce Stx1, and belong to a restricted number of serogroups: O5, O26, O111, O118 (204). EHEC O118 strains, in particular, have frequently been isolated from diarrhoeic calves in Germany (203), and their zoonotic transmission to humans has been demonstrated (7). In investigations conducted in

Germany (203) and Brazil (114), STEC were isolated more frequently from diarrhoeic calves than from healthy calves. Conversely, other studies have reported higher rates of STEC faecal carriage in healthy than in diarrheic animals (10,159). The results of the investigations on the prevalence of STEC and/or EHEC O157 in cattle are clearly influenced by the sampling and detection methods that are used. *E. coli* O157:H7 can be detected using sorbitol MacConkey agar, since unlike other *E. coli* serotypes, it is sorbitol negative; however, it is difficult to detect non-O157 STEC based on phenotypic traits. Methods based on detection of the Shiga toxins or the Shiga toxin genes must be used. The use of specific immunoconcentration procedures for EHEC O157 (193, 209) strongly enhances the sensitivity of the isolation methods. Therefore, the studies based on such procedures reported prevalence rates for EHEC O157 much higher that those reported in investigations aimed at revealing all the STEC, regardless of their serotype. Antibody-coated magnetic beads used for immunoconcentration procedures are not available for most non-

O157 STEC strains. It is therefore difficult to determine whether the STEC/EHEC prevalence results reported reflect true differences in colonisation rates with O157 and non-O157 strains or are the consequence of the different methodologies adopted.

2.2. Other ruminant species

STEC, including EHEC O157 and other serogroups associated with human infections like O91, O128 and O146, have been frequently isolated from the intestinal content of sheep (82,158,190). EHEC O157 has also been found in both meat (30)and milk (164) and sheep are now considered as an important reservoir for human infection. EHEC O157 has also been isolated from goats (155) and goat milk has been associated with an outbreak (8). Small ruminant flocks may also have a relevant role in spreading STEC contamination in the environment (87,142). The water buffalo is another potential source for STEC infections. A recent survey conducted in southern Italy (unpublished results) (25) showed that buffalo dairy herds were frequently colonised by EHEC O157(1);

yet the organism was not found in a study conducted on mozzarella cheese prepared with unpasteurised buffalo milk(33).

STEC can be found in wild ruminants, and the possible role of these animals as reservoirs for domestic ruminants sharing the same environment has been suggested. EHEC O157 has been repeatedly isolated from deer (161)and the consumption of deer venison has been associated with human infections (98,157); these episodes also underline the risk of products derived from private slaughtering.

2.3. Other non- ruminant mammals

STEC have been sporadically isolated from mammals other than ruminants, but in many cases it is not clear whether they represented actual hosts or merely vectors transiently colonised after contact with ruminant feces excrections (197). EHEC O157 strains isolated from companion animals, such as horses (28,187) and dogs living in a farm environment and have been associated with human infections,. The presence of STEC, including EHEC O157, has been recently described in both wild and farmed rabbits (67,108, 156),

and the possible role of the rabbit as a source of human infections requires further studies. Pigs are not considered to be a major source of EHEC O157 and other STEC associated with human infections. Prevalence rates of EHEC O157 faecal carriage ranging from 0.2 to 2% have been reported in pigs slaughtered in European countries (17,83, 94) Japan (136) and the United States (53). The low carriage rate observed in these studies could be the result of accidental exposure of pig herds to EHEC O157 through contamination of feedstuff or the environment with ruminant manure in farms that do not comply with good husbandry practices. However, investigations conducted in South America (18,162) showed a surprisingly high rate of EHEC O157 faecal carriage (8–10%) in slaughtered pigs. These marked differences in prevalence may be due to differences in pig husbandry and slaughtering practices.

2.4. Birds

STEC have also been isolated from birds. The isolation of EHEC O157 (195) and non-O157 STEC (118) from gull droppings has been reported. The gulls were not considered as a true reservoir of STEC but rather as potential vectors for their dissemination since a low carriage was found and since the birds fed on contaminated sites. STEC strains producing a particular variant of Stx2, designated Stx2f (167), have been frequently isolated from feral pigeons (41). Most of these strains possessed genes encoding for other virulence factors, such as the intimin protein and the cytolethal distending toxin (133). It is difficult to establish whether Sxt2f–producing strains may represent a cause of avian disease or even a potential health hazard for humans. Pigeons seem to be a natural reservoir for these particular STEC strains, which could be host adapted. STEC have not been found in live chickens (6, 83) even though EHEC O157 strains have been isolated from retail poultry products (47, 108) and from the intestinal content of a turkey (83).

Moreover, the organism has been shown to colonise the caecum of chicks for long periods following experimental infection (3)

3. ECOLOGY IN CATTLE FARMING

Many studies have been performed to increase our understanding of the on-farm ecology of EHEC O157, in order to develop strategies for preventing or reducing carriage and shedding of the organism by cattle.

3.1. Shedding and persistence

Faecal shedding of EHEC O157 appears to be transient in cattle. It can last approximately one month in a same animal (5), and colonisation can be more prolonged in the winter than in the summer months (170). The load of EHEC O157 in calf faeces can range from 10^2 to 10^5 cfu/gram of faecal matter (170, 210) . In a recent study conducted on a breeding farm in Japan (201), faecal shedding of EHEC O157 and EHEC O26 persisted up to 10 and three weeks, respectively. The magnitude of faecal shedding was

approximately 10^4 cfu/gram for EHEC O157 and 10^2 cfu/gram for EHEC O26. Some strains of STEC can persist for many years in the same cattle herd, and this may explain the role of cattle as a reservoir. Moreover, the introduction of new STEC strains via feed and drinking water is always possible (78). The importance of the farm environment as a potential source or reservoir of EHEC O157 has been extensively addressed. Lejeune et al. (113) found a 13% prevalence of EHEC O157 in commercial feedlot cattle throughout the finishing feeding period prior to slaughter, with the predominance and the persistence of four specific clonal types over a period of four months. In the USA, a same well-identified EHEC O157 strain persisted in a farm environment for more than two years (170). The strain was isolated from cattle but also from other animals, including birds or flies and from feed and drinking water. Bad husbandry conditions, such as a wet ground with faecal matter and urine accumulation, seem to favour the persistence of EHEC O157 in feedlot cattle (174). Cleaning methods and housing conditions are also important. In a cattle herd, a higher prevalence

of EHEC O157 was observed when the ground was washed with a jet of water, rather than by dry scraping (66). In a Swedish study, among calves that carried and shed EHEC O157 in their faeces in the spring, only those kept in a cowshed during the summer remained positive four months later, while the organism was not isolated from the calves grazing in a pasture during the same period (95). The persistence of STEC in calves kept in a cowshed might result from continuous contact between the animals and/or with their environment, which allows regular re-infection.

3.2. Effects of the diet

There is no clear relationship between feed composition and STEC faecal shedding in cattle. Some authors formulated the hypothesis that a grain-rich diet may induce mechanisms of STEC acid resistance in the rumen that favour STEC survival and faecal shedding (19, 44). However, different studies conducted on hay-fed and grain-fed cattle produced conflicting results. Sometimes hay-fed cattle shed EHEC O157 longer than the grain-fed animals (86,

130), and sometimes grain-fed animals shed EHEC O157 longer than cattle grazing a pasture (95). Other studies have reported no differences between the two categories of animals (70, 74). The effect of diet on the shedding of EHEC O157 was also investigated by the experimental infection of sheep (104): hayfed sheep shed the bacterium twice as long as, and in larger numbers, than sheep fed with a mixture of corn and pelleted alfalfa. The effect of fasting has also been investigated, and was shown to have only a small effect on faecal shedding and rumen proliferation of EHEC O157 in calves (79). The presence of EHEC O157 in cattle feed has been recently investigated in the USA. EHEC O157 was detected in 75 of 504 feed samples (14.9%) collected from 54 feedlots, and no correlation between the presence of EHEC O157 and generic coliform counts in feed was observed (45). Faecal contamination of grass followed by poor silage management may be a factor favouring the persistence of EHEC O157 carriage in ruminants (55). On the contrary, EHEC O157 did not survive a good silage fermentation process, indicating that properly ensiled and correctly stored grass is unlikely to be a

vector for the transmission of this pathogen among cattle (24). Experimental inoculation of lactic acid-producing bacteria in silage, which decreases pH more rapidly during ensiling, appears to hasten the elimination of EHEC O157 from the silage (2).

The quality of drinking water is another important issue related to the on farm ecology of EHEC O157. The organism can survive and even grow in the sediments of a drinking trough contaminated with faeces, which may serve as a long-term reservoir and source of infection for cattle (52, 77, 111, 112, 170, 191).

3.3. Persistence in faeces and manure

STEC appear to be well adapted to survive in animal faeces, where they can remain viable for periods ranging from several weeks to many months (122). Experimental contamination of cattle faeces showed a good survival capacity when faeces were maintained between 15°C and 18°C (62, 120). The temperature and the water activity of the faeces influenced the duration of EHEC O157 survival, which was longer (more than 70 days) when faecal

samples spiked with the organism were stored at 5°C, and had a water activity of 0.98 at the end of the storage (196). When cattle faeces contaminated with 10^8 cfu/gram of EHEC O157 were kept on the surface of grazing land, the count decreased by 4.0–5.0 log 10 cfu/gram within 50 days, but the organism was still detectable in the surrounding soil for up to 99 days (15). Persistence of STEC in the soil (63, 64) favours the infection of cattle and makes environmental exposure a risk factor for human infection (35, 87, 142, 176). Animal wastes and effluents from farming operations, including manure and slurries, are frequently applied as a fertiliser to land used for crop or silage production and cattle grazing. The presence and persistence of STEC, and in particular of EHEC O157, in these products may therefore be an important factor in the initial infection and re-infection of cattle (91, 105). On the contrary, when cattle manure or slurry are applied on farmland in the production of food crops that are to be consumed in the raw or minimally processed state, appropriate handling of these products is necessary to control the spread of potentially present STEC and limit the risks of human

infection (76, 89). Cattle manure composting before its spread onto land may reduce the risk of transmission of STEC through contaminated vegetables (92, 93, 115). When STEC-containing manure is applied on land, concomitant periods of heavy rainfall can cause the transport of bacteria to both deeper layers of the soil by leaching and drains and rivers by run-off (56, 63). EHEC O157 has been isolated from surface waters (110, 123) and an increased risk of waterborne infections was recorded immediately after cattle manure spreading (141). Waterborne episodes of STEC infection have been increasingly reported (43, 122, 145, 184)but the causative agent has rarely been isolated from the related water samples (1, 26). Cattle husbandry is likely the major source of environmental contamination with STEC. However, it should also be considered that the presence of STEC may result from the release of contaminated wastewaters of human origin into the environment (107) or spreading of contaminated sewage- sludge onto the land (193).

4. EMERGING MODES OF TRANSMISSION

During the 1980s, most of the outbreaks of STEC O157 infection were food borne and the food vehicles implicated were usually inadequately cooked hamburgers or other beef products and unpasteurised milk (71). In the past ten years, remarkable changes in the epidemiology of human infections have occurred. In addition to foods of bovine origin, several outbreaks have been associated with low pH products like fermented salami, mayonnaise and yogurt (126) This has highlighted the tolerance of *E. coli* O157 to acidic pH and its ability to survive the processes of fermentation and drying. In addition, waterborne outbreaks and outbreaks associated with other types of environment related exposures have been increasingly reported (122, 184). The dispersion of untreated manure in the environment can cause the contamination of different items, which can then act as secondary vehicles of human infections (32, 122).

4.1. Fruit and vegetables

An increasing spectrum of fruits and vegetables fertilised with ruminant manure or contaminated during harvesting or processing has been involved in outbreaks (122, 184). Contaminated sprouts have caused episodes of salmonellosis and represent an emerging source of EHEC O157 (127). Viable organisms have been observed in the inner tissues of sprouts grown from experimentally contaminated seeds (90) and this is a matter of particular concern, since disinfection of the sprout surface could not insure the safety of this ready-to-eat food. Other fresh produce like lettuce, tomatoes, coleslaw, and others (122, 184) are established or potential vehicles of STEC infection. Unpasteurised fruit juices, increasingly popular among consumers, represent another safety concern, if the acidic tolerance and the low infectious dose of EHEC O157 are considered. Apple juice, in particular, has been frequently involved in outbreaks (122,184).

4.2. Environment-related exposures

An increasing number of outbreaks of EHEC O157 infection have occurred among persons involved in outdoor activities, usually in summer months (184). Swimming in contaminated waters appears to be an important issue (121), as well as the contamination of drinking water supplies in rural settings, such as springs or wells. Water contamination frequently occurs due to runoff from land contaminated with animal faeces (43, 145). Outbreaks have also occurred among persons attending open-air events, such as fairs, music festivals, parties, and visits to farms or petting zoos (184). The sources of infection implicated include well water, exposure to mud contaminated with cattle faeces, and direct contact with animals on display. Visiting a farm is now considered an important risk factor for acquiring severe EHEC infections (139, 150). Conversely, EHEC O157 has been frequently isolated from farm workers in the absence of disease suggesting that farm residents may develop immunity, possibly by exposure to the more common and less virulent STEC non-O157 (4, 183, 207).

5. CONTROL STRATEGIES

As for other zoonotic agents, having animals and raw products that are free from STEC is not possible in practice. However, their occurrence can be minimised by applying high standards of hygiene in all the steps of the food production chain. At the farm level, classical eradication strategies based on the elimination of positive animals is not feasible, due to the high prevalence of colonisation, its transient nature, and the technical difficulties in detecting low levels of the organism in animal faeces. Many approaches have been attempted to reduce the intestinal colonisation in cattle. These include interventions associated with the diet of the animals, the administration of probiotics as competitive microflora (20, 183), and the use of bacteriophages active on EHEC O157 (106). These approaches have produced inconclusive and sometimes conflicting results. Moreover the feed regimens and the treatments adopted in experimental trials are often difficult to apply to farming practices.

Recently, experimental vaccines aimed at reducing the shedding of EHEC O157 in cattle were developed. Subcutaneous administrations of type III secreted proteins, (mainly EspA, EspB EspD and Tir) are able to decrease shedding of EHEC O157 by cattle (153). Transgenic tobacco plant cells that express the host cell-binding domain of EHEC O157 intimin have also been tested successfully in a mouse model (96). Although transgenic plants are not likely to be used for cattle vaccination, at least in Europe, this latter result suggests that an intimin-based vaccination strategy could be successful. However, as for other infectious diseases, good hygiene and management practices remain at the present the best way to reduce the spread and persistence of EHEC O157 on the farm. As discussed in the above paragraphs, these may include cleaning the water troughs where EHEC O157 can survive and even grow (77, 111, 112, 170, 171, 191), reducing faecal contamination and humidification of feed, and a correct preparation of silage. Other factors that could favour colonisation and shedding of EHEC O157 like sudden modifications in the diet and the stress derived

from movement or overcrowding should be reduced. Since environmental contamination may have an important role in the transmission of the infection to humans, the handling of the animal dejections represents an important issue. STEC can survive in bovine faeces for a considerable time (122), therefore manure and slurries should be properly composted to ensure sterilisation or at least the reduction of the microbial load (92, 93, 115). As far as the transmission through the direct contact with animals is concerned, both farmers and people visiting farms should apply hygiene practices. In particular, farms receiving school visits must ensure that adults always supervise children, facilities for hand washing are easily available, and areas for food consumption are clearly separated from those where the animals are kept. At the abattoir level, no specific procedures for STEC elimination can be applied. However, good hygiene and manufacturing practices as well as implementation of HACCP will contribute to reducing faecal contamination of carcasses. The general principles of food hygiene will also be effective in preventing EHEC infections at the

processing and retail levels of the food chain. In particular, cross contamination between raw and ready to eat products must be avoided, bearing in mind that several large outbreaks (34, 189) have originated from gross failures in this basic point. Microbiological testing of meat lots consumed by persons who have become ill suggests that the infectious dose for EHEC O157 might be very low (71, 140). This represents a strong argument for enforcing zero tolerance for this organism in processed food and for markedly decreasing contamination of raw ground beef.

6. THE EVOLUTION OF EHEC

EHEC strains are not a homogenous pathogroup, and it has been suggested that they fall into at least four divergent clonal groups (25). One clonal group includes EHEC strains of serotype O157:H7 and the closely related EPEC of serotype O55:H7. A second clonal group includes EHEC strains of serotype O111:H8 and O26:H11. A third group includes EHEC strains of serotype O103:H2 and O45:H2. A fourth group includes many different O types, usually associated with the H21 flagellar antigen. The most common serotypes are O113:H21, OX3:H21, and O91:H21. Except for the last clonal group, bacteria from the other clonal groups invariably carry the *eae* gene, but otherwise they display a diverse array of virulence. The mechanisms underlying the evolution and emergence of EHEC clones are not well understood but it is now clear that the composition of the *E. coli* genome is highly dynamic. Such fluid gain and loss of genetic material is well illustrated by the comparison of the genomic sequence of EHEC O157 with the non-

pathogenic laboratory strain K-12 genome. A 4.1-Mb sequence is highly conserved between the two strains and may represent the fundamental backbone of the *E. coli* chromosome. In contrast, the remaining 1.4-Mb sequence comprises EHEC O157-specific sequences. Molecular analysis suggests that EHEC acquired the majority of their virulence factors by horizontal transfer of genetic material, and the acquisition of the LEE pathogenicity island (PAI) and the Stx genes were two crucial steps in the evolution of EHEC O157 from a commensal ancestor. Genetic analyses suggest that EHEC O157 separated from a common ancestor of *E. coli* K-12 as long as 4.5 million years ago. The stepwise model of Feng et al. (54) makes specific predictions about the history of descent and the order of acquisition of virulence factors. Such a model predicts that EHEC O157:H7 and O157:H– were derived from an EPEC-like O55:H7 ancestor that carried the LEE located at the *selC* site and acquired the bacteriophages carrying the Stx-encoding genes. As far as the LEE is concerned, however, it is not clear how often this transfer took place and which parts of the locus were involved. Like the

large EHEC-haemolysin plasmid, the LEE is clearly a mosaic structure, which arose from multiple recombination events with foreign DNA. Interestingly, LEE can be found in or next to tRNA genes at different locations on the chromosomes of different EPEC and EHEC clonal types, suggesting that it may have been acquired on more than one occasion. Despite the wealth of information available, further comparative studies are needed to decipher definitively the evolution of virulence in EHEC. However, the presence of 24 prophages and prophage-like elements that occupy more than half of the EHEC O157-specific sequences suggest a predominant role of bacteriophages. These prophages, including the Stx-transducing phages, exhibited extensive structural and positional diversity, implying that variation of bacteriophages is a major factor in generating genomic diversity among the EHEC O157 lineage. In addition, it was recently shown that these prophages do not only code for Stx, but also for effector molecules which are recognized by the type III secretion system encoded by

the LEE. Phages could be the major contributors in the evolution of EHEC virulence and in the emergence of new EHEC clones. (25)

7. PATHOGENESIS AND VIRULENCE FACTORS

An important objective of the studies conducted in recent years on virulence and pathogenicity has been to define the combination of virulence genes and the mechanisms that make a STEC strain an EHEC fully pathogenic to humans. The production of Stx appears to be essential but not solely responsible for the pathogenic effects. As a matter of fact, EHEC associated with severe human disease are usually capable of colonising the intestinal mucosa with a characteristic “attaching and effacing” mechanism and possess virulence plasmids, while these properties are significantly less common among STEC strains isolated from healthy cattle. An increasing number of additional virulence factors has been described, and they are usually carried by mobile genetic elements

like plasmids and PAIs, large genetic elements carrying virulence genes and inserted in chromosomal loci encoding tRNA.(25)

7.1. The pathogenesis process

EHEC are highly infectious to human beings. The infection is usually acquired by ingestion of contaminated food or water or by person-to-person spread through close contact. Outbreaks or incidents of illness are believed to result from a very low infective dose, e.g. < 100 cells, but people may carry EHEC as part of their transient gut microflora without disease.

When these organisms do cause illness, very serious clinical manifestations can occur, including haemorrhagic colitis and HUS. This latter condition usually occurs in children under five years of age and is the major cause of acute renal failure in children. The pathogen and host factors that contribute to the clinical manifestations of EHEC infection are the subject of considerable ongoing investigations and the pathogenesis process is still not fully understood. Stx production is a prerequisite for EHEC mediated

diseases. The toxin is able to pass through the intestinal epithelium to reach its target on endothelial cells lining small blood vessels that supply the gut, kidney and other viscera. In addition to exacerbating the intestinal damage associated with infection, Stx are responsible for life-threatening post-diarrhoeal complications due to their action on glomerular and brain microvascular endothelial cells and the activation of prothrombotic and proinflammatory cascades that lead to the development of HUS and central nervous system complications. While the mechanism of action of Stx and the resultant cytotoxicity are well described, the pathogenic mechanism(s) leading to the profound vascular damage seen in HUS is less well understood. Possible contributors to pathogenesis may include bacterial lipopolysaccharides and the proinflammatory cytokines tumour necrosis factor α and interleukin-1β.

Besides Stx production, colonisation of the host intestinal mucosa is another key determinant of virulence, and several virulence factors involved in the process have now been characterised. In conclusion, not all the strains of STEC are able to cause haemorrhagic colitis or

HUS, and those that do carry virulence determinants in addition to Stx. However, it is still not clear why only the subset of STEC defined as EHEC have narrow host specificity and are human pathogens. (25)

7.2. Shiga toxins

Stx are considered to be the major virulence factor of EHEC and comprise a family of structurally related cytotoxins with similar biological activity. The two main groups consist of Stx1, which is nearly identical to the toxin of *S. dysenteriae* type 1, and Stx2, which shares less than 60% amino acid sequence with Stx1. The genetic information for the production of Stx1 and Stx2 is located in the genome of lambdoid prophages integrated in the STEC chromosome. Several variants of Stx2 with altered antigenic or biological characteristics have been described, and variants of Stx1 (Stx1d and Stx1c) have also been described. Stx2 variants have been termed Stx2c, Stx2d, Stx2e and Stx2f. Besides these toxins, which have been reported in a relevant number of strains, several

reports on other Stx2 variants produced by single strains have been published. Epidemiological studies have revealed that Stx2 is more often associated with severe human disease than Stx1. Among the Stx2 variants, Stx2 and Stx2c have been frequently found in strains isolated from patients with HUS, while strains producing Stx2d are usually isolated from cases of uncomplicated diarrhoea. Other variants are produced by strains of animal origin and are rarely observed in human isolates: Stx2e is mainly found in STEC causing oedema disease in pigs, and Stx2f appears to be closely associated with STEC of avian origin. (25)

7.3. The Locus of Enterocyte Effacement (LEE)

Most STEC included in the EHEC group colonise the intestinal mucosa with a mechanism that subverts the epithelial cell function and induces a characteristic histopathologic lesion defined as "attaching and effacing"(A/E). The A/E lesion is due
to marked cytoskeletal changes and is characterised by effacement of microvilli and intimate adherence between the bacteria and the

epithelial cell membrane, with accumulation of polymerised actin directly beneath the adherent bacteria.

The complex mechanism of A/E adhesion is genetically governed by a large PAI defined as the LEE, and epidemiological studies have shown that LEE-positive strains are highly associated with severe human disease. LEE consists of three functionally different modules. The first encodes a type III secretion system (TTSS) that exports effector molecules. The second encodes the secreted proteins EspA, B, and D, which function as part of the type III secretion apparatus. The third encodes the adhesin "intimin" and the "translocated intimin receptor" (Tir), which is translocated into the host cell plasma membrane by the TTSS.

Intimin mediates the intimate attachment of EHEC and also of enteropathogenic *E. coli* (EPEC) to the host cell, and its important role in the pathogenic process has been demonstrated. The intimin encoding genes (*eae*) present a considerable heterogeneity in their 3' end that encodes the C-terminal 280 amino acids involved in

binding to the enterocytes and Tir, and the corresponding changes in the amino acid sequence also represent antigenic variations.

Based on the sequence and antigenic differences in this C-terminal cell-binding domain, several distinct intimin types have been identified and classified with a nomenclature system based on the Greek alphabet. The main types are termed α,β, ε, γ. Intimin α is generally found in EPEC, while types γ and ε are closely associated with EHEC. Intimin γ is produced by serogroups O157, O111, and O145, while intimin ε by serogroups O103 and O121. Intimin β can be found in both EPEC and EHEC, the most important EHEC serogroup producing intimin β being O26. Several other less frequent *eae* gene variants have been described, and a PCR typing scheme for their identification has been developed. It has been hypothesised that the wide variability in the polypeptide cell-binding domain of intimin could play a role in the tissue tropism of the different intimin-producing *E. coli*. EPEC, which produce β intimins, can colonise almost all regions of the small bowel, while

binding of γ intimin-positive EHEC strains is restricted to the follicle-associated epithelium of the Peyers patches.

In addition to Tir, LEE-positive EHEC use the TTSS to inject several effector proteins directly into the eukaryotic cell, where the normal cellular functions are subverted to the benefit of the bacteria. To date, seven EPEC and EHEC effector molecules have been shown to be injected into the host cell by the TTSS. Five translocated effectors are encoded by LEE: Tir/EspE, Map, EspF, EspG and EspH. Two effectors are encoded outside the LEE by lambdoid prophages: Cif and NleA/EspI. Blast analysis revealed that homologues of NleA/EspI are encoded by an Stx1-converting phage in EHEC of serotype O84:H4 (unpublished results) or by the phage coding for Cif. Other potential type III effectors encoded outside the LEE have also been identified using a proteomic approach. These non-LEE effectors are encoded by lambda-like phages or by putative PAI, such as PAI O#122, also termed SpLE3, integrated near tRNA *pheV* in EHEC O157. The set of translocated effector molecules tends to be unique to each pathogen: it reflects

the needs and specific niches of each bacterial species and could also determine the clinical manifestation of disease in the host. The repertoire of LEE encoded effectors does not explain the full spectrum of pathologic phenomena induced by infections with EHEC in the host. The newly identified non-LEE-encoded effectors will open up new areas of investigation to increase our understanding of EHEC-mediated diseases. (25)

7.4. Other mechanisms of intestinal adhesion

LEE-negative STEC are rarely isolated from cases of bloody diarrhoea or HUS and are usually not included among EHEC. A few exceptions have been reported in the literature, in which other mechanisms of adhesion could have allowed the strains to colonise the intestinal mucosa as efficiently as strains having the A/E phenotype, and hence to cause disease. STEC O111:H2 from an outbreak of HUS were shown to display aggregative adhesion to HEp-2 cells instead of the localised adhesion typical of A/E *E. coli* and to possess the genetic markers of enteroaggregative *E. coli*

instead of the LEE. Tissue culture adherent STEC of serotype O113:H21 have been isolated from patients with HUS. STEC O113 strains produce an autoagglutinating adhesin, encoded by a genetic locus, termed *saa,* which could have a role in the colonisation of the host intestinal mucosa.(25)

7.5. Other pathogenicity islands

Genetic analysis of the complete DNA sequence of EHEC O157:H7 showed that almost 20% of its chromosome is constituted of foreign DNA, which is not present in the chromosome of *E. coli* K-12, and which was probably acquired from other bacterial species through horizontal gene transfer. Similarly to LEE, other regions of this foreign DNA can be considered as putative PAI since they carry virulence-associated genes, show a lower GC content, and are inserted in tRNA loci. Some of these PAI are not restricted to EHEC O157, but have been observed in other EHEC serogroups and in *E. coli* strains belonging to other diarrhoeagenic groups. In particular, a PAI termed O#122 is present in most EHEC and EPEC, but not in

other groups of *E. coli*. In many clones, PAI O#122 and LEE are physically linked to form a larger mosaic PAI, and that could be the basis of the association with EHEC and EPEC. In EHEC O157, PAI O#122 is located apart from the LEE, and it has been hypothesised that the two islands were separated after being acquired as a unique large PAI, following events of chromosomal rearrangement. PAI O#122 carries *efa1/lifA*, a 10-kb virulence locus that has been involved with both the repression of host lymphocyte activation response and the adhesion to cultured cells. In vivo, the presence of this gene has been associated with the capability of colonising the intestinal tract of cattle and of inducing diarrhoea in young calves. The PAI O#122 of EHEC O157 possess only the 5' region of *efa1/lifA*, but even that portion of the gene appears to have a role in its adherence properties.

Another PAI first described in pathogenic *Yersinia* species and termed high-pathogenicity island (HPI) has been reported in EHEC. It encodes the pesticin receptor FyuA and the siderophore

yersiniabactin, and has been detected in EHEC O26 strains, but not in EHEC belonging to serogroups O157, O103 and O111.(25)

7.6 Biofilm formation in *E. coli*.

Biofilms are structured bacterial communities attached to a surface and encased in a self-produced polymeric substance. Cells within biofilms have an increased tolerance to antimicrobial agents compared to planktonic cells, therefore, formation of biofilms is problematic in food processing environments. In *E. coli,* quorum sensing mechanisms are believed to be involved in biofilm formation (109). In addition, a number of surface structures, including curli, flagella, and fimbriae (192-147a). Dudley et al., (48) identified an IncI1 plasmid in an enteroaggregative *E. coli* strain, which was named, pSERB1, encodes a type IV pilus that was involved with adherence to mammalian cells, plasmid conjugation, and adherence to surfaces. Fourteen genes are found within the type 4 (thin pilus) pilus locus, which are designated *pilI* through *pilV*. Deletion of the *pilS* gene that encodes the major type IV pilin

subunit resulted in reduced conjugal transfer of the plasmid, reduced adherence to polystyrene, and a reduction in mature biofilm formation.

7.7. Plasmids

EHEC O157 possesses a large virulence plasmid of approximately 93-kb termed pO157.

The nucleotide sequence of 93-kb plasmid showed that it encodes 35 proteins, some of which are presumably involved in the pathogenesis of EHEC infections. The *hly* operon encodes four ORFs necessary for the synthesis and transport of the enterohaemolysin and confers to EHEC the enterohaemolytic phenotype. The *hly* operon is considered as the best marker of the presence of pO157 and is also present in large plasmids that can be detected in most non-O157 EHEC strains. pO157 also carries a type II secretion system related to the pullulanase secretion pathway of *Klebsiella*, but its function has yet to be elucidated. Other putative virulence factors harboured by this plasmid comprise a katalase-

peroxidase and a serine protease, encoded by *katP* and *espP* genes, respectively. The involvement of these factors in EHEC pathogenesis is unclear, but antibodies to the *espP* product have been found in convalescent-phase sera from children who suffered from EHEC infection. pO157 is a dynamic structure, which includes different mobile genetic elements such as transposons, prophages, and parts of other plasmids assembled together by recombination events. As a consequence, the plasmid-encoded determinants are unevenly distributed among EHEC O157 strains. The *hly* operon and the type II secretion system are present in almost all the isolates, while *katP* and *espP* can be detected in two thirds of the strains. Another virulence gene, termed *toxB,* has been recently described in pO157 and it appears to be present in all the EHEC O157 isolates (186). The product of *toxB* seems to be involved in the colonisation of the host gut by influencing the expression of the LEE encoded type III secreted proteins and by inhibiting the activation of host lymphocytes. These biological activities are similar to those conferred by the *efa1/lifA*gene carried by PAI O#122. Both *toxB* and

efa1/lifA are large genes, which show a 50% mean homology in the amino acid sequence, although there are no significant homologies at the nucleotide level. It is interesting to note that EHEC O157, which possess *toxB*, carries only a small portion of *efa1/lifA* corresponding to the 5' region of the gene. Conversely, most non-O157 EHEC have the entire *efa1/lifA* but did not present *toxB* sequences (unpublished results) (25). Large plasmids resembling pO157 can be found in most non-O157 EHEC strains. These plasmids usually carry the *hly* operon, while other markers like the type II secretion system, *katP* and *espP* can be found in less than 50% of the isolates. These findings probably reflect the remarkable plasticity of this group of large virulence plasmids.

8. CURRENT STUDIES ON THE EHEC VIRULENCE PLASMID AND RESEARCH OBJECTIVES

8.1 O157:H7 and O157:H- outbreaks

EHEC strains harbor a large plasmid that possesses genes associated with virulence. An *E. coli* O157:H7 strain associated with an outbreak that occurred in Japan in 1996 possessed two plasmids, 93 and 3.3 kb in size (118). Analysis of the complete sequence of the large plasmid designated as pO157 showed similarity to F-factor and to R100, a transmissible drug resistance plasmid. It encodes genes involved in virulence as described previously, including the EHEC hemolysin, HlyA, a catalase peroxidase, KatP, a serine protease, EspP, ToxB, which is involved in adherence, and a type II secretion system (*etpC-O*) involved in secretion of pathogenic factors.

In European countries, particularly Germany, and in some non-European countries, sorbitol-fermenting *E. coli* O157:H- strains are an important cause of diarrheal illness and HUS. Brunder et al. (22)

sequenced the large plasmid of sorbitol-fermenting *E. coli* O157:H- strain 3072/96, designated pSFO157. This plasmid was 121,239 kb in size, ca. 32% larger than pO157, and it lacked the *katP*, *espP*, and *toxB* genes that are found in pO157. In another study, Brunder et al. (21) analyzed the large plasmids found in a number of STEC serotypes and in other diarrheagenic *E. coli*. They found that there was considerable variation among the plasmids with respect to the gene arrangement and the presence of specific virulence genes.

8.2 E.coli O26:H11 outbreaks

Shiga toxin-producing *E. coli* O26:H11 is the most important non-O157 EHEC and has been associated with many outbreaks and sporadic cases of hemorrhagic colitis and hemolytic uremic syndrome. An *E. coli* O26:H11 multi-state outbreak in Germany caused by *E. coli* O26:H11 associated with a beef product known as "Seemerrolle" affected 11 people (198). An outbreak due to an EHEC O26:H11 strain that possessed the stx_1 and *eae* genes occurred in Denmark in 2007 and was linked to an organic

fermented cured beef sausage (50). Other outbreaks caused by *E. coli* O26:H11 linked to contaminated food and water or from undetermined sources have also been reported (131, 85, 84, 124). *E. coli* O26:H11 strains associated with illness possessed OI 122 and HPI (9).

8.3 toxB gene

toxB has recently been described as a new virulence gene located on the large virulence plasmid of EHEC O157. Its presence has been associated with an enhancement of bacterial adhesion to cultured cells and with the inhibition of the host lymphocyte activation. Moreover, it has been shown to influence the expression and secretion of the LEE-encoded proteins. *toxB* is 9.5 kb in size and the presence of its complete coding sequence has been demonstrated only in the two pO157 plasmids which have been fully sequenced so far. Little is known about the frequency of this gene among EHEC O157 strains or its presence in other EHEC and EPEC strains. The available information refers to the presence of DNA fragments

corresponding to limited regions of *toxB* in some EHEC serogroups and in the plasmid of an EPEC O111 strain (186). The *toxB* gene in EHEC and EPEC strains belonging to different serogroups by using a combination of three PCRs and two DNA probes, altogether spanning most of the full length sequence of the gene, has recently been investigated. All of the EHEC O157 strains tested reacted with the three PCR primer pairs and the two probes, thus indicating the presence of a complete *toxB* gene regardless of whether the isolates were from human disease or from animal sources. Moreover, the strains belonged to different phage types and had been isolated in different years and different Italian regions, thus indicating that they were not clonally related. This suggests that *toxB* is a stable component of the pO157 plasmid. The presence of a presumably entire *toxB* gene was not restricted to EHEC O157, as positive reactions with all of the genetic tools employed in the study were also observed in a considerable proportion (50%) of EHEC O26 strains and in a few other EHEC (O118 and O123) and EPEC (O26 and O86) strains. *E. coli* O26 probably represents the most

important non-O157 EHEC serogroup in human infections (186). Furthermore, EHEC O118 and O123 have been associated with severe infections in calves (186). So, the presence of *toxB* in EHEC serogroups causing severe infections in both humans and calves may support the hypothesis that this gene has an important role in the pathogenesis of EHEC infections. Conversely, *toxB* sequences were not found in other EHEC serogroups, like O111 and O103 that are often associated with severe human infections. Sequencing of the 5' region of the *toxB* gene of an EPEC O26 strain indicated an 86% homology with the corresponding region of the EHEC O157 *toxB* gene (186). Several EHEC and EPEC isolates belonging to different serogroups showed positive reactions with at least one of the PCRs and/or probes. This finding could be explained by the presence of either *toxB*-like genes with a higher degree of polymorphism or truncated forms of *toxB*, like that present in the plasmid of the EPEC O111 strain B171 (GenBank accession no. AB024946) (186). The studies indicate that the *toxB* gene harbored on pO157 is consistently present in EHEC O157 strains, regardless

of their human or bovine source. DNA sequences closely related to *toxB* are frequent in EHEC O26 and have also been detected in a few other EHEC and EPEC serogroups.(186)

8.4 Research objectives

Despite great efforts by various investigators, further studies on the mechanisms of pathogenesis and the evolution of EHEC are waranted. Understanding the factors that govern the development of severe disease in human beings, and the colonisation of animal hosts will provide insights for more effective interventions on both of these aspects. Moreover, defining the combination of virulence genes and the mechanisms that make a STEC strain fully pathogenic will be pivotal for improving the efficacy of the diagnostics of human infections, surveillance of animal reservoirs, and the assessment of public health risks. The epidemiology of EHEC infections has remarkably changed during the past ten years. The organisms have been reported in a large variety of domestic and wild animal species, and an increasing number of diverse food

vehicles have been associated with human infections. New routes of transmission have emerged, like contact with animals during farm visits and a wide variety of environment-related exposures. In particular, the isolation of EHEC from a growing spectrum of animal species, which can either act as true natural hosts or merely as occasional vectors, suggests that investigations on episodes of human disease with a potential link to a rural environment should be conducted with an open mind. Furthermore, previously identified and unidentified animal reservoirs, or food or environmental vehicles should be considered and tested for the presence of STEC/EHEC, including EHEC O26:H11.

So, the objective of this study was to sequence and analyze the virulence plasmid in *E. coli* O26:H11:K60 ED21, a clinical isolate, to determine the similarity to other EHEC virulence plasmids. In addition to the large virulence plasmid, the strain possessed 5 other plasmids; therefore all six plasmids were sequenced and analyzed. Preliminary results are presented.

9. MATERIALS AND METHODS

9.1 Bacterial strain and growth conditions.

E. coli O26:H11:K60 CL19(H30), isolated from an infant with diarrhea (103) was obtained from Dr. Mohammed Karmali at the. Laboratory of Foodborne Zoonoses, Public Health Agency of Canada, and then renamed strain ED21 at the Istituto Superiore di Sanitá. This strain has been used as a reference strain for Stx1 production (152). For the current study, it was routinely grown in Luria Bertani (LB) broth (Becton Dickinson, Sparks, MD) or LB agar at 37°C.

9.2 PCR assays to determine the presence of virulence genes.

To confirm the identity of the *E. coli* O26:H11 ED21 strain, the culture was plated onto LB agar, and colonies were tested for the presence of virulence genes and genes in the *E. coli* O26 O antigen cluster. The PCR was used to determine if *E. coli* O26:H11 ED21 possessed the EHEC hemolysin gene, *hly*, using primer set MFS1F

and MFS1R (59), the *toxB* gene using primers described by Tozzoli et al. (186), the stx_1 and stx_2 genes using primer sets described by Fratamico et al. (60), the *eae* gene using primer described by Gannon et al. (64 a.) and the *E. coli* O26 *wzx* and *wzy* genes found in the O26 O antigen gene cluster (38). The DNA sequences of the primers used are shown in Table 1. A colony from LB agar was resuspended in 200 µl of the PrepMan Ultra reagent (Applied Biosystems) and was heated at 100°C for 10 min, followed by cooling and centrifugation at 16,000 x *g* for 2 min. The supernatant containing the template DNA was used in the PCR. For the PCR , the Qiagen Multitplex PCR Kit was used (Qiagen, Valencia, CA), and 0.5 µM of each of the primers and 2.5 µl of template DNA were added, The cycling conditions consisted of an enzyme activation step at 95°C for 15 min, followed by 35 cycles of 94°C for 0.5 min, annealing at 55°C (*toxB*), 57°C (*hly*), 57°C (*eae*), 51°C (O26 *wzy* and *wzx*) for 1.5 min and extension at 72°C for 1.5 min. There was a final extension step at 72°C for 10 min.

Table 1. PCR primers used in this study

Gene	Primer name	Primer sequence	Size of PCR product	Reference
hly$_{933}$	MFS1-F	ACGATGTGGTTTATTCTGGA	166	Fratamico et al., 1995
	MFS1-R	CTTCACGTCACCATACATAT		
stx$_1$	SLT1-F	TGTAACTGGAAAGGTGGAGTATAC	210	Fratamico et al., 2004
	SLT1-R	GCTATTCTGAGTCAACGAAAAATAAC		
stx$_2$	SLT2-F	GTTTTTCTTCGGTATCCTATTCC	484	Fratamico et al., 2004
	SLT2-R	GATGCATCTCTGGTCATTGTATTAC		
eaeA$_{GEN}$	EAE-F	GTGGCGAATACTGGCGAGACT	890	Gannon at al., 1997
	EAE-R	CCCCATTCTTTTTCACCGTCG		
O26 wzx	O26wzx-F	GCGCTGCAATTGCTTATGTA	152	DebRoy et al., 2004
	O26wzx-R	TTTCCCCGCAATTTATTCAG		
O26 *wzy*	O26wzy-F	TAAATTGCGGGGAAAGAATG	276	DebRoy et al., 2004
	O26wzy-R	GACTTCATGGGTACCGCCTA		
toxB	toxB.911F	ATACCTACCTGCTCTGGATTGA	600	Tozzoli et al., 2005
	toxB.1468R	TTCTTACCTGATCTGATGCAGC		

9.3 Pulsed field gel electrophoresis (PFGE).

Since initially, the PCR assays occasionally gave conflicting results, PFGE was performed on several colonies obtained from LB agar plates to confirm the purity and identity of the strain analyzed, *E. coli* O26:H11 ED21. The bacterial suspension was embedded in

agarose, lysed, washed, and digested with the restriction enzyme, *XbaI* (New England Biolabs, Beverly, MA) overnight (12-16 h) at 37°C essentially as described in the Centers for Disease Control and Prevention (Atlanta, GA) "One-Day (24-28 h) Standardized Laboratory Protocol for Molecular Subtyping of *Escherichia coli* O157:H7, non-typhoidal *Salmonella* serotypes, and *Shigella sonnei* by Pulsed Field Gel Electrophoresis (PFGE)" (http://www.cdc.gov/pulsenet/protocols.htm) (see detailed protocol described below). Electrophoresis was performed in a 1% agarose gel using 0.5X Tris-borate-EDTA buffer on a Chef Mapper XA (BioRad Laboratories, Hercules, CA), which was stained for 30 min at room temperature with ethidium bromide (Invitrogen, Carlsbad, CA) and photographed. *Salmonella choleraesuis* subspecies Braenderup (ATCC# BAA-664) was included as a reference. Pattern images were acquired using a BioRad Gel Doc system with the Multi-Analyst software program (Bio-Rad; v. 1.1) and analyzed using Bionumerics software program version 2.0 (Applied Maths BVBA, Saint-Martens-Latem-Belgium).

One-Day (24-28 h) Standardized Laboratory Protocol for Molecular Subtyping of *Escherichia coli* O157:H7, non-typhoidal *Salmonella* serotypes, and *Shigella sonnei* by Pulsed Field Gel Electrophoresis (PFGE).

PREPARATION OF PFGE PLUGS FROM AGAR CULTURES

Day 0

Streak an isolated colony from test cultures to Trypticase Soy Agar with 5% defibrinated sheep blood (TSA-SB) plates (or comparable media) for confluent growth; stab or streak small screw cap tubes of TSA, HIA, or similar medium, using the same inoculating needle/loop. This will ensure that the same colony can be retested if necessary. Incubate cultures at 37ºC for 14-18 h.

3. Prepare 1% SeaKem Gold:1% SDS agarose in TE Buffer (10 mM Tris:1 mM EDTA, pH 8.0) for PFGE plugs as follows:

a. Weigh 0.50 g (or 0.25 g) SeaKem Gold (SKG) into 250 ml screw-cap flask.

b. Add 47.0 ml (or 23.5 ml) TE Buffer; swirl gently to disperse agarose.

c. Remove cap, cover loosely with clear film, and microwave for 30-sec; mix gently and repeat for 10-sec intervals until agarose is completely dissolved. Place flask in 55-60ºC water bath for 5 minutes before adding SDS.

d. Add 2.5 ml (or 1.25 ml) of 20% SDS (pre-heated to 55ºC) and mix well.

e. Recap flask and return to 55- 60ºC water bath until ready to use.

4. Label small tubes (12-mm x 75-mm Falcon tubes or equivalent) with culture numbers.

5. Prepare Cell Suspension Buffer (100 mM Tris:100 mM EDTA, pH 8.0)

6. Transfer ≈2 ml of Cell Suspension Buffer (CSB) to small labeled tubes. Use a sterile polyester- fiber or cotton swab that has been moistened with sterile CSB to remove some of the growth from agar plate; suspend cells in CSB by spinning swab gently so

cells will be evenly dispersed and formation of aerosols is minimized.

Note: The minimum volume of the cell suspension needed will depend on size of the cuvettes or tubes used to measure the cell concentration and are dependent on the manufacturer's specifications for the spectrophotometer, turbidity meter, or colorimeter. Keep suspensions on ice if you have more than 6 cultures to process or refrigerate cell suspensions if you cannot adjust their concentration immediately.

7. Adjust concentration of cell suspensions to one of values given below by diluting with sterile CSB or by adding additional cells.

a. Spectrophotometer: 610 nm wavelength, absorbance (Optical Density) of 1.35 (range of 1.3-1.4)

b. Dade Microscan Turbidity Meter: 0.48 - 0.52 (measured in Falcon 2054 tubes) 0.68 - 0.72 (measured in Falcon 2057 tubes)

c. bioMérieux Vitek colorimeter: ≈14-15% transmittance (measured in Falcon 2054 tubes)

CASTING PLUGS

Label wells of PFGE plug molds with culture number. When reusable plug molds are used, put strip of tape on lower part of reusable plug mold before labeling wells.

Note 1: Unused plug agarose can be kept at room temperature and reused 1-2 times. Microwave on low- medium power for 10 -15 sec and mix; repeat for 5 -10 sec intervals until agarose is completely melted.

Note 2: Proteinase K solutions (20 mg/ml) are available commercially, or a stock solution of Proteinase K can be prepared from the powder in sterile Ultrapure (Reagent Grade Type 1) water, aliquoted in 300- 500 µl amounts, and kept frozen. Just before use, thaw appropriate number of vials needed for the samples; keep Proteinase K solutions on ice. Discard any thawed Proteinase K stock solution that was prepared from powder by the user at end of work day. Store commercially prepared Proteinase K solutions according to directions provided by the supplier.

1. Transfer 400 µl (0.4 ml) adjusted cell suspensions to labeled 1.5-ml microcentrifuge tubes. If cell suspensions are at room temperature, agarose can be added directly without pre-warming cell suspensions. If cell suspensions are cold, place tubes containing cell suspensions in plastic holders (floats); incubate in a 37ºC water bath for a few minutes.

2. Add 20 µl of Proteinase K (20 mg/ml stock) to each tube and mix gently with pipet tip. (200 µl are needed for 10 cell suspensions.)

3. Add 400 µl (0.4 ml) melted 1% SeaKem Gold:1% SDS agarose to the 0.4-ml cell suspension; mix by gently pipetting mixture up and down a few times. Maintain temperature of melted agarose by keeping flask in beaker of warm water (55-60ºC).

4. Immediately, dispense part of mixture into appropriate well(s) of reusable plug mold. Do not allow bubbles to form. Two plugs of each sample can be made from these amounts of cell suspension and agarose. Allow plugs to solidify at room temperature for 10-15 min. They can also be placed in the refrigerator (4ºC) for 5 minutes.

Note: If disposable plug molds are used for making plugs with 1% SeaKem Gold:1% SDS agarose, use 200 µl cell suspension, 10 µl of Proteinase K (20 mg/ml stock) and 200 µl of agarose; up to 4 plugs can be made from these amounts of cell suspension and agarose.

LYSIS OF CELLS IN AGAROSE PLUGS

Note: Two plugs (reusable plug molds) or 3 - 4 plugs (disposable plug molds) of the same strain can be lysed in the same 50-ml tube.

1. Label 50-ml polypropylene screw-cap or 50-ml Oak Ridge tubes with culture numbers.

2. Prepare Cell Lysis Buffer (50 mM Tris:50 mM EDTA, pH 8.0 + 1% Sarcosyl) as follows:

25 ml of 1 M Tris, pH 8.0

50 ml of 0.5 M EDTA, pH 8.0

50 ml of 10 % Sarcosyl (N-Lauroylsarcosine, Sodium salt)

Dilute to 500 ml with sterile Ultrapure (Reagent Grade Type 1) water

3. Calculate the total volume of Cell Lysis/Proteinase K Buffer needed as follows:

a. 5 ml Cell Lysis Buffer (50 mM Tris:50 mM EDTA, pH 8.0 + 1% Sarcosyl) is needed per tube (e. g., 5 ml x 10 tubes = 50 ml).

b. 25 µl Proteinase K stock solution (20 mg/ml) is needed per tube of the cell lysis buffer

c. Measure correct volumes into appropriate size test tube or flask and mix well.

Note: The final concentration of Proteinase K in the lysis buffer is 0.1 mg/ml, and is different from the concentration that was added to the cell suspension (0.5 mg/ml).

4. Add 5 ml of Proteinase K/Cell Lysis Buffer to each labeled 50 ml tube.

5. Trim excess agarose from top of plugs with scalpel or razor blade (optional). Open reusable plug mold and transfer plugs from

mold with a 6-mm wide spatula to appropriately labeled tube. If disposable plug molds are used, remove white tape from bottom of mold and push out plug(s) into appropriately labeled tube. Be sure plugs are under buffer and not on side of tube.

Note: The excess agarose, plug mold, spatula, etc. are contaminated. Discard or disinfect appropriately.

6. Remove tape from reusable mold. Place both sections of plug mold, spatulas, and scalpel in 70% isopropanol (IPA) or other suitable disinfectant. Soak them for 15 minutes before washing them. Discard disposable plug molds or disinfect them in 10% bleach for 30-60 minutes if they will be washed and reused.

7. Place tubes in rack and incubate in a 54ºC shaker water bath or incubator for 1.5 – 2h with constant and vigorous agitation (150-175 rpm). If lysing in water bath, be sure water level is above level of lysis buffer in tubes.

The N-Lauroylsarcosine, Sodium salt can be added directly to the other ingredients and allowed to dissolve.

8. Pre-heat enough sterile Ultrapure (Reagent Grade Type 1) water to 50ºC so that plugs can be washed two times with 10-15 ml water (200-250 ml for 10 tubes).

WASHING OF AGAROSE PLUGS AFTER CELL LYSIS

Lower the temperature of the shaker water bath or incubator to 50ºC.

1. Remove tubes from water bath or incubator, and carefully pour off lysis buffer into an appropriate discard container; plugs can be held in tubes with a screened cap or spatula.

Note: It is important to remove all of the liquid during this and subsequent wash steps by touching edge of tube or screened cap on an absorbent paper towel.

2. Add at 10-15 ml sterile Ultrapure (Reagent Grade Type 1) water that has been pre-heated to 50ºC to each tube and shake the tubes in a 50ºC water bath or incubator for 10-15 min.

3. Pour off water from the plugs and repeat wash step with pre-heated water (Step 2) one more time.

a. Pre-heat enough sterile TE Buffer (10 mM Tris:1 mM EDTA, pH 8.0) in a 50ºC water bath so that plugs can be washed four times with 10-15 ml TE (300-350 ml for 10 tubes) after beginning last water wash.

4. Pour off water, add 10-15 ml pre-heated (50ºC) sterile TE Buffer, and shake the tubes in 50ºC water bath or incubator for 10-15 min.

5. Pour off TE and repeat wash step with pre-heated TE three more times.

6. Decant last wash and add 5-10 ml sterile TE. Continue with step 1 in "Restriction Digestion" section or store plugs in TE Buffer at 4ºC until needed. Plugs can be transferred to smaller tubes for storage.

Note: If restriction digestion is to be done the same day, complete Steps 1-3 of next section (RESTRICTION DIGESTION OF DNA IN AGAROSE PLUGS WITH XbaI) during last TE wash step for optimal use of time.

RESTRICTION DIGESTION OF DNA IN AGAROSE PLUGS WITH *Xba*I

Note: A small slice of the plug or the entire plug (made in disposable plug molds) can be digested with the restriction enzyme. Restriction digestion of a small slice of the plug is recommended because less enzyme is required and other slices of the plug can be subjected to restriction analysis with other enzymes, such as AvrII (BlnI), SpeI, etc. This is important when the PFGE patterns obtained with the primary enzyme from two or more isolates are indistinguishable, and confirmation is needed to determine that the PFGE patterns of these isolates are also indistinguishable with additional enzymes.

1. Label 1.5-ml microcentrifuge tubes with culture numbers; label 3 (10-well gel) or 4 (15-well gel) tubes for *Salmonella* ser. Braenderup H9812 standards.

Optional Pre-Restriction Incubation Step: Dilute 10X H buffer (Roche Molecular Biochemicals or equivalent) 1:10 with sterile Ultrapure (Reagent Grade Type 1) water according to the following table.

Reagent	µl/Plug Slice	µl/10 Plug Slices	µl/15 Plug Slices
Sterile Reagent Grade Water	180 µl	1800 µl	2700 µl
H Buffer	20 µl	200 µl	300 µl
Total Volume	200 µl	2000 µl	3000 µl

a. Add 200 µl diluted H buffer (1X) to labeled 1.5-ml microcentrifuge tubes.

b. Carefully remove plug from TE with spatula and place in a sterile disposable Petri dish or on large glass slide.

c. Cut a 2.0- to 2.5-mm-wide slice from test samples with a single edge razor blade (or scalpel, cover slip, etc.) and transfer to tube containing diluted H buffer. Be sure plug slice is under buffer. Replace rest of plug in original tube that contains 5 ml TE buffer.

Store at 4ºC.

Note: The shape and size of the plug slice that is cut will depend on the size of the comb teeth that are used for casting the gel. PulseNet recommends that the combs with larger teeth (10-mm-wide teeth) be used to cast the gels because computer analysis of the gel lanes is more accurate and less tedious than analysis of gel lanes cast with combs with the smaller teeth (5.5-mm). The number of slices that can be cut from the plugs will depend on the skill and experience of the operator, integrity of the plug, and whether the slices are cut vertically or horizontally (plugs made in disposable molds).

d. Cut three or four 2.0-mm-wide slices from plug of the S. ser. Braenderup H9812 standard and transfer to tubes with diluted H buffer. Be sure plug slices are under buffer. Replace rest of plug in original tube that contains 5 ml TE buffer. Store at 4ºC.

e. Incubate sample and control plug slices in 37ºC water bath for 5-10 min or at room temperature for 10-15 min.

f. After incubation, remove buffer from plug slice using a pipet fitted with 200-250 µl tip all the way to bottom of tube and aspirate buffer. Be careful not to cut plug slice with pipet tip and that plug slice is not discarded with pipet tip.

2. Dilute 10X H buffer 1:10 with sterile Ultrapure (Reagent Grade Type 1) water and add *Xba*I restriction enzyme (50 U/sample) according to the following table. Mix in the same tube that was used for the diluted H buffer.

Reagent[4]	µl/Plug Slice	Ml/10 Plug Slices	µl/15 Plug Slices
Sterile Reagent Grade Water	175 µl	1750 µl	2625 µl
H Buffer	20 µl	200 µl	300 µl
Enzyme(10 U/µl)	5 µl	50 µl	75 µl
Total Volume	200 µl	2000 µl	3000 µl

Note: Keep vial of restriction enzyme on ice or in insulated storage box (-20˚C) at all times.

3. Add 200 µl restriction of enzyme mixture to each tube. Close tube and mix by tapping gently; be sure plug slices are under enzyme mixture.

4. Incubate sample and control plug slices in 37˚C water bath for 1.5-2 h.

5. If plug slices will be loaded into the wells (Option B, page 9), continue with Steps 1-4 of the next section (CASTING AGAROSE GEL) approximately 1 h before restriction digest reaction is finished so the gel can solidify for at least 30 minutes before loading the restricted PFGE plugs.

CASTING AGAROSE GEL

A. Loading Restricted Plug Slices on the Comb:

1. Confirm that water bath is equilibrated to 55- 60ºC.

2. Make volume of 0.5X Tris-Borate EDTA Buffer (TBE) that is needed for both the gel and electrophoresis running buffer according to one of the following tables.

5X TBE:

Reagent	Volume in milliliters (ml)					
5X TBE	200	210	220	230	240	250
Reagent Grade Water	1800	1890	1980	2070	2160	2250
Total Volume of 0.5X TBE	2000	2100	2200	2300	2400	2500

10X TBE:

Reagent	Volume in milliliters (ml)					
10X TBE	100	105	110	115	120	125
Reagent Grade Water	1900	1995	2090	2185	2280	2375
Total Volume of 0.5X TBE	2000	2100	2200	2300	2400	2500

3. Make 1% SeaKem Gold (SKG) Agarose in 0.5X TBE as follows:

a. Weigh appropriate amount of SKG into 500 ml screw-cap flask.

b. Add appropriate amount of 0.5X TBE; swirl gently to disperse agarose.

c. Remove cap, cover loosely with clear film, and microwave for 60-sec; mix gently and repeat for 15-sec intervals until agarose is completely dissolved.

d. Recap flask and place in 55-60ºC water bath.

Mix 1.0 g agarose with 100 ml 0.5X TBE for 14-cm-wide gel form (10 or 15 wells) Mix 1.5 g agarose with 150 ml 0.5X TBE for 21-cm-wide gel form (≥15 wells)

4. A small volume (2-5 ml) of melted and cooled (50-60ºC) 1% SKG 1% SKG agarose may be wanted to seal wells after plugs are loaded. Prepare 50 ml by melting 0.5 g agarose with 50 ml 0.5X TBE in 250 ml screw-cap flask as described above. Unused SKG agarose can be kept at room temperature, melted, and reused several

times. Microwave for 15-20 sec and mix; repeat for 10-sec intervals until agarose is completely melted. Place in 55-60ºC water bath until ready to use. Alternatively, save approximately 5 ml of the melted agarose used to cast the gel in a pre-heated (55-60ºC) 50 ml flask and place in 55-60ºC water bath until used.

Note: Confirm that gel form is level on leveling table, that front of comb holder and teeth face the bottom of gel, and that the comb teeth touch the gel platform.

5. Remove restricted plug slices from 37ºC water bath. Remove enzyme/buffer mixture and add 200 µl 0.5X TBE. Incubate at room temperature for 5 min.

6. Remove plug slices from tubes; put comb on bench top and load plug slices on the bottom of the comb teeth as follows:

   a. Load S. ser. Braenderup H9812 standards on teeth (lanes) 1, 5, 10 (10-well gel) or on teeth 1, 5, 10, 15 (15-well gel).

   b. Load samples on remaining teeth.

7. Remove excess buffer with tissue. Allow plug slices to air dry on the comb for ≈5 minutes or seal them to the comb with 1% SKG agarose (55-60ºC).

8. Position comb in gel form and confirm that the plugs slices are correctly aligned on the bottom of the comb teeth, that the lower edge of the plug slice is flush against the black platform, and there are no bubbles (if allowed to air dry).

9. Carefully pour the agarose (cooled to 55-60ºC) into the gel form.

10. Put black gel frame in electrophoresis chamber. Add 2 -2.2 L freshly prepared 0.5X TBE. Close cover of unit. (The amount of buffer needed depends on whether residual buffer was left in tubing or if unit was flushed with water after the last gel was run.)

11. Turn on cooling module (14ºC), power supply, and pump (setting of ≈70 for a flow of 1 liter/minute).

12. Remove comb after gel solidifies for 30-45 minutes.

13. Fill in wells of gel with melted and cooled (55- 60ºC) 1% SKG Agarose (optional). Unscrew and remove end gates from gel form;

remove excess agarose from sides and bottom of casting platform with a tissue. Keep gel on casting platform and carefully place gel inside black gel frame in electrophoresis chamber. Close cover of chamber.

B. Loading Restricted Plug Slices into the Wells:

1. Follow steps 1-4 in Option A on pages 7 and 8 (Loading Restricted Plug Slices on the Comb).

Note: Confirm that gel form is level on gel-leveling table before pouring gel, that front of comb holder and teeth face bottom of gel, and the bottom of the comb is 2 -mm above the surface of the gel platform.

2. Cool melted SKG agarose in 55-60ºC water bath for 15-20 min; carefully pour agarose into gel form (casting stand) fitted with comb. Be sure there are no bubbles.

3. Put black gel frame in electrophoresis chamber. Add 2-2.2 L freshly prepared 0.5X TBE. Close cover of unit. (The amount of

buffer depends on whether residual buffer was left in tubing, or if unit was flushed with water after the last gel was run.)

4. Turn on cooling module (14ºC), power supply, and pump (setting of ≈ 70 for a flow of 1 liter/minute) approximately 30 min before gel is to be run.

5. Remove restricted plug slices from the 37ºC water bath. Remove enzyme/buffer mixture and add 200 µl 0.5X TBE. Incubate at room temperature for 5 minutes.

6. Remove comb after gel solidifies for at least 30 minutes.

7. Remove restricted plug slices from tubes with tapered end of spatula and load into appropriate wells. Gently push plugs to bottom and front of wells with wide end of spatula. Manipulate position with spatula and be sure that are no bubbles.

a. Load S. ser. Braenderup H9812 standards in wells (lanes) 1, 5, 10 (10-well gel) or in wells 1, 5, 10, 15 (15-well gel).

b. Load samples in remaining wells.

Note: Loading the plug slices can be tedious; each person has to develop his/her own technique for consistently placing the plug slices in the wells so the lanes will be straight and the bands sharp.

8. Fill in wells of gel with melted 1% SKG Agarose (equilibrated to 55- 60ºC). Allow to harden for 3-5 min. Unscrew and remove end gates from gel form; remove excess agarose from sides and bottom of casting platform with a tissue. Keep gel on casting platform and carefully place gel inside black gel frame in electrophoresis chamber. Close cover of chamber.

ELECTROPHORESIS CONDITIONS

1a. Select following conditions on CHEF Mapper for Escherichia coli O157:H7 and *Shigella sonnei* strains restricted with *Xba*I or *Avr*II (*Bln*I):

Auto Algorithm

30 kb - low MW

600 kb - high MW

Select default values except where noted by pressing "enter".

Change run time to 18 - 19 h (See note below)

(Default values: Initial switch time = 2.16 s; Final switch time = 54.17 s)

1b. Select following conditions on CHEF-DR III

Initial switch time: 2.2 s

Final switch time: 54.2 s

Voltage: 6 V

Included Angle: 120°

Run time: 18-19 h (See note below)

1c. Select following conditions on CHEF-DR II

Initial A time: 2.2 s

Final A time: 54.2 s

Start ratio: 1.0 (if applicable)

Voltage: 200 V

Run time: 19-20 h (See note below)

2a. Select following conditions on CHEF Mapper for non-typhoidal Salmonella strains restricted

with *Xba*I or *Avr*II (*Bln*I):

Auto Algorithm

30 kb - low MW

700 kb - high MW

Select default values except where noted by pressing "Enter".

Change run time to 18 - 19 h (See note below)

(Default values: Initial switch time = 2.16 s; Final switch time = 63.8 s)

2b. Select following conditions on CHEF DR-III

Initial switch time: 2.2 s

Final switch time: 63.8 s

Voltage: 6 V

Included Angle: 120°

The same electrophoresis conditions are used for gels of *Shigella sonnei* restricted with *Not*I. Other species of *Shigella* tested at the

CDC usually give satisfactory results when these electrophoresis conditions are used; adjustments to the initial and final switch times may be required to optimize separation of the bands.

Run time: 18-19 h (See note below)

2c. Select following conditions on CHEF DR-II.

Initial A time: 2.2s

Final A time: 63.8 s

Start Ratio: 1.0 (if applicable)

Voltage: 200 V

Run time: 19-20 h (See note below)

Note: The electrophoresis running times recommended above are based on the equipment and reagents used at the CDC. Run times may be different in your laboratory and will have to be optimized for your gels so that the lowest band in the *S.* ser. Braenderup H9812 standard migrates 1.0 - 1.5 cm from the bottom of the gel.

Day 2

STAINING AND DOCUMENTATION OF PFGE AGAROSE GEL

1. When electrophoresis run is over, turn off equipment; remove and stain gel with ethidium bromide. Dilute 40 µl of ethidium bromide stock solution (10 mg/ml) with 400 ml of reagent grade water (this volume is for a staining box that is approximately 14-cm x 24-cm; a larger container may require a larger amount of staining solution). Stain gel for 20 - 30 min in covered container.

Note: Ethidium bromide is toxic and a mutagen; the solution can be kept in dark bottle and reused 3-5 times before discarding according to your institution's guidelines for hazardous waste or use the destaining bags recommended for disposal of ethidium bromide (Section 10).

2. Destain gel in approximately 500 ml reagent grade water for 60 - 90 min; change water every 20 minutes. Capture image on Gel Doc 1000, Gel Doc 2000, or equivalent documentation system. If

background interferes with resolution, destain for an additional 30-60 min.

Note: If both a digital image and conventional photograph are wanted, photograph gel first before capturing digital image.

3. Follow directions given with the imaging equipment to save gel image as an *.img or *.1sc file; convert this file to *.tif file for analysis with the BioNumerics software program.

4. Drain buffer from electrophoresis chamber and discard. Rinse chamber with 2 L reagent grade water or, if unit is not going to be used for several days, flush lines with water by letting pump run for 5-10 min before draining water from chamber and hoses.

Please note the following if PFGE results do not have to be available within 24-28 hours:

1. Plugs can be lysed for longer periods of time (3-16 hours).

2. The washing steps with TE to remove the lysis buffer from the PFGE plugs can be done for longer periods of time (30-45 min) and at lower temperatures (37°C or room temperature). They can be

started on Day 1 and finished on Day 2 after overnight refrigeration of the plugs in TE.

3. The restriction digestion can be done for longer periods of time (3-16 hours).

4. If the lowest band in the H9812 standard does not migrate within 1 -1.5 cm of the bottom of the gel, the run time will need to be determined empirically for the conditions in each laboratory.

NOTE: CLIA LABORATORY PROCEDURE MANUAL REQUIREMENTS

Efforts have been made to assure that the procedures described in this protocol have been written in accordance with the 1988 Clinical Laboratory Improvement Amendments (CLIA) requirements for a procedure manual (42 CFR 493.1211). However, due to the format required for training, the procedures will require some modifications and additions to customize them for your particular laboratory operation.

9.4 Plasmid purification and library construction.

Plasmid DNA from *E. coli* ED21 was purified using the Qiagen Plasmid Midi Kit (Qiagen, Valencia, CA), and plasmids were visualized following agarose (0.8%) gel electrophoresis. The gel contained ethidium bromide at a concentration of 0.5 µg/ml. Three libraries were constructed:

1. The plasmid DNA preparation from *E. coli* ED21 was digested with Sau 3A I (0,50 units/µg DNA) at 37°C for 16h. The fragments were purified from 0,7% agarose gel by using the QIAquick Gel Extraction Kit (Qiagen) and ligated using T4 DNA ligase (New England BioLabs) with *Bam*HI digested pBC SK(+) vector (Fermentas, Glen Burnie, MD) (calf intestine phosphatase treated), and then, the plasmid was transformed into *E. coli* K12 DH5α . (Invitrogen, Carlsbad, CA). by electroporation (BioRad Pulse Controller - cuvette size O.2 cm, capacitance 25 microfarads, resistance 25 ohms, and voltage 2500 V).

The resulting clones were grown on LB agar at 37°C supplemented with chloramphenicol (170 μg/ml). One hundred microliters of 10 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) prepared in sterile water and 100 μl of 2% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) prepared in dimethylformamide were spread onto the LB chloramphenicol (170 μg/ml) agar, which was allowed to dry prior to plating.

2. Digestion with *Eco*RV + *Sph*I (New England BioLabs) (0,50 units/μg DNA) at 37°C for 16h. The fragments were purified from a 0,7% agarose gel using the QIAquick Gel Extraction Kit, ligated using T4 DNA ligase (New England BioLabs) into *Sma*I+*Sph*I digested pUC19 (Fermentas) and transformed into *E. coli* K12 DH5α (Invitrogen). The cells were plated onto LB agar + ampicillin (50 μg/ml) supplemented with 100 μl of 10 mM IPTG and 100 μl of 2% X-gal.

3. Digestion with *Sma*I + *Sph*I (New England BioLabs) (0,50 units/µg DNA) at 37°C for 16h. The resulting fragments were purified from a 0,7% agarose gel by using the QIAquick Gel Extraction Kit, ligated using T4 DNA Ligase (New England BioLabs) with *Sma*I+*Sph*I digested pUC19 (Fermentas), transformed into *E. coli* K12 DH5α (Invitrogen), and plated onto LB agar + ampicillin (50 µg/ml) supplemented with the 100 µl of 10 mM IPTG and 100 µl of 2% X-gal.

9.5 Subculturing Colonies in Liquid Culture and Plasmid Purification.

Individual bacterial white colonies selected from the LB agar plates described above, were transferred to liquid culture medium in two 48-well blocks and incubated overnight with shaking on the Orbital Shaker at 37°C. The BioRobot 9600 (Qiagen) and Protocol: The QIAprep 96 Turbo BioRobot Kit was used to extract high purity

DNA, which involved lysing the bacteria and purifying the plasmid DNA. This method is used for purification of both low and high copy number plasmids. First the cells are lysed, with detritus removed during filtration, the DNA is then bound onto a membrane, and finally, the DNA is washed and eluted into a 96-well plate.

9.6 Cycle Sequencing Reaction and Clean up

The BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) was used for performing fluorescence-based cycle sequencing reactions on clones. BigDye sequencing reactions were conducted by using M13 forward, reverse and T7 primers. The master mix composition and amplification conditions are shown in table 2 and table 3. The pGEM DNA was used as control for each reaction. Post-reaction cleanup of excess unincorporated dye-terminators was performed using a CleanSEQ Kit® (Agencourt).

Table 2.

MASTER MIX	1:8 dilution mix single reaction
Milli-Q water	10 µL
2.5x Buffer	7 µL
RR mix	1 µL
Primer (3.2 pmol/µl)	1.2 µL
Total	19 µL
Template(s) (80-150 ng/µl)	1.2 µL

Table 3

THERMOCYCLING AMPLIFICATION
Initial heat 96°C 5 min
Cycling conditions (30 cycles):
96°C 10 sec
55°C 5 sec
60°C 4 min
Soak (hold) at 4°C ∞

9.7 DNA sequencing, analysis, and annotation. Sequencing was performed on an Applied Biosystems 3730 DNA Analyzer and an Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequence assembly and analysis of sequence data was performed using Sequencher software (Gene Codes Corp., Ann Arbor Mich. USA) and BLAST.

9.8 Biofilm assay

E. coli O26:H11 ED21 was cultured overnight in LB broth. One hundred microliters of the culture were added to 25 ml of LB broth with no salt in 50-ml sterile plastic tubes. Microscope glass slides that were washed with Liqui-nox (Alconox, White Plains, NY), rinsed with distilled water, and sterilized by autoclaving were positioned vertically in the tubes, which were incubated at room temperature for 72 h. The culture was then removed, and the slides were gently washed once with sterile saline. The biofilm on the slide was fixed by the addition of 2.5% glutaraldehyde in 0.1M imidazole buffer (pH 7.0) for two hours at room temperature, followed by dehydration in a graded series of ethanol solutions (50%, 80% and absolute) and critical point drying from liquid carbon dioxide. The section of the slide containing the biofilm was cut and was sputter coated with a thin layer of gold, mounted directly on a multiple sample holder accessory, and examined and imaged in a model Quanta 200 scanning electron microscope (FEI Co., Inc., Hillsboro,

OR) operated at high vacuum in the secondary electron imaging mode at various instrumental magnifications for digital imaging.

10. RESULTS

10.1 Screening for virulence genes and O26-specific genes by the PCR.

PCR results showed that *E. coli* O26:H11:K60 strain ED21 possessed the chromosomal phage-encoded stx_1 gene (fig. 5), the *eae* gene (fig.2) found in the chromosomal LEE locus, the *tox B* (fig.4) gene, *hly* gene (fig.3) the *wzx* and *wzy* genes (fig.1) . PCR results were negative for the presence of the stx_2 gene. Thus, the PCR results confirmed that the strain possessed the stx_1 gene as described by Konowalchuk et al. (103), it possessed the *toxB* and *hly* genes, which are associated with the EHEC virulence plasmid, and it possessed the *eae* gene, which encodes the intimin protein.

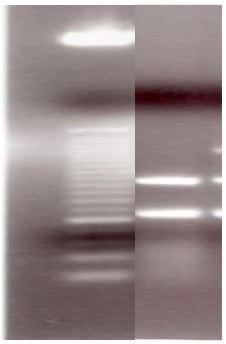


*Fig1. E. coli O*26
Lane 1: 25-bp ladder
Lane 2: *wzx* 152 bp
wzy 276 bp

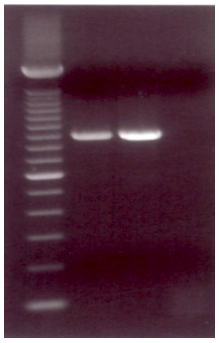


*Fig 2.*EHEC/EPEC
Lane 1:100-bp ladder
Lane2 *eae* 890 bp
Lane 3: *eae* 890 bp

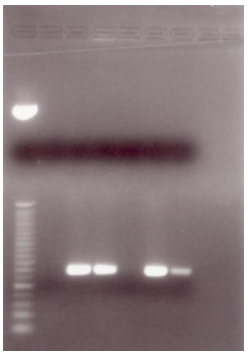


Fig 3.
Lane1: 100-bp ladder
Lane 3:*hly* 166 bp
Lane 4:*hly* 166 bp
Lane 6:*hly* 166 bp
Lane 7:*hly* 166 bp

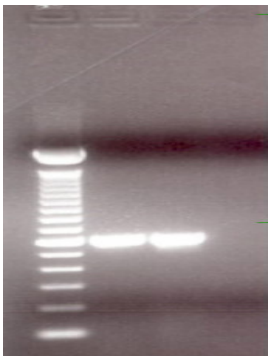


Fig 4
Lane 1:100-bp ladder
Lane2*: toxB* 600 bp

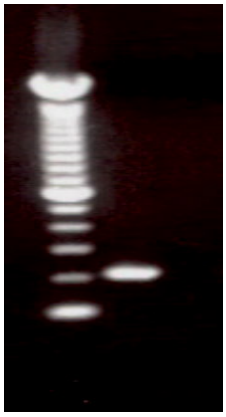


Fig 5
Lane 1: 100-bp ladder
Lane 2: stx_1 210 bp

10.2 PFGE analysis.

PFGE was performed on several colonies from the *E. coli* O26:H11:K60 culture. All of the colonies selected for analysis showed the same PFGE digestion pattern, indicating that they were from the same clone, and the culture was pure (Fig. 6). The

similarity in the patterns was confirmed using Bionumerics software.

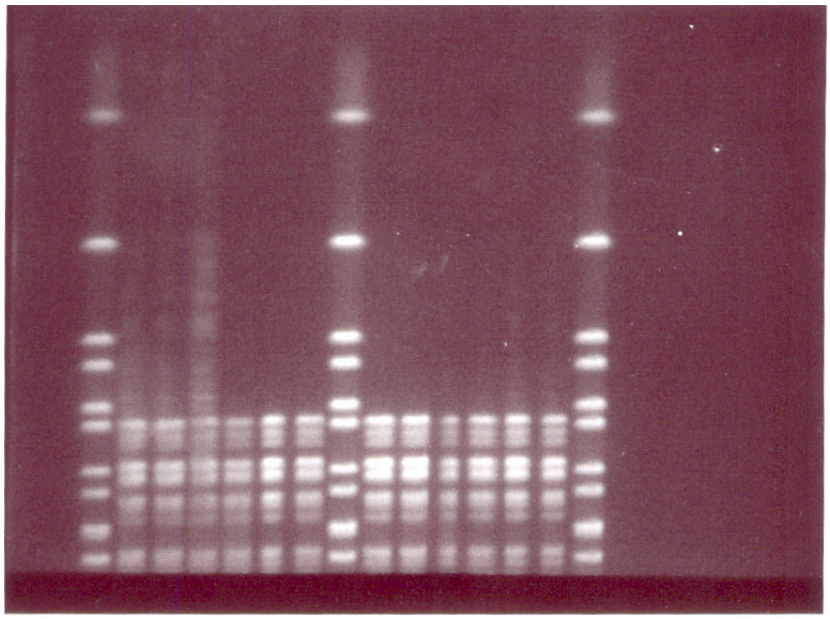


Fig.6 PFGE on *E. coli O*26:H11:K60 ED21 colonies

10.3 Plasmids in *E. coli* O26:H11 ED21.

Plasmid preparations visualized on agarose gels initially showed that there was only one large plasmid, approximately 75 to 95 kb in size. However, when the agarose gel was allowed to run at a lower voltage and for a longer time, it appeared that the strain may possess two large plasmids, in addition to several smaller plasmids. The 1kb

DNA marker, Boston BioProducts (Worcester, MA) was used. The strain appears to contain 6 plasmids of different sizes (1,5 - 3,2- 4,1- 6,8- 72,4 and >90Kb see Fig. 7)

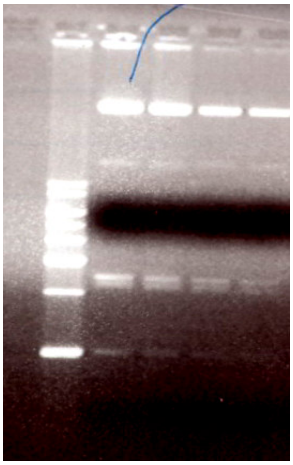


Fig. 7
Plasmid purification from *E. coli* O26:H11 ED21 colonies

10.4 Sequencing and analysis of plasmids in *E. coli* O26:H11:K60 ED21.

The large virulence plasmid is greater than 90 kb in size, and analyses conducted thus far indicate that it contains the following virulence genes: *hlyA, katP, espP, toxB*, genes encoding a type III secretion system, and genes encoding a type IV pilus. Plasmid 1 appears to contain the *repA* gene, autoregulating structural gene

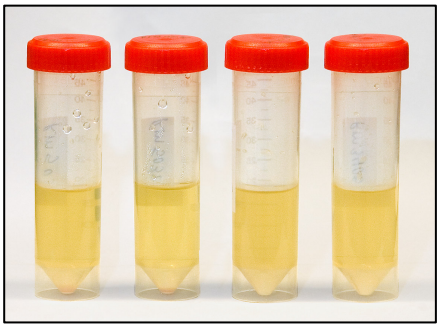
responsible for replication and copy number control. The function of genes encoded on plasmids 2, 3, and 4 could be related to transport of toxins and other proteins; however, this is yet to be determined. In addition to the presence of virulence genes, the large virulence plasmid in ED21 also contains genes that encode the type IV or thin pilus, *pilI-pilV*. This pilus is involved in surface interactions among bacteria, adherence to epithelial cells, and biofilm formation. (Table 4)

	Size (kb)	Replicon	No. of Genes	Virulence Genes	Resistance Genes
Plasmid 1	**1.5**	**RepA**	**1**	**None**	**None**
Plasmid 2	**3.2**	**None?**	**1**	**None?**	**None**
Plasmid 3	**4.1**	**None?**	**3**	**None?**	**None**
Plasmid 4	**6.8**	**Yes**	**5**	**None?**	**None**
Plasmid 5	**72.4**	**Yes**	**?**	**None?**	**Yes**
Plasmid 6	**>90**	**Yes**	**?**	**Yes**	**Yes**

Table 4

10.5 Biofilm formation of *E. coli* O26:H11:K60 ED21.

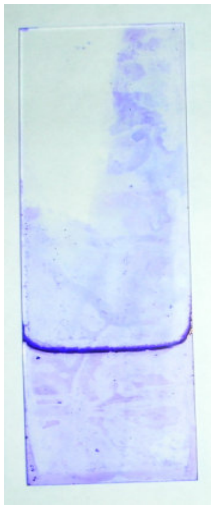
ED21 produced a strong biofilm as observed by visual examination and comparison to other strong biofilm-forming *E. coli* strains in our collection. The biofilm was observed at the air liquid interface. LB medium with no salt was used to form the biofilm, since previous studies in our laboratory indicated that this medium is suitable for *E. coli* strains that form biofilms.



Incubation of glass slides with bacterial suspension for 48 h at room temperature

↓

Staining of biofilm with crystal violet



←

Fig 8.Biofilm formed by *E. coli* O26:H11 ED21 on a glass slide

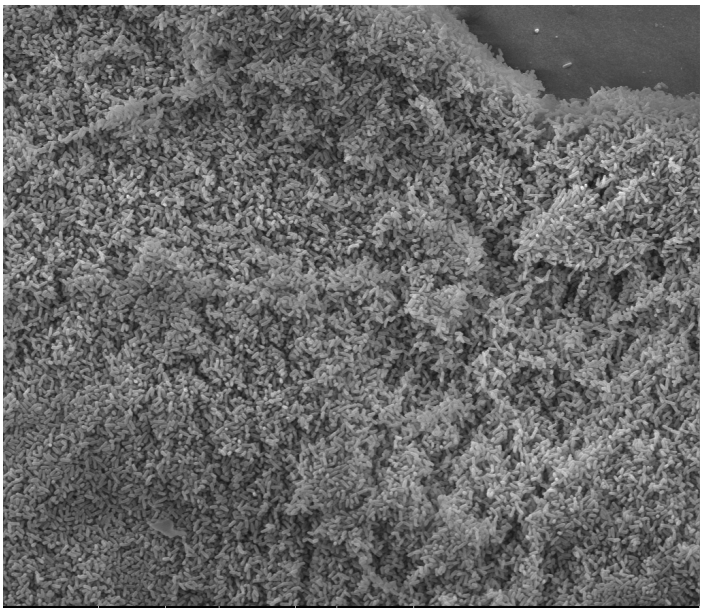


Fig.9 Biofilm. Scanning electron microscopy. 1000X

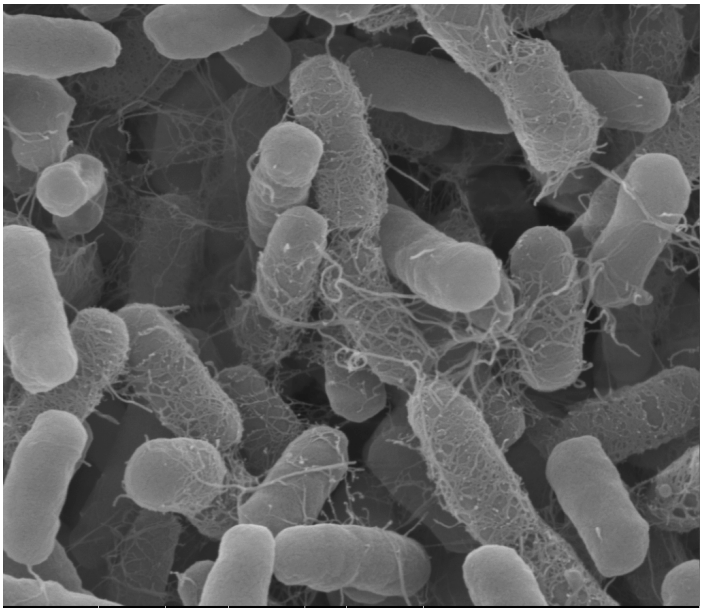


Fig.10 Biofilm. Scanning electron microscopy. 25000X

11. DISCUSSION

Enterohemorrhagic *E. coli* (EHEC), particularly, *E. coli* serotype O157:H7, are important food-borne pathogens responsible for many outbreaks of hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS). HUS is the leading cause of acute renal failure in children. Other important EHEC serogroups include O26, O103, O111, O145, and others. EHEC possess several virulence factors, and the production of Shiga toxin is the most critical. The toxin cleaves a specific adenine base from the 28S rRNA, resulting in inhibition of protein synthesis. Shiga toxin-producing *E. coli* (STEC) that cause HC and HUS are referred to as EHEC (69). EHEC also possess a pathogenicity island called the locus of enterocyte effacement (LEE), which encodes proteins necessary for attaching and effacing lesions, including intimin (Eae), an outer membrane protein, translocated intimin receptor (Tir), a type III secretion apparatus, and effector proteins translocated by the secretion system. Several other candidate pathogenicity islands, including O island (OI) 122 have also been found in EHEC;

however, their role in disease is not fully clear. The presence of OI 122 in non-O157 EHEC has been linked to the ability of these strains to cause HUS and outbreaks (200). EHEC may also possess genes that encode fimbrial or nonfimbrial adhesins, proteases, or other toxins, including cytolethal distending toxin (Cdt) or the enteroaggregative *E. coli* heat-stable enterotoxin (EAST1) (69). Other virulence genes include the plasmid-encoded hemolysin (Hly) and the ToxB protein, which contributes to adherence.

Shiga toxin-producing *E. coli* O26:H11 is the most important non-O157 EHEC and has been associated with a many outbreaks and sporadic cases of hemorrhagic colitis and hemolytic uremic syndrome. The objective of this study was to sequence and analyze the virulence plasmid in *E. coli* O26:H11:K60 ED21, a clinical isolate, obtained from the Istituto Superiore di Sanità to determine the similarity to other EHEC virulence plasmids. At first, to confirm the identity of the *E. coli* O26:H11 ED21 strain, the colonies were tested for the presence of virulence genes and genes in the *E. coli*

O26 O antigen cluster by PCR assays. Since initially, the PCR assays occasionally gave conflicting results, PFGE was performed on several colonies obtained from LB agar plates to confirm the purity and identity of the strain analyzed, *E. coli* O26:H11 ED21. After plasmid purification, three libraries were constructed and sequenced. The strain harboured six plasmids, which were purified and sequenced. Plasmid 1 appears to contain the *repA* gene, an autoregulating structural gene responsible for replication and copy number control. The function of genes encoded on plasmids 2, 3, and 4 could be related to transport of toxins and other proteins; however, this is yet to be determined. The large virulence plasmid is greater than 90 kb in size, and it contains the virulence genes: *hlyA, katP, espP, and toxB* and also genes encoding a type III secretion system and a type IV pilus (thin pilus), *pilI-pilV*. This pilus is involved in surface interactions among bacteria, adherence to epithelial cells, and biofilm formation. In fact, ED21 produced a strong biofilm as observed by visual examination and comparison to other strong biofilm-forming *E. coli* strains in our collection. A

comparison between the *E. coli* O26:H11:K60 ED21 strain and outbreak strain *E. coli* O157:H7 933 (table 5) shows high similarity in the virulence genotype patterns, indicating that strain O26:H11:K60 ED21 has virulence potential.

It is important to know the sequence of virulence genes and other genes that can be used for identification of EHEC O26, to enable the design of multiplex PCR assays to rapidly detect and identify EHEC O26 strains. The *E. coli* O26 *wzx* and/or *wzy*, genes in the *E. coli* O26 O antigen gene cluster, could also be included in the multiplex PCR assays in addition to virulence genes to identify serogroup O26 and determine if the O26 strain has genes typical of EHEC. Sequencing of EHEC plasmids, including the large virulence plasmid also helps us to identify additional biomarkers and to determine their evolutionary origin. The results presented in this work are not yet published, and some further confirmation of the data is needed.

Despite great efforts by various investigators, further studies on the mechanisms of pathogenesis and the evolution of EHEC are

waranted. Understanding the factors that govern the development of severe disease in human beings, and the colonisation of animal hosts will provide insights for more effective interventions on both of these aspects. Moreover, defining the combination of virulence genes and the mechanisms that make a STEC strain fully pathogenic will be pivotal for improving the efficacy of the diagnostics of human infections, surveillance of animal reservoirs, and the assessment of public health risks. The epidemiology of EHEC infections has remarkably changed during the past ten years. The organisms have been reported in a large variety of domestic and wild animal species, and an increasing number of diverse food vehicles have been associated with human infections. New routes of transmission have emerged, like contact with animals during farm visits and a wide variety of environment-related exposures. In particular, the isolation of EHEC from a growing spectrum of animal species, which can either act as true natural hosts or merely as occasional vectors, suggests that investigations on episodes of human disease with a potential link to a rural environment should be

conducted with an open mind. Furthermore, previously identified and unidentified animal reservoirs, or food or environmental vehicles should be considered and tested for the presence of STEC/EHEC, including EHEC O26:H11.

Virulence genes	O157:H7 933	O26:H11:K60 ED21
eae*	YES	YES
stx_1*	YES	YES
stx_2*	YES	NO
toxB**	YES	YES
hlyA**	YES	YES
katP**	YES	YES
espP**	YES	YES

Table 5
****: Chromosomal biomarkers**
*****: Plasmid biomarkers**

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