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PREVALENCE OF SHIGA-LIKE TOXIN-PRODUCING *ESCHERICHIA COLI* IN "SARDA" SLAUGHTERED SHEEP: DIRECT DETECTION OF VIRULENCE GENES AND MOLECULAR CHARACTERIZATION OF NON-O157 ISOLATES

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INTRODUCTION

Food-borne diseases are caused by pathogenic microbes such as bacteria, viruses, and parasites, or their toxins present in contaminated foods. Every year they cause illness and death in a large amount of people, great economic cost and human suffering. Many pathogen microbes are commonly found in the intestines of healthy food producing animals and the risks of contamination are present from farm to fork. During slaughter, meat can be contaminated by coming into contact with small amounts of intestinal contents and at the food processing stage microbes can be introduced by cross-contamination from another raw agricultural product or from infected humans handling the food (EFSA, 2009).

Verotoxin-producing *E. coli* (VTEC) is an emerging cause of food-borne disease in human, mainly due to O157:H7 serotype, that has been identified as a causative agent both of community outbreaks and of sporadic cases of hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS), thrombotic thrombocytopenic

purpura (TTP) and death (Griffin et al., 1991; Bhong et al. 2008) in several countries.

Consumption of foods of bovine origin, such as undercooked meat and raw milk, has been linked epidemiologically with several outbreaks of VTEC O157 infections, suggesting an important role of cattle as a reservoir of the pathogen (Griffin et al., 1991; Chapman, 1995).

Healthy cattle are considered to be the principal reservoir of *E. coli* O157 and faeces and hides of cattle are considered to be the main sources of *E. coli* O157 contamination of carcasses during slaughter (Aslam et al., 2003; Barkocy-Gallagher et al., 2003; Elder et al., 2000). Transmission can also occur following direct contact with shedding animals (Chapman, 2000) and the direct transmission from cattle to humans (Louie et al., 1999).

The relevance of O157 serotype in human disease is linked to:

- severe human disease;
- low infection dose;

- large diffusion in the food-producing animals environment;

- asymptomatic carrier ruminants.

However, other numerous VTEC serotypes have been associated with worldwide human outbreaks. These non-O157 serotype can possess the same range of virulence factors of VTEC O157:H7 and the estimation of the true incidence of disease due to strains other than O157 is not simple by the need of suitable detection methods (Nataro J.P. and Kaper J.B., 1998).

Recent evidence indicated that sheep harbor O157:H7 (Chapmann P.A. et al., 1996; Kudva I.T. et al., 1996, 1997) and non-O157:H7 (Beutin L. et al., 1993, 1995) VTEC at rates similar to those reported for cattle and suggested their potential role as a food safety risk factor (Hussein H.S. et al., 2000). Worldwide, sheep have been shown to shed several non-O157 VTEC in their feces, and the possibility of cross-contamination of lamb, mutton, carcasses at the abattoir and of their products share a food safety risk similar to that of beef. Because the importance of food safety has increased dramatically in recent years, it is important to understand the types and trends of VTEC shedding by meat animals, including sheep.

EFSA has identified a restricted range of serotypes (O157, followed by O26, O103, O91, O145 and O111) associated with public health risks. For these seropathotypes, monitoring should be applied based on the periodical analysis of human disease and epidemiological data.

Monitoring data on the prevalence and concentration of VTEC in ruminants' faeces, fleeces and carcasses after chilling at the abattoir would assist in the assessment of risk to consumers. Co-ordinated sampling of raw meat cuts or trim for the prevalence and concentration of VTEC provide suitable comparisons between EU Member States.

Ruminant meat and minced meat products (in particular those that are likely to be consumed without cooking), ready-to-eat fermented meats, fresh vegetable and salads, in addition to unpasteurised milk and dairy products derived therefore should be the object of targeted surveys

conducted on a co-ordinated basis through Member States.

In the present study we will report the results of a survey carried out with the aim to evaluate VTEC prevalence and seropathotypes at sheep abattoirs and on the farms and to investigate VTEC routes by phenotypic and molecular methods.

The experimental study will be preceded by a review about: the new EU approach to the control of zoonosis, the characteristics of Pathogenic *E. coli*, a resuming on VTEC, pathogenesis and epidemiology, its detection and typing methods, the official surveillance systems and the risk factors analysis along the ovine production chain.

FOODBORNE MICROBIOLOGICAL RISKS: ZOONOSES SURVEILLANCE ACCORDING TO EU LEGISLATION

The European Food Safety Authority (EFSA) was established and funded by the European Community as an independent agency in 2002 following a series of food-linked disease episodes that caused concerns about food safety in the European community. The aim of the authority is to fully protect consumers, it is responsible for examining the data on zoonoses, antimicrobial resistance and food-borne

outbreaks collected from the Member States in accordance with Directive 2003/99/EC and for preparing the Community Summary Report based on these results.

Zoonoses are defined by the World Health Organisation as "…diseases and infections which are naturally transmitted between vertebrate animals and man…". Zoonoses are transmitted directly or through ingestion of contaminated foodstuffs and may be bacterial, viral, or parasitic, or may involve unconventional agents (BSE): more than 200 diseases are classified as zoonoses.

Zoonoses transmissible through food may cause human suffering, as well as relevant economic losses to food production and the food industry; zoonoses transmitted through sources other than food, especially from wild animals and pet animal populations, are also matter of concern.

In order to prevent zoonoses from occurring it is important to identify which animals and foodstuffs are the main sources of infections. For this purpose European Union (EU) recommends all EU Member States to collect and analyse information to

be used to improve control measures aimed to protect human health and assuring the highest standards of food safety.

The European Union's food policy must be built around high food safety standards, which serve to protect and promote the health of the consumer.

EU legislation for the monitoring of zoonoses and zoonotic agents is regulated by the **Directive 2003/99/CE** of 17 November 2003 (amending Council Decision 90/424/EEC and repealing Council Directive 92/117/EEC) and defines the Community system for the monitoring of zoonoses, which obliges EU Member States to collect relevant data, considering verotoxigenic *Escherichia coli* (VTEC) in animals and foodstuffs.

The objectives of this directive are:

- monitoring of zoonoses and zoonotic agents;
- monitoring of related antimicrobial resistance;
- epidemiological investigation of food-borne outbreaks;
- exchange of information related to zoonoses and zoonotic agents.

The Regulation cite: "...monitoring should take place on a harmonised basis. This would make it possible to evaluate trends and sources of zoonoses and zoonotic agents within the Community. Priority should be given to those zoonoses posing the greatest risk to human health. However, the monitoring should also facilitate the detection of emerging or newly emerging zoonotic diseases and new strains of zoonotic organisms..." and also "...in addition to general monitoring, specific needs may be recognised which may necessitate the establishment of coordinate monitoring programmes."

Based on the opinion of the Scientific Panel on Biological Hazards on the monitoring of VTEC and the identification of the types of human pathogenic VTEC (EFSA, 2007; 2009), harmonized technical specifications were proposed for the monitoring and reporting of VTEC in populations of animals and relevant food categories. These technical specifications would facilitate a better analysis of the situation in the Member States and the Community.

The Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs "…lays down the microbiological criteria for

certain micro-organisms and the implementing rules to be complied with by food business operators when implementing the general and specific hygiene measures referred to in Article 4 of Regulation (EC) No 852/2004". This Regulation laid down food safety criteria for certain important foodborne bacteria, their toxins and metabolites, such as *Salmonella*, *Listeria monocytogenes*, *Enterobacter sakazakii*, staphylococcal enterotoxins and histamine in specific foodstuffs.

In this Regulation the Scientific Committee on Veterinary Measures relating to Public Health (SCVPH) issued an opinion on verotoxigenic *E. coli* (VTEC) in foodstuffs: "…applying an end-product microbiological standard for VTEC O157 is unlikely to deliver meaningful reductions in the associated risk for the consumers. However, microbiological guidelines aimed at reducing the faecal contamination along the food chain can contribute to a reduction in public health risks, including VTEC."

The SCVPH identified the following food categories where VTEC represents a hazard to public health: raw or undercooked beef and possibly meat from other ruminants, minced meat and fermented beef and products thereof, raw milk and raw

milk products, fresh produce, in particular sprouted seeds, and unpasteurised fruit and vegetable juices.

SURVEILLANCE OF ZOONOSES IN EUROPE

FRAMEWORK OF REPORTING

The Community system for monitoring and collection of information on zoonoses is based on the Zoonoses Directive 2003/99/EC, which obligates the European Union Member States to collect relevant and comparable data of: 1) zoonoses; 2) zoonotic agents; 3) antimicrobial resistance; 4) foodborne outbreaks.

In addition, Member States shall assess trends and sources of these agents and outbreaks in their territory, and transmit to the European Commission, every year, a report covering the data collected. The European Food Safety Authority (EFSA) is assigned the tasks of examining the data collected and publishing the Community Summary Report.

The Decision 2119/98/EC on setting up a network for the epidemiological surveillance and control of communicable diseases in the Community, established

the data collection on human communicable diseases from the Member States. The Decisions foresee that data from the networks shall be used in the Community Summary Report on Zoonoses. In such report the data related to the occurrence of zoonotic agents in animals, foodstuffs and feed as well as to antimicrobial resistance in these agents are collected in the framework of Directive 2003/99/EC. This applies also to information of foodborne outbreaks.

Since 2005, the European Centre for Disease Prevention and Control (ECDC) has provided the data on zoonotic infections in humans, as well as the analyses of these, for the Community Summary Report. The data used for analysis were derived from several disease networks: the new European Surveillance System (TESSy); and two Dedicated Surveillance Networks (DSN): Enter-Net for *Salmonella* and *E. coli* verotoxigenic (VTEC) surveillance, and Euro-TB for tuberculosis surveillance.

ENTER-NET (Enteric Pathogen Network) is the european surveillance system for *Salmonella* and *E. coli* (VTEC) infections in humans. It is coordinated by the Health Protection Agency (HPA), comprises 23 European countries and

collaborate with non-European countries (Japan, Canada, Australia, South Africa). Italy is represented in the network by the Istituto Superiore di Sanità (ISS).

ENTER-NET has the following objectives:

- obtain descriptive data on isolates of *Salmonella,* VTEC and other enteric bacteria on the EU territory in a short time since isolation;
- describe the frequency of serotypes and virulence characteristics isolates;
- analyze surveillance data to recognize early warning of potential epidemics also based on the typing of isolates;
- identify and compare any outbreaks involving more than one nation;
- implement the surveillance system of veterinary and environmental isolates in order to realize an integrated surveillance system.

The Rapid Alert System for Food and Feed (RASFF) is a system which has been in place since 1979 and whose purpose is to provide the control authorities with an effective tool for exchange of information on measures taken to ensure food safety.

The legal basis of the RASFF is the Regulation EC/178/2002 that laid down the general principles and requirements of food law, established the European Food Safety Authority and laid down the procedures in matters of food safety. The Annual Report on the RASFF provides useful data on the number of notifications received every year, as well as details on the origin of the notifications, the products and countries involved, and the identified risks. It also details the follow-up actions carried out in response to various food safety problems.

To assist the members of the network, information is classified under two different headings:

- Alert notifications: are sent when the food or feed presenting the risk is on the market and when immediate action is required. Alerts are triggered by the Member State that detects the problem and has initiated the relevant measures, such as withdrawal/recall. The notification aims to give all the members of the network the information to verify whether the concerned product is on their market, so that they also can take the necessary measures. The Member States

have their own mechanisms to carry out such actions, including the provision of detailed information through the media if necessary.

• Information notifications: concern a food or feed for which a risk has been identified, but for which the other members of the network do not have to take immediate action, because the product has not reached their market. These notifications mostly concern food and feed consignments that have been tested and rejected at the external borders of the EU.

The Pathogenic *Escherichia coli* Network (PEN) is another important reality with the aim of monitoring these pathogens and provide scientific data. It is a co-ordination action project, co-ordinated by the Ashtown Food Research Centre (Teagasc) of Dublin, that consists of a durable multidisciplinary network of 35 international research groups working on pathogenic *E. coli*, with the ultimate aim of reducing the burden of related illness. Experts from these partner institutions along with other international experts come together at international devoted conferences. Overall this co-ordination action will provide a forum for different disciplines (which traditionally work separately) and experts from different geographical locations

around Europe and the world to exchange information, and technologies to achieve a decreased incidence of pathogenic *E. coli* on farms, reduce contamination rates at the processing and retail stages and result in decreasing incidence of human infection. The information gathered and conclusions of the work-packages should also inform future food safety legislation in the European Union including the legal requirements for pre-requisite controls, HACCP and microbiological criteria for meat carcasses and meat products.

THE OPINIONS OF THE SCIENTIFIC PANEL ON BIOLOGICAL HAZARDS

When in 2006 European Centre for Disease Prevention and Control (ECDC) analysed the data on zoonoses received from the Member States, it became apparent that the information available on Verotoxigenic *Escherichia coli* (VTEC) were not sufficient to make possible a proper analysis of the importance of the findings of VTEC from foodstuffs and animal populations to the human VTEC cases. There was, in particular, lack of information on the VTEC serotypes and virulence factors

of the VTEC isolates from food and animals, and due to this, it was often not possible to estimate whether the VTEC isolates from foodstuffs and animals were pathogenic to humans. According to the Opinion of the Scientific Committee on Veterinary Measures Relating to Public Health on Verotoxigenic *E. coli* (VTEC) in foodstuffs, only a small fraction of all VTEC-types isolated from animals, food, or the environment, are associated with human illness. Also, the use of different analytical methods, some of which were only able to detect serotype VTEC O157, hampered the analyses.

For this reason the **Scientific Opinion of the Panel on Biological Hazards (BIOHAZ) - Monitoring of verotoxigenic *Escherichia coli* (VTEC) and identification of human pathogenic VTEC** has the aim to identify the strains and/or serotypes of VTEC which are pathogenic to humans, to give advice regarding the analytical methods, including testing for virulence factors, to be used to, and recommends the monitoring methods in animal populations and foodstuffs that are most optimal from the public health point of view.

According to a risk-based sampling strategy, the **Technical specifications for the monitoring and reporting of verotoxigenic *Escherichia coli* (VTEC) on animals and food (VTEC surveys on animals and food)** describe an entire survey design, aiming at estimating at slaughter the prevalence of VTEC O157 contamination, primarily on hide of young cattle and secondarily on sheep fleeces. It recommend that all Member States carry out monitoring at minimum three-year intervals. Member States may extend the monitoring to the serogroups of VTEC O26, O103, O111 and O145, identified in some Member States as causes of human infections. Regarding foodstuffs, general guidelines are proposed for carrying out specific surveys on the food categories that are most likely to be sources of VTEC O157 and non-O157 infections in humans.

Furthermore, a Manual for Reporting on Zoonoses, Zoonotic Agents and Antimicrobial Resistance in animals, food and feed provides guidance under the framework of Directive 2003/99/EC. The objective is to harmonise and streamline the reporting made by the Member States in a way that the data collected would be relevant and easy to be analysed at the Community level. The manual is in particular

intended to be used when reporting the data through the web reporting application run by the EFSA.

GENUS *ESCHERICHIA*: GENERAL CHARACTERISTICS

Escherichia coli is a Gram-negative bacterium of the family *Enterobacteriaceae* and include bacillary form micro-organisms, measuring 1.1 to 1.5 x 2.0 to 6.0 µm, often enveloped, able to move by the presence of peritrichous flagella, oxidase-negative, did not liquefy gelatin and ferment lactose. Within the

genus *Escherichia* is acknowledged the existence of five species: *E. coli, E. blattae, E. Ferguson, E. Hermann and E. vulneris* (Brenner and Farmer, 2005).

The optimum temperature for growth is 37°C but can grow in a range between 15°C and 45°C. They can withstand at temperatures of 55°C for 60 minutes and 60°C for 15 minutes (Brenner and Farmer, 2005).

Selective media as deoxycholate agar and eosin methylene blue agar (EMBa) are optimal for growth of bacteria belonging to this Genus.

The species *Escherichia coli* is the predominant facultative anaerobe of the intestinal flora of humans, domesticated and wild animals (Naylor et.al 2005). In human range the organism typically colonizes the infant gastrointestinal tract within hours of life, and, thereafter the host derive mutual benefit (Drasar et al, 1974).

Commensal *E. coli* strains usually remains harmlessly confined to the intestinal lumen and rarely cause disease except in immunocompromised hosts or where the normal gastrointestinal barriers are breached, as in peritonitis, even normal "nonpathogenic" strains of *E. coli* can cause infection (Nataro et al., 1998). The niche of commensal *E. coli* is the mucous layer of the mammalian colon. The

bacterium is a highly successful competitor at this crowded site, comprising the most abundant facultative anaerobe of the human intestinal microflora. Sweeney et al. (1996) suggested that *E. coli* might exploit its ability to utilize gluconate in the colon more efficiently than other resident species, thereby allowing it to occupy a highly specific metabolic niche.

THE EVOLUTION AND EMERGENCE OF PATHOGENIC *ESCHERICHIA COLI*

Pathogenic *E. coli* are a large group several highly adapted *E. coli* clones that differs from the other commensal *E. coli* of intestinal tract. The acquisition of specific virulence attributes confers an increased ability to adapt to new niches and allows them to cause a broad spectrum of disease. These virulence attributes are frequently encoded on genetic elements that can be mobilized into different strains to create novel combinations of virulence factors, or on genetic elements that might once have been mobile, but have now evolved to become 'locked' into the genome. Only the most successful combinations of virulence factors have persisted to become

specific ‘pathotypes’ of *E. coli* that are capable of causing disease in healthy individuals (Kaper et al.2004).

The finding that independent lineages harbor the same virulence factors and cause clinically similar disease indicates that certain pathotypes have evolved multiple times in different clonal groups (Reid et al., 2000).

The evolution of *E. coli* as a gastrointestinal pathogen is comparatively recent (Pupo et al., 1997). Studies have shown that pathogenic strains are highly developed from diverse commensal *E. coli* because of the insertion and deletion of genes over time. Diversification may have started 9 million years ago with the acquisition of islands of DNA from other bacteria by horizontal gene transfer (e.g. via bacteriophages, transposons and plasmids), a process that continues today. Thus the evolution of *E. coli* is associated with the continuous generation of novel genetic variants.

Bacteria must survive under continuously changing environmental conditions necessitating adaptation. In addition to an immediate response to changing conditions at the gene expression level, bacteria rely on the development of genetic diversity.

Thus mutation and the capture of new genetic material by horizontal gene transfer mechanisms generate genome flexibility and increased ability to adapt and survive. These transfer events occurred independently of each other with different sets of virulence genes. In addition to gene transfer, genomic instability is a phenomenon which has gained considerable interest recently (Clarke et al. 2001). In fact, these genes are clustered on large mobile genomic islands enabling bacteria to rapidly develop new bacterial variants and adapt to specific ecological niches. Successful new genotypes tend to stabilize allowing for the further genetic variation and ultimately the development of new genera and new species. This process is the same for both pathogenic and non-pathogenic organisms. The former acquire genes encoding virulence factors such as capsules, adhesins and toxins which enable them to survive and multiply in a particular host resulting in disease (P.E.N., 2006).

Verocytotoxin-producing *E. coli* (VTEC) are a good example of the evolution and emergence of pathogenic *E. coli*. These bacteria comprise over 100 serotypes of *E. coli* (Scheutz and Strockbine, 2005) and some strains are a major cause of hemorrhagic colitis (HC) and hemolytic uraemic syndrome (HUS) worldwide. The

verocytotoxins are encoded on bacteriophages that integrate into the chromosome of the host *E. coli*. There is some evidence to suggest that VTEC evolved from Enteropathogenic *E. coli* (EPEC) through a series of genetic events, most notably the acquisition of bacteriophages encoding the verocytotoxins. It is probable, for example, that VTEC O157:H7 evolved from EPEC O55:H7 but this may not hold true for all VTEC (Whittam et al., 1993). From an evolutionary point of view, and because of the occurrence of examples like these, it can be assumed that all these genetic elements are variations of genomic islands (Hacker et al, 1999). Since genomic islands show similar structural features, it is likely that they have been transferred in recent times by horizontal processes. The genomic islands may contribute to the fitness (fitness islands) or metabolic flexibility (metabolic islands) of the organisms, or they may increase their pathogenic potential (PAIs). The particular function of an island will thus depend strongly on the genetic background of the individual strains (Karch et al., 1999).

Competition between bacteria and the dominance of certain *E. coli* serotypes is suggested to be one explanation for the shedding of one predominant *E. coli* strain

over time bacterial evolution is an ongoing process and there is little doubt that new, more virulent pathogenic clones of *E. coli* will emerge in the future (Midgley et al., 1999).

CLASSIFICATION FOR PATHOGENIC *E. COLI*

The most recent and useful classification for pathogenic *E. coli* provides for the division in pathotypes: a group of strains of a single species that cause a common disease using a common set of virulence factors (Kaper et al., 2004).

Bacteria of the same pathogenic clone represent a monophyletic branch of an evolutionary tree and typically carry many of the same mobile genetic elements, including those that determine virulence.

Among the intestinal pathogens there are six well-described pathotypes: enterohaemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAggEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC) (Nataro et al., 1998). The latest

class of diarrhoeagenic *E. coli* to be described is cytolethal distending toxin (CDT)-producing *E. coli* (Clarke et al., 2001).

Urinary tract infections (UTIs) are the most common extraintestinal *E. coli* infections and are caused by uropathogenic *E. coli* (UPEC). An increasingly common cause of extraintestinal infections is the pathotype responsible for meningitis and sepsis—meningitis-associated *E. coli* (MNEC). The *E. coli* pathotypes implicated in extraintestinal infections have recently been called ExPEC (Russo et al., 2000). An additional animal pathotype, known as avian pathogenic *E. coli* (APEC), causes extraintestinal infections — primarily respiratory infections, pericarditis, and septicaemia of poultry (Kaper et al., 2004).

The various pathotypes of *E. coli* tend to be clonal groups that are characterized by shared O (lipopolysaccharide, LPS) and H (flagellar) antigens that define serogroups (O antigen only) or serotypes (O and H antigens). Sometimes K (capsular) antigens can be used (Whittam, 1996).

Phylogenetic analyses have shown that *E. coli* strains fall into four main phylogenetic groups (A, B1, B2, and D) (Selander et al., 1987) and that virulent

extra-intestinal strains belong mainly to group B2 and, to a lesser extent, to group D, whereas most commensal strains belong to group A (Bingen et al., 1998; Picard et al., 1999).

Enterohaemorrhagic *E. coli* (EHEC)

Enterohaemorrhagic *Escherichia coli* (EHEC) is the pathogen group that is the cause of a potentially fatal foodborne or waterborne illness, whose clinical spectrum includes a severe bloody diarrhoea without fever and described as haemorrhagic colitis (HC) (Riley et al., 1983). Possible sequelae of this infection include renal failure, thrombocytopaenia and microangiopathic haemolytic anaemia, designated as haemolytic uraemic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP).

The principal reservoir of EHEC is the bovine intestinal tract and initial outbreaks were associated with consumption of undercooked hamburgers. Subsequently, a wide variety of food items have been associated with disease, including sausages, unpasteurized milk, lettuce, cantaloupe melon, apple juice and radish sprouts (Kaper et al., 2004).

EHEC belong to different O serogroups, but those of serogroup O157 are the most important in human disease (Paton & Paton, 1998, Karch et al.,2001), but infections caused by EHEC strains belonging to serogroups other than O157, such as O26, O55, O91, O111, O103, O145, and O146 have been increasingly reported (Caprioli et al., 1997, Griffin and Tauxe, 1991).

Although more than 200 serotypes of *E. coli* can produce Stx, most of these serotypes do not contain the LEE pathogenicity island and are not associated with human disease. This has led to the use of Shiga toxin-producing *E. coli* (STEC) or verotoxin-producing *E. coli* (VTEC) as general terms for any *E. coli* strain that produces Stx (Konowalchuk et al., 1977), while the term "enterohemorrhagic *E. coli*" EHEC denotes strains that cause HC and HUS, express Stx, cause A/E lesions on epithelial cells, and possess a ca. 60-MDa plasmid that encodes for a enterohemolysin (hlyA) (Levine, 1987; Levine and Edelman R., 1984). Thus, EHEC denotes a subset of VTEC and includes a clinical connotation that is not implied with VTEC. Whereas not all VTEC strains are believed to be pathogens, all EHEC strains by the above definition are considered to be pathogens (Nataro and Kaper, 1998).

EHEC can be subdivided in typical and atypical. Typical EHEC strains, such as O157, O26, O103, O111 and O145 were shown to express one or more types of Stx, the A/E phenotype, and the plasmid-encoded EHEC hemolysin (Burk et al., 2003). In humans, typical EHEC strains are isolated frequently from young children, whereas atypical EHEC strains are predominant in older patients (Beutin et al., 2004; Friedrich et al., 2002). Atypical EHEC strains express Stx2 and/or Stx2d but do not carry the LEE and belong to serotypes other than those of typical EHEC strains (Nataro and Kaper, 1998; Teel et al., 2002). Strains of serotypes O91:H21, O113:H21 and O104:H21 have been associated with sporadic cases of hemorrhagic diseases than typical EHEC, but are a frequent cause of diarrhoea (Brooks et al., 2005; Eklund et al., 2001; Nataro and Kaper, 1998).

In recent years, new serotypes of EHEC have also emerged, such as O103:H25 (Schimmer et al., 2008), O118:H16 (Maidhof et al., 2002), O45:H2 (Brooks et al., 2005) or O55:H7 (EFSA, 2007) that have been identified as an important cause of food-borne infections in humans.

Since some strains cause damage in some animal species only, it was proposed the concept of species specificity, and it would be reasonable to refer to such strains as host-specific EHEC, e.g. bovine EHEC (McNally et al., 2001).

The main features of EHEC epidemiology include a reservoir in the intestinal tract of cattle and other animals; transmission by a wide variety of food items, with beef being a major vehicle of infection; and a very low infectious dose, enabling high rates of attack and of person-to-person transmission. These characteristics are described in more details in the chapter on VTEC.

Enteropathogenic *E. coli* (EPEC)

Enteropathogenic *E. coli* (EPEC) cause infantile diarrhoea in young children and were the first *E. coli* pathotype to be recognised. EPEC remain a serious problem in developing countries through outbreaks of infantile diarrhoea, largely disappeared from industrialised nations (Naylor et al., 2005).

EPEC, that belong to serogroups O55, O86, O111, O119, O125, O126, O127, O128 and O142, cause typical A/E lesions, intimately attaching to intestinal

epithelial cells and causing cytoskeletal changes, like the accumulation of polymerized actin directly beneath the adherent bacteria (Levine, 1987; Donnenberg and Kaper, 1992). The microvilli of the intestine are effaced and pedestal-like structures on which the bacteria rest amd frequently rise up from the epithelial cells. Like EHEC, the ability to induce A/E histopathology is encoded by genes on a 35-kb pathogenicity island (PAI) called the locus of enterocyte effacement (LEE).

According to their virulence markers, EPEC strains are subdivided into typical and atypical EPEC. Typical EPEC (tEPEC) harbor a plasmid which carries genes for regulation of LEE functions (EAF, EPEC adherence factor) and another one for production of bundle-forming pili (BFP). BFP mediate the first contact of bacteria with the host cell which attach in a localized type of adherence to epithelial cells. Atypical EPEC (aEPEC) strains are negative for both, the EAF plasmid and BFP and show diffuse or localized like adherence patterns (Nataro and Kaper, 1998; Trabulsi et al., 2002). Atypical EPEC may possess other virulence factors (e.g. Ehx) (Ramachandran et al., 2003). Human infections with tEPEC are still a major cause

of neonatal mortality in regions with poor sanitation and hygiene (Trabulsi et al., 2002). They also differ in genetic characteristics and serotypes (Naylor et al., 2005).

Typical EPEC were rarely isolated from animals and humans are the major natural reservoir for these pathogens (Goffaux et al., 2000; Nakazato et al., 2004). In contrast, aEPEC strains were isolated from both animals and humans (Aktan et al., 2004; Goffaux et al., 2000; Cid et al., 2001; Beutin et al., 2003; Regua-Mangia et al., 2004) and in consequence, animals could serve as a source of human infections. EPEC were also associated with diarrhoea in young animals such as dogs, cats, lambs, goat kids and calves (Beutin et al., 1999; Goffaux et al., 2000; Nakazato et al., 2004; Peeters et al., 1994).

EPEC strains do not multiply intracellularly or escape from a phagocytic vacuole and thus do not appear to be specifically adapted for intracellular survival (Nataro and Kaper, 1998) but the characteristic intestinal histopathology associated with EPEC infections is the 'attaching and effacing' (A/E) lesion, the bacteria intimately attach to intestinal epithelial cells and cause striking cytoskeletal changes, including the accumulation of polymerized actin directly beneath the adherent

bacteria. The microvilli of the intestine are effaced and pedestal-like structures on which the bacteria perch frequently rise up from the epithelial cell (Nataro and Kaper, 1998).

A three-stage model has been suggested for the pathophysiologic mechanism to cause the attaching and effacing lesion (Donnenberg and Kaper, 1992). This is homologous to the EHEC A/E pathophysiologic mechanism, except that EPEC express intimin type α and have a different adherence factor (EAF) encoded by a gene carried by a 60-MDa plasmid (Baldini et al., 1986). The identity of the factor mediating localized adherence was reported in 1991 by Girón et al., who described 7-nm-diameter fimbriae produced by EPEC strains which tended to aggregate and form bundles, thereby suggesting the name "bundle-forming pilus" (BFP).

Some EPEC strains produce a low-molecular-weight stable enterotoxin called EAST1 encoded by *astA* gene. EPEC strains carrying *astA* gene, are more frequently isolated from adult human than EPEC strains lacking this gene (Savarino et al., 1996).

Expression of EPEC virulence factors is regulated by a trans-acting protein. A cluster of three open reading frames designated *perA*, *perB*, and *perC* (for plasmid-encoded regulator) encode proteins that form a regulatory complex, which regulates the LEE-encoded regulator, activates the transcription of several genes in the bacterial chromosome and on the EAF plasmid (Kaper and Gómez-Duarte, 1997). The Per regulator increases expression of *eae* (Gómez-Duarte and Kaper, 1995) and *espB* genes (Gomez et al., 1993).

The presence of disseminated A/E lesions on the bowel mucosa leads to the impressive loss of the absorptive microvilli and the decreasing of monolayer resistance, which could result in diarrhoea via malabsorption. Diarrhoea probably results from multiple mechanisms, including active chloride secretion via a pathway involving PKC that could account for the rapid onset of diarrhoea.

A variety of intracellular mediators of intestinal ion transport, such as calcium, PKC, inositol phosphates, and tyrosine kinase, are involved. There have been recent reports of EPEC actively altering ion transport in epithelial cells, decreasing transmembrane potential in epithelial cells and suggesting that EPEC

stimulates either an influx of positive ions or an efflux of negative ions across the membrane (Stein et al., 1996). As noted above, attachment of EPEC to cultured human intestinal epithelial monolayers induces the transmigration of polymorphonuclear leukocytes PMNs (Savkovic et al., 1996) and this can, in turn, result in increased short-circuit current (Isc) due to chloride secretion (Madara et al., 1993), thereby suggesting another mechanism of diarrhoea due to EPEC. These multiple mechanisms could all be involved in diarrhoea due to EPEC. The active chloride secretion, perhaps mediated via a pathway involving PKC, could account for the rapid onset of diarrhoea.

The prolonged diarrhoea seen in some patients could result from malabsorption due to loss of the brush border, increased intestinal permeability, intestinal inflammation and loss of absorptive surface area resulting from microvillus effacement (Nataro and Kaper, 1998).

Several studies have shown a strong correlation of isolation of EPEC from infants with diarrhoea compared to healthy infants. The correlation is strongest with infants younger than 6 months. In children older than 2 years, EPEC can be isolated

from healthy and sick individuals, but a statistically significant correlation with disease is usually not found (Levine et al., 1984). However, several outbreaks of diarrhoea due to EPEC have been reported in healthy adults (Viljanen et al., 1990), presumably due to ingestion of a large inoculum from a common source. Sporadic disease has also been seen in some adults with compromising factors (Levine et al., 1984).

The reason for the relative resistance of adults and older children is not known, but loss of specific receptors with age is one possibility (Nataro and Kaper, 1998).

EPEC strains are no longer as important a cause of diarrhea in developed countries as they were. Otherwise EPEC is a major cause of infant diarrhoea in developing countries where sanitation and water quality may be poor, where they are more frequently isolated from infants with diarrhoea (Donnenberg, 1995). Studies carried out in Brazil (Gomes et al., 1991), Mexico (Cravioto et al., 1988), and South Africa (Robins-Browne et al., 1982) have shown that 30 to 40% of infant diarrhoea can be attributed to EPEC.

Transmission is fecal-oral, with contaminated hands, contaminated weaning foods or contaminated fomites serving as vehicles, thus symptomatic or asymptomatic children and asymptomatic adult carriers, including mothers and people who handle infants, act as reservoirs (Levine et al., 1984; Wu and Peng, 1992).

Several studies showed that the infectious dose of EPEC in healthy adults can been estimated to be 10^8 organisms (Donnenberg et al.1993). A high prevalence of aEPEC in both infants and older children in contrast to a stronger tendency for infants to be infected with Tepec, has been reported. Patients infected with aEPEC are far more likely to experience diarrhoea which lasts more than 14 days, the point long recognized as a clinical watershed that heralds increased risk of illness and death. Recent data suggest that atypical EPEC are more prevalent than typical EPEC in both developing and developed countries. Data from several studies have shown overall that atypical EPEC was responsible for 78% (131/169) of all EPEC cases in children younger than 5 years with diarrhoea (Ochoa et al., 2008).

The major O groups within this pathotype which are linked to human illness include O55, O86, O111, O119, O126, O127, O128 and O142 (Bolton et al., 2008).

In general, symptoms appear about 12-36 hours after ingestion and include vomiting and acute profuse watery diarrhoea accompanied by low grade fever. Stools are rarely bloody. In infants the disease can be severe lasting longer than 2 weeks.

Faecal leukocytes are seen only occasionally, and proximal small intestinal mucosal biopsy specimens often, but not always, show intimately adherent bacteria and the classic A/E histopathology (Sherman et al., 1989). The presence of the A/E lesion is associated with disarrangement of the digestive-absorptive enzyme system, leading to malabsorption of nutrients (Hill et al., 1991).

Mortality of 30% was reported in developing countries (Senerwa et al., 1989), while in developed countries with the full range of modern treatment available, mortality is much lower although deaths can still result (Rothbaum et al., 1982).

EPEC is also associated with disease in cattle, rabbits, pigs and dogs but the involved serogroups differ from those causing human illness. Moreover, while for

typical EPEC, the only reservoir are humans; for atypical EPEC, both humans and different animals can be reservoirs. An association between serotype O26:H11 and calves is well known and EAF negative serotype O128:H2 is rather frequent in rabbits and dogs (Bolton et al., 2008).

Lesions in animals resemble the human EPEC, however, compared to the adhesion of human EPEC, the adherence factors important in the pathogenesis of animal EPEC are not so well understood.

Since some of the genes found in EPEC (e.g. *eae*) are also present in EHEC strains it is necessary to confirm the presence of *eae* by molecular techniques in order to distinguish between these pathotypes (P.E.N., 2007).

Enteroinvasive *E. coli* (EIEC)

Enteroinvasive *E. coli* (EIEC) are an important cause of diarrheal disease. They are responsible for a dysentery-like illness and, therefore, share some of the virulence properties associated with Shigella dysenteriae (Hart et al., 1993). In fact EIEC strains are biochemically, genetically, and pathogenetically related closely to

Shigella spp: generally lysine decarboxylase negative, nonmotile, and lactose negative (Brenner et al., 1973).

EIEC are enteropathogens and cause a significant rate of morbidity and mortality in young children in developed countries although, they are more important in developing countries where sanitation and hygiene levels are of a poor standard.

EIEC attach to and subsequently invade the colonic enterocytes by endocytosis. The current model of EIEC pathogenesis comprises: (1) epithelial cell penetration, (2) lysis of the endocytic vacuole, (3) intracellular multiplication, (4) directional movement through the cytoplasm and (5) extension into adjacent epithelial cells (Sansonetti, 1992). The bacteria multiply within these cells eventually causing their death. The bacteria are released back into the colon after which they invade other cells. This process results in an inflammatory response accompanied by necrosis and ulceration of the large bowel leading to release of blood and mucous in the stools (Hart et al., 1993). When the infection is severe, this sequence of events elicits a strong inflammatory reaction which is manifested grossly as ulceration in the colonic mucosa.

The ability of EIEC to invade, survive and multiply within colonic enterocytes is dependent upon the presence of a 120–140 MDa plasmid (pInv) which encodes all the genes necessary for such virulence (Hart et al., 1993) including outer membrane proteins required for invasion (Levine, 1987). To cause this kind of lesions *mxi* and *spa* genes are necessary, because encode for type III secretion apparatus (Allaoui et al., 1995; Venkatesan et al., 1992), required for the secretion of multiple proteins which are necessary for full pathogenicity. The Ipa proteins (IpaA to IpaD) are secreted proteins, of which IpaC has been shown to promote the uptake of pathogen into the eukaryotic cell (Marquart et al., 1996), whereas IpaB is thought to function in the lysis of the phagocytic vacuole (High et al., 1992) and in the induction of apoptosis in macrophages (Zychlinsky et al., 1994). These pathogens are able to elaborate one or more secretory enterotoxins that may play roles in diarrheal pathogenesis. A plasmid-borne gene from EIEC (designated *sen*) encodes a novel protein with a predicted size of 63 kDa, cause of watery diarrhea that precedes the onset of scanty dysenteric stools containing blood and mucus.

In sporadic cases, many EIEC strains are probably misidentified as *Shigella* spp or nonpathogenic *E. coli* strains and few minor biochemical tests are able to distinguished EIEC from *Shigella* spp. Documented EIEC outbreaks are usually foodborne or waterborne (Tulloch et al, 1973; Smith et al., 1983), although person-to-person transmission does occur (Harris et al., 1985).

Enterotoxigenic *E. coli* (ETEC)

Enterotoxigenic *E. coli* (ETEC) is an important and global cause of severe, watery diarrhoea in the offspring of some animal species such as suckling calves weaned piglets. Interestingly, it is very rare or almost non-existent in other important farm animals like rabbits, horses or poultry, for which there is no good explanation at present, as these animals seem to have receptors and be responsive to enterotoxins and to adhesins of some ETEC. In humans, ETEC is recognised as one of the most frequent causes of childhood diarrhoea in the developing countries and as an important causative agent of traveller's diarrhoea (Bouckenooghe et al., 2002; Dalton et al., 1999).

ETEC are able to adhere to the small intestinal epithelium without inducing significant morphological changes, and to secrete proteins (enterotoxins) that alter the functions of enterocytes by increasing secretion and reducing absorption. Thus the main virulence attributes of ETEC are adhesions (fimbriae and pili) and enterotoxins. Pathogenesis also involves host factors, which are receptors for adhesins and for enterotoxins, conferring species specifity in the infections. Therefore, animal ETEC strains do not represent real hazards to human and cannot be regarded as zoonotic (Nagy et al., 2005).

The symptoms of ETEC infection are due to the production of one or both of two types of enterotoxins by the bacterium, namely heat stable enterotoxins (ST) and heat labile enterotoxins (LT). ST's are small, monomeric plasmid-mediated toxins comprising two unrelated classes, namely STa (or ST-I) and STb. The LT toxins are oligomeric and consist of two major serogroups, namely LT-I and LT-II (Nataro & Kaper, 1998).

LT-I is related to the cholera toxin (Hart et al., 1993); it is composed of one A subunit and five identical B subunits (Nataro and Kaper, 1998). The A subunit is

responsible for the enzymatic activity of the toxin by activating adenylate cyclise resulting in an intracellular increase in cyclic adenosine monophosphate (cAMP) concentrations. This leads to a decrease in sodium absorption by villous cells and subsequent active chloride secretion by crypt cells thereby resulting in osmotic diarrhoea toxin (Hart et al., 1993; Nataro & Kaper, 1998). LT-I is expressed by *E. coli* strains that are pathogenic for both humans and animals. LT-II is found primarily in animal *E. coli* isolates and rarely in human isolates, but in neither animals nor humans has it been associated with disease (Nataro and Kaper, 1998).

ETEC colonizes the surface of the small bowel mucosa and elaborates enterotoxins, which give rise to intestinal secretion. Colonization is mediated by one or more proteinaceous fimbrial or fibrillar colonization factors (CFs), which are designated by CFA (colonization factor antigen), CS (coli surface antigen) or PCF (putative colonization factor) followed by a number. More than 20 antigenically diverse CFs have been characterized, yet epidemiological studies indicate that approximately 75% of human ETEC express either CFA/I, CFA/II or CFA/IV51. ETEC are also an important cause of diarrhoeal disease in animals and these animal

strains express fimbrial intestinal colonization factors, such as K88 and K99, which are not found in human ETEC strains (Kaper et al., 2004).

They are associated with poor hygiene and sanitation and are therefore of most importance in developing countries. In such countries the number of diarrhoeal episodes due to ETEC is equivalent to that caused by EPEC. Most of these cases probably occur due to the ingestion of water or food contaminated with ETEC and outbreaks can occur after person-to-person spread. The illness is known as traveler's diarrhoea because many individuals visiting developing countries suffer from ETEC infection during their stay or upon their return. It is usually an acute illness with symptoms consisting of loose stools, nausea, vomiting and abdominal cramps. In developing countries the most severe form of acute infection is a cholera-like illness which occurs in areas where *Vibrio cholerae* is already endemic. It may occur in all age groups and is difficult to distinguish clinically from true cholera. ETEC is not a usual problem in developed countries although it has been linked with outbreaks of infantile enteritis in hospitals and also with larger outbreaks due to contaminated water or food (Adhikari et al., 1985; Daniels et al., 2000).

So the high prevalence of ETEC in developing countries might have a protective effect against this important disease, and indicates that infectious diseases might exist in a complex evolutionary balance with their human populations (Kaper et al., 2004).

Enteroaggregative *E. coli* (EAggEC)

Enteroaggregative *E. coli* (EAggEC) are a more recent addition to the diarrhoeagenic classes of *E. coli* and are so named due to their adherence pattern to cultured epithelial cells. They are defined as *E. coli* that do not secrete LT or ST and that adhere to HEp-2 cells in a pattern known as autoaggregative, in which bacteria adhere to each other in a 'stacked-brick' configuration. This definition encompasses both pathogenic and nonpathogenic clones, which share factors conferring a common phenotype (Nataro & Kaper, 1998).

EAggEC are increasingly recognized as a cause of often persistent diarrhoea in children and adults in both developing and developed countries, and have been identified as the cause of several outbreaks worldwide. (Law and Chart, 1998; Kaper et al., 2004). Furthermore, EAggEC are a cause of diarrhoea in travelers who have

visited endemic countries (Gascon et al., 1998). However, a high rate of asymptomatic carriage often occurs with EAggEC infection and therefore the presence of other pathogens should be excluded (Law and Chart, 1998).

The basic strategy of EAEC infection seems to comprise colonization of the intestinal mucosa, predominantly the colonic one, followed by secretion of enterotoxins and cytotoxins

Their pathogenicity is due to a toxin known as the enteroaggregative heat-stable enterotoxin (EAST1) (Elliot and Nataro, 1995) and their aggregative adherence phenotype is mediated by at least two plasmid-encoded fimbriae (AAFI and AAFII) (Czeczulin et al., 1997). The EAST1 enterotoxin is partly homologous and functionally similar to, but distinct from ETEC heat stable enterotoxin, and is thought to be responsible for the symptoms of infection. The production of a heat labile toxin which is antigenically related to EHEC hemolysin has also been reported (Baldwin et al., 1992). It is conceivable that EAST1 could contribute to watery diarrhoea in EAST1-positive strains; however, the EAST1 gene (*astA*) can also be

found in many commensal *E. coli* isolates, and therefore the role of EAST1 in diarrhoea is not clear (Menard and Dubreuil, 2002).

Nataro and Kaper (1998) proposed a three-stage model pathogenicity. Stage I involves initial adherence to the intestinal mucosa and/or the mucus layer. AAF/I and AAF/II are the leading candidates for factors that may facilitate initial colonization. Stage II involves enhanced mucus production, apparently leading to deposition of a thick mucus-containing biofilm encrusted with EAggEC. The blanket may promote persistent colonization and perhaps nutrient malabsorption. Stage III, suggested from histopathologic and molecular evidence, includes the elaboration of an EAggEC cytotoxin which results in damage to intestinal cells. It is tempting to speculate that malnourished hosts may be particularly impaired in their ability to repair this damage, leading to the persistent-diarrhoea syndrome (Nataro & Kaper, 1998).

EAggEC induces mild, but significant, mucosal damage, that are most severe in colonic sections (Hicks et al., 1996). The most dramatic histopathological finding in infected animal models is the presence of a thick layer of autoaggregating bacteria adhering loosely to the mucosal surface (Benjamin et al., 1995).

The role of excess mucus production in EAggEC pathogenesis is unclear; however, the formation of a heavy biofilm may be related to the diarrhoeagenicity of the organism and, perhaps, to its ability to cause persistent colonization and diarrhoea. In addition to the formation of the characteristic mucus biofilm, experimental evidence suggests that EAggEC infection is accompanied by cytotoxic effects on the intestinal mucosa (Nataro et al., 1995).

Diffusely Adherent *E. coli* (DAEC)

Diffusely adherent *E. coli* (DAEC) have been described as a cause of diarrhoea in both developed and developing countries. They are so named because their virulence is mediated by α hemolysin and cytotoxic necrotising factor 1, and because of their diffuse adherence pattern to cultured epithelial cells (Elliot and Nataro, 1995; Gunzburg et al., 1993; Elliot et al., 1998).

Individuals infected with DAEC may experience mucoid watery stools and suffer from fever and vomiting with a mean duration of illness of about 8 days. Their pathogenic mechanisms are poorly understood although it is thought that DAEC may produce one or more toxins, one of which is a hemolysin (Elliot and Nataro, 1995).

Approximately 75% of DAEC strains produce a fimbrial adhesin called F1845 or a related adhesin; DAEC strains induce a cytopathic effect that is characterized by the development of long cellular extensions, which wrap around the adherent bacteria (Bilge et al., 1989).

Several studies have implicated DAEC strains as agents of diarrhoea, while other studies have not recovered DAEC strains more frequently from diarrheal patients than from asymptomatic controls. An age-dependent susceptibility may explain this observation, because when populations are stratified by age, the association of DAEC with diarrhea is found only in children older than infants (Gunzberg et al., 1993; Levine et al., 1993).

Cytolethal distending toxin-producing *E. coli*

Cytolethal distending toxin (CDT)-producing *E. coli* are the latest and less known class of diarrheagenic *E. coli* to be described. These *E. coli* have been reported in a number of studies (Bouzari and Varghese, 1990; Bouzari et al., 1992;

Albert et al., 1996) and cause the distension and eventual disintegration of cells of certain lines (Pickett et al., 1994). In one study (Albert et al., 1996), CDT-producing *E. coli* additionally possessed the virulence factors of EPEC or EAggEC.

Uropathogenic *E. coli* (UPEC)

E. coli is by far the most common infecting agent at the urinary tract, that is among the most commonly subjected to bacterial infection. The subset of *E. coli* that causes uncomplicated urinary tract infections (UTI), cystitis and acute pyelonephritis is distinct from the commensal *E. coli* strains that comprise most of the *E. coli* populating the lower colon of humans (Kaper et al., 2004).

E. coli from a small number of O serogroups (six O groups cause 75% of UTIs) have phenotypes that are epidemiologically associated with cystitis and cute pyelonephritis in the normal urinary tract, which include expression of P fimbriae, haemolysin, aerobactin, serum resistance and encapsulation (Manges et al. 2001). Although many UTI isolates seem to be clonal strains, there is no single phenotypic profile that causes UTIs. Specific adhesins, including P (Pap), type 1 and other fimbriae are involved in colonization (Johnson, 1991).

Virulence factors, including haemolysin, cytotoxic necrotizing factor and an autotransported protease (Sat), are found in differing percentages among various subgroups of UPEC (Kaper et al., 2004). It has been suggested that infection begins with the colonization of the bowel with a uropathogenic strain in addition to the commensal flora. This strain, by virtue of factors that are encoded in pathogenicity islands, is capable of infecting an immunocompetent host, as it colonizes the periurethral area and ascends the urethra to the bladder. Between 4 and 24 hours after infection, the new environment in the bladder selects for the expression of type 1 fimbriae, necessary for the attachment to epithelial cells (Gunther et al., 2001). Invasion of the bladder epithelium is accompanied with formation of pod-like bulges on the bladder surface that contain bacteria encased in a polysaccharide-rich matrix surrounded by a shell of uroplakin (Anderson et al. 2003).

In some cases, the barrier that is provided by the one-cell-thick proximal tubules can be breached and bacteria can penetrate the endothelial cell to enter the bloodstream, leading to bacteraemia (Kaper et al., 2004).

VEROTOXIN-PRODUCING *E. COLI* (VTEC) AND SHIGA-LIKE TOXIN-PRODUCING *E. COLI* (STEC)

Verotoxin-producing *E. coli* (VTEC) are characterized by the production of a family of toxins which has cytotoxic activity on African-Green Monkey kidney (Vero) cells. The toxins are often termed Vero cytotoxin (VT) but may be also be known as Shiga-like toxin (Stx) (Karmali et al., 1983) thus VTEC are named Shiga-like toxin-producing *E. coli* (STEC) too. Most of them contain LEE chromosomal

region, that encodes effector proteins that lead to attaching and effacing (A/E) lesions (Donnenberg et al., 1993).

VTEC are a serious public health concern because cause food poisoning and severe and potentially fatal illnesses. They are associated with large outbreaks and with HUS, which is the foremost cause of acute renal failure in children. Karmali et al. (1983) suggested that VT was of direct significance in the genesis of HUS.

Not all serotypes of VTEC are equally pathogenic: there is much evidence of genetic diversity within serotypes, which can affect virulence determinants and, ultimately, pathogenicity (Nataro and Kaper, 1998). Such differences can be manifested, for example, in the infectious dose of the organism, the level and type of toxin produced, the extent of gastrointestinal colonization, the rate of toxin delivery to the endothelial cells and/or the severity of ensuing disease.

Seropathotype is an emerging concept that classifies VTEC into five main groups (A to E) based on the incidence of the serogroup in human disease, association with outbreaks versus sporadic infection, their capacity to cause HUS or HC, and the presence of virulence markers (Karmali, 2003; Wickham et al., 2006).

Seropathotype A strains (*E. coli* O157) have a high relative incidence, commonly cause outbreaks and are associated with HUS. Seropathotype B includes O26:H11, O103:H2/NM, O111:NM and O145:NM together with O121:H19 as they have a moderate incidence and are uncommon in outbreaks but are associated with HUS. Seropathotype C includes O91, O104 and O113 strains all of H-type 21 and associated with HUS, but these strains are of low incidence and rarely cause outbreaks. Seropathotypes D and E are not HUS-associated, are uncommon in humans or are found only in non-human sources. This concept is likely to be further refined and will provide a valuable tool in the future for the assessment of the human pathogenic potential of different VTEC serotypes (P.E.N., 2008).

Physiology

VTEC has a good tolerance to the general stresses such as acidity and dryness, and can survive for long time periods in soil, manure, and water or during food processing and distribution (Loukiadis et al., 2006; Maule, 2000).

Temperature: the optimum temperature for growth is 37 ° C, while the minimum range is approximately between 8 and 10ºC (Buchanan et al., 1997). These

bacteria can survive at chill and freezing temperatures and growth of the organism can potentially occur at temperatures of ≥7°C (Palumbo et al., 1995). Pasteurisation and adequate cooking will inactivate the pathogen (Rhee et al., 2003). The thermal resistance of serogroup O157 is broadly similar to other non-O157 serogroups with some observed inter strain differences (Duffy et al., 2006).

pH: although the growth of the organism occurs at pH values ranging from 5.5 to 7.5, values below can allow its survival (Buchanan et al., 1997). When the pH falls below the minimum value (minimum pH for growth from 4.0 to 4.5) populations of *E. coli* O157: H7 decreased over time. There is some evidence that emergent non-O157 serovars of VTEC are less acid tolerant than O157 (Bergholz and Whittam, 2007). This organism can adapt to acidic conditions to allow it to survive at pH 3.4 for several days (Benjamin and Datta, 1995; Leyer et al., 1995).

The survival of the organism in acidic foods (fruit juices, fermented sausages) is extremely important, since *E. coli* persists in these foods and cause illness episodes.

Water activity: optimal a_w for the growth of *E. coli* O157:H7 is 0,995 (Brenner and Farmer, 2005).

Vero cytotoxin

Shigatoxin (Stx) was first purified from *Shigella dysenteriae* and HUS can also result from infection with this species, although not with other *Shigella* spp. The cytotoxic effect of Shiga toxin on intestinal epithelial cells causes the characteristic bloody diarrhea associated with STEC infection. The Stx family includes two subgroups (Stx1 and Stx2) which share approximately 55% and 57% sequence identity in the A and B subunits respectively (Melton-Celsa and O'Brien, 1998). The type and/or amount of Shiga toxin produced determine the capacity of the organism to cause human disease.

A single EHEC strain may express Stx1 only, Stx2 only, or both toxins or even multiple forms of Stx2.

Genetic variants within members of the Stx1 and Stx2 families were defined according to differences in toxicity, toxin receptor, and amino acid composition of

StxA and StxB subunits (Paton and Paton., 1998; Scheutz et al., 2001). Some Stx types, such as Stx2 and the elastase (mucus)-activatable Stx2d type, are highly associated with HC and HUS (Friedrich et al., 2002; Bielaszewska et al., 2006).

Recent studies suggest that STEC strains isolated from ovine and bovine feces contain genetically distinct Shiga toxin subtypes. For example, stx1c and stx2d (Pierard) subtypes are commonly associated with STEC populations recovered from the feces of healthy sheep but not from the feces of healthy cattle (Brett et al., 2003; Ramachandran et al., 2003). STEC carrying stx1c and stx2d (Pierard) subtypes are more frequently isolated from patients with uncomplicated diarrhoea than from the feces of patients experiencing HC or HUS (Friedrich et al., 2003; Pierard et al., 1998). Thus, the allele stx1c may be a marker that identifies STEC strains that cause milder disease (Beutin et al., 2004; Zhang et al., 2002). The allele stx2e has been identified in STEC strains recovered from the feces of swine but not among STEC strains recovered from ruminant species (Fratamico et al., 2004).

Stx2g was recently described as a new, rarely occurring Stx2 variant in bovine VTEC that is not activatable by intestinal mucus (Leung et al, 2003). Stx2g

STEC strains are present in food samples and humans can be exposed to these STEC types along the food chain. While stx2f STEC appear to be uncommon in meat and milk samples (Beutin et al., 2007).

Strains producing Stx1 only, have also been associated with human illness, including HUS (Eklund et al., 2001) and not all non-O157 STEC strains that produce Stx cause HUS. This variability in virulence may have led to an underestimate of the pathogenicity of this diverse set of strains. However, based on data from Europe and Australia, a subset of non-O157 STEC strains are as virulent as *E. coli* O157 with epidemiological evidence of similar incubation periods, symptom onsets, symptom profiles and comparable proportions of case-patients who develop HUS (Wickham et al., 2006).

The structural genes for Stx1 and Stx2 are found on lysogenic lambdoid bacteriophages and the genes for Stx2 are chromosomally encoded. Production of Stx1 from *E. coli* and *S. dysenteriae* is repressed by iron and reduced temperature, but expression of Stx2 is unaffected by these factors (Nataro and Kaper, 1998).

The basic A-B subunit structure is conserved across all members of the Shiga toxin family. For the prototype toxin of this family, Shiga toxin, the single 32-kDa A subunit is proteolytically nicked to yield a ca. 28-kDa peptide (A1) and a 4-kDa peptide (A2); these peptides remain linked by a disulfide bond.

The A1 peptide contains the enzymatic activity, and the A2 peptide serves to bind the A subunit to a pentamer of five identical 7.7-kDa B subunits. The B pentamer binds the toxin to a specific glycolipid receptor, globotriaosylceramide or Gb3, which is located on replicating progenitor epithelial cells in the crypts adjacent to submucosa in the bovine gastrointestinal tract. These receptors are absent on human gastrointestinal epithelia (Hoey et al., 2003). Gb3-positive bovine epithelial cells are resistant to a wide range of concentrations of Shiga toxin 1 (Stx1) (Gyles et al., 1998). In fact, in Gb3-positive bovine epithelial cells, Stx1 is internalized, processed by lysosomes and degraded. Otherwise, in Stx1-sensitive cells the toxin is transported to the endoplasmic reticulum, nuclear membrane, and nucleus and the Shiga toxin A subunit catalytically cleaves the glycoside bond within 28S rRNA, leading to disruption of protein synthesis. These observations may confer some

selective advantage in cattle and probably have contributed to dissemination of STEC among cattle populations (Hoey et al., 2003).

The Stx2e variant uses Gb4 as the receptor. Stx2e is classically associated with pig edema disease rather than human disease, but occasional strains that express only this variant are isolated from patients with HUS or diarrhoea (Pierard et al., 1991; Thomas et al., 1994).

After binding to the Gb3 receptor (Lingwood et al., 1998) on the target endothelial cell, the toxins are internalized by receptor-mediated endocytosis (Sandvig and van Deurs, 1996), an A subunit fragment with N-glycosidae is released into the cytosol and removes an adenine group from position 2324 from the eukarytotic 28S RNA from the 60S ribosomal subunit (Endo et al., 1988). This results in the inhibition of protein synthesis (O'Brien et al., 1992) and precipitates apoptosis in eukaryotic cells (Monnens et al., 1998). Cytokines, especially TNF-a and IL1-b, which are thought to be produced by Stx stimulation of monocytes, potentiate toxin action on endothelial cells through upregulation of the cellular receptor; Gb3 (Monnens et al., 1998). The eventual outcome of Stx action on

endothelial cells is thrombotic microangiopathy in the renal glomeruli and other organs.

This damage can lead to HUS, which is characterized by haemolytic anaemia, thrombocytopoenia and potentially fatal acute renal failure. Stx is also able to induce apoptosis in intestinal epithelial cells (Jones et al., 2000).

Stx also mediates local damage in the colon, which results in bloody diarrhoea, haemorrhagic colitis, necrosis and intestinal perforation (Kaper et al., 2004).

Stx can travel by the bloodstream to the kidney, where it damages renal endothelial cells and occludes the microvasculature through a combination of direct toxicity and induction of local cytokine and chemokine production, resulting in renal inflammation (Andreoli et al., 2002).

Locus of enterocyte effacement (LEE)

The ability to induce this A/E histopathology is encoded by genes on a 35-kb pathogenicity island (PAI) called "locus of enterocyte effacement" (LEE) (McDaniel

et al., 1995). Homologues of LEE are also found in other human and animal pathogens that produce the A/E histopathology, including EHEC, rabbit EPEC (REPEC), diarrheagenic *H. alvei* and *Citrobacter rodentium*, which induces colonic hyperplasia in mice. Collectively these are known as attaching–effacing *E. coli* (AEEC), although this term is not widely used (Naylor et al., 2005).

This large region of chromosomal DNA is not present in *E. coli* strains in the normal flora, *E. coli* K-12, or ETEC. The G+C content of the LEE is ca. 38%, which is strikingly lower than the 50 to 51% G+C content of the total *E. coli* genome, thus suggesting horizontal transfer of this pathogenicity island into *E. coli* from another species (McDaniel et al. 1995). The low G+C content of the LEE, together with other features, such as the carriage of virulence genes, the large size, and the insertion in chromosomal loci encoding tRNAs, indicates that this locus is a pathogenicity island (PAI) (Sperandio et. al., 1998; Wieler et al., 1997).

The LEE region contains:

- the *sep* and *esc* genes encoding a type III secretion system (Jarvis et al., 1995);

- the *eae* gene encoding an adhesin called intimin that is responsible for the intimate attachment of bacteria to the epithelial cell (Jerse and Kaper, 1991);

- the *espA/B/D* genes, which encode proteins secreted by a type III secretion system, including EspA, which forms a filamentous secretion tube and EspB and EspD, which are believed to facilitate pore formation at the host surface and thereby complete the conduit for delivery of proteins from the bacterium into the host cell cytoplasm (Donnenberg et al., 1993b; Kenny et al., 1996; Lai et al., 1997; Knutton et al., 1998);

- the *tir* gene, which encodes the translocated intimin receptor, the receptor for intimin (Kenny et al., 1997);

- the *ler* gene (LEE-encoded regulator), which encodes a positive regulator of LEE genes (Mellies et al., 1999).

The region is constituted by 41 open reading frames (ORFs) that are highly conserved at the DNA and protein levels for the type III secretion genes, but were more variable for the *esp*, *eae*, and *tir* genes. The majority of the LEE genes were reported to be in five major polycistronic operons named: LEE1, LEE2, LEE3, LEE4

and LEE5 (Griffin et al., 1991; Perna et al., 1998; Mellies et al., 1999; Elliot et al., 1999).

The operons LEE1, LEE2, and LEE3 encode the components of a type III secretion system, while the LEE4 operon encode proteins which are secreted by the type III secretion machinery (Griffin et al., 1991; Mellies et al., 1999;). Of particular interest is LEE 5, which contains the *eae* gene, which encodes the outer membrane adhesin intimin (Nataro and Kaper, 1998). This operon contains also genes that encode for the translocated intimin receptor known as Tir (Kenny et al., 1997) or EspE (Deibel et al., 1998) and the Tir chaperone, CesT (Elliott et al., 1999).

Karch et al., (1997) identified a high incidence of *eae* positive STEC in HUS patients, particularly children (Beutin et al., 1998; Pradel et al., 2000) suggesting that the presence of this gene is associated with increased virulence in STEC. Moreover, this gene is not a universal requirement for virulence (Wieler et al., 1996), and pathogenic strains associated with serious clinical outcomes not carrying this gene have been isolated (Keskimäki et al., 1997; Pradel et al., 2000; Eklund et al., 2001). Paton et al. (2001) reported that Saa is encoded by a gene located on the large

plasmid of some eae-negative STEC strains, that had caused HUS outbreaks. This plasmid gene encodes for an auto-agglutinating adhesion designated Saa (STEC autoagglutinating adhesion). Subsequent investigation by these researchers found homologues of *saa* in several LEE-negative STEC serotypes associated with HUS patients (Paton et al., 2001).

Attaching and effacing lesions

The A/E lesion of EHEC serotypes resembles that associated with enteropathogenic *E. coli* (EPEC) and consists of the destruction of microvilli, an intimate effacing adherence of the organism to the enterocyte membrane, and changes in the cytoskeletal structure of the enterocyte associated with the accumulation of polymerized actin and other cytoskeletal proteins beneath the site of bacterial attachment (Nataro and Kaper, 1998).

Typical A/E lesions have been observed in the intestine of a number of species in the neonatal period, including pigs (Staley et al., 1969), rabbits (Cantey

and Blake, 1977), calves (Hall et al., 1985), goats (Drolet et al., 1994b) and lambs (Janke et al., 1989). Such lesions have been reported in older animals including: calves (Hall et al., 1985) and adult cattle (Pearson et al., 1999), young goats, pigs and dogs (Duhamel et al., 1992; Drolet et al., 1994a; Higgins et al., 1997), young and adult cats (Pospischil et al., 1987) and human infants (Ulshen et al., 1980). In most of these cases, animals were asymptomatic.

The pathophysiologic mechanism by which EHEC cause the attaching and effacing lesion in the intestinal tract of calves is the same to the first described three-stage model proposed for human strains of EPEC consisting of localized adherence, signal transduction, and intimate adherence (Donnenberg and Kaper, 1992; Donnenberg et al., 1993a, b). The temporal sequence of these stages is not certain and, indeed, the different stages may occur concurrently.

-Localized adherence: rod-like fimbriae and fibrillae with subunit sizes of 16.5, 15.5, and 14.7 kDa are produced by these strains, these thin fibers resembling fimbriae appear to link bacterial cells and epithelial cells, while rope-like structures may be primarily involved in bacterium-bacterium interactions (Nataro and Kaper,

1998). Indeed the most highly conserved feature of diarrheagenic *E. coli* strains is their ability to colonize the intestinal mucosal surface despite peristalsis and competition for nutrients by the indigenous flora of the gut (including other *E. coli* strains). The presence of surface adherence fimbriae is a property of virtually all *E. coli* strains, including non-pathogenic varieties. However, diarrhoeagenic *E. coli* strains possess specific fimbrial antigens that enhance their intestinal colonizing ability and allow adherence to the small bowel mucosa, a site that is not normally colonized (Levine et al, 1984).

-Signal transduction: Adherence to epithelial cells induces a variety of signal transduction pathways in the intestinal cell. In this stage is crucial the role played by intimin, multiple secreted proteins (EspA, EspB, and EspD, for 'EPEC-secreted proteins') and by others proteins secreted by a type III secretion system encoded by LEE. In fact mutation of these factors abolishes these multiple signalling events (Nataro & Kaper, 1998).

Infection induces increases in the intracellular calcium levels [Ca^{2+}] in cultured epithelial cells to which they are attached. The calcium originates from

intracellular stores rather than from an influx of extracellular calcium, and buffering of intracellular calcium greatly reduces the polymerization of actin and formation of the A/E lesion (Baldwin et al., 1993). The increase in [Ca^{2+}] has been hypothesized to produce the cytoskeletal changes induced by EHEC via activation of a calcium-dependent, actin-severing protein which could break down actin in the microvillus core (Baldwin et al., 1991). Furthermore, since increases in intracellular calcium can inhibit Na^{+} and Cl^{-} absorption and stimulate chloride secretion in enterocytes, these data also suggest that changes in [Ca^{2+}] may mediate the intestinal secretory response to EHEC (Field et al., 1989).

There is evidence that calcium is released from 1,4,5-inositol trisphosphate (IP3)-sensitive stores (Baldwin et al., 1991) and several studies have shown that binding of EHEC to cultured epithelial cells triggers the release of inositol phosphates including IP3 and IP4 in infected cells (Foubister et al., 1994). The increase in the amount of inositol phosphates is consistent with the recently reported activation of phospholipase Cγ1 by EHEC attached to epithelial cells (Kenny et al., 1997). Adherence of EHEC to epithelial cells results in the phosphorylation of

several epithelial cell proteins on serine and threonine residues, the most prominent of which is myosin light chain (Manjarrez-Hernandez et al., 1992). Activation of at least two kinases, protein kinase C (PKC) and myosin light chain kinase has been shown (Crane et al., 1997).

Activation of PKC induces rapid changes in intestinal water and electrolyte secretion (Rao et al., 1990) and phosphorylation of myosin light chain can lead to increased permeability of tight junctions, thereby suggesting additional potential mechanisms of diarrhoea due to EHEC (Manjarrez-Hernandez et al. 1996).

The major tyrosine-phosphorylated protein is a 90-kDa protein named Tir (translocated intimin receptor), it is inserted into the host-cell membrane in a hairpin loop topology with the central loop of the molecule exposed to the host cell surface and accessible for interaction with intimin (DeVinney et al., 1999; Kenny et al., 1997). This protein is part of the A/E lesion, and it serves as a receptor for the intimin, thus, the signal transduction induced in epithelial cells by EHEC activates receptor binding activity as well as subsequent cytoskeletal rearrangements (Kenny et al., 1997). When Tir is not tyrosine-phosphorylated, it cannot serve as a receptor.

The intracellular amino and carboxyl termini of Tir interact with a number of focal adhesion and cytoskeletal proteins and contribute to pedestal formation (Campellone and Leong, 2003).

The carboxyl-terminal domain of intimin mediates the interaction with Tir; however, interactions between intimin and cellular coreceptors include β1-integrins and surface-localized nucleolin as well (Frankel et al., 1996; Sinclair and O'Brien, 2002).

-Intimate adherence. Intimate adherence of EHEC to epithelial cells is mediated by intimin. This is a 94kDa protein encoded by *eae* gene and it is present in all EHEC, EPEC, and all strains capable of producing the A/E histopathology but it is absent from *E. coli* strains in the normal flora, ETEC strains, and other bacteria that do not produce the A/E lesion (Jerse and Kaper, 1991; Donnenberg and Kaper, 1992).

It has been suggested that the presence of several distinct *eae* genes could account for the ability of the intimin-producing strains to colonize different tissue and/or different hosts (Fitzhenry et al., 2002). Although, intimin is unlikely to be the

only factor that determines host specificity (Hartland et al., 2000). However, it is suggested that intimins are associated with tissue specific tropism (Phillips et al., 2000) and that the intimins themselves are an evolutionarily divergent group of proteins (Zhang et al., 2002). On the basis of antigenic variation, PCR analysis and sequencing, at least 17 different intimin types and subtypes (α1, α2, β1, ξR/β2B, δ/κ/ β2O, γ1, θ/γ2, ε1, υR/ε2, ζ, η, ι1, μR/ι2, λ, μB, υB and ξB) and most commonly STEC produce intimin γ and ε have been identified (Pelayo et al., 1999; Orden et al., 2003a; Blanco et al., 2004a,b,c). Although various intimin variants have been detected among *E. coli* strains isolated from cattle and sheep, the intimin subtype may be a good prognostic indicator of the potential of STEC isolates derived from ruminant sources to cause disease in humans (Ramachandran et al., 2003).

The type β seems to be the most widespread type of the *eae* gene among human and animal AEEC strains, including important human and ruminant diarrhoeagenic serogroups such as EPEC O26, O111 and O128 and EHEC O26 (Oswald et al., 2000). The *eaeβ* gene is the most prevalent among AEEC isolated from healthy and diarrhoeic calves, lambs and goats, which show significantly higher

percentages of positivity than adults. These data suggest that AEEC strains with the *eaeβ* gene may play a role in diarrhoeal disease in newborn ruminants (Orden et al 2003a,b).

The types ζ and ι of the *eae* gene have been recently identified and were first sequenced from bovine and human AEEC strains, respectively. Both types do not occur frequently in AEEC strains isolated from ruminants and humans with diarrhoea, although *eaeι* is isolated with relative frequency from diarrhoeic lambs AEEC and bovine AEEC in healthy cattle and in diarrhoeic calves (Orden et al 2003a; Zhang et al., 2002). The types *eaeα*, ε, κ and λ are principally restricted to human (Zhang et al., 2002).

Intimin protein has 31% identity and 50% similarity to the invasin protein of *Yersinia* species (Isberg et al., 1987). Comparison of the intimin proteins of EPEC strain E2348/69 and EHEC O157:H7 strain EDL933 reveals a striking pattern of sequence conservation among intimin proteins (Yu and Kaper, 1992). Although the overall protein identity is 83%, the sequence divergence is concentrated in the C-terminal region.

The highly divergent C-terminal region is the portion of the molecule that binds to receptors on the epithelial cell and the different intimin sequences can confer different colonization patterns within the intestine, while the N-terminal region shown high conservation (Frankel et al., 1994). This could account for the fact that EPEC is a small bowel pathogen while EHEC is a large bowel pathogen (Yu and Kaper, 1992.).

AEEC binds through the interaction of intimin with Tir inserted in the membrane and numerous cytoskeletal proteins accumulate underneath the attached bacteria (Kaper et al., 2004).

At the end, the A/E lesion is characterized by intimate adherence between the bacterium and the host epithelial cell membrane, with an intervening gap of about 10 nm, plus effacement of enterocyte microvilli. Beneath the adherent bacterium, a cytoskeletal rearrangement, including the accumulation of filamentous actin (F-actin), can be observed. The bacteria often sit upon a pedestal-like structure, which can extend up to 10 mm away from the epithelial cell surface (Kaper et al., 1998).

Fluorescent-actin staining (FAS) test shows high concentrations of polymerized filamentous actin (F-actin) in the composition of the A/E lesion. In addition to F-actin, the composition of the A/E lesion includes other cytoskeletal components such as α-actinin, talin, ezrin, and myosin light chain. At the tip of the pedestals beneath the plasma membrane, proteins that are phosphorylated on a tyrosine residue in response of infection are located (Finlay et al., 1992).

Studies with electron micrographs on HEp-2 cell showed that microvilli are effaced and the underlying cytoskeleton is reorganized at the sites where individual AEEC are intimately associated with the membrane. Thus, AEEC induce ultrastructural changes in tissue culture cells which resemble those induced by AEEC in the gastro-intestinal mucosa (Finlay et al., 1992).

A/E lesion is the phenotype that seems to be the primary contributor to colonization in all ruminant species including sheep and goats (Woodward et al., 2003). The clinical outcome of infection in humans and animals seems to be directly related to the immune status of the host, and host susceptibility to the effects of colonization.

The production of intimin and other virulence factors is affected by the temperature, composition of the growth medium, and growth phase, with the optimal conditions often reflecting those encountered inside the human body (Kenny and Finlay, 1995; Knutton et al., 1997). Studies by Cornick et al. and Woodward et al. demonstrated that intimin contributes significantly to the colonization and persistence of *E. coli* O157:H7 in sheep (Cornick et al. 2002; Woodward et al. 2003).

Intimin has not only functions as a ligand for epithelial cell adhesion, but also stimulates mucosal TH1 immune responses and intestinal crypt hyperplasia (Higgins et al., 1999).

Other virulence factors

Studies suggest that the longterm persistence of *E. coli* O157:H7 in small ruminants may be significantly affected by bacterial factors in addition to intimin and Tir, that have a fundamental role in colonization and/or persistence of the gut (Woodward et al. 2003; La Ragione et al. 2005, 2006)

Most of EHEC A/E lesion-involved virulence factors belong to type three secretion system (TTSS). The genes encoding for the TTSS were initially named *sep*, for secretion of EPEC proteins, because they were found in EPEC strains before (Jarvis et al.1995). The nomenclature for these genes has recently been revised to correspond with type III secretion systems in *Yersinia* and other species (Nataro and Kaper, 1998). Those genes with homologous to *Yersinia ysc* genes are now called *esc*, and those type III secretion genes with no homologous will continue to be called *sep*.

AEEC TTSS is similar to other specialized protein secretion systems found in a variety of gram-negative human, animal, and plant pathogens. The TTSSs are responsible for secretion and translocation of critical virulence determinants such as *Shigella* Ipa proteins, *Yersinia* Yops, and proteins involved in invasion by *Salmonella* spp (Mecsas et al., 1996).

In AEEC the TTSS is made up of proteins in the bacterial inner and outer membranes and a needle complex that allows injection of effector proteins, including EspB, EspD and translocated intimin receptor (Tir) through the host cell membrane.

The needle complex consists of a short EscF needle elongated by a filament made up of EspA (Wilson et al., 2001).

At least four Esp proteins are secreted extracellularly, and three of these, EspA (25 kDa) (Kenny et al., 1996), EspB (38 kDa; formerly called EaeB) (Donnenberg et al., 1993; Foubister et al., 1994; Kenny et al., 1995) and EspD (40 kDa) (Lai et al., 1997) are essential for the A/E histopathology. Mutation of the *espA*, *espB*, or *espD* gene abolishes the signal transduction in epithelial cells produced by wild-type strains and the A/E histopathology.

Esp A, B and D are thought to create a bifunctional adhesin and a pore-forming channel, through which proteins, including the translocated intimin receptor Tir, are translocated into a host enterocyte. Tir ultimately localizes to the host cell membrane, where it functions as a receptor for intimin in the formation of an intimate attachment (Frankel et al., 1998).

EspB binds to eukaryotic cells and is involved in activation of host cell signal transduction pathways (Rosenshine et al., 1992). Secretion of the EspB protein is essential for signal transduction in host cells and A/E lesion formation (Foubister

et al., 1994). Three types of the *espB* gene have been identified: α, β and γ (China et al., 1999; Cid et al., 2001). The variability in genes encoding proteins that interact directly with the host, as intimin and EspB, suggests that these variable proteins are subject to selection for evasion of the host immune system (Frankel et al., 1998).

A fourth protein of ca. 110 kDa, called EspP, is homologous to members of the autotransporter protein family, it is considered a enterotoxin (Mellies et al., 2001). Mutation of the *espP* gene does not affect signal transduction, A/E histopathology, or any other obvious pathogenic phenotype of EHEC (Stein et al., 1996). EspC does not require the type III secretion system for delivery into the extracellular milieu (Jarvis et al, 1995; Stein et al., 1996). This protein shows amino acid homology to members of the immunoglobulin A (IgA) protease family of autotransporters which include, among others, the IgA protease of *Neisseria gonorrhoeae* (Pohlner et al ., 1987), Tsh of avian-pathogenic *E. coli* (Provence et al., 1994), Pic of enteroaggregative *E. coli* (EAEC) (Henderson et al., 1999), Pet of EAggEC (Eslava et al., 1998), and EspC of enteropatoghenic *E. coli* (EPEC) (Brunder et al., 1997).

This protein belongs to subfamily of autotransporters termed SPATE, for serine protease autotransporters of *Enterobacteriaceae* (Henderson et al., 1998), but its function is not clear, most likely it plays an accessory role in AEEC pathogenesis, presumably as an enterotoxin (Mellies et al., 2001).

It has been observed that in EHEC, EspD is essential for the formation of surface appendages, is integrated in the cytoplasmic membranes of target cells, and might participate in the translocation of effector molecules (Kresse et al., 1999).

Another recently discovered Esp protein is EspJ that inhibits macrophage phagocytosis and is not required for A/E lesion formation. EspJ appears to have properties that might enhance virulence, inhibiting an aspect of the host immune response, but, based on the limited evidence available, EspJ may also limit persistence (Marchès et al., 2008).

LEE-negative serotypes are also associated with serious human disease, suggesting that, in addition to LEE, other possibly PAIs, may also enhance the virulence potential of VTEC strains (Nataro and Kaper, 1998). Genome sequencing of two epidemic strains of *E. coli* O157:H7, EDL 933 (Perna et al., 2001) and the

Sakai strain (Hayashi et al., 2001), has revealed several additional candidate PAIs which, in the EDL 933 genome, include O island 1 (OI-1), OI-43, OI-48, OI-115, OI-122, OI-140, OI-141, and OI-154.

The presence of PAI O122 (23.029 bp) seems to be a peculiar feature of AEEC, strains, since it was detected in most of the EHEC and EPEC strains but not in the other groups of *E. coli* examined. Nicholls et al. (2001) showed that it confers a sevenfold increase in the ability of EHEC strains to adhere to cell monolayers.

It consists of three distinct modules separated by mobile genetic elements (Karmali et al., 2003). The first module encodes Z4321, a gene product with 46% homology to the *phoP*-activated gene C product (PagC) that enables survival in macrophages of *Salmonella enterica* serovar Typhimurium (Pulkkinen and Miller, 1991). This module is present in strains ranging from strains carrying a complete OI-122 with all three modules to incomplete strains carrying only this module.

Module 2 carries the Z4326 (*sen*) gene, whose product is 39% homologous to *Shigella* enterotoxin, and genes encoding two proteins, Z4328 and Z4329, with 89

and 86% sequence homology to non-LEEencoded (Nle) effectors of *Citrobacter rodentium*, NleB and NleE, respectively (Nataro et al., 1995; Karmali et al., 2003).

The third module encodes Z4332 and Z4333, which are enterohemorrhagic *E. coli* (EHEC) factors for adherence (Efa1 and Efa2) (Nicholls et al., 2001) which is also referred to as lymphocyte inhibition factor *lifA* (Klapproth et al., 2000). OI-122 and LEE are functionally related; the LEE-encoded type III secretory apparatus is required for secretion of the OI-122 non-LEE-encoded effectors NleB and NleE encoded in module 2.

The family of Nle comprise as many as 32 molecules distributed among 20 families encoded on prophages, and include NleC and NleD (OI-36), NleF, NleG, and NleA (OI-71), and NleB and NleE (OI-122) (Deng et al., 2004; Tobe et al., 2006; Coburn et al., 2007).

Among the *nle* genes, those encoded in the O-island 122 (OI-122) and O-island 71 (OI-71) have been proposed to distinguish EHEC O157 and non-O157 strains (Coombes et al., 2008; Konczy et al., 2008). It has been proposed by Coombes et al. (2008) that the presence of these genes can be used to make a

"molecular risk assessment" to predict the potential virulence of strains. In particular *nleA* (*espI*) gene has been found widely in EHEC (O157 and non-O157) and EPEC and appears to be associated with virulence in humans (Mundy et al., 2004; Creuzburg and Schmidt, 2007).

Iha (adherence-conferring protein similar to *Vibrio cholerae* IrgA) is a OI-43 and OI-48 encoded factor that play a role in adherence (adhesins) (Toma et al., 2004)

Protein kinase C (PKC), phospholipase Cγ, myosin light-chain kinase and (MAP) kinases are activated during adhesion to enterocytes, which leads to several downstream effects, including increased permeability due to loosened tight junctions (Vallance and Finlay, 2000) and migration of polymorphonuclear leukocytes (Savkovic et al., 1996). This transmigration across intestinal epithelial cells has been shown for invasive organisms such as *Salmonella* spp (McCormick et al., 1993) but is unusual for a primarily non-invasive organism such as EHEC and EPEC (Nataro and Kaper, 1998). During attaching to epithelial cells, a nuclear factor (NF)-κB is activated, leading to production of a interleukin (IL-8) and an inflammatory response

that involves transmigration of polymorphonuclear leukocytes (PMNs) to the luminal surface and activation of the adenosine receptor (Kaper et al., 2004).

The cytolethal distending toxin V (CDT-V), occurs in *E. coli* O157:H7 and non-O157 strains (Janka et al., 2003). It causes an arrest in the G2/M phase in human brain microvascular endothelial cells and, moreover, death of endothelial cells (Bielaszewska et al., 2005).

EHEC strains carry functional or mutated forms of a lambdoid phage encoded protein named Cif. It triggers an irreversible cytopathic effect in HeLa cells, which is characterized by the progressive recruitment of focal adhesions, the assembly of stress fibres and arrest of the cell cycle (Nougayrede et al., 2001; Marchès et al., 2003). It has been hypothesized that Cif-dependent arrest of the host cell cycle in a population with rapid turnover, such as enterocytes, may aid colonization and persistence (Marchès et al., 2003).

Fimbriae and flagella

Pathogenic *E. coli* strains possess specific adherence factors that allow them to colonize sites that *E. coli* does not normally inhabit, such as the small intestine and the urethra. Most frequently these adhesins form distinct morphological structures called fimbriae (also called pili) or fibrillae, which can belong to one of several different classes. Fimbriae are rod-like structures of 5–10 nm diameter that are distinct from flagella. Fibrillae are 2–4 nm in diameter, and are either long and wiry or curly and flexible (Cassels and Wolf, 1995).

Fimbriae have been shown to be important in the pathogenesis of a number of VTEC (La Ragione et al., 2000; Lane and Mobley, 2007), and recent studies have revealed the presence of at least 16 fimbrial gene clusters in *E. coli* O157:H7. Long polar fimbriae (LPF), originally found to be important for the pathogenesis of *Salmonella typhimurium* (Bäumler et al., 1996), represent one potential adherence determinant in *E. coli* O157:H7, which contains two loci homologous to *lpf* of *S. Typhimurium* (Perna et al., 2001).

Long polar fimbriae encoded in unique O islands LPF OI-141 and OI-154 are present in *E. coli* O157 and play a role in adherence (Tarr et al., 2001; Torres et al., 2002). On the other hand, Doughty et al. (????) suggested that LPF encoded in OI-154 functions as an adhesin in LEE-negative STEC isolates. For other AEEC subtypes, mainly BFP has an important role in initial attachment (Knutton et al., 1987).

Flagellum-driven motility has been shown to be associated with adherence to mucus in the intestinal tract by many diverse *E. coli* pathotypes (Allen-Vercoe and Woodward, 1999; La Ragione et al., 2000; Wright et al., 2005) Erdem et al. (2007) recently demonstrated that flagella from EHEC and EPEC may mediate adherence

Flagella, including H7, have been recognized as potent inflammatory ligands, inducing inflammatory responses through engagement with a number of receptors (Zhou et al., 2003).

However, *E. coli* O157:H- are almost always non-motile, but are still able to cause clinical disease in humans (Monday et al., 2004), indicating that flagella are not essential for EHEC virulence in the human host.

Plasmid-mediated factors

The feature that distinguish EHEC from other VTEC is the possess of a highly conserved 97-kb plasmid (pO157), which carries genes that encode for several putative virulence factors, including the serine protease (EspP) (see above), a enterohemolysin (Ehly), a bifunctional catalase peroxidase (KatP), an adhesin (ToxB), a subtilase cytotoxin (SubAB), an immunomodulator (Lif) and secretion proteins (Etp).

KatP is a catalase-peroxidase, it is a part of bacterial defence mechanisms against oxidative stress, which involve at least 30 proteins, depending on the stimulus applied (Farr and Kogoma, 1991).

The adhesin ToxB is a large 362-kDa protein, that shares sequence similarity with the large *Clostridium* toxin family, and to the EPEC LifA protein (Klapproth et al. 2000) and the Efa-1 protein. ToxB is required for full expression of adherence of O157:H7 strain Sakai (Tatsuno et al., 2001).

SubAB has been detected in a number of VTEC serogroups, (O26, O91, O111, O113, O128, and O157) mainly in eae-negative strains, it is involved in the generation of HUS disease in animal experiments (Paton et al., 2004).

The precise role of these genes in the virulence of VTEC has not been fully elucidated (McNally et al., 2001).

Moreover enterohemolysin is the more investigated and an association between the carriage, the production of this factor and the *eae* gene has been suggested (Eklund et al., 2001).

Ehly belongs to the RTX toxin family, members of which are expressed by uropathogenic *E. coli*, *Pasteurella haemolytica*, and other human and animal pathogens (Bauer and Welch, 1996). It has been found in nearly all O157:H7 strains and is widely distributed among non-O157 Stx-producing *E. coli* strains (Nicholls et al., 2001).

The role of enterohemolysin is to cause lysis of erythrocytes with release of heme and hemoglobin, which enhance the growth of *E. coli* O157:H7 and could serve as a source of iron.

As a matter of fact, the growth of *E. coli* O157:H7 is stimulated by the presence of heme and haemoglobin, and the lysis of erythrocytes by one or more of the hemolysins reported for this pathogen could release these sources of iron, thereby aiding infection (Law and Kelly, 1995).

In addition to lysing erythrocytes, the toxin lyses bovine but not human leukocytes (Bauer and Welch, 1996). Ehly1 and Ehly2 are other two genetically distinct phage-encoded hemolysins, and they have been reported to be produced by many Stx-producing *E. coli* strains (Beutin et al., 1993; Stroeher et al, 1993) but there are no data to suggest in vivo expression or any role in pathogenesis for these hemolysins.

It has been suggested that expression of EHEC virulence factors is regulated by a trans-acting protein analogous to Per system of EPEC (Gomez-Duarte and Kaper, 1995).

Escherichia coli O157:H7

Although many VTEC serotype have been associated with human illness, the vast majority of reported outbreaks and sporadic cases of VTEC-infection in humans have been associated with serotype O157:H7. In 1982, for the first time *E. coli O157:H7* was associated to outbreaks of HC infection in humans within a batch of undercooked hamburgers (Riley et al., 1983). Subsequently the isolated strains were shown to produce Shiga toxins, encoded on a temperate bacteriophage (O'Brien et al., 1982, 1984).

E. coli O157:H7 is the more investigated of VTEC. In recent years, the annual incidence of reported clinical EHEC O157:H7 infection in humans in the United States has been around 1 per 100,000 (Centers for Disease Control and Prevention, 2008), with 2,621 cases reported in 2005. Twenty-six countries (mainly European but including Japan) reported a total of 2,937 EHEC O157:H7 infections to Enter-Net in 2005 (Enter-Net, 2007). Out of these cases, 21% developed HUS, but it is possible that many less severe cases went unreported. EHEC O157:H7 still predominate in many countries such as the UK, USA and Japan, while in Europe,

non-O157 serogroups like O111, O26, O103 and O145 are more frequently detected (WHO, 1998; Eklund et al., 2001).

Ruminants, mainly cattle, are the main reservoirs of VTEC. Cattle are the major reservoir of the pathogen which is carried in and excreted from their gastrointestinal tract without any symptoms of disease (Chapman et al., 2001; Hancock et al., 1998; Renter et al., 2003).

E. coli O157:H7 induces disease in young calves and it is generally considered avirulent in older cattle. In contrast, there are isolated reports of non-O157-mediated disease in older cattle. This means that this O157:H7 serotype lacks certain factors required for virulence in such animals (Pearson et al., 1999; Wada et al., 1994).

Faeces and hides of cattle are considered to be the main sources of *E. coli* O157 contamination of carcasses during slaughter (Aslam et al., 2003; Elder et al., 2000).

However, several other species including rabbits, zebu cattle, deer, water buffalo, pigs, chickens and seagulls have also been implicated as carriers of *E. coli*

O157:H7 (Griffin et al., 1991; Pritchard et al., 2001; Kaddu-Mulindwa et al., 2001; Eriksson et al., 2003; Galiero et al., 2005; Dipineto et al., 2006; Foster et al., 2006; Scaife et al., 2006; Garcia-Sanchez et al., 2007; Cornick & VuKhac, 2008). In particular, there is much evidence of the carriage of toxigenic *E. coli* O157 by small domestic ruminants as sheep and goats (Chapman et al., 1997; Heuvelink et al., 1998; Meng et al., 1998; Fegan and Desmarchelier, 1999; Ogden et al., 2005). Furthermore, human cases and outbreaks of VTEC O157:H7 disease have often been linked to open farms or petting zoos, where the organism has been isolated from animals including small ruminants (Shukla et al., 1995; Chapman et al., 2000; Pritchard et al., 2000; Heuvelink et al., 2002; Payne et al., 2003; Stirling et al., 2008). Other cases have been linked to small ruminant dairy products (Bielaszewska et al., 1997; Steen et al., 2001; McIntyre et al., 2002; Espie et al., 2006). With the increasing popularity of petting farms, the intensification of goat and sheep farming and the wider availability of sheep and goat products worldwide (including milk and unpasteurized cheeses), small ruminants are gaining attention as potentially significant reservoirs of *E. coli* O157:H7.

The infectious dose in humans has been estimated to be less than 100 c.f.u. (Griffin, 1995) and efficient transmission between animals is possible even at very low doses (Besser et al., 2001). The pathogen is mainly transmitted to humans via contamination of food by animal faeces, thus the consumption of undercooked minced beef and the cross-contaminated cooked meats are the primary ways of spread of the disease (Griffin and Tauxe, 1991; Zhao et al., 1995; Hancock et al., 1997). Other studies indicated that *E. coli* O157:H7 was also widespread in meat sources other than beef (Doyle and Schoeni, 1987). Transmission to humans by raw milk or raw milk homemade cheese has been demonstrated (Blanco et al., 2001). In addition, human disease outbreaks can be related to the consumption of plant products, including apple cider and vegetables such as lettuce, radishes, alfalfa sprouts and spinach (Besser et al., 1993; Fukushima et al., 1999; Ferguson et al., 2005; Maki, 2006). The human infection via water or direct contact with ruminant faeces is possible too (Licence et al., 2001; Strachan N.J.C. et al., 2001) and interpersonal spread can also be considered as a significant factor in some outbreaks

(Ryan et al., 1986). Therefore, *E. coli* O157:H7 may also be considered as an environmental pathogen (Strachan et al., 2006).

Non-O157 *Escherichia coli*

Analysis of data on human VTEC infection from European countries reported thorough the Enter-Net Surveillance database (Enter-Net, 2004) indicated an overall increase in VTEC infections of 15% between 2000 and 2004. While *E. coli* O157:H7 remained the most common serotype in this period, non-O157 serogroups increased by 52%. Even with this dramatic increase, non-O157 serogroups still remain under-diagnosed and their true significance is not yet fully recognised (Fisher, 2006).

Some non-O157 VTEC possess the same range of virulence factors as *E. coli* O157:H7, including production of Shiga toxin, LEE and the other plasmid mediated factors. More than 200 non-O157 VTEC serotypes have been identified from all sources (Beutin et al., 1998; Johnson et al., 1996) and although many lack the full complement of known virulence factors found in strains that cause serious disease, over than 100 have been associated with disease in humans (EFSA, 2007).

Estimation of the true incidence of disease due to non-O157 strains is not simple by the need to detect these infections by the presence of *stx* genes, since these serotypes are usually sorbitol positive and there are no convenient media such as sorbitol MacConkey agar (see below) that will reliably screen for them (Nataro and Kaper, 1998).

For these serogroups, the amount of information on VTEC monitoring in food and animals provided by reporting countries is relatively sparse. Therefore, based on the data available, it is difficult to assess the potential human health risk of the presence of VTEC in animals and food. In order to improve the quality of the data from VTEC monitoring, the EU and EFSA's Task Force on Zoonoses Data Collection, have undertaken the task of developing guidelines and technical specifications for monitoring and reporting VTEC in animals and food. The recent scientific opinion from EFSA's Biological Hazards panel on the monitoring of verotoxigenic *Escherichia coli* (VTEC) refers that the serogroups associated with public health risks are O26, O103, O91, O145 and O111, and however these isolates are not necessary pathogenic when recovered from food or live animals.

Karmali et al., (2003) suggest that the serotypes vary in frequency with the country and year, indicating serogroups O26, O91, O103, O111, O113, O121 and O145 as more common non-O157 regarded as especially likely to cause severe human infections (Boerlin et al., 1999; Karmali et al., 2003).

Non-O157 serogroups have been reported from both sporadic cases as well as outbreaks, most notably O111 in Italy (Caprioli et al., 1994), Japan (Watanabe et al., 1996) and Australia (Cameron et al., 1995). Outbreaks due to other serotypes include O145:H- in Japan (Kudoh et al., 1994), O103:H2 in France (Mariani-Kurdjian et al., 1993), O123 in Germany (Bettelheim, 2000).

In addition, O91 serogroup isolates from healthy sheep was associated to HUS cases in Germany (Bockemühl et al., 1992) and Australia (Goldwater and Bettelheim, 1994) and to episodes of diarrhoea and haemorrhagic colitis in the UK (Scotland et al., 1988; Willshaw et al., 1992).

The non-O157 serotypes occasionally associated with illness in humans are: O26:H–, O26:H11, O91:H–, O103:H2, O111:H–, O113:H21, O118:H16, O128:H2, O145:H-, O146:H21 and many others (Blanco et al., 2001; Bettelheim, 2003). The

majority of these human strains may originate from animal sources and VTEC are not considered to be normal inhabitants of the human gut (Stephan et al., 2000).

Non-O157 STEC have been found in ground beef and on cattle hides and feces at levels comparable to those for *E. coli* O157. Bovine faeces may be a source of environmental contamination (e.g., soil or water) which can lead to secondary contamination of produce growing in fields (Dean-Nystrom et al., 1997; Hussein and Sakuma, 2005).

Studies carried out on US dairy cattle have reported non-O157 STEC prevalence from 0% to 19% (Wells et al., 1991; Cray et al., 1996; Thran et al., 2001). Prevalence of non-O157 STEC in beef cattle are reported at 19.4% (feces) and 56.3% (hides) respectively.Pathogen prevalence on hides may reflect several sources of contamination, such as soil, feces from other animals and the environment. Prevalence on pre-evisceration carcasses was 58%, dropping to 9% post-processing (Barkocy-Gallagher et al., 2003). Similarly, Arthur et al. (2002) reported that 53.9% of beef carcasses in large processing plants carried at least one type of non-O157 STEC prior to evisceration, but that the prevalence could be reduced to 8.3% with

various intervention strategies. In a study by Samadpour et al. (2006) were reported non-O157 STEC in 2.3% of 1,750 retail raw ground beef samples, compared to *E. coli* O157, found in 1.1% of samples tested.

Some VTEC may cause A/E lesions and induce disease in cattle (Pearson et al., 1999; Gunning et al., 2001), pigs (Higgins et al., 1997), sheep (Woodward et al., 2003) and goats (Duhamel et al., 1992).

Ruminant derived VTEC are potential human pathogens. It is possible that only some variants may actually be capable of causing human illnesses. It has been shown that certain verotoxin subtypes are more likely to be associated with more virulent VTEC serogroups such as O26, O103, O111, O145, and O157 (Piérard et al., 1997) and also there appears to be a relationship between the verotoxin produced and the VTEC serotype (Djordjevic et al., 2001, Ramachandran et al., 2001). Further studies may confirm that certain serotypes are associated with certain hosts and tend to carry unique virulence factors. In fact, it has been shown that the *eae* gene has been associated with many of the more important human pathogenic VTEC serotypes, including O111:H− and O157:H7 (Sperandio et al., 1998).

Non-O157 VTEC should not be overlooked in human disease investigations because these strains are more prevalent than O157 VTEC in the faeces of meat-producing animals, indicating that humans are more likely to become exposed to these VTEC strains as contaminants in foods (Arthur et al., 2002; Djordjevic et al., 2001). The O157 VTEC strains are not commonly isolated from the faeces of healthy cattle and sheep in many parts of the world (Blanco et al., 2003; Djordjevic et al., 2001). Moreover, 100 VTEC serotypes have been recovered from patients with HUS (http://www.microbionet.com.au/frames/feature/vtec/brief01.html); and finally 435 STEC serotypes have been recovered from human infections (http://www.lugo.usc.es/_ecoli/).

TABLE No.1: EFSA 2009: VTEC serogroups by country, 2007 (TESSy data)

Country	Serogroups										
	O157	**NT**	**O26**	**O103**	**O91**	**O145**	**O111**	**O128**	**O113**	**O146**	**Other**
Austria	17	41	1	3	2	7	2			2	7
Belgium	25	3	5	2	1	2	2		1	2	4
Denmark	25	1	28	16	9	5	4	8	5	8	47
Estonia	2							1			
Finland	9	3									
France	14	29	10		1		1	1			1
Germany	66	577	61	46	26	13	12	9	8	1	51
Hungary	1										
Ireland	94	5	13			1	1	1			
Italy	5	20	1				1				
Luxembourg	1										
Malta	4			3							
Netherlands	80	1	3	1	1						
Poland	2			6							
Slovakia	3	3									
Slovenia											4
Spain	18										
Sweden	85	138	13		3	1		1	2	1	12
United Kingdom	1,120	21	1			2					4
Total (19 MSs)	**1,571**	**842**	**136**	**77**	**43**	**31**	**23**	**21**	**16**	**14**	**130**

Clinical symptoms

Since the early 1990s there has been a general global increase in the number of EHEC infections and recent large outbreaks have led to an increased interest in the pathogen (Clark et al., 1997; Cowden et al., 2001; Yamamoto et al., 2001).

STEC causes symptoms ranging from mild non-bloody diarrhoea in healthy adults to more significant health outcomes, sometimes proving fatal, in young, old or immunocompromised individuals. In such susceptible individuals, STEC infection generally causes diarrhoea and abdominal cramps, with little or no fever, and resolves itself in 5 to 10 days. However, in some instances, more serious sequelae including hemorrhagic colitis, hemolytic uremic syndrome and thrombotic thrombocytopenic purpura (TTP) can develop (Coia, 1998).

Hemorrhagic colitis is a form of gastroenteritis in which STEC attach to the large intestine and secrete Shiga toxin, leading to bloody diarrhea as a result of damage to the lining of the large intestine. If the toxins are subsequently absorbed into the bloodstream, they can also affect other organs, such as the kidneys. HC can occur in people of all ages but is most common in children and the elderly.

Symptoms include the sudden onset of severe abdominal cramps along with watery diarrhoea that typically becomes bloody within 24 hours. The diarrhoea usually lasts from 1 to 8 days. Fever is usually absent or mild but occasionally can exceed 38.9°C. The prognosis for this disease is good; rarely, death may occur in elderly patients.

HUS is characterized by acute renal failure, hemolytic anaemia and thrombocytopaenia. Between 2% and 7% of infected individuals usually develop HUS and it is more common in children. The infectious dose has been estimated to be less than 100 organisms as determined from outbreak investigation (Nataro and Kaper, 1998). The incubation period is usually from 1 to 6 days but may be up to 14 days and the infection is usually self-limiting, often resolving within 8 days. Some HUS patients develop complications of the nervous system or brain damage. There is no specific treatment for the infection. Therapy is symptomatic only and antibiotic therapy, as in many gastrointestinal infections, is contraindicated (Elliot et al., 2001).

TTP is a blood disorder more commonly found in non-elderly adults, characterized by fever, a low platelet count, low red blood cell count (caused by premature breakdown of the cells), compromised kidney function, and neurological

abnormalities. Symptoms include fluctuating neurological symptoms, such as bizarre behavior, altered mental status, stroke or headaches, kidney failure, fever, thrombocytopenia leading to bruising or frank purpura, and microangiopathic hemolytic anemia. The mortality rate associated with TTP approached 100% until the 1980s. Today, the survival rate is 80-90% with early diagnosis and treatment with plasma infusion and plasma exchange, however, mortality remains at approximately 95% for untreated cases. Up to one third of patients who survive the initial episode experience a relapse within the following 10 years (Elkins et al., 1996).

EPIDEMIOLOGY

Incidence in animal

VTEC has become more significant in developed countries in recent years due to the occurrence of numerous outbreaks (Schmidt et al.,1997). However, due to the lack of valid methods for detection of non-O157 serogroups, it is necessary to distinguish the epidemiological situation of the O157 and of non-O157 strains.

In fact, while the epidemiology of *E. coli* O157 is well established, the epidemiology of non-O157 VTEC is less well understood, because many laboratories do not have the means to isolate, identify and characterize the large variety of VTEC. While it is clear that non-O157 EHEC serotypes such as O26:H11, O103:H2, O111:H8, O121:H19 and O145:H28 are associated with HUS, in general the pathogenicity of individual non-O157 VTEC remains difficult to predict.

During 2007, EFSA report showed that in bovine animals the average VTEC prevalence in reporting MEMBER STATES was 3.7% and the proportion of VTEC

O157 positive animals was 3%. The reported occurrence of VTEC ranged from 0% to 22.1% in MEMBER STATES investigations (EFSA, 2009).

Prevalence of *E. coli* O157 in individual cattle varies considerably, with rates of 1.8% in Japan (Miyao et al., 1998), 1.9% in Australia (Cobbold and Desmarchelier, 2000), 1.5% in Brazil (Cerqueira et al., 1999), 0–7.4% in the United States (Faith et al., 1996) and 1–4.2% in England and Wales (Chapman et al., 1993; Richards et al., 1998; Paiba et al., 2003). Another study showed that individual- and herd-level prevalence reported in scottish beef herds were around 8.7% and 24%, respectively (Synge and Paiba, 2000). Different sampling techniques have hampered valid comparisons between geographical locations and consequently there is some controversy over prevalence rates.

Although cattle are regarded as a natural host for O157:H7 VTEC, these prevalence are generally very low. Non-O157 were found to occur more frequently in cattle and could be detected in relatively high numbers in fecal samples (Beutin et al., 1997). Similar observations were made for sheep and goats which harbor other types of VTEC than cattle (Beutin et al., 1995). These findings suggest that some

VTEC types are adapted for colonizing well the intestine of their animal hosts and become residents over long time periods (Beutin et al., 1997).

The prevalence of EHEC is higher in younger animals and in animals subject to transit, feed changes and antimicrobial therapy, possibly due to disturbance in the resident intestinal microflora (Hancock et al., 1998). The shedding patterns are characterized by sharp bursts of EHEC excretion in a high percentage of animals separated by longer periods of very low prevalence.

Furthermore it has been observed that only a small proportion of positive animals are in fact shedding high levels of *E. coli* O157:H7. These high-level shedders (‘‘supershedders’’) have been hypothesised to be disseminating the organism and contaminating other individuals. The *E. coli* O157 carriage of supershedders is been defined as $\geq 10^3$ c.f.u./ml^{-1} or g^{-1} and these animals have been estimated as a minority of a positive population (Low et al., 2005).

Distal sites in the intestine such as the caecum, colon and rectum are believed to be the principal sites of *E. coli* O157:H7 colonization in ruminants (Cray and Moon, 1995; Wales et al., 2001a, b; Grauke et al., 2002).

In calves, natural and experimental infection with VTEC may result in acute enteritis or subclinical infection, depending on serotype and ill-defined host-specific factors. It is notable that most of the literature reveals the clinical effects of different *E. coli* serogroups to be moderate and that *E. coli* O157 strains cause no clinical signs in experimentally infected or naturally colonised animals (Naylor et al., 2005).

Animals colonized by EHEC are hard to identify since infections in weaned calves and adult cattle and sheep are asymptomatic (Brown et al., 1997; Cornick et al., 2000; Wray et al., 2000). Histological analysis of intestinal epithelia from calves and cattle infected with *E. coli* O157:H7 reveals intimate bacterial adherence in some but not all cases and a mild inflammation characterized by diffuse infiltration of neutrophils into the lamina propria (Wales et al, 2005).

However, several reviews describe A/E lesions confined to the large intestine by AEEC associated or not with disease. The clinical signs include diarrhoea usually with bloody faeces, mucosal inflammation and sometimes a fibrino-haemorrhagic exudates (Wales et al, 2005). It appears that certain strains of *E. coli*, particularly

serogroups O26, O111 and O118 are virulent to cattle, particularly young calves (Mainil et al., 1987).

In general, pigs, poultry and other non-ruminants are not considered to be a source of STEC and sometimes, sporadic reports may derive from inadvertent exposure to infected ruminants (Caprioli et al., 2005).

Evaluation of the potential pathogenicity of VTEC isolates requires both the detection of a range of virulence factors and serotype determination. Thus, there is a public health need for a rapid, specific and cost-effective tool for the detection of genes involved in serotype determination and associated with virulence. Such a tool is also required for establishing monitoring programs to follow VTEC contamination in animals and foodstuffs, as part of the European Food Safety Authority (EFSA) program (EFSA, 2007; EFSA, 2009).

Factors influencing the microbial carriage in animals

In several studies it has been shown that the highest prevalence of *E. coli* O157:H7 was seen in late summer and early autumn. *E. coli* O157 peaked

significantly in the summer (12.9%), then declined through the autumn (6.8%) to a low of 0.3% in the winter, before rising in the spring (3.9%). However, for non-O157 VTEC, highest detection rates were in the spring and autumn (22.5% and 27.1%, respectively) compared to 13.9% and 14.0% in the summer and winter, respectively (Barkocy-Gallagher et al., 2003). A similar trend was observed for *E. coli* O157 in Canada in which prevalence in faeces was 19.7% in the summer, 0.7% in the winter and slightly under 5% in the spring and autumn (Van Donkersgoed et al. 1999).

The magnitude of seasonal variation of temperature and that of *E. coli* O157 prevalence appear to show some correlation. Peak shedding in the summer and early autumn has been also observed in two studies carried out in Italy, with monthly prevalences between 2.7% and 23.7% (Conedera et al., 2001) and 0% and 33% (Bonardi et al., 1999).

Higher rainfall and increased muddiness in the spring and autumn may facilitate transmission while high temperatures and dryer conditions in the summer may cause more rapid inactivation of environmental pathogens (Williams et al., 2005; Echeverry et al., 2006).

Several studies indicate that it may be possible to reduce the carriage of EHEC by ruminants by manipulating the diet. Different feeds may influence the magnitude and duration of shedding of VTEC by altering the rate of epithelial cell proliferation in the lower gastrointestinal tract (Magnuson et al., 2000).

Dairy cattle with three or more hours access to pasture per day were approximately twice as likely to be carriers of VTEC than those without access (Kuhnert et al., 2005). Indoor housing is positively associated with *E. coli* O157 shedding, as is the spreading of slurry on pasture while the spreading of manure on pasture is negatively associated with *E. coli* O157 prevalence (Gunn et al. 2007).

Increased prevalence and durability of *E. coli* O157 faecal shedding was demonstrated in cattle fed barley compared to those fed corn (Berg et al. 2004; Buchko et al., 2000).

The difference to the fermentation properties of the starch from the grains could be the reason of different prevalence, although the possibility of contamination of the feed could not be eliminated. Corn starch is less readily fermented in the rumen and continues to be fermented in the lower gastrointestinal tract, lowering the

pH and selectively favouring *E. coli* O157 (Berg et al., 2004). The faecal pH was significantly lower in the corn-fed animals: mean faecal pH 5.4–6.3 compared to 6.6–7.5 (Buchko et al., 2000). Furthermore, fermentation conditions give rise to elevated volatile fatty acid concentrations, adverse pH and/or changes in the composition of the resident microflora (Russell et al., 2000).

Incidence in animal at slaughterhouse

Previuolsy published prevalence rates in slaughtered cattle (Chapman et al., 1997; Omisakin et al., 2003; Paiba et al., 2002) have suggested that fewer than 10% of cattle are shedders of *E. coli* O157. In contrast, in a study carried out in the USA, it was found to be 28% overall (Elder et al., 2000). Considering the average from multiple studies, the within herd prevalence of VTEC on the hides of cattle is higher than that in the faeces (Rhoades et al., 2009). A single animal can contaminate directly with its faeces or via the ground and lairage fixtures the hides of many other animals in the herd (Small et al., 2002). This relationship was observed by Barkocy-Gallagher et al. (2003), who detected *E. coli* O157:H7 on 60.6% of cattle presented for slaughter, while the faecal prevalence was 5.9% (Barkocy-Gallagher et al. 2003).

However, American surveys reported that the prevalence of *E. coli* O157 in faeces (27.8%) was higher than that in the corresponding hide samples (10.7%) from positive animals at slaughter (Woerner et al., 2006).

The brisket is most likely to be contaminated from the ground when the cattle rest, whereas the flanks are more likely to be cross-contaminated by other animals, and they should be considered as sampling sites (Elder et al., 2000).

Studies in the UK and Ireland, on pasture-fed animals revealed a lower hide prevalence of *E. coli* O157: 28.8% and 7.3% of bovine hides were contaminated respectively (Reid et al., 2002; O'Brien et al., 2005). Pasture-fed animals experience less animal-to-animal contact than those in feedlots: ground contamination is less concentrated when stocking density is lower (Rhoades et al., 2009).

A significant decrease in hide prevalence of *E. coli* O157 in the winter (29.4%) is linked to lowest faecal prevalence (Barkocy-Gallagher et al., 2003).

Incidence in food

The peak of outbreaks is mostly due to an increase in the consumption of fast foods and to the use of such food preparation that holds a greater risk of foods being improperly cooked.

Most cases are caused by ingestion of contaminated foods, particularly foods of bovine origin. Ingestion of undercooked hamburgers, prepared in a restaurant or homemade, has been highlighted as a particularly important cause of outbreaks (Griffin and Tauxe, 1991).

Other foods, including roast beef and raw milk, and porcine, avian, and sheep meats have been also directly linked to outbreaks (Griffin, 1995).

Traditionally, the safety of fermented meats was assured by the low pH achieved during the fermentation process. However, the emergence of STEC, which exhibit significant acid-resistance and can thereby survive the fermentation process if present in the raw meat ingredients, meant that traditional fermentation processes were no longer sufficient to assure the safety of such products (Riordan et al., 1998).

However, most fermented meat production processes include a heat-lethality step. It has beem shown that *E. coli* O157:H7 has a notable ability to grow in foods of low pH as fermented hard salami, under conditions, where other pathogens would not survive (C.D.C., 1995).

VTEC does not grow below 7°C and so should not proliferate in beef products stored below this temperature. In minced beef stored at 7.2°C for 72 h, a significant growth was not observed and a 1.0 log c.f.u. g^{-1} increase was observed at 10°C (Mann and Brashears, 2006). Good survival on beef has been reported at 7°C for 11 days and -18°C for 84 days (Uyttendaele et al., 2001; Dykes et al., 2001). *E. coli* O157 has no notable heat resistance: reported D-values in lean minced beef include 0.16 and 20 min at 63 and 55°C, respectively (Smith et al., 2001) and 0.39 and 21.1 min at 65°C and 55°C, respectively (Juneja et al., 1997).

The Community Summary Report on Trends and Sources of Zoonoses and Zoonotic Agents in the European Union in 2007 by EFSA reported low occurrence of VTEC in food generally. In fresh bovine meat the proportion of samples positive for VTEC was 0.3% at EU level and 0.1% for the serogroup VTEC O157. The

prevalence of VTEC ranged from 0% to 2.9% and the prevalence of VTEC O157 ranged from 0% to 1.6%. Some MEMBER STATES reported also the recovery of O26, O103, O111, and O113 serogroups from bovine meat: such serogroups are frequently isolated from human VTEC cases. Several MEMBER STATES tested vegetables for VTEC and no samples were found positive (EFSA, 2009).

In an US survey on VTEC in raw meats from grocery stores, no O157:H7 isolates were recovered but a high prevalence of non-O157 *E. coli* was found in beef (23%), pork (4%), lamb (48%), veal (63%), chicken (12%), turkey (7%), fish (10%), and shellfish (5%) (Samadpour et al., 1994). The possibility of cross-contamination and deficiencies hygiene practices in the stores were considered.

Another study showed that 38 of 183 *E. coli* O157 foodborne outbreaks identified (1982 through 2002) were attributable to produce, with 26 outbreaks traced to leafy green vegetables (Rangel et al., 2005). The spinach outbreak in 2006 and two following lettuce-linked outbreaks have heightened consumer awareness of the potential for foodborne illness associated with produce. Contamination can arise from seeds, irrigation water or the use of untreated animal manure harboring the

pathogen. Due to the nature of the contamination, the pathogen may become established in the structure of the plant during its development, making it impossible to eliminate by washing (Itoh et al., 1998). Pathogens can exceed 107 c.f.u. per gram of sprouts produced from inoculated seeds during sprout production, without adversely affecting appearance.

The Food and Drug Administration (FDA) recommends that raw alfalfa sprouts should be considered potentially contaminated and avoided by persons at high-risk such as the young, elderly and immunocompromised.

Raw milk may be contaminated by bacteria present on the cow's udders, hide or on equipment. The sale of raw milk is prohibited or strictly limited in most US states (Bren, 2004), and is subject to restrictions established by Order of December 10, 2008 of Ministry Of Labour, Health and Social Policies in Italy.

Fruit used for juice is typically of poorer quality than that sold as eating-quality fruit. It has been demonstrated that *E. coli* O157 can grow on dropped, damaged or blemished fruit (Dingman, 2000), thereby providing an avenue for pathogen entry (Riordan et al., 2000). In addition, dropped fruit potentially

contaminated with manure may be used in juice-making, and though all produce is washed prior to processing, it is very difficult to completely remove pathogens from fruit with traditional washing procedures (Annous et al., 2001). The low pH associated with products such as apple juice, which had traditionally assured their safety, is insufficient to eliminate surviving STEC. A number of outbreaks linked to the consumption of unpasteurized juice (Besser et al., 1993) led the FDA in 2001 to release Juice HACCP regulations, essentially requiring all juice to be either pasteurized or sold with a warning label.

Incidence in humans

STEC have been identified as the primary cause (up to 90%) of HUS in temperate climates and less commonly *Shigella, Salmonella, Yersinia,* and *Campylobacter* have been implicated.

The number of infections and incidence of O157 STEC per 100,000 population of human infection are 513 and 1.12 respectively, while for non-O157 STEC 205 and 0.45 (FoodNet, 2009). These values remain relatively lower than in

cattle. One possible explanation for this is a difference in the human pathogenic potential between strains of bovine origin.

In 2007, a total of 2,905 confirmed human VTEC cases were reported from 23 EU MEMBER STATES. The EU notification rate was 0.6 per population of 100,000. The most commonly identified VTEC serogroup was O157 (54%). The notification rate was highest in 0 to 4 year old children and this group also accounted for almost 60% of the 103 HUS cases reported, mainly associated with VTEC O157 infections (EFSA, 2009).

During 2003, a total of 178 cases of HUS were reported from 32 US States. Out of these, 118 (66%) occurred among children aged <10 years (CDC, 2005). Lynn et al., (2005) reported an increase in pediatric HUS cases in England and Wales from 50 in 1985, to 1087 in 1997 (Lynn et al., 2005). The increased incidence was attributed to improved laboratory techniques, increased reporting, and recognition of the importance of elucidating the causes of diarrheal disease.

HUS is the most common cause of long-term renal failure in children in the US and Britain (Boyce et al., 1995; Chapman, 1995) and has a reported mortality rate

of 5-10% in the US (Corrigan and Boineau, 2001). Dunne et al. (2000) reported an annual incidence of 10.6 cases per million to 1997 through 1999 for children under 16. Approximately 85% of children with classic HUS recover completely with supportive therapy. However, 15-20% of children may develop hypertension 3-5 years after the onset of disease. Adult patients with HUS have a lower mortality rate; however, the renal prognosis is poor in patients who are not treated. Up to 80% of adults with HUS will ultimately require long-term dialysis or renal transplantation.

The most extensive assessment to date was published by Brooks et al. in 2005, who summarized data from a convenience sampling of sporadic cases from a 20-year-period (1983 to 2002). The top six serogroups identified (O26, O45, O103, O111, O121, O145) accounted for 71% of all the isolates while O26, O103, and O111 accounted for more than 50% of isolates. The non-O157 STEC isolates, as a group, were similar to *E. coli* O157 in seasonality, presence in children, and distribution of Shiga toxin genes. However, serogroup O111 was the only serogroup associated with HUS. Studies carried out in the US lead to the conclusions that non-

O157 STEC serotypes were at least as prevalent as serogroup O157 in diarrheal samples (Fey et al., 2000).

In Italy, human VTEC infections are less common than in the USA or UK. Despite this, a surveillance system for HUS in children has been established since 1988, showing an average incidence of 0.2 cases per annum per 100,000 population

aged 0-15 years between 1988 and 1997 (Caprioli et al., 1994; Caprioli and Tozzi, 1998). Serogroups O157 was by far the most common identified in these outbreaks (Caprioli et al., 1994).

METHODS FOR DETECTION, ISOLATION AND IDENTIFICATION OF VTEC

No single method or approach can be used to detect or isolate all of the *E. coli* pathotypes of concern. Consequently, standard or validated alternative methods have been developed to specifically detect or isolate VTEC O157 from food and animals. For the other serotypes, there are no universally accepted and validated methods, but pragmatic approaches have been produced.

There is no standard protocol for enumeration of VTEC O157 or other VTEC serotypes in food or environmental samples, and, although quantitative data are essential to better understand the human health risks, enumeration of VTEC is generally not conducted as part of routine monitoring or testing programs. Recent advances in molecular detection methods combine the traditional detection methods and target both serotype specific genes, *vtx*, and other virulence genes. However, isolation of VTEC, and subsequent strain characterization is still needed in order to ensure that the detected genes are present on the same bacteria (EFSA, 2007).

Methods to detect VTEC

Cell cultures

The gold standard for the detection of VT employs Vero cells assay. It has been applied to the detection of free VT in faecal specimens to provide evidence of infection and to enrichment cultures inoculated with foods, animal faeces or environmental samples. However, the most usual application of the vero cell assay is for confirmation of toxin production by pure cultures (Konowalchuk et al., 1977).

Immunologically based methods

Immunological methods are now widely used for the detection of VT. The methods utilize VT-specific poly- or monoclonal antibodies. The assay formats include enzyme-linked immunosorbent assays (ELISA) and reversed passive latex agglutination (RPLA).

The Center for Disease Control and Prevention (CDC) evaluated the commercial latex reagents, concluding that they are good alternatives to standard serologic methods for identifying the *E. coli* O157 and H7 antigens when the

manufacturer directions are closely followed. Additional controls with non-O157 antiserum and latex control reagents should be included to rule out non-specific agglutination.

Additionally assays have been also developed and utilize a combination of receptor-specified interactions and antibodies towards VTs (De Boer and Heuvelink, 2000; Baylis et al., 2001; Scheutz et al., 2001; Bettelheim and Beutin, 2003).

Immunoassays can be applied to pure and mixed cultures as enrichment broth inoculated with food or faeces, usually after overnight incubation. Colonies can be submitted to further examination after VT detecting. These methods are generally reliable and most assays are easy to implement in laboratories and do not require expensive equipment.

DNA-based methods

The demonstration of vtx and other virulence associated gene fragments as well as serotype specific gene fragments is a widely used method. Detection of these

targets is achieved by the use of DNA-DNA hybridization probes or by amplification of VT-specific DNA. Numerous DNA-DNA hybridization assays, using oligo- or polynucleotide probes have been described and different formats have been used including dot-blot and replica assays, liquid-based assays, and more recently micro-array chips. Amplification of specific DNA is most frequently achieved by PCR: these techniques involves the use of internal sequence-based probes (particularly in real-time PCR), full DNA sequencing, and the use of fragment analysis following restriction endonuclease digestion.

The DNA-based detection methods can be applied to nucleic acid from pure or mixed cultures (enrichment broth inoculated with food or faeces), as well as colonies growing on solid isolation media. The amplification-based techniques are rapid and have the advantage that it is possible to simultaneously investigate cultures for several genes at the same time. However, when testing mixed cultures, the detected genes might not originate from the same VTEC strain. By using DNA-based methods it is also possible to differentiate between the different VT subtypes.

PCR primers have been developed successfully for several of the categories of diarrheagenic *E. coli*. Advantages of PCR include great sensitivity and *in situ* detection of target templates. However, substances within faeces have been shown to interfere with the PCR, thus decreasing its sensitivity (Stacy-Phipps et al., 1995). Several methods have been used successfully to remove such inhibitors, including Sepharose spin column chromatography and adsorption of nucleic acids onto glass resin (Stacy-Phipps et al., 1995; Lou et al., 1997).

Furthermore, several quantitative PCR methods are used to support in subculturing of selected enrichment broths with priority given to the highest target concentration since there is a correlation between the number of VT copies and the success of isolation of VTEC from an enrichment broth. DNA based methods have the disadvantage of being unable to distinguish between DNA from viable and non-viable cells.

The detection by PCR of Stx-producing strains of any serotype in a clinical specimen is assumed to be significant, while the mere presence of such strains of non-O157 serogroups in food or other nonclinical samples is of uncertain

significance. Frequent loss of *stx* genes upon subculture can also occur (Karch et al., 1995). For these reasons, probes and PCR techniques for additional VTEC virulence factors can often provide additional clinically relevant information that may also have great epidemiological value (Nataro and Kaper, 1998). For example, testing for *eae* and *ehxA* confirms the presence of the LEE pathogenicity island and the large virulence plasmid pO157, respectively, both of which are more commonly found in STEC strains associated with severe human disease (Boerlin et al., 1999; Gannon et al., 1993; Schmidt et al., 1995).

Some early studies used a single primer pair to detect both *stx1* and *stx2* (Karch and Meyer, 1989; Paton et al., 1993), but the most actual methods now include two primer pairs to distinguish the different-sized products for *stx1* and *stx2*. Not all primer pairs designed for *stx2* will detect all variants of this gene (Ramotar et al., 1995), and specific primer pairs for *stx2c* (Tyler et al., 1991; Rüssmann et al., 1994) and *stx2e* (Thomas et al ., 1994) have been designed.

Real-time PCR is increasingly used as a rapid, sensitive and specific molecular diagnostic technique for the testing and identification of VTEC from biological and environmental samples (Bellin et al., 2001).

Methods to detect specific serotypes and immunoconcetration separation

The methods based on the specific detection of VTEC strains belonging to serotype O157, and also to some of the most common serotypes considered to be pathogenic to humans are based on:

• The use of selective/differential media such as Sorbitol MacConkey (SMAC) agar detecting non-sorbitol fermenting or the rhamnose MacConkey agar to detect VTEC O26 and/or β-glucuronidase-negative *E. coli* O157 strains,

The inability of *E. coli* O157:H7 to produce b-glucuronidase can be tested by using media supplemented with 4-methylumbelliferyl-β-Dglucuronide (MUG) (Thompson et al., 1990). Hydrolysis of MUG by most non-O157:H7 *E. coli* strains produces a fluorescent compound, and this substrate has been incorporated into some

agar media: Rainbow Agar O157 (Biolog Inc., Hayward, Calif.) Fluorocult *E. coli* O157 agar (Merck, Darmstadt, Germany).

• Serotype-specific enrichment procedures, based on immunocapture techniques: these include the use of serotype-specific LPS-antibodies coated to paramagnetic beads (immunomagnetic separation, IMS) or other supports. Originally developed for VTEC O157, these reagents are now available for other main VTEC serotypes, including O26, O103, O111, and O145.

The IMS Dynabeads (Dynal, Invitrogen, Norway) procedure is widely used, it consists of several handlings, including a number of washing steps to remove non-(specifically) bound bacteria and matrix components. Prior to plating onto selective isolation media, VTEC cells present in the enrichment culture are selectively concentrated by magnetic beads with *E. coli* O-specific antibodies covalently bound onto their surface. The IMS procedure can be performed both manually and automatically (Islam et al., 2006). IMS is applicable for isolation of VTEC O157 from artificially mixed bacterial cultures, inoculated meat samples, and inoculated as well as naturally contaminated bovine faeces (Fratamico et al., 1992; Chapman et al.,

1994; Wright et al., 1994; Bennett et al., 1996; Heuvelink et al., 1997). When applied to artificially contaminated samples, the methods usually enable the detection of less than 1 c.f.u. per gram of sample (EFSA, 2007).

Karch et al. (1996) evaluated the IMS as the most sensitive of all detection techniques, even more sensitive than PCR, which required approximately 10^5 c.f.u. of O157 organisms per g of stool to yield a positive result, compared to 10^2 for IMS.

However, recent experimental studies shown that IMS has a higher recovery rate for sorbitol-negative O157 and lower affinity for O111 and O145 serotypes (Verstraete et al., 2010).

Another example of immunoconcentration is the fully automated Vitek Immunodiagnostic Assay System (VIDAS) (bioMérieux, Marcy l'Etoile, France). The VIDAS Immuno-Concentration (ICE) kit includes two ready-to-use components. One is a Solid Phase Receptacle (SPR), which serves as the solid phase as well as the pipetting device for the assay. The interior of the SPR is coated with anti-*E. coli* O-specific antibodies adsorbed on its surface. The other component is a strip which contains all the wash and release solutions. An aliquot of the enrichment culture is

manually transferred into the strip and subsequently the sample is cycled in and out of the SPR. *E. coli* O157 present in the broth will bind to the anti-*E. coli* O157 antibodies coating the interior of the SPR. Unbound sample components are then washed away. A final enzymatic step releases the captured *E. coli* O157 into a specific well from which they can be plated onto selective agar (Islam et al., 2006). *E. coli* serotyping can be performed by agglutination reactions using antisera raised in rabbits against 179 O and 56 H serogroup antigens standard references strains. Serotyping, however, can generally only be performed in specialized laboratories, is labor-intensive, and may require several days to complete. Moreover, cross-reactivity of antisera with multiple O or H serogroups frequently occurs (Fratamico et al., 2003).

The presence of specific serotypes can be also detected by PCR amplification of O-antigen-specific DNA sequences.

Isolation and enrichment of VTEC O157

Isolation

VTEC O157 are usually both unable to ferment sorbitol within 24 hours of incubation and lack β-glucuronidase activity unlike other *E. coli* (March and Ratnam, 1986; Thompson et al., 1990). These characteristics are exploited in the routine selective isolation of VTEC O157. The most widely used solid medium for the detection of non-sorbitol fermenting VTEC O157 is sorbitol MacConkey (SMAC) agar. Sorbitol-nonfermenting colonies, indicative of *E. coli* O157:H7, are colorless on this medium. Media that simultaneously indicate sorbitol fermentation and β-glucuronidase activity have also been developed, including different chromogenic media. The addiction of selective supplements, as cefixime, a third generation cephalosporine, and potassium tellurite (e.g. CT-SMAC) (Zadik et al., 1993) increases the selectivity of the different solid media. However, some VTEC O157 strains are sensitive to cefixime and potassium tellurite and therefore may not be detected on CT-SMAC agar (MacRae et al., 1997).

Individual colonies suspected to be VTEC O157 should be tested for the O157 antigen by using VTEC O157 antiserum or latex agglutination reagents. Isolates agglutinating with O157 antiserum should be confirmed as *E. coli* by biochemical reactions, since other species and VTEC non-O157 can cross-react with O157 antiserum. The confirmation of VT production or the presence of *vtx* genes it is necessary in all cases (EFSA, 2007).

Human infections with sorbitol fermenting (SF) VTEC O157 have been increasingly recognised in many EU MEMBER STATES (EFSA, 2007). Although the methods used for VTEC O157 are not validated for the detection of SF VTEC O157, these organisms can be detected by testing sorbitol fermenting colonies grown on solid media that do not present typical VTEC O157 colonies for the O157 antigen.

Enrichment techniques

Enrichment methods are applied to animal faeces, food and environmental samples because usually contain low numbers of VTEC O157 together with an abundant microbial flora.

Human clinical stool specimens require a selective enrichment step when they contain low levels of VTEC only. The most widely used media for the enrichment of VTEC O157 are tryptone soya broth (TSB) (mainly for food) and buffered peptone water (BPW) (for human and animal faeces). These broths may be supplemented with different selective agents such as novobiocin, vancomycin, cefsulodin, cefixime, and bile salts (Doyle and Schoeni, 1987; Chapman et al., 1994).

There are two different lines of thought with regard to temperature (37°C versus 42°C) and the time (6-8 hours incubation versus overnight incubation) of incubation for all types of samples.

In fact, the required incubation period depends on the competing microflora. Standard methods for food include the analysis of both the 6- and 18-h incubation enrichment cultures. A 6-8 hour incubation of the enrichment broth increases the sensitivity when analysing matrices with a high number of background flora and it is recommended when testing matrices where *E. coli* has a short-lag time before onset of growth, as for example with minced meat products. However, in case of stressed or sublethally-injured VTEC O157, a more long incubation time is necessary.

Isolation and enrichment of VTEC non-O157

Isolation

Procedure of subculturing onto solid media where single colonies are then tested for the presence of different O antigens by slide-agglutination with O-specific sera or pools of sera is suitable for the detection of non-O157 serotypes.

Enrichment and immuno-separation techniques

The limited specificity of culture methods for non-O157 VTEC serotypes, has led to the provision of beads coated with antibodies to the O antigen for O26, O103, O111, and O145 serotypes. IMS-based detection of serotypes other than O157 are similar to those for the detection of *E. coli* O157: enrichment, IMS followed by seeding onto selective indicative agars, although these have not yet been sufficiently validated. Selective agents to improve the isolation of VTEC O157 (e.g. novobiocin) may inhibit the growth of some VTEC non-O157 (Vimont et al., 2007).

However, many VTEC non-O157 serotypes (O5:H-, O26:H-, O26:H11, O91:H21, O111:H-, O111:H8, O104:H11, O113:H21 and O157:H8) are capable of growing on media supplemented with vancomycin, cefixime, and cefsulodin.

MacConkey agar with rhamnose addiction and supplemented with cefixime and potassium tellurite (CR-SMAC) is the optimal agar for the growing of VTEC O26, giving the most effective suppression of background microflora (Catarame et al., 2003; Murinda et al., 2004). Serotype-specific detection of VTEC O26 was achieved by selecting cefixime-tellurite-resistant, MUG-fluorescent, rhamnose-non-fermenting colonies.

A nonselective, but differential plating medium is enterohaemolysin agar (washed sheep blood agar supplemented with calcium) which may be suitable for isolation of all VTEC strains including O157 (Beutin et al., 1989). Enterohaemolysin producing *E. coli* grows on this medium by small turbid zones of haemolysis around the colonies occurring after 18 to 24 h incubation at 37°C. To improve the selectivity of the medium, antibiotics such as novobiocin and cefsulodin may be used.

Alternative methods to detect specific serotypes

Typing and subtyping of strains is a valuable aid to identify outbreaks, detect them at an earlier stage, trace their sources, and increase the specificity of case definition (Nataro and Kaper, 1998). The main methods currently used for typing and subtyping include pulsed field gel electrophoresis (PFGE) (Gerner-Smidt et al., 2006), phage-typing (Ahmed et al., 1987) and Multiple Loci VNTR Analysis (MLVA) (Hyytia-Trees et al., 2006). PulseNet USA, established in 1996, is a network of federal and state public health agencies that use a standardized PFGE protocol to subtype *E. coli* O157 (Gerner-Smidt et al., 2006). The PulseNet network has been implemented in Canada, Europe, the Asia Pacific region, and Latin America who, along with the USA, work together to share molecular epidemiological information (Gerner-Smidt et al., 2006).

Several non-culture-based assays can be applied to detect specific VTEC serotypes as ELISA, immunoblots and dipsticks. Specific immunoassays for VTEC non-O157 serotypes are very limited, cause false-positive results may be generated because of cross-reaction with surface antigens of other bacteria. Thus, it is not

possible to rely solely on these rapid immunological tests to identify VTEC serotypes without cultural isolation and further characterization (EFSA, 2007).

Phage typing can separate O157:H7 strains into 66 different phage types (Frost et al., 1993), but this technique is available only in reference centers that possess the typing phage.

PCR techniques are able to target a limited number of other VTEC serotypes identified as clinically important to humans as O26, O111, O145 and O103 (Paton and Paton, 1999; DebRoy et al, 2004; O'Hanlon et al, 2004; Perelle et al., 2007).

Standard methods available for food

Standard methods for VTEC O157

Horizontal methods applicable for culture-based detection of VTEC O157 in all types of foods and feeding stuffs have been issued by the Nordic Committee on Food Analysis (NMKL) and the International Organization for Standardization (ISO). Both methods (NMKL No. 164, 1999 and ISO 16654: 2001) prescribe the use of TSB supplemented with bile salts and novobiocin (mTSBn) for the pre-enrichment

step, with an incubation period of 6-8 hours as well as 18-24 hours at 41.5°C. Furthermore, they recommend to execute an immunomagnetic affinity purification step and to subculture the immunomagnetic particles with adhering bacteria onto CT-SMAC and the user's choice of a second selective isolation agar. These two standard methods are widely being used.

Standard methods for non-VTEC serotypes

At present, an international standard method for the detection and isolation of VTEC non-O157 is not available. However, under the authority of Working Group 6 of the Technical Committee 275 of the European Normalisation Committee (CEN TC275/WG6) the preparation of an European Standard proposal based on a PCR-based horizontal method is still in progress.

The German organisation for standardisation (DIN) has published two documents on the detection of VTEC in food derived from animals. DIN 10118:2004 specifies a method based on immunochemical detection of VTs in enrichment cultures and isolation of presumptive VTEC from VT-positive cultures by immunoblot, followed by confirmation of the positive colonies. The technical rule

BVL L 07.18-1 (2002) describes a method for detection, isolation and characterisation of VTEC from minced meat by PCR and colony DNA hybridization.

The French Food Safety Agency (AFSSA) recommends a PCR-based method that is suitable for detecting VTEC belonging to the main pathogenic serotypes, including O157.

According to definition by AFSSA, VTEC strains have to: harbour *vtx* genes (*vtx1* and or *vtx2*) and the *eae* gene and belong to one of the following serotypes: O157, O26, O111, O103, or O145. Screening is based on two PCR steps: the first one allows the detection of the *vtx* genes, and the second one the genes encoding the LPS of the five major VTEC serotypes associated with human diseases: O157, O26, O103, O111 and O145. The isolates positive for *vtx* genes and one or more of these O antigen genes are further submitted to an IMS step for the respected O antigen(s) detected by PCR.

Methods for detection in animal faeces and environmental samples

Since there is no internationally recognised standard method for the isolation of VTEC from animals, the ISO 16654 and the NMKL No. 164 methods have been adapted to faeces and have been used in many studies published in the literature.

The OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2004 describes an IMS-based method for screening animal faeces for VTEC O157.

The detection of VTEC in drinking, source, waste and recreational waters is preformed for specific purposes such as source tracing and epidemiological studies and is carried out by filtration followed by enrichment and immuno-magnetic separation in an analogous method to that for food. However, transient contamination that caused disease may not be detected at the time of sampling.

Quantification of VTEC

There is no standard protocol for enumeration of VTEC O157 or other VTEC serotypes from food or environmental samples. Although quantitative data are

essential to better understand the human health risks, enumeration of these pathogens is generally not carried out as part of routine monitoring. For bovine and beef samples, there are limited published studies in the literature reporting successful enumeration of VTEC O157 (Arthur et al., 2004; O'Brien et al., 2005; Carney et al., 2006; LeJeune et al., 2006). These approaches included the subculturing dilutions of the sample directly onto SMAC or CT-SMAC. The detection limit of this technique is generally low and stressed or sublethally injured bacteria may not be recovered.

An alternative approach is to use an MPN (most probable number) method (Fegan et al., 2004).

Some studies have attempted to estimate numbers of O111, O26, O145, O103 VTEC serogroups from bovine and ovine samples, but the absence of a media that clearly differentiates colonies of different serotypes means that this presents considerable technical difficulties since many colonies must be identified using serological or molecular methods. This indicates that enumeration of VTEC as part of a routine monitoring program would be very complicated.

CONTROL OF VTEC IN FOODCHAIN

Identifying and modifying management strategies associated with faecal shedding could reduce animal exposure and transmission. An advantage of this approach is that it tends to target management practices related to faecal-oral transmission, and thus could have an impact on enteric pathogens in general, rather than specifically VTEC. Dietary changes in food animals during the days before slaughter may reduce fecal shedding of bacteria. Feed deprivation could result in increased shedding of *E. coli*, a sudden decrease in hay intake increased *E. coli* load in cattle feces (Brownlie and Grau, 1967) and diet change from alfalfa pellet to poor quality forage increased fecal shedding of *E. coli* O157:H7 in experimentally infected sheep (Kudva et al., 1995). However, current knowledge of the factors affecting the transmission, colonization, and shedding of *E. coli* O157:H7 is insufficient to develope pre-harvest pathogen reduction strategies.

Another option for controlling *E. coli* O157 in cattle is to increase herd resistance to infection, using probiotics, vaccination, antimicrobials, sodium chlorate, and bacteriophages (Callaway et al., 2004; Lonergan and Brashears, 2005; Sargeant

et al., 2007), although all these approaches require further investigations in order to verify their effectiveness.

At present, on-farm control programs are voluntary and tend to consist of general good production practices that are not targeted specifically at VTEC control (Haines, 2004).

For these reasons, most of the control interventions have to be carried out on animals and on carcasses in abattoir.

Prior to pelt removal, the underlying carcass tissue is considered to be sterile due to the inability of microorganisms to penetrate the fleece/hide (Gill, 1998; McEvoy et al., 2004). When this sterile tissue is exposed during the slaughter process, the carcass is inevitably contaminated with bacteria which may include human pathogenic species.

The two main sources of pathogenic bacteria on cattle and sheep carcasses are the fleece and viscera but contamination from the latter is rare and less significant when compared to the one associated to the fleece (Mulcock, 1965; Gerrand, 1975; Grau et al., 1986). The transfer of bacteria from the fleece to the carcass may be the

result of direct contact between the fleece and the carcass or occur indirectly via the knives and carcass manipulation during cutting prior to pelt removal (Biss and Hathaway, 1996). Contamination of carcass often occurs during dressing: primarily during the skinning but also during the evisceration. In addition, air and/or water-borne contamination is also possible. (Elder et al, 2000).

The Regulation EC 853/2004 lays down specific hygiene rules with regard to animal origin foodstuff for food business operators, but does not give indication on how microbiological parameters to be met. These parameters are specified in the Regulation EC 2073/2005, raw meat and meat products section. With regards to process hygiene in slaughterhouses, Reg. EC 2073/2005 contains microbiological criteria for carcasses: *Enterobacteriaceae* (according to ISO 21528-2) and aerobic colony count (according to ISO 4833) must be sought. Sampling is carried out in the post-slaughter phase and prior to cooling.

In case of unacceptable results, an improvement in slaughter hygiene and the review of process controls are compulsory.

In order to set a microbiological standard for end-products and to significantly reduce the risks for consumers, the application of VTEC O157 as parameter is sometimes inappropriate. However, the definition of microbiological guidelines aimed at reducing the faecal contamination along the food chain can help to reduce risks to public health, including VTEC.

TABLE NO.2: Regulation EC 2073/2005: meat process hygiene criteria

Food category	Micro-organisms	Sampling plan (1)		Limits (2)		Analytical reference method (3)	Stage where the criterion applies	Action in case of unsatisfactory results
		n	c	m	M			
2.1.1 Carcases of cattle, sheep, goats and horses (4)	Aerobic colony count			3,5 log cfu/cm^2 daily mean log	5,0 log cfu/cm^2 daily mean log	ISO 4833	Carcases after dressing but before chilling	Improvements in slaughter hygiene and review of process controls
	Enterobacteriaceae			1,5 log cfu/cm^2 daily mean log	2,5 log cfu/cm^2 daily mean log	ISO 21528-2	Carcases after dressing but before chilling	Improvements in slaughter hygiene and review of process controls

The hygienic condition of carcasses and fleece when presented for slaughtering are of fundamental importance, and a HACCP based policy of cleaning must be applied. This should include slaughter of high-risk animals at the end of the day, reduction of the slaughter-line speed, thorough cleaning of operator hands, arms and aprons before and during the pelt removal process (Hudson et al., 1998), use of the inverted dressing procedure (Bell and Hathaway,1996; Whyte et al., 2002), greater spacing between carcasses and in some cases rejection of the animals.

Carcass chilling is not likely to have any significant effect on *E. coli* prevalence or counts (McEvoy et al., 2004). The concentration of VTEC should not increase when chill conditions are well controlled, but cross contamination may occur to cuts and surfaces with distribution of the pathogen throughout the ground meat.

Interventions include the use of chemicals or antimicrobial products, knife trimming, vacuuming, washing, stream pasteurization, multiple hurdle interventions, gamma irradiation, low-dose low-penetrating radiation, and good manufacturing practices in the processing line (Koohmaraie et al., 2005; Edwards and Fung, 2006).

In many countries, public health regulations lay down minimal cooking temperatures and outline good food hygiene practices in restaurants and commercial food processing facilities. At home, high-risk food-handling, preparation, and consumption practices are common, revealing the need for food safety education of consumers. Programs aimed at consumer food-safety education, point up the need for frequent washing of hands and surface areas in contact with food, separation of foods during storage and preparation to avoid cross-contamination, proper cooking

temperatures to kill pathogens that may be present, and prompt refrigeration of purchased and leftover foods (www.fightbac.org).

MONITORING SCHEMES

According to the EC Zoonoses Directive 2003/99, EFSA proposed technical specifications for monitoring schemes for VTEC: "Scientific Opinion of the Panel on Biological Hazards (BIOHAZ) - Monitoring of verotoxigenic *Escherichia coli* (VTEC) and identification of human pathogenic VTEC" and the "Technical specifications for the monitoring and reporting of verotoxigenic *Escherichia coli* (VTEC) on animals and food (VTEC surveys on animals and food)". The aim of these reports is to obtain comparable data on the occurrence of human pathogenic VTEC in animal populations and food such that epidemiological relationships can be established to link reservoirs of human infection (particularly for VTEC non-O157) to their sources as well as measuring the effects of any interventions designed to prevent this group of bacteria from entering the food chain.

At the beginning, monitoring is concentrate on VTEC O157, but should be extended to other serotypes (O26, O103, O111 and O145) that are identified as pathogenic for humans, based on the periodical analysis of human disease and epidemiological data.

However, recently new methods are constantly being developed and as new methods are standardised at EU level it may be possible to expand the monitoring programme to include other relevant serogroups (e.g. O91).

VTEC isolates from animals, food and the environment, as well as those from human infections, should be characterised by serotyping (including molecular serotyping), phage typing for VTEC O157, *vtx* gene subtyping and detection of *eae* genes using harmonised methods. Strain characterisation could be completed by molecular typing (PFGE, MLVA) and comparison of isolates from human and non-human sources may be useful in providing data on the spread of specific strains in the future.

Monitoring of cattle is of primary importance (Hussein and Bollinger, 2005). After cattle, sheep and goats are the second most important reservoirs (Rey et al.,

2003; Ogden et al., 2004; Lenehan et al., 2007). Since data on the occurrence VTEC non-O157 is scarce, EFSA recommends cattle, sheep and goats should be initially investigated as possible reservoirs for these serotypes.

Water buffalo, game animals and wild ruminants, should be submitted to additional monitoring according to the national differences in animal populations, farming practices and food consumption patterns.

Monitoring should be carried out at various levels:

- At farm level to provide information on the spread of VTEC in the environment.
- At the abattoir. In fact, good hygiene practices, according to process hygiene criteria of Regulation (EC) 2073/2005 (*Enterobacteriaceae* and in generic *E. coli*), is likely to be the most effective method for reducing the public health risks for VTEC infection. However, compliance with the hygiene criteria does not guarantee the absence of VTEC or its presence in low counts.
- Slaughtered cattle at 3-24 months and sheep at 4-12 months age, are eligible for sampling

Samples that should be considered for monitoring are faeces of the animals after the slaughter, hide and fleece and pre-chill carcasses. In particular, data on the presence of VTEC on hides or fleece provide a starting point for quantitative microbial risk assessment models (Duffy et al., 2006).

Sampling of the animal faeces at the abattoir reflects what is present in the animal population being presented for slaughter and generate valuable information on the occurrence of super-shedder animals.

Cattle hide and small ruminants fleece represents a key source of VTEC contamination into the slaughter plants reflecting the cross-contamination during transport and lairage. (Elder et al., 2000; O'Brien et al., 2005; Arthur et al., 2007; Lenehan et al., 2007; Mather et al., 2007).

Sampling of cattle and small ruminant carcasses will provide an indication of VTEC contamination on muscles. The microbiological sampling procedure for fresh meat carcasses, set out in Commission Decision 2001/471 and Regulation (EC) No 2073/2005, can be well applied to VTEC.

- At the processing of meat including deboning and trimming. Sampling raw meat cuts or trim reflects cross-contamination which occurs during deboning and trimming meat from the primal cuts and the effect of washing and chilling.
- Foodstuffs subjected to faecal contamination from animals. Although compliance with the hygiene criteria does not guarantee the absence of VTEC at concentrations sufficient to cause human disease, good hygiene practices at processing plants, including monitoring for microbiological indicators (*Enterobacteriaceae* and in generic *E. coli*) are likely to be the most effective method for reducing the public health risks for VTEC infection. Therefore, monitoring take into account compliance with the criteria of the Regulation (EC) No 2073/2005, the presence of VTEC in high risk foodstuffs and other risk-based supporting data. Furthermore, application of efficient validated HACCP-procedures for production of raw ready-to-eat meat, meat preparations and other foods is important to reduce the public health risks for VTEC infection. At the same time, sampling of raw minced meat, meat products, ready-to-eat fermented meats, fresh produce, unpasteurised milk and derived dairy products.
- Water and environmental sources are useful only for epidemiological investigations.

National Reference Laboratories for *E. coli* (NRLs) are the laboratories responsible for the analyses of the samples. However, the competent authorities may decide to designate other laboratories involved in official controls of food and animal samples to perform the analyses, under the supervision of the NRLs. These laboratories should have demonstrated experience of using the required detection methods, and must have an accredited quality assurance system complying with ISO 17025 (ISO 2005).

The competent authority responsible for the preparation of the yearly national report on zoonoses pursuant to Article 9 of Directive 2003/99/EC should ensure that the results of the surveys are collected, evaluated and reported to the annual Zoonoses report to the Commission and EFSA.

VTEC IN SHEEP

Healthy sheep carry VTEC at similar rates to cattle but examples of disease-associated AEEC in sheep are rare and none refers to a VT-producing strains.

Although the last EU MEMBER STATES report showed that the highest reported proportions of VTEC positive sheep were 1.4% (EFSA, 2009), several studies report higher prevalence rates: 45% in Australia (Bettelheim et al., 2000) 67% in Germany (Beutin et al., 1993) 68 % in Spain (Blanco et al., 2003).

VTEC O157:H7 strains have been detected in sheep faeces or on carcasses at slaughter (Blanco, 2001b), but sheep have been mainly cited as reservoirs for a diverse number of non-O157 serogroups associated with disease in humans. Thus small ruminants can play an important role to shedding these pathogens through the faeces (Blanco et al., 2003; La Ragione et al., 2008). Since mutton and lamb carcasses can be contaminated with faecal *E. coli*, the possibility that VTEC are present on the carcasses can not be excluded.

As for cattle, the occurrence in sheep shows a seasonal variation, with the warm season giving the highest incidence (Kudva et al., 1996, 1997).

Age-related differences in VTEC serotypes range can be detected: 6 weeks old lambs had the highest prevalence of VTEC and the greatest variety of serotypes. Lambs, especially during natural suckling, are more easily contaminated with VTEC and can present scattered lesions in the intestine, particularly in the large one. Furthermore, it has been suggested that lambs may have distinct populations of VTEC (Djordjevic et al., 2004). The differences in VTEC serotypes may be related to the effect of age on the physiological differences in the digestive tract, on the diet, or on the immune response to these organisms.

The general health status of the ovine host may have a major influence on persistence and high-level shedding of *E. coli* O157:H7, and it may be hypothesized that concomitant infection by *Cryptosporidium parvum* or immunosuppressive viruses (border disease virus or maedi-visna virus) could influence AEEC colonization and persistence. Furthermore, colostrum deprivation increases

susceptibility to colonization by this pathogen in young lambs (La Ragione et al., 2005b, 2006).

Colonization of the gastrointestinal tract may occur throughout the distal region and be diffuse, not preferentially targeted to the rectoanal junction (RAJ) as in cattle. However within the flock some animals are considered to be persistent shedders and have *E. coli* O157 organisms throughout the entire gastrointestinal tract, rather than just the large intestine (Woodward et al., 2003).

Most commonly found serotypes in sheep

Studies carried out by Beutin et al. (1997) compared the clonality of natural populations of *Escherichia coli* strains in cattle and sheep, and concluded that the two species were completely different with respect to the O:H types of the VTEC flora, indicating a host serotype specificity. In the same way, the serovars of positive *E. coli* isolated from sheep are dissimilar to those from goats (Cid et al., 2001; de la Fuente et al., 2002; Aktan et al., 2004).

Ovine VTEC strains belonging to a total of 50 O serogroups and 101 O:H serotypes have been isolated in Spanish studies (Rey et al., 2003; Table 1). However, only a reduced number of serotypes (O5:H-, O91:H-, O128:H2, O146:H8 and O146:H21) has been the most frequent and commonly found in sheep populations from different countries.

Worldwide, sheep have been shown to shed several non-O157:H7 VTEC in their faeces. For example, serotypes O5:H−, O69:H8, O75:H−, O75:H40, O91:H−, O123:H−, and O163:H19 were detected in Australia (Bettelheim et al., 2000; Djordjevic et al., 2001). In the USA, other non-O157:H7 VTEC (i.e., O5:H−, O6:H10, O6:H49, O88:H−, O91:H−, O128:H−, O128:H2, and O146:H21) were also detected (Kudva et al., 1997; McCluskey et al., 1999). In Germany, serotypes O5:H−, O6:H10, O30:H12, O71:H12, O77:H4, O90:H21, O90:H24, O91:H−, O119:H25, O123:H10, O125:H14, O125:H26, O128:H2, O136:H20, O146:H8, and O146:H21 were also reported (Beutin et al.,1993,1997). The predominant ovine serotype in Germany, Spain and USA is O91:H-, and several of these VTEC serotypes (e.g., O5:H−, O88:H−, O91:H−, O128:H−, O128:H2, O146:H8,

O146:H21, and O163:H19) have been associated with sporadic cases or most severe outbreaks of human illnesses (Stockbine N.A. et al., 1998).

The ecology of *E. coli* O157:H7 in sheep has been extensively studied by Woodward and colleagues (Wales et al., 2001a, b; Woodward et al., 2003), and A/E lesions were only detected in the 6 day old animals, but no clinical disease was observed (Wales et al., 2005).

The distal intestine including the caecum, colon and rectum, has been suggested as preferred site of colonization of *E. coli* O157:H7 in ewes, although detection of A/E lesions is rare (La Ragione et al., 2005a, 2006).

More frequently, A/E lesions induced by *E. coli* O157:H7 have been identified in the small and large intestines of lambs (Wales et al.,2002; Woodward et al., 2003) and O157:H7 organisms have been detected in the terminal rectum (Grauke et al., 2002).

Sheep AEEC isolates carry various intimin types (Aktan et al., 2004; Cookson et al., 2007), and it may be hypothesized that different intimin types facilitate tropism to different specific sites in the gastrointestinal tract, as for cattle

and humans, or perhaps that some intimin subtypes play no role in the colonization of AEEC in sheep.

TABLE NO. 1. O:H serotypes of VTEC isolated from sheep. (Rey et al., 2003)

O2:H?	O75:**H8**,H40,H55,**H-**	O119:**H25**,**H-**
O4:**H10**,H25,**H-**	O77:**H4**	O120:H-
O5:H28,**H-**	O79:**H14**	O123:H10,H-
O6:**H2**,H10,**H12**,**H28**,**H49**,**H-**	O84:H16,**H-**	O125:H14,H26,**H-**
O8:**H9**,H16,H49	O85:H49	O126:**H8**
O9:**H-**	O87:H2,H16	O128:**H2**,**H8**,**H10**,H16,H21,**H-**
O11	O88:**H-**	O136:H20,H40,H-
O15:**H-**	O90:H21,H24,**H-**	O141:**H-**
O21:H21,H28	O91:H2,**H21**,H28,H29,**H-**	O146:**H8**,**H21**,**H-**
O26:**H11**	O98:H7	O153:**H25**,**H-**
O30:H12	O103:**H2**,H38	O156:H11,H25,**H-**
O35:H7	O104:**H7**	O157:**H7**,H21,**H-**
O42:H?	O106:H2,H20	O163:H11,**H19**,**H-**
O49:**H-**	O110:**H-**	O165:**H-**
O52:H12,H45	O112:**H21**,H-	O166:**H28**,H49,H-
O55:H2,**H-**	O117:**H-**	O174(OX3):**H2**,**H21**
O71:H12,**H-**	O118:**H12**	

EXPERIMENTAL PLAN

The aim of the present study was to evaluate the role of the sheep as VTEC reservoir and to detect the prevalent serogroups and serotypes over the sheep chain production.

The results of the present study will be useful to explain the epidemiological trend related to the prevalence of the VTEC in lambs and adult sheep from Sarda breed. Moreover the definition of virulence profile of the strains will be useful to define the risk related to the spread of the pathogens in the environment and in the food.

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The application of phenotipical and molecular typing methods will contribute to define the prevalent serotypes and subtypes of VTEC in the sheep and sheep products and to trace the contamination route in relation to the animal and environment-linked variables over the sheep chain production.

The experimental plan of the present study consisted of two different parts. Part I was carried out next to the Animal Biology Department of the University of Sassari (Italy). Part II was carried out next to the Food Safety Department of the Ashtown Food Research Centre (Teagasc) of Dublin. In Figure No. 1, a flow diagram of the detection of VTEC performed in this study is reported.

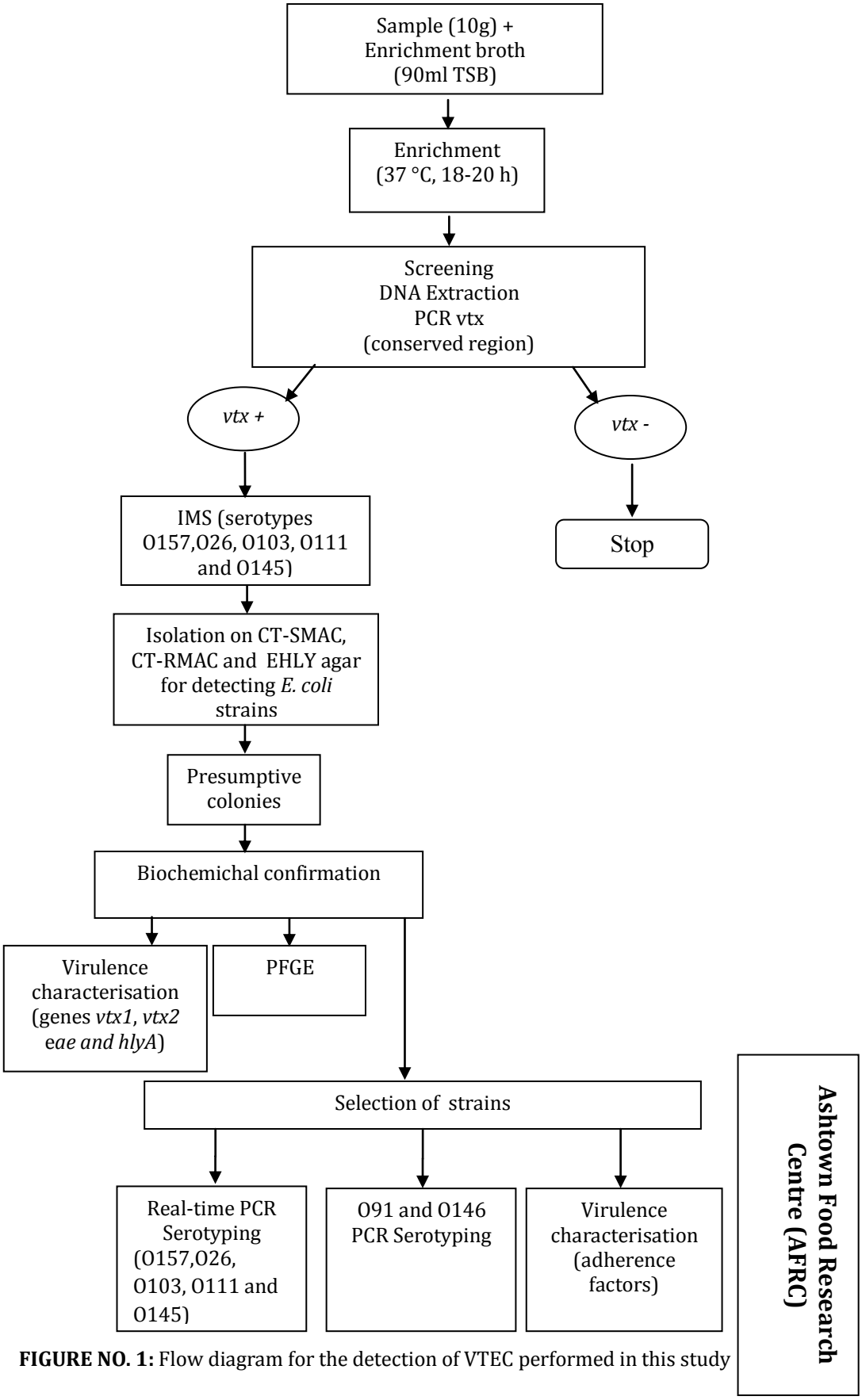


FIGURE NO. 1: Flow diagram for the detection of VTEC performed in this study

MATERIALS AND METHODS

PART I

ANIMAL BIOLOGY DEPARTMENT, UNIVERSITY OF SASSARI (ITALY)

Nineteen sampling sessions were carried out from January 2009 to May 2010. Specimens were collected from three industrial capacity slaughterhouses (*Sa*, *Sb*, *Sc*) located in Sardinia, in the Sassari and Nuoro provinces. Specimens were taken regularly from local ewes (n.50) and suckling lambs (n.45) from 60 flocks. During each visit 5 subjects were randomly sampled.

Plant *Sa* was visited 7 times (140 samples), plants *Sb* and *Sc* 6 times (120 samples each).

A total of 380 specimens were collected from different matrices:

- *gut mucosa* (n.95): immediately after evisceration a 10g rate was removed from colon and rectum using sterile scalpel;

- *hide* (n.95): a 20 cm^2 sized sample was taken nearby the incision line from the brisket and the belly of the carcass, using scissors, tweezers and sterile scalpel;
- *carcass surfaces* (n.95): after skinning, samples were collected with non-destructive methods by rubbing a sterile disposable polyurethane sponge (8cm x 2cm x 1cm), over an area of 300 cm^2 on three sites (chest, abdomen, thigh);
- *feaces* (n.95): after evisceration, a 30 g rate was taken from rectum using sterile spoons.

Hides and sponges were combined into a single pool of each representative sample.

All specimens were placed immediately in sterile containers, stored at refrigeration temperature (4°C), transported to the laboratory and subjected to analysis for determination of *E. coli*.

PRELIMINARY SCREENING OF VTEC BY PCR

In order to make a preliminary screening of VTEC in collected samples, a simplex PCR was performed on all the ovine specimens.

A rate of 10 g of each sample was subjected to selective enrichment in the ratio 1:10 using modified Tryptone Soya Broth (Oxoid, Italia, Milano) supplemented with novobiocin (20 mg/l) (Sigma-Aldrich, Italia) and incubated at 37 °C for 18-20 h.

Step 1: *DNA extraction*

A rate of 1 ml of each enrichment was dispensed into 1.5 ml microcentrifuge tubes for DNA extraction. The supernatant was removed after a preliminary centrifugation (5' at 10.000X) and the pellet suspended in 300µl of Chelex® 100 resin (BioRad, USA). After a second incubation at 56ºC for 20', the microtubes were placed in a water bath at 100ºC for 8'.Finally, the microtubes were left on ice for 2' and immediately centrifuged for 5' at 10.000X at 4°C. Supernatant was transferred into a new microtube. The obtained DNA was kept on ice until use.

Step 2: *DNA amplification*

According to the EU Food PCR Project method (EU-project: QLK1-1999-00226) with some modifications in the temperatures used for amplification, the specific target sequence of VTEC was performed in order to determining the genes *stx1* and *stx2*.

MK1 and MK2 primers were used:

MK 1: 5'- TTT ACG ATA GAC TTC TCG AC -3'

MK 2: 5'- CAC ATA TAA ATT ATT TCG CTC-3'

This set of primers leads to an amplified of 230 bp approximately of a common sequence in the genes *stx1* and *stx2*.

In order to improve the application of the method, two reference strains, *E. coli* O157:H7 EDL933 ATCC 35150 and *E. coli* O157:H7 NCTC 12900, were use used as positive and negative control respectively. In order to prevent and control contamination each PCR test included:

a) reagent control (without DNA);

b) control of Chelex 100 resins used for DNA extraction.

The amplification program was:

- Pre-incubation: 94°C for 1',

n.35 cicles of:

- Denaturing: 94°C for 30'',
- Annealing: 48 °C for 30'',
- Extension: 72 °C per 30'',

Final incubation: 72° C for 4'

Step 3: electrophoresis conditions

After setting the final temperature at 10°C, amplifieds were subjected to electrophoresis by agarose gel electrophoresis in 1.5% Tris-acetate-EDTA (TAE) 1X (Invitrogen, USA). A 50µl volume of PCR reaction was prepared for each sample. Images of the stained gels were visualized and acquired by using a Gel Doc digital photo-documentation system (Bio-Rad Lab., Hercules, CA, USA).

IMMUNO-MAGNETIC SEPARATION (IMS) AND MEDIA SELECTIVE STREAKING

All positive samples for the *stx1*/*stx2* PCR were subjected to immuno-magnetic separation tecnique (IMS). 1ml of enrichment broth was added to 20 µl of magnetic beads coated with antibodies against *E. coli* O157 and O26 (Dynabeads anti-*E. coli* O157, and anti-*E.coli* O26, Dynal, Oslo) in a 1.5 ml microcentrifuge tube. Furthermore, 26 positive samples were tested with the Dynabeads anti-*E. coli* O103, anti-*E. coli* O111 and anti-*E. coli* O145. The beads were suspended, mixed, separated in a magnetic particle concentrator (MPC-10, Dynal) and washed, as described previously (Chapman et al., 1994). After the final wash and separation, the beads were suspended in 100 ml of PBS-Tween buffer.

Dynal beads anti-*E. coli* O157 were inoculated onto cefixime tellurite sorbitol MacConkey agar (CT-SMAC), Dynal beads anti-*E. coli* O26 onto cefixime tellurite rhamnose MacConkey agar (CT-RMAC), Dynal beads anti-*E. coli* O103, anti-*E.*

coli O111 e anti-*E. coli* O145 onto Enterohaemolysin Agar (EHLY AGAR, Oxoid) and incubated overnight at 37ºC.

Sorbitol non-fermenting, rhamnose non-fermenting and colonies that showed a clear halo of hemolysis were selected and streaked onto Tryptone Soy Agar (TSB, LAB 4; LABM) plates in order to obtain well-isolated pure colonies.

IDENTIFICATION TEST

All isolated colonies were subjected to phenotypic-biochemical identification by the use of MiniApi System with API 20E galleries (bioMérieux) according to the producer.

LATEX AGGLUTINATION TEST

Presumptive *E. coli* O157 colonies (non-sorbitol fermenting) and reference strains (*E. coli* O157:H7 EDL933 ATCC 35150 and *E. coli* O157:H7 NCTC 12900) were

subjected to slide agglutination with Dry Spot *E. coli* O157 Latex Test (Oxoid, Italy) sensitized with *E. coli* O157 antibodies.

The test was performed on slides and, if positive, the agglutination was visible within one minute from the execution.

MULTIPLEX PCR FOR THE DETECTION OF stx1, stx2, eae, hlyA GENES

All isolates were tested for the detection of *stx1,stx2, eae* and *hlyA* genes as described by Paton and Paton (1998) .

Working protocol provides the following steps:

Step 1: DNA collection

- Isolates were streaked on plates of Tryptone Soy Agar (TSA, Biolife),

- A single colony was taken by 1µl loop, and suspended in 100µl of sterile milliQ H_2O, which is increased to 100°C for 10 minutes.

Step 2: DNA amplification

The sequences of primers and amplification size (bp) are indicated in Table n.1.

For the amplification of *vtx1* and *vtx2* genes, stx1F/stx1R and stx2F/stx2R primers were used respectively. The couple of primers stx2F/stx2R recognizes all vtx2 variants except of vtx2f. While eaeAF and eaeAR primers recognize all polymorphic variants of *eae* gene. Furthermore, hlyAF and hlyAR primers recognize a portion of the EHEC *hlyA* gene.

In order to test the method, two reference strains, *E. coli* O157:H7 EDL933 ATCC 35150 and *E. coli* O157:H7 NCTC 12900, were used as positive and negative control respectively.

In order to prevent and control contamination, each PCR test included:

a) reagent control (without DNA),

b) control of Chelex 100 resins used for DNA extraction.

Samples were subjected to 35 PCR cycles, each consisting of:

- Denaturation 95 °C for 1',
- Annealing 65 °C for 2'', for the first 10 cycles
- Decrementing 60°C by cycle 15
- Extension 72 °C for 1.5', incrementing to 2.5' from cycles 25 to 35

- Final incubation 72°C for 4'.

Step 3: electrophoresis conditions

After bringing the final temperature at 10°C, amplifieds were subjected agarose gel electrophoresis in 2.5% Tris-acetate-EDTA (TAE) 1X (Invitrogen, USA). A 50 µl volume of PCR reaction was prepared for each sample. Images of the stained gels were visualized and acquired by using a Gel Doc digital photo-documentation system (Bio-Rad Lab., Hercules, CA, USA).

PULSED FIELD GEL ELECTROPHORESIS

Traceability of *E.coli* in the slaughterhouses was determined by Pulsed Field Gel Electrophoresis (PFGE), using the PFGE-PulseNet protocol (Efrain et al., 2006). Electrophoresis conditions settled up in the CHEF Mapper XA Pulsed Electrophoresis System (Bio-Rad) were as follows:

TABLE 3: Electrophoresis conditions of the PFGE-PulseNet protocol

Run Time	21 h
Initial Switch Time	2,2 s
Final Switch Time	54,2 s
Included Angle	120°
Voltage	6V/cm
Ramping factor	Linear

Gel Images were visualized and acquired by using a Gel Doc digital photo-documentation system (Bio-Rad Lab., Hercules, CA, USA). The XbaI (New England Biolabs) restriction profiles were analyzed by the GelCompar II software (Applied Maths) and submitted to similarity analysis through Dice coefficient (tolerance 1%; optimization 1%) and UPGMA algorythm. PFGE analysis was carried out on a subset of 21 *E.coli* strains isolated from 14 different animals (7 lambs and 7 ewes) sampled in six different slaughtering days at the three slaughterhouses (*Sa, Sb, Sc*)

included in the survey. The subset of *E.coli* strains was selected according to the representativeness of serotype, source of isolation and origin of the samples.

PART II

ASHTOWN FOOD RESEARCH CENTRE, TEAGASC, DUBLIN (IRELAND)

In order to complete the characterization of a subset of *E.coli* isolates, 37 strains were further analyzed at the Food Safety Department of the Ashtown Food Research Centre (Teagasc) of Dublin. The following determinations with molecular methods were carried out:

- Detection of O157, O26, O103, O111, O145 serogroups by Real Time PCR
- O91 and O146 Serotyping
- Detection of virulence and adherence genes

Preliminary Step: *DNA extraction*

All the strains (n.37) were streaked on Luria-Bertani (L.B.) agar (Sigma-Aldrich, St. Louis, MO, U.S.A.), suspended in 10 ml of L.B. broth (Sigma-Aldrich) and stored at -4°C and -80°C on protect beads (Technical Service Consultants Ltd, UK). In order to verify membership at the *E. coli* species, colonies were streaked on Eosin methylene blue agar (E.M.B.) media (Oxoid, Basingstoke, U.K.).

DNA was extracted from bacterial pellets using the DNeasy Blood & Tissue Kit (Qiagen Crawley, West Sussex, U.K.), according to protocol for extraction of DNA from Gram-negative bacteria provided by the manufacturers and eluted in 100µl of AE buffer. The DNA purity and concentration from each sample was tested using NanoDrop® ND-1000 spectrophotometer (NanoDrop Tecnologies, Wilmington, DE, U.S.A.).

DETECTION OF SEROGROUPS BY REAL-TIME PCR

Presumptive *E. coli* O157, O26, O103, O111 and O145 colonies were subjected to Real-time PCR analysis to detect the presence of the serogroup specific gene O157 (*per*), O26 (*wzx*), O103 (*wzx*), O111 (*wyz*), O145 (*lhp1*) (Table 2).

Single PCR amplifications were performed in the Lightcycler 480 Roche instrument (Roche Diagnostics, UK). Each reaction consisted of 5 Lightcycler® 480 SYBR Green Mater mix, 0.5 µl forward primer (final concentration 2 pmol/µl), 0.5 µl reverse primer (final concentration 2 pmol/µl), 3 µl PCR grade water and 1 µl DNA (≤ 200ng/reaction). Each run included a positive control, a negative control and a no template control (containing all reagents, substituting PCR grade water for the DNA template). The PCR amplification programs for each serogroup specific target were carried out as outlined in Table No.3 and the fluorescence was detected in the 530 nm channel.

Qualitative detection analysis was performed on Lightcycler® 480 software. The qualitative detection module analysed the sampled for the presence based on whether the data shows target amplification. Melting temperature analysis (T_m

calling analysis) was also performed on each sample using the same software, to identify characteristic melting profile of specific DNA products.

O91 AND O146 SEROTYPING BY PCR

Two separate PCR assay were performed to identify strains belonging to O91 and O146 serogroups among the isolates.

The O91 serotyping PCR was performed on DNA extracted using the Promega GoTaq mix (Promega Corporation) to detect the *wbsD* gene that allows identification of the O91 serogroup, using specific primers (Eurofins MWG Synthesis GmbH) (Table No.4 and 5; Perelle et al., 2002).

Each reaction consisted of 12,5µl of Promega GoTaq mix (Promega Corporation), 1µl of each primer (final concentration-0.25µM) and 1µl of template DNA (≤ 200ng/reaction) made up to 25µl by adding PCR grade water.

A positive and negative DNA control, from a O91:H21 strain and a O157:H7 strain respectively, and a blank control were included in each amplification set. The

PCR reaction was conducted under the conditions described previously (Perelle et al., 2002).

Duplex PCR reactions were performed on DNA extracted to detect *wzx* and *wzy* genes of *E. coli* O146 using Promega GoTaq mix (Promega Corporation) and specific primers (Eurofins) (Table No.4; Liu et al., 2007).

Each reaction consisted of 12,5µl of Promega GoTaq mix (Promega Corporation), 0.5µl of each primer (final concentration-0.25µM) and 1µl of template DNA (≤ 200ng/reaction) made up to 25µl by adding PCR grade water.

A positive and negative DNA control, from a O146:H21 strain and a O157:H7 strain respectively, and a blank control (containing all reagents, substituting PCR grade water for the DNA template) were included in each amplification set. The PCR reaction was conducted under the conditions described previously (Table No. 4 and 5. Liu et al., 2007).

Some PCR products were purified according to manufacturers' instructions using the Roche High Pure PCR Product Purification Kit (Roche Diagnostics, UK) and were sequenced commercially (Eurofins MWG Operon Germany). The DNA

samples were at the 2ng/µl concentration in a total volume of 15µl (<300bp), the DNA purity and concentration from the samples were tested using NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies).

DETECTION OF VIRULENCE AND ADHERENCE GENES BY PCR

Multiplex PCR and serotyping PCR positive strains were tested in 21 PCRs to detect other virulence genes that have an important role in pathogenicity, using specific primers (Eurofins MWG Synthesis GmbH) (Table No.4).

Each reaction consisted of 12,5µl of Promega GoTaq mix (Promega Corporation), 1µl of each primer (final concentration-0.25µM) and 1µl of template DNA (≤ 200ng/reaction) made up to 25µl by adding PCR grade water.

A positive DNA control (O157:H7 reference strain) and a blank control (containing all reagents, substituting PCR grade water for the DNA template) were included in each amplification set, except for the reaction for the detection of *saa* and *sub-AB* genes, where an O113:H21 serotype strain was used.

The concentration of primers and reaction conditions for each gene investigated were based on previous work which is stated in the publications referenced in Table No.4. and 5

TABLE NO.4: Oligonucleotide primer sequences for O91 and O146serotyping and detection of virulence and adherence genes.

Primer name	Sequence (5'-3')	Reference	Size
vt1-F	5'-ATAAATCGCCATTCGTTGACTAC-3'	(Paton and Paton, 1998)	180
vt1-R	5'-AGAACGCCCACTGAGATCATC-3'		
vt2-F	5'-GGCACTGTCTGAAACTGCTCC-3'		255
vt2-R	5'-TGCCCAGTTATCTGACATTCTG-3'		
eaeA-F	5'-GACCCGGCACAAGCATAAGC-3'		384
eaeA-R	5'-CCACCTGCAGCAACAAGAGG-3'		
hlyA-F	5'-GCATCATCAAGCGTACGTTCC-3'		534
hlyA-R	5'-AATGAGCCAAGCTGGTTAAGCT-3'		
O91-P47B	5'-GCTGACCTTCATGATCTGTTGA-3'	(Perelle et al., 2002)	291
O91-P47C	5'-TAATTTAACCCGTAGAATCGCTGC-3'		
O146 wzxF	5'-AGGGTGACCATCAACACACTTGGA-3'	(Liu et al., 2007)	640
O146 wzxR	5'-AGTTCAATACTGTCGCAGCTCCTC-3'		
O146 wzyF	5'-ATTCGGGTAACGACCCTGTGTTGA-3'	(Liu et al., 2007)	378
O146 wzyR	5'-AGACTGCTAATGCAAGGAACATGG-3'		
pagC-F	5'-ATGAGTGGTTCAAGACTGG-3'	(Konczy et al., 2008)	521
pagC-R	5'-CCAACTCCAACAGTAAATCC-3'		
Sen-F	5'-GGATGGAACCATACCTGG-3'	(Karmali et al., 2003)	551
Sen-R	5'-CGCAATCAATTGCTAATGC-3'		
efa1-F	5'-CTCCCAGAGATAATTTTGAGG-3'	(Karmali et al., 2003)	504
efa1-R	5'-CAACTGTATGCGAATAGTACTC-3'		
efa2-F	5'-CTGTCAGACGATGACATTGG-3'	(Karmali et al., 2003)	547
efa2-R	5'-GAAGGATGGGCATTGTGTC-3'		
TIR-F	5'-GTGGCGCATTGATTCTTGG-3'	(Higgins et al., 2003)	53
TIR-R	5'-CCGGCTGATTTTTTCGATGA-3'		
espA-F	5'-CACGTCTTGAGGAAGTTTGG-3'	(McNally et al., 2001)	299
espA-R	5'-CCGTTGTTAATGTGAGTGCG-3'		
espB-F	5'-CGATGGTTAATTCCGCTTCG-3'	(McNally et al., 2001)	304
espB-R	5'-GCCTGCTGAATCTGATACCT-3'		
espP-F	5'-AAACAGCAGGCACTTGAACG-3'	(McNally et al., 2001)	301
espP-R	5'-AGACAGTTCCAGCGACAACC-3'		
katP-F	5'-CTTCCTGTTCTGATTCTTCTGG-3'	(Brunder et al., 1996)	2125
katP-R	5'-AACTTATTTCTCGCATCATCC-3'		
saa-F	5'-CGTGATGAACAGGCTATTGC-3'	(Paton & Paton, 2002)	119
saa-R	5'-ATGGACATGCCTGTGGCAAC-3'		
iha-F	5'-CAGTTCAGTTTCGCATTCACC-3'	(Toma et al., 2004)	1305
iha-R	5'-GTATGGCTCTGATGCGATG-3'		
toxB-F	5'-ATACCTACCTGCTCTGGATTGA-3'	(Toma et al., 2004)	605
toxB-R	5'-TTCTTACCTGATCTGATGCAGC-3'		

lpfOI41-F lpfOI41-R	5'-CTGCGCATTGCCGTAAC-3' 5'-ATTTACAGGCGAGATCGTG-3'	(Toma et al., 2004)	412
OI154-FCT OI154-RCT	5'-GCAGGTCACCTACAGGCGGC-3' 5'-CTGCGAGTCGGCGTTAGCTG-3'	(Toma et al., 2004)	525
RTsubAB-F RTsubAB-R	5'-GCAGATAAATACCCTTCACTTG-3' 5'-ATCACCAGTCCACTCAGCC-3'	(Slanec et al., 2009)	232
cif-int-s cif-int-as	5'-AACAGATGGCAACAGACTGG-3' 5'-AGTCAATGCTTTATGCGTCAT-3'	(Slanec et al., 2009)	383
cdtV-F cdtV-R	5'-TTCATTGTTCGCCTCCTG-3' 5'-TTTATAAGCTGGTATCCTG-3'	(Slanec et al., 2009)	755
nleB-F nleB-R	5'-CATGTTGAAGGCTGGAASTTTGT-3' 5'-CCGCTACAGGGCGATATGTT-3'	(Bugarel et al., 2010)	73
nleE-F nleE-R	5'-AGAAGCGTT TGAACCTATTTCCA-3' 5'-TTGGGCGTTTTCCGGATAT-3'	(Bugarel et al., 2010)	82
nleF-F nleF-R	5'-TGAGGTGAGAAATGAAAATACTGATG-3' 5'-CTATCCCTGTCCTCTATCGTCATTC-3'	(Bugarel et al., 2010)	74
nleA-F nleA-R	5'-AGATAACYCTAATACTAAATATGCC-3' 5'-GCCCAACCATTGCRCCGATATGAGG-3'	(Bugarel et al., 2010)	136

TABLE NO. 5: Cycling parameters of PCR amplification of serotyping and virulence genes of *E. coli* strains.

GENE OF INTEREST	CYCLING PARAMETERS			REFERENCE
	DENATURATION[1]	ANNEALING	EXTENSION[2]	
O91 wbsD	94°C (30s)	65°C (30s)	72°C (30s)	Perelle et al., 2002
O146 *wzx/wzy*	94°C (30s)	57°C (60s)	72°C (30s)	Liu et al., 2007
katP	94°C (30s)	56°C (60s)	72°C (60s)	Brunder et al., 1996
pagC	92°C (60s)	58°C (60s)	72°C (30s)	Konczy et al., 2008
saa	95°C (60s)	65°C (2m)	72°C (90s)	Paton & Paton, 2002
OI-154/OI-141	95°C (2m)	50°C (40s)	68°C (60s)	Toma et al., 2004
subAB	94°C (30s)	56°C (30s)	72°C (42s)	Slanec et al., 2009
cdt-V	94°C (60s)	50°C (60s)	72°C (60s)	Slanec et al., 2009
cif-int	94°C (30s)	57°C (60s)	72°C (30s)	Slanec et al., 2009
iha	94°C (30s)	56°C (60s)	72°C (90s)	Toma et al., 2004
nleA/B/F/E	94°C (30)	52°C (40s)	72°C (60s)	Bugarel et al., 2010
efa1/2/sen	94°C (30s)	56°C (60s)	72°C (2.5m)	Karmali et al., 2003
tir	95°C (15s)	55°C (90m)	72°C (2m)	Higgins et al., 2003
espA/B	94°C (45s)	57°C (45s)	72°C (90s)	McNally et al., 2001
espP	94°C (45s)	59°C (45s)	72°C (90s)	McNally et al., 2001
toxB	94°C (60s)	52°C (60s)	72°C (90s)	Toma et al., 2004

[1] Ten minute for the first cycle

[2] Seven minutes for the last cycle

TABLE NO.6: Serogroup specific primers.

Serogroup target gene	Primer name	Sequence	Product size (bp)	T_m (°C)	Reference
O157 (*per*)	O157F	5'-TCTGCGCTGCTATAGGATTAGC-3'	202	80.20	O'Hanlon et al., (2004)
	O157R	5'-CTTGTTTCGATGAGTTTATCTGCA-3'			
O111 (*wzy*)	O111F	5'-CTTTTTTTGAACCTACAGCAAGTAA-3'	204	78.30	O'Hanlon et al., (2004)
	O111R	5'-GATAAACCAATGCTCCTATCACAC-3'			
O26 (*wzx*)	O26F	5'-CGCGACGGCAGAGAAAATT-3'	135	77.00	Perelle et al., (2004)
	O26R	5'-AGCAGGCTTTTATATTCTCCAACTTT-3'			
O103 (*wzx*)	O103F	5'-CAAGGTGATTACGAAAATGCATGT-3'	99	77.80	Perelle et al., (2005)
	O103R	5'-GAAAAAAGCACCCCCGTACTTAT-3'			
O145 (*Ihp1*)	O145F	5'-CGATAATATTTACCCCACCAGTACAG-3'	132	87.00	Perelle et al., (2004)
	O145R	5'-GCCGCCGCAATGCTTGCGATAT-3'			

TABLE NO.7: Conditions for serogroup specific Real-time PCR assays.

Program PCR SYBR- Green protocol	Target Temperature (°C)	Hold Time (s)	Slope (°C/s)	Acquisition Mode
Denaturation (1 cycle)	95	600	20	None
Amplification (35 cycles)	95	15	20	None
	*54	5	20	None
	72	12	20	Single
Melting curves analysis (1 cycle)	95	0	20	None
	65	15	20	None
	95	0	0.1	Continous
Cooling (1 cycle)	40	30	20	None

*54 °C for *E. coli* O111 and O157; 57°C for *E. coli* O26 and O103; 60°C for *E. coli* O145

RESULTS AND DISCUSSION

PART I

The preliminary PCR screening method showed a global VTEC prevalence of 32.6% (31 of out 95 animals). Animals were positive in at least one different matrice. Prevalence was higher in ewes than in lambs (42% vs. 22.2%).

The analyzed enrichment broths revealed an overall prevalence of VTEC of 11.1%. Considering the individual sources, the prevalence were: 18.9% for the fleeces, 14.7% for the carcass swabs and 10.5% for the gut mucosa. Conversely the feces samples were always negative (Table No.8)

In particular, the highest prevalence of VTEC was detected in ewe's fleeces samples (26%).. In the ewe's carcass swabs and in the gut mucosa the prevalence were 18% and 12% respectively.

Slaughterhouse C (*Sc)* showed the highest prevalence (61.9%), followed by *Sa* (23.8%) and *Sb* (14.3%) In February was collected the highest number of positive samples, from carcass swabs sampled in plant *Sc*..

Positive sheep were from No.17 (28.3%) different flocks, mainly from the province of Nuoro (No.9, 60% of the total flocks), followed by the province of Sassari (No.6, 20%), Olbia-Tempio (No.1) and Ogliastra (No.1).

TABLE 8 . Prevalence (%) of VTEC in sheep samples in relation to source and animal category.

	Prevalence (Pos/N)		
	Total	Ewe	Lamb
Sheep Samples			
Fleece	18.9 (18/95)	26 (13/50)	11.1 (5/45)
Carcass swab	14.7 (14/95)	18 (9/50)	11.1 (5/45)
Mucosal gut	10.5 (10/95)	12 (6/50)	8.9 (4/45)
Faeces	ND*	ND	ND
Total	11.1 (42/380)	14 (28/200)	7.8 (14/180)

ND* : under the detection limit of the method

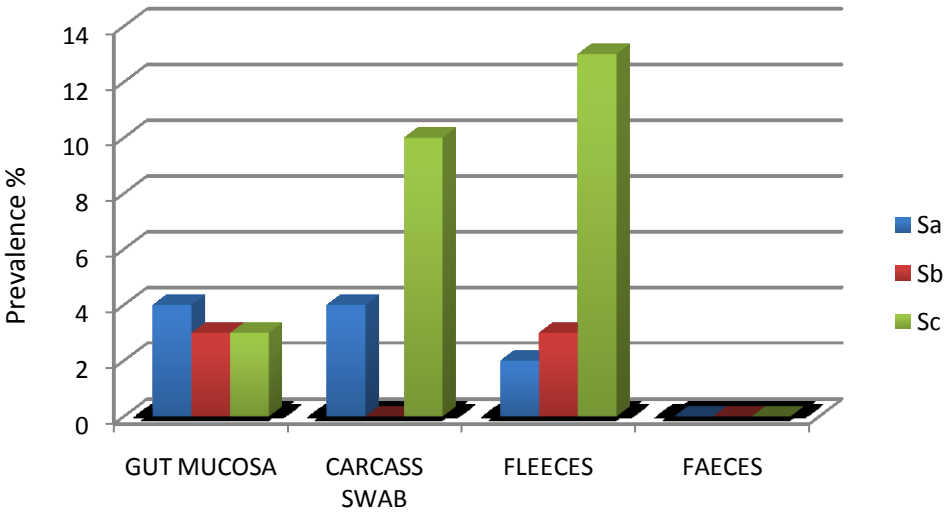


FIGURE NO.2: Prevalence (%) of VTEC in slaughtered sheep in relation to source and plant.

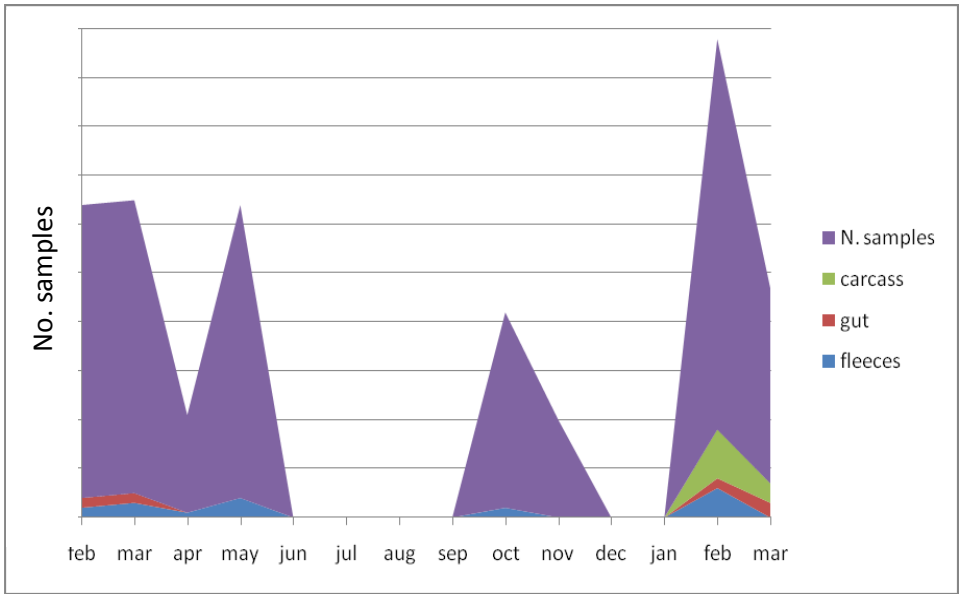


FIGURE NO.3: Number of positive samples at the screening PCR method per source for each month. Feces samples were always negative.

STRAIN ISOLATION

The procedure of streaking on selective media (CT-SMAC, CT-RMAC) and on EHLY agar allowed to isolate a total of 124 supposed (before serotyping) *E. coli* belonging to different serotypes (O157, O26, O103, O111, O145) from lamb and sheep sources (Table No.9).

In particular were isolated:

a) CT-SMAC: No.29 alleged *E. coli* O157 from fleeces (No.13), gut mucosa (No.9) and carcass surfaces (No.7);

b) CT-RMAC: n.21 alleged *E. coli* O26 from fleeces (No.8), gut mucosa (No.4) carcass swabs (No.9);

c) EHLY agar: n.26 supposed *E. coli* O103 (fleeces No.6, gut mucosa No.6, carcass No.14), No.24 supposed *E. coli* O111 (fleeces No.5, gut mucosa No.6, carcass No.13) and No.24 supposed *E. coli* O145 (fleeces No.4, gut mucosa No.6, carcass No.14).

The distribution among the abattoirs was the following:

- plant *Sa*: 7 O157, 3 O26, 1 O103, 0 O111, 1 O145 supposed serotypes;
- plant *Sb*: 7 O157, 7 O26, 7 O103, 7 O111, 7 O145 supposed serotypes;
- plant Sc: 15 O157, 11 O26, 18 O103, 17 O111, 16 O145 supposed serotypes.

Only three strains, one suspected O145 and two O103, isolated from the surface of carcasses, did not show the characteristic haemolysis on EHLY agar plates.

The isolation rates were 57.1% for CT-SMAC, 42.9% for CT-RMAC, while in EHLY AGAR the isolation occurred for all examined samples.

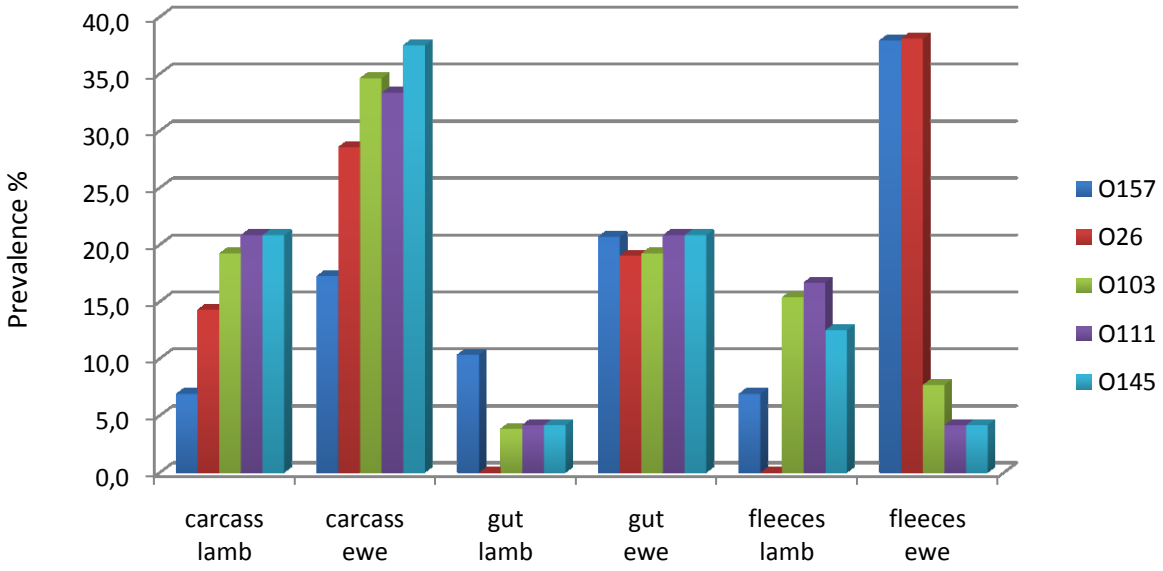


FIGURE NO.4: Prevalence of supposed *E. coli* serotype isolated, by IMS, on CT-SMAC, CT-RMAC and EHLY agar in relation to animal category and sources.

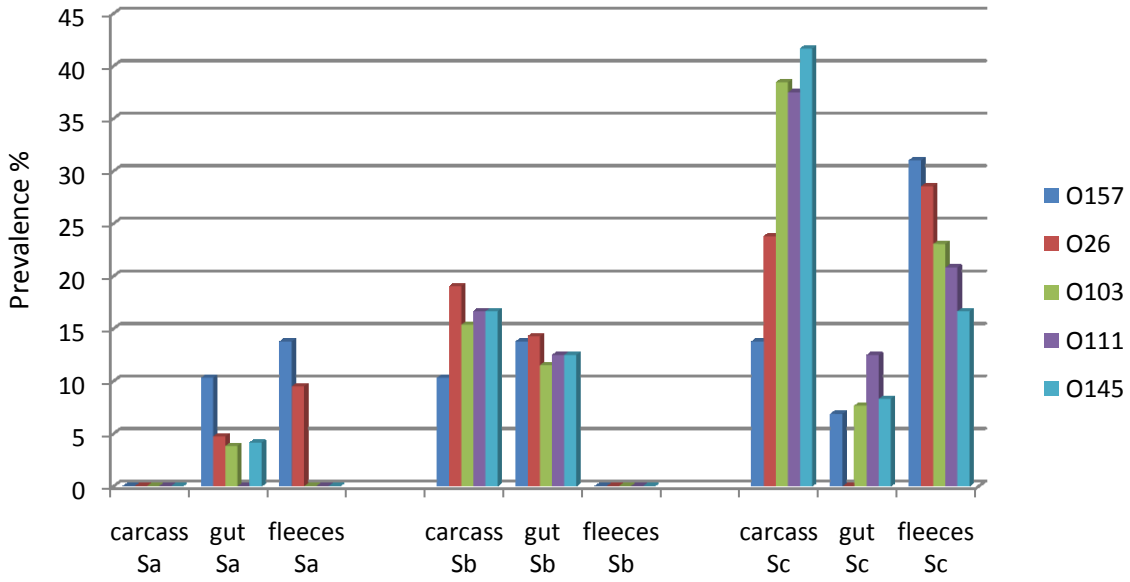


FIGURE NO.5: Prevalence of supposed *E. coli* serotype isolated, by IMS, on CT-SMAC, CT-RMAC and EHLY agar in relation to plants and sources.

LATEX AGGLUTINATION TEST

All examined strains were negative at the latex agglutination test.

PHENOTYPIC IDENTIFICATION OF SPECIES

All isolates were recognized as belonging to the *E. coli* species by the phenotypic method miniApi 20E (bioMérieux).

DETECTION OF *vtx1, vtx2, eae, hlyA* GENES

Table 9 and 10 showed the prevalence and the virulence profile of isolates.

Multiplex PCR allowed to detect almost one of the virulence genes in 62.9% (No.78) of the isolates.

Twenty eight of the 124 (22.6%) strains carried one of *vtx* genes and can be ascribed to VTEC pathogroup. Among these, 24 (85.7%) harbored *eae* gene too. Eleven (8.9%) VTEC strains were collected from lambs and 17 (13.7%) from ewes.

VTEC prevalence was higher in lamb (28.2%) than ewe (12.9%) matrices, showing the highest values in carcass swabs samples (10.3%) and lamb gut mucosa (10.3%).

Fifteen (12.1% of total isolates, 53.6% of VTEC) strains, mainly isolated from carcass swabs (7.3%, No.9), can be considered EHEC, as they harbor intimin-, enterohemolisin- and one of the verocitotoxin-encoding genes. The complete virulence profile (*vtx1, vtx2, eae* and *hlyA*) was detected in 8 (6.5%) strains.

Seventeen isolates showed both *vtx1* and *vtx2* genes, apart from the other virulence considered genes, while 3 (2.4%) of the remaining isolates showed only the *vtx1* gene, and 8 (6.5%) only the *vtx2* gene. In the latter 8 isolates a constant relationship between the presence of *vtx2* and *eae* genes was observed.

Overall, the *vtx2* gene was detected in No.25 (20.2%) strains, mainly recovered from ewes (No. 16, 12.9%).

The *eae/hlyA* virulence profile was the most frequently detected (21%, No.26), mainly from ewes (17.7%, No.22). The higher prevalence was observed in

carcass swabs (10.5%, No.13), especially in samples collected from slaughterhouse *Sc* (15.3%, No.19).

The *eae* was the more commonly isolated gene (57.3%, No.71), mainly from carcass swabs (27.4%, No.34). Strains carrying exclusively *eae* gene were recovered mainly from lamb carcasses of plant *Sc* (13.7%, No.17).

Six of the 12 isolates from plant *Sa* were VTEC; among these 2 (16.7%) strains were EHEC.

Nine of 35 isolates (25.7%) from plant *Sb* were VTEC; among these 4 (11.4%) were EHEC.

Thirteen of 77 (16.9%) strains isolates from abattoir *Sc* were VTEC, 9 (11.7%) of which were EHEC.

The prevalence of EPEC was 37.9% (No. 47). Highest prevalences were found in plant *Sc* (48.1%, No 37), followed by *Sa* (33.3%, No. 4) and *Sb* (25.7%, No.9)

TABLE NO.9: Results of Multiplex PCR: prevalence (%) of VTEC strains and of virulence genes in relation to category and sources (number of positives inside brackets)

				Genes %			
	No		**VTEC+ %**	***Vtx1***	***Vtx2***	***eae***	***hlyA***
Ewe	85	Carcass	8.2 (7)	3.5 (3)	8.2 (7)	22.4 (19)	20 (17)
		Fleeces	5.9 (5)	3.5 (3)	4.7 (4)	14.1 (12)	8.2 (7)
		Gut	5.9 (5)	5.9 (5)	5.9 (5)	11.8 (10)	10.6 (9)
		Total	13.7 (17)	12.9 (11)	18.8 (16)	48.2 (41)	38.8 (33)
Lamb	39	Carcass	10.3 (4)	7.7 (3)	10.3 (4)	38.5 (15)	15.4 (6)
		Fleeces	7.7 (3)	7.7 (3)	7.7 (3)	28.2 (11)	7.7 (3)
		Gut	10.3 (4)	7.7 (3)	5.1 (2)	10.3 (4)	7.7 (3)
		Total	8.9 (11)	23.1 (9)	23.1 (9)	76.9 (30)	30.8 (12)
Total **(Ewe and Lamb)**	124	Carcass	8.9 (11)	4.8 (6)	8.9 (11)	27.4 (34)	18.5 (23)
		Fleeces	6.5 (8)	4.8 (6)	5.6 (7)	18.5 (23)	8.1 (10)
		Gut	7.3 (9)	6.5 (8)	5.6 (7)	11.5 (14)	9.7 (12)
		Total	22.6 (28)	16.1 (20)	20.2 (25)	57.3 (71)	36.3 (45)

TABLE NO.10: Results of Multiplex PCR: prevalence (%) of EHEC strains category and sources (number of positives inside brackets)

	No	**EHEC+%**	***Carcass swab***	***Fleeces***	***Gut mucosa***
Ewe	85	9.4 (8)	5.9 (5)	1.2 (1)	2.4 (2)
Lamb	39	17.9 (7)	10.6 (4)	2.3 (1)	5.1 (2)
Total	124	12.1 (15)	7.3 (9)	1.6 (2)	3.2 (4)

TABLE NO.11A: Prevalence (%) and virulence profiles of lamb and ewe isolates.

		vtx1/vtx2/eae/hlyA	vtx1/vtx2eae	vtx1/vtx2/hlyA	vtx1/eae/hlyA	vtx2/eae/hlyA	vtx1/vtx2	vtx1/eae
Lamb	Gut	-	-	2.6 (1/39)	2.6 (1/39)	2.6(1/39)	-	-
	Carcass	7.7 (3/39)	-	-	-	2.6 (1/39)	-	-
	Fleeces	2.6 (1/39)	5.1 (2/39)	-	-	-	-	-
Ewe	Gut	2.4 (2/85)	1.2 (1/85)	-	-	-	2.4 (2/85)	-
	Carcass	2.4 (2/85)	1.2 (1/85)	-	-	3.5 (3/85)	-	-
	Fleeces	-	2.4 (2/85)	-	-	1.2 (1/85)	-	1.2 (1/85)
Total		6.5 (8/124)	4.8 (6/124)	0.8 (1/124)	0.8 (1/124)	4.8 (6/124)	1.6 (2/124)	0.8 (1/124)

TABLE NO.11B: Prevalence (%) and virulence profiles of lamb and ewe isolates.

		vtx2/eae	*eae/hlyA*	*vtx1/hlyA*	*vtx2/hlyA*	*vtx1*	*vtx2*	*eae*	*hlyA*
Lamb	Gut	-	-	-	-	2.6 (1/39)	-	5.1 (2/39)	-
	Carcass	-	5.1 (2/39)	-	-	-	-	23.1 (9/39)	-
	Fleeces	-	5.1 (2/39)	-	-	-	-	15.4 (6/39)	-
Ewe	Gut	-	5.9 (5/85)	-	-	-	-	2.4 (2/85)	1.2 (1/85)
	Carcass	-	12.9 (11/85)	-	-	-	-	1.2 (1/85)	2.4 (2/85)
	Fleeces	-	7.1 (6/85)	-	-	-	-	1.2 (1/85)	-
Total		-	21 (26/124)	-	-	0.8 (1/124)	-	16.9 (21/124)	2.4 (3/124)

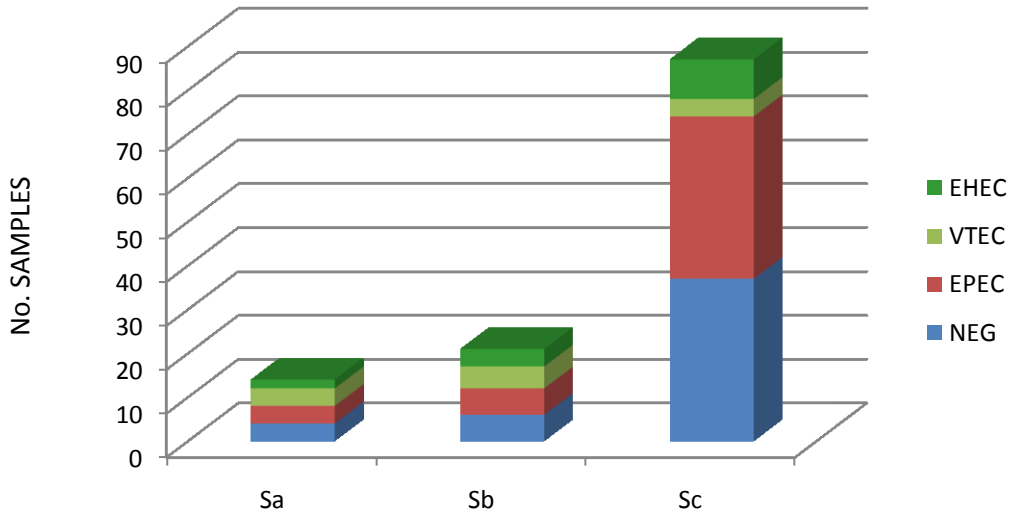


FIGURE NO.6: Distribution (n.) of the *E. coli* pathogroups in the slaughterhouses.

TABLE NO.12: Prevalence (%) of *E. coli* pathotypes in relation to categories and samples in unusual slaughterhouses (*Sa*, *Sb* and *Sc)*

	Ewe					Lamb				
Sa	**No. isolates**	VTEC	EHEC	EPEC	Untypable	**No. isolates**	VTEC	EHEC	EPEC	Untypable
Carcass	-	-	-	-	-	-	-	-	-	-
Fleeces	5	60 (3)	-	20 (1)	20 (1)	1	100 (1)	100 (1)	-	-
Gut mucosa	4	-	-	75 (3)	25 (1)	2	100 (2)	50 (1)	-	-
Total	9	33.3 (3)	-	44.4 (4)	22.2 (2)	3	100 (3)	66.6 (2)	-	-
Sb		VTEC	EHEC	EPEC	Untypable		VTEC	EHEC	EPEC	Untypable
Carcass	19	21.1 (4)	10.5 (2)	21.1 (4)	57.9 (11)	-	-	-	-	-
Fleeces	-	-	-	-	-	-	-	-	-	-
Gut mucosa	15	26.7 (4)	6.7 (1)	13.3 (2)	60 (9)	1	100 (1)	100 (1)	-	-
Total	34	23.5 (8)	8.8 (3)	17.6 (6)	58.8 (20)	1	100 (1)	100 (1)	-	-
Sc		VTEC	EHEC	EPEC	Untypable		VTEC	EHEC	EPEC	Untypable
Carcass	18	16.7 (3)	16.7 (3)	38.9 (7)	44.4 (8)	18	20 (4)	20 (4)	55 (11)	16.7 (3)
Fleeces	18	11.1 (2)	5.6 (1)	33.3 (6)	55.6 (10)	12	16.7 (2)	-	66.7 (8)	16.7 (2)
Gut mucosa	6	16.7 (1)	16.7 (1)	33.3 (2)	50 (3)	3	33.3 (1)	-	66.7 (2)	-
Total	42	14.3 (6)	11.9 (5)	35.7 (15)	50 (21)	33	21.2 (7)	12.1 (4)	63.6 (21)	15.1 (5)

PFGE

PFGE pointed out the presence of 20 different *Xba*I restriction patterns or pulsotypes (Table No.13, Figure No.7). The analysis of the restriction patterns allowed us to highlight the distribution of the several pulsotypes only in *Sc*, the plant with the major rate of isolated strains (70%). A high heterogeneity of pulsotypes was pointed out, with 14 different pulsotypes (Figure No.8), not shared with strains isolated from the other plants. Strains isolated from lambs and ewes belonged to different pulsotypes. No permanent pulsotypes were observed during the different samplings, suggesting a continuous supply of new pulostypes inside the plants through ewes and lambs. Altogether, the distribution of pulsotypes in the plants was as follows: in lambs, 13 different pulsotypes were observed: (2-3-6-7-8-9-10-12-13-14-15-18-19). In the ewes, strains belonged to 7 different pulsotypes (1-4-5-11-16-17-20). The analysis of similarity allowed to divide the 21 strains of *E. coli* in 4 different clusters (similarity ≥ 60%) called A-B-C-D. Two strains showed similarity values of <60% and therefore were not included in any cluster (Figure No. 7). The distribution within the clusters was rather heterogeneous with respect to the slaughterhouses, sources and serotypes.

TABLE NO.13: Features of the 21 *E.coli* strains submitted to PFGE

Code of the strain	Serotype	Matrix	Slaughterhouse	Pulsotype
VT50	O157	Fleece ewe	C	5
VT43	O111	Carcass lamb	C	6
VT32	O26	Gut mucosa ewe	B	4
VT109	O26	Carcass lamb	C	3
VT126	O157	Gut mucosa lamb	A	2
VT99	O26	Fleece ewe	C	1
VT124	O26	Fleece ewe	C	1
VT123	O157	Gut mucosa lamb	B	8
VT59	O145	Fleece lamb	C	7
VT28	O103	Fleece lamb	C	9
VT81	O103	Fleece lamb	C	15
VT83	O145	Carcass lamb	C	13
VT82	O111	Fleece lamb	C	12
VT84	O111	Carcass lamb	C	14
VT108	O111	Fleece ewe	C	11
VT56	O157	Gut mucosa ewe	B	16
VT76	O157	Gut mucosa ewe	B	17
VT91	O103	Carcass lamb	C	19
VT12	O111	Fleece lamb	C	18
VT65	O111	Fleece lamb	C	10
VT125	O157	Fleece lamb	A	20

FIGURE NO.7 UPGMA Clustering of the 21 E.coli isolates

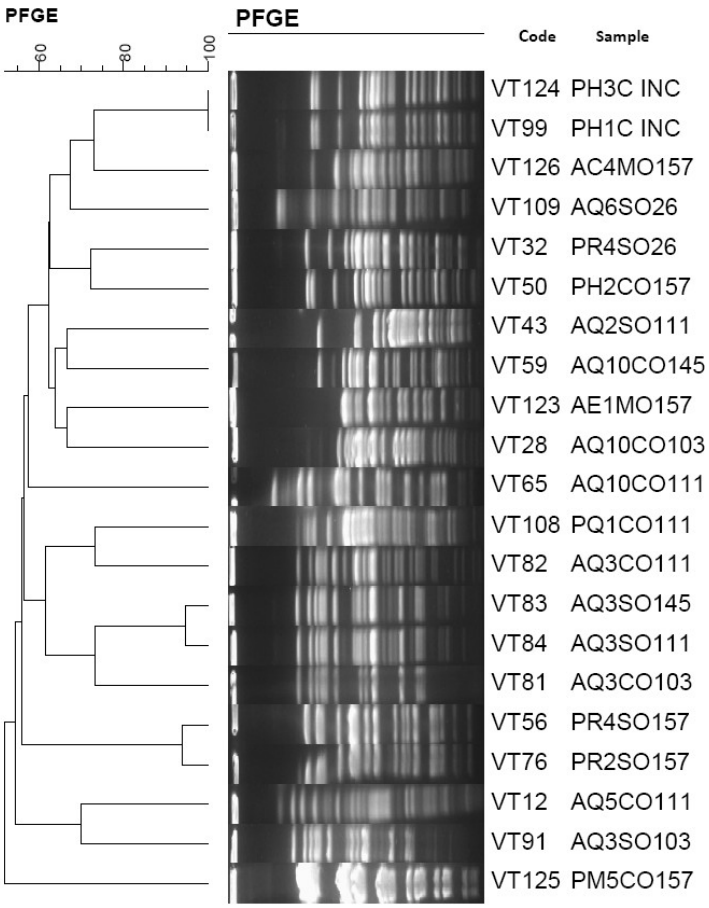
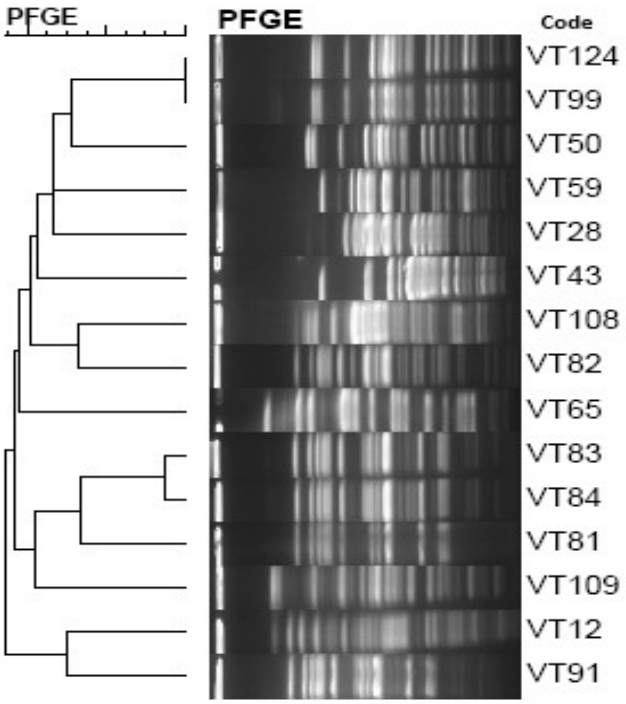


FIGURE NO.8 UPGMA Clustering of the *E.coli* isolates from plant *Sc*



DISCUSSION

The prevalence, of VTEC in different sources of healthy sheep determined by direct PCR screening, was quite high. In 32.6% of the sheep, VTEC was observed at one or more sampling sites, according to other similar studies (Zschöck et al., 2000; Blanco et al., 2003; Rey et al., 2003; Zweifel et al., 2003).

The majority of VTEC isolates were from the fleece (18.9%): during sampling procedures, visible faecal contamination was often observed in most of these samples, indicating the high risk for cross-contamination connected to these matrices. Indeed, Elder et al. (2000) considered hides and fleeces as a major source of spread of pathogenic *E. coli* contamination in slaughterhouse.

However, contamination by the environment, the tools and operators can not be excluded. In a survey on the prevalence of pathogens in sheep lairages, Small et al. (2002) identified some critical points during the pre-slaughtering phases where the cross-contamination can occur, e.g. unloading ramp, pen floor and water trough, resulting by an 'indirect mixing' of animals from different groups or farms. The

critical points identified above may contribute to the pathogen's persistence in the abattoirs.

We have observed a significant increase in prevalence over the last three months of sampling, with regard to the contamination of carcass surfaces. These values do not exclude the possibility of cross-contamination by the presence of asymptomatic carriers, in the lack of application of good hygiene practice.

Although unusual, no positive feces samples were obtaine also by others researchers (Johnsen et al. 2001, Lenaham et al, 2007). The detection of VTEC in feces is complicated, because of the presence of PCR inhibitor factors, as bile salts or other competitive factors (Widjojoatmodjo et al., 1992). Furthermore, a very high natural microflora should be considered in this source and as a consequence, antibiotics are used to support the isolation of specific pathogens. In this way, sensibility of the enrichment procedure is improved, but injured and stressed pathogen cells are not capable of survival and growth in these conditions. Also the enrichment method used in the present study may not therefore have been

sufficiently sensitive to detect the low levels of the microrganism and, in particular, to detect the injured cells.

In general the VTEC presence in the slaughterhouse environment depend on the microbial flora carried by the slaughtered sheep during the same day. The different prevalence observed between *Sc* and other slaughterhouses could be linked to the slaughtering and hygienic practices application by the workers, the moment of the sampling and the animal origin.

Overall, the molecular method showed a high sensitivity and was very useful as a rapid screening test for direct detection of VTEC in foodstuff and providing an interesting insight in the epidemiological profile and the traceability of VTEC in the sheep foodchain.

PCR method was also more sensitive than isolation by IMS, as confirmation test. Nevertheless the IMS and the cultivation on selective media are essentials to specifically confirm the positivity detected by molecular methods, directly through the isolation of strains and determination of the their virulence profile. Ogden et al. (2004) explained the reason for the low sensitivity of the IMS method with oily

nature of sheep feces that impedes the magnetic separation process. Multiplex PCR has proved to be a valuable tool to evaluate the virulence profiles of the isolates.The method allowed to determine the pathogroups of the isolates, identifying strains belonging to VTEC (22.6%) and especially many of the EPEC group (47.9%). The finding of VTEC and EPEC (AEEC) strains, mainly on carcass swabs, has a major public health interest. Aktan et al. (2004) suggested a better adaptation of AEEC in sheep than other species. Alternatively, sheep are exposed to AEEC more frequently than other species but in this case the source of the AEEC is unclear. These hypotheses need other investigations. In this way, sheep are considered a source of serologically and genetically diverse intimin-harbouring *E. coli* strains.

Prevalence of VTEC (28.2%) and EPEC (53.8%) are higher in lambs than ewes, the difference may be related to the effect of age on the physiological differences in the digestive tract, on the diet, or on the immune response to these organisms (Djordjevic et al., 2004).

The finding of strains belonged mostly to the group of EPEC pathogroup have a significant interest from the epidemiological point of view. The detection of

vtx genes among isolated strains was less than expected and compared with other studies (Sanchez et al., 2010; Rey et al., 2003).

The differences can be due to:

- different sensibility of the determination methods;
- different considered sources
- a recycling of cattle and sheep strains, since most farmers that keep sheep also have cattle. These animal husbandry practices may contribute to the survival and recycling of *E. coli* within the grazing herds, and the colonization of sheep with new and unusual strains (Cornick et al. 2000)
- genetic rearrangements by stx-encoding bacteriophages (Beutin et al., 1997).

However, VTEC were detected mainly from carcasses and fleeces, with an high risk of contamination for humans. VTEC isolates often harbored the *eae* gene (85.7%), giving them expression of full virulence for humans. Furthermore, about 36% of VTEC strains isolated in this study from healthy sheep were *hlyA*-positive. As this gene is considered a potential virulence factor for humans (Beutin et al.,

1995), their presence in this percentage of VTEC strains from sheep might increase the pathogenicity of these strains for humans. Of particular interest is the fact that No.15 (53.6%) *eae*-positive VTEC strains were also *hlyA*-positive, and that most of them were recovered from carcasses.

Sarda sheep seem to hold pathogenic *E. coli* mostly belonging to the group of EPEC, however EHEC and VTEC are present, which have prominent virulence features and can contaminate sheep foodstuff.

PART II

CONFIRMATION ON MEDIA

All the 37 selected strains showed a metallic green sheen on EMB agar.

DETECTION OF SEROGROUPS BY REAL-TIME PCR

The results of real-time PCR showed that none of the isolates were confirmed as the serogroup previously identified with IMS technique. Figure No. 9 illustrates an O157 serogroup assay: no specific amplification occurred except for the positive control.

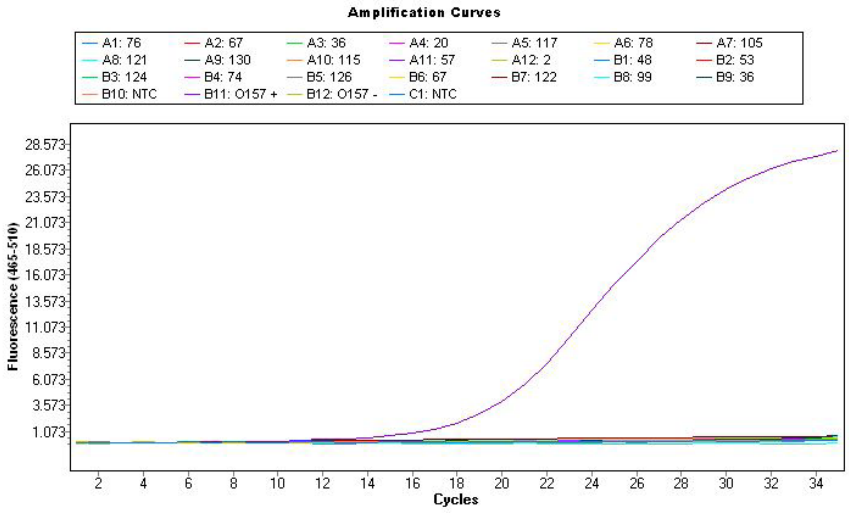


FIGURE NO. 9: O157 serogroup real-time PCR Lightcycler® 480 Software Report

DETECTION OF VIRULENCE AND ADHERENCE GENES

The 37 selected isolates were: No.11 VTEC, (of which 5 EHEC), 12 EPEC and 14 untypable strains.

The complete virulence profiles of the tested strains are presented in Table No. 12 and 13.

Genes encoding for Type III Secretion System (TTSS) proteins

No. 13 of 37 isolates harbored the three most important genes belonging to TTSS (*tir, espA, espB*). They were sampled from No.6 lambs and No.7 ewes, and 7 (63.6%) of them were considered VTEC and No.5 (31.3%) EPEC.

The presence of the gene encoding for intimin receptor Tir, in No.15 isolates (40.5%) was always associated with the occurrence of *eae* gene.

pO157-borne genes

There was no correlation between the presence of the different pO157-borne genes.

Gene encoding for EspP was the most frequently detected in No. 6 (54.5%) of the VTEC strains, in No.4 (33.3%) of the EPEC strains and No.7 (50%) of the untypable

strains. The second frequently recovered pO157-borne gene was *toxB*, in No. 6 (54.5%) of the VTEC strains, in No.4 (33.3%) of the EPEC strains. The presence of this gene was associated 6 times with the other pO157-borne gene *hlyA*. The other two searched genes *subAB* and *katP* were found only in No.2 isolates (No.1, 9.1% VTEC and No.1, 7.1% EPEC strains) and No.1 (9.1%) VTEC strain respectively.

OI 122-borne genes

The complete range of virulence genes (*pagC, sen, nleB nleE, efa1, efa2*) composing the pathogenic island OI 122 was found in No.4 (10.8%) isolates. Three (27.3%) of these belong to VTEC and No.1 (8.3%) to EPEC pathogroup. The most prevalent detected gene was *sen* (91.9%, No. 34). All VTEC (No.11) and EHEC (No.5) strains carried this gene, while it was present in No. 14 (75%) of the EPEC isolates. Genes encoding for *efa1* and *efa2* were present in No.27 (73%) and No.28 (75.7%) strains respectively, mainly from VTECs (No.8, 72.7% and No10, 90.9%).

OI 71-borne genes

Genes encoding for non-LEE encoded proteins F and A were detected in No.2 (18.2%) VTEC strains, No.4 (33.3%) EPEC and No.5 (35.7%) untypable strains. The virulence in humans associated *nleA* gene was carried by No.15 (40.5%) isolates.

OI 141 borne genes

Most of the tested strains harbored the LPF gene cluster in OI 141 (No.33, 89.2%). All the detected EHEC isolates (No.5, 100%) carried the *lpfA OI-141* gene, while it was found in No.10 of 12 (83.3%) EPEC strains, and in No.13 of 14 (92.9%) of the strains that were not classified as VTEC or EPEC.

OI 154 borne genes

The LPF gene cluster in OI 154 was detected in No.29 (78.4%) of the tested strains. No.4 (80%) of which belonged to EHEC, No. 10 (83.3%) to EPEC pathogroup and No.11 (78.6%) to the unsortable strains.

OI 43 and OI 48-borne gene

Gene encoding for Iha adeshin was observed in No.1 (20%) EHEC and No.2 (16.7%) strains assigned to EPEC pathogroup.

saa gene

Only No.2 (5.4%) of the tested strains harbored the *saa* gene encoding for the autoagglutinating adhesion. This strain was *subAB* positive confirming studies reported by Paton (2001).

cif-int gene

Cycle-inhibiting factor (Cif) occurred in No.2 (27.3%) of the EHEC strains and in No. 5 (41.7%) of the isolates that belong to EPEC pathogroup.

cdt-V gene

No.10 (27%) of the isolates showed the presence of *cdt-V* gene (No.4, 36.4% of the VTECs, No.5, 41.7% of the EPECs and No.1, 7.1% of the untypable strains).

O91 AND O146 SEROTYPING

None of the isolates was positive to the PCR specific for O146 serogroup, while No. 21 of the 37 strains belonged to O91 serogroup. Three of these were randomly selected and their DNA products were submitted to sequencing, showing a E value Score Percent Identity with O91 *E. coli* of 100%, 99% and 100% respectively. Most of O91 *E. coli* were collected from adult sheep (No.17, 81%) and

mainly from carcass source (No.11, 52.4%). Slaughterhouses *Sc* showed the highest prevalence of this serogroup (No.12, 57.1%), followed by *Sb* (No.8, 38.1%) and *Sa* (No.1, 4.8%).

Taking into account multiplex PCR for the detection of *vtx1*, *vtx2*, *eae* and *hlyA* genes, No.5 (23.8%) of these were VTEC, No.1 (4.8%) EHEC, No.4 (19%) and No.12 (57.1%) negatives for presence of these virulence genes (Table No.14).

DETECTION OF VIRULENCE AND ADHERENCE GENES BY PCR IN O91 E. COLI

Genes encoding for Type III Secretion System (TTSS) proteins

The occurrence of virulence profiles of O91 serotype strains is presented in Table No. 12.

Genes encoding Tir, EspA and EspB were present concomitantly in No. 5 (23.8% of O91 *E. coli*) isolates, No.2 of which were VTEC. The most frequently found gene was *EspA* (No.16,76.2%), while strains that harbored gene *tir* were No.11 (52.4%) and only No.4 (36.4%) of these were associated with *eae* gene.

pO157-borne genes

There was no significant correspondence between the finding of the pO157-borne genes in *E. coli* O91 serotype. Only No. 1 (20%) of VTEC strains carried genes encoding for serine protease EspP, the adhesin ToxB and the subtilase cytotoxin SubAB. Most of the isolates carried *espP* gene (No.12, 57.1%), while none of these harbored *katP* gene.

OI 122-borne genes

We obtained many positive strains for *sen*, *efa1* and *efa2* but negative for *pagC,* that was detected in No.1 (4.8%) isolates only.

The most complete OI 122 genes profile (*sen+, efa1+, efa2+, nleB+, nleE+*) was observed in No. 2 (9.5%) strains, No. 1 of which belonged to VTEC pathogroup. 95.2% (No.20) of the isolates harbored the *sen* gene, while *efa1* were present along with *efa2* in No.11 (52.4%) O91 *E. coli*. Module 2 genes *nleB* and *nleE* were carried by No.3 (14.3%) of O91 strains.

OI 71-borne genes

Six (28.6%) strains isolated from ewes showed *nleF* and *nleA* genes concomitantly, No. 5 (41.7%) were negative for *vtx* and *eae* gene and No. 1 (25%) was an EPEC strain.

Overall O91 *E. coli* carrying *nleA* gene were No.9 (42.9%), and they lacked of the *vtx* genes.

OI 141 borne genes

The majority of isolates (No. 18, 85.7%) harbored the *lpfA OI 141* gene (No.4, 80% of VTEC; No.1, 100% of EHEC; No.3, 75% of EPEC and No.11, 91,7% of the negative strains for the presence of *vtx* and *eae* genes).

OI 154 borne genes

No. 16 (76.2%) of the isolated strains had the LPF gene cluster in OI 154, No.3 (60%) of these can be ascribed to VTEC pathogroup and No.9 (75%) did not belong to VTEC or EPEC pathogroups.

OI 43 and OI 48-borne gene

Only No. 1 EPEC strain harbored the *iha* gene (4.8% of the tested strains, 25% of the EPEC strains).

saa gene

Gene *saa* was carried by No.1 VTEC exclusively (4.8% of the tested strains, 20% of the VTEC strains). This isolate showed a complete genes profile encoding for TTSS, and an almost complete range of genes belonging to OI-122 and pO157.

cif-int gene

None of the isolates encoded the cycle-inhibiting factor (Cif).

cdt-V gene

No. 1 O91 *E. coli* harbored the cdt-V gene (4.8% of the tested strains, 11.1% of the untypeable strains).

TABLE NO.14: Virulence profile of 16 *E. coli* isolates.

		TTSS			OI 122						OI 71		OI 141	OI 154	OI 43/ OI 48	pO157							
Samples	**Patho group**	***tir***	***espA***	***espB***	***pagC***	***sen***	***efa1***	***efa2***	***nleB***	***nleE***	***nleF***	***nleA***	***lpfA OI-141***	***lpfA OI-154***	***iha***	***hlyA***	***espP***	***katP***	***toxB***	***subAB***	***cd-tv***	***cif-int***	***saa***
1	EPEC	-	pos	pos	-	pos	-	pos	-	pos	pos	pos	pos	-	pos	-	-	-	pos	pos	-	-	pos
2	EHEC	pos	pos	pos	pos	pos	pos	pos	pos	pos	-	-	pos	-	pos	pos	-	-	pos	-	-	pos	-
3	Untyp	pos	pos	pos	-	pos	pos	pos	-	-	-	-	pos	pos	-	-	pos	-	pos	-	-	pos	-
4	EPEC	pos	pos	-	-	pos	pos	-	pos	pos	-	-	pos	pos	-	pos	pos	-	pos	-	pos	pos	-
5	EPEC	pos	-	-	-	pos	-	-	-	-	pos	pos	-	pos	-	-	-	-	-	-	pos	pos	-
6	VTEC	pos	pos	pos	-	pos	pos	pos	pos	pos	pos	pos	pos	pos	-	-	-	-	pos	-	pos	-	-
7	EPEC	pos	pos	-	-	-	pos	-	-	-	pos	-	pos	pos	-	-	-	-	-	-	-	-	-
8	EHEC	pos	pos	pos	-	pos	pos	pos	pos	pos	-	-	pos	pos	-	-	pos	-	pos	-	pos	-	-
9	EPEC	pos	-	-	-	-	pos	-	-	-	-	-	pos	pos	-	-	-	-	-	-	pos	-	-
10	EPEC	pos	-	-	-	pos	pos	pos	pos	pos	pos	-	pos	-	-	pos	-	-	-	-	-	pos	-
11	EPEC	pos	pos	-	pos	pos	pos	pos	pos	pos	-	-	pos	pos	-	pos	-	-	pos	-	pos	pos	-
12	EHEC	-	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	-	pos	-	-	pos	-	-	pos	-
13	Untyp	pos	pos	pos	pos	pos	pos	-	pos	pos	pos	-	pos	pos	-	pos	pos	-	-	-	-	-	-
14	EHEC	pos	pos	pos	pos	pos	-	pos	pos	-	pos	-	pos	pos	-	pos	pos	-	-	-	pos	-	-
15	EPEC	pos	pos	pos	-	pos	pos	pos	pos	pos	pos	pos	pos	pos	-	pos	-	-	pos	-	pos	pos	-
16	VTEC	pos	pos	pos	pos	pos	pos	pos	pos	pos	-	pos	pos	pos	-	pos	-	pos	pos	-	pos	pos	-

TABLE NO.15: Virulence profile of 21 O91 *E. coli* isolates

		TTSS			OI 122						OI 71		OI 141	OI 154	OI 43/ OI 48	pO157								
Samples	**Patho group**	***tir***	***espA***	***espB***	***pagC***	***sen***	***efa1***	***efa2***	***nleB***	***nleE***	***nleF***	***nleA***	***lpfA OI-141***	***lpfA OI-154***	***iha***	***hlyA***	***espP***	***katP***	***toxB***	***subAB***	***cd-tv***	***cif-int***	***saa***
1	Untyp	-	pos	-	-	pos	-	pos	-	pos	pos	pos	pos	pos	-	-	-	-	-	-	-	-	-
2	Untyp	pos	pos	-	-	pos	pos	pos	-	-	-	pos	pos	pos	-	-	pos	-	-	-	-	-	-
3	VTEC	-	-	pos	-	pos	pos	-	-	-	-	-	-	pos	-	-	pos	-	-	-	-	-	-
4	Untyp	-	pos	pos	-	pos	-	-	pos	-	-	-	pos	pos	-	-	-	-	-	-	-	-	-
5	VTEC	pos	pos	pos	-	pos	pos	pos	pos	pos	pos	-	pos	-	-	-	pos	-	pos	pos	-	-	pos
6	Untyp	-	pos	pos	-	pos	pos	pos	pos	pos	-	-	-	pos	-	-	-	-	-	-	-	-	-
7	Untyp	-	-	pos	-	pos	pos	pos	-	-	-	pos	pos	pos	-	-	pos	-	-	-	-	-	-
8	Untyp	pos	pos	pos	-	pos	pos	-	pos	pos	-	-	pos	-	-	-	-	-	pos	-	-	pos	-
9	Untyp	-	pos	-	pos	pos	pos	pos	-	-	-	-	pos	pos	-	-	pos	-	-	-	-	-	-
10	Untyp	pos	pos	-	-	pos	-	pos	-	-	pos	pos	pos	pos	-	-	-	-	-	-	-	-	-
11	Untyp	pos	-	-	-	pos	-	pos	-	-	-	-	pos	pos	-	-	-	-	-	-	-	-	-
12	Untyp	pos	pos	-	-	pos	pos	pos	-	-	pos	pos	pos	-	-	-	pos	-	-	-	-	-	-
13	VTEC	-	pos	-	-	pos	pos	pos	-	pos	-	-	pos	-	-	-	pos	-	-	-	-	-	-
14	Untyp	pos	pos	pos	-	pos	pos	pos	-	pos	pos	pos	pos	pos	-	-	-	-	-	-	-	-	-
15	Untyp	pos	pos	pos	-	pos	pos	pos	-	-	pos	pos	pos	-	-	-	pos	-	-	-	-	-	-
16	EHEC	pos	pos	pos	-	pos	-	pos	-	pos	-	-	pos	pos	-	pos	-	-	-	-	-	-	-
17	VTEC	-	pos	-	-	pos	-	pos	-	-	-	-	pos	pos	-	-	pos	-	-	-	-	-	-
18	EPEC	-	pos	pos	-	pos	pos	-	-	pos	-	-	pos	pos	-	pos	pos	-	-	-	-	-	-
19	EPEC	pos	-	pos	-	pos	pos	pos	-	-	pos	pos	pos	pos	pos	pos	pos	-	-	-	-	-	-
20	EPEC	pos	-	pos	-	-	-	pos	-	-	-	pos	-	pos	-	-	pos	-	-	-	-	-	-
21	EPEC	-	pos	-	-	pos	pos	pos	-	-	-	-	pos	pos	-	-	-	-	-	-	-	-	-

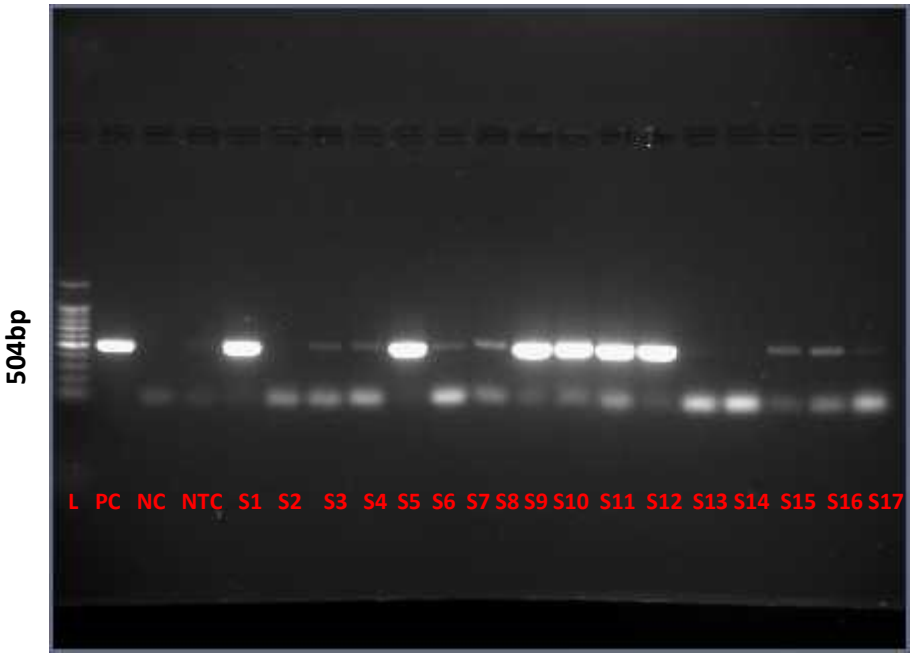


FIGURE NO.11: *efa1* gene detecting PCR,
L: Ladder 100bp, PC: Positive Control, NC: Negative Control, NTC: Negative Template Control , S1-S17: DNA samples

DISCUSSION

The real-time PCR analysis have not confirmed the results of the IMS technique: none of the isolated strains belonged to O157:H7 serogroup or to the other most frequently mentioned as a cause of human disease. This result pointed out the limits of the immunomagnetic method but on the other hand, suggested the possibility to study in depth the presence of other serogroups in the Sarda sheep. Indeed the identification of 21 O91 *E. coli* support the theory that sheep is the reservoir of different from the more common serogroups. In many studies O91 was the predominant serogroup detected in sheep and frequently it was associated with human diseases (Beutin et al., 1993; Rey et al., 2000; Djordjevic et al., 2001; Kudva et al., 1997; Fach et al., 2001).

The detection of several accessory virulence factors leads to the fact that the isolates are potentially able to colonize the intestinal mucosa. Adherence virulence factors were more numerous in some strains and allowed to distinguish the presence of different PAI.

We detected the genes encoding for TTSS in 35.1% of the tested strains, whose actions result in characteristic attaching and effacing lesions on enterocytes. Moreover, microrganisms able to encodes for the intimin-receptor Tir were numerous (67.6%): this protein have the major role in colonization and persistence in ruminants (Karmali et al. 2010).

The presence of genes belonging to different OI in isolated strains, gives them additional pathogenicity, making them potentially able to colonize the enterocytes (Toma et al., 2004).

Morabito et al. (2003) states that the presence of OI 122 seems to be a peculiar feature of AEEC strains, since it was detected in most of the EHEC and EPEC strains but not in the other groups of *E. coli*. We observed complete OI 122 borne genes in 10.8% of the isolates. In other cases, the profile was incomplete, but the genes and *sen, efa1, efa2* were found frequently. This fact may indicate a possible gene recombination, and it appears reasonable that the transmission of *E. coli* from one sheep to another can be accompanied by genetic re-arrangements. A major cause of such re-arrangements could be stx-encoding bacteriophages, which might excise

and reintegrate at different sites on the bacterial chromosome (Beutin et al., 1997). *E. coli* strains are subject to constant genes recombination, they can undergo ephemeral mutations via loss and gain of Stx-encoding phages, which leads to different pathotypes (Fröhlicher et al., 2008).

Finally, the finding of several genes coding for accessory toxins (*cd-tv*, *cif-int*, *nleA*) is to be considered as a possible source of damage to intestinal cells in animals and humans (Marchès et al., 2003; Creuzburg and Schmidt, 2007).

CONCLUSIONS

The results of the present study provide an epidemiological survey on the presence and traceability of VTEC in the meat sheep chain.

The preliminary PCR screening method showed a good sensibility and represent an useful rapid test to the direct detention of the VTEC in food.

The molecular methods resulted more effective than those used for the strain isolation (IMS), but the latter are essential to the strain characterization and the definition of virulence profile. In particular, multiplex PCR has proved to be a valid tool to evaluate the virulence profiles of the isolates.

The pathogroup determination of the *E.coli* isolates from Sarda sheep, revealed that, as reported by others authors (Aktan et al., 2004), sheep are exposed to EPEC, EHEC, and VTEC. Moreover the detection of pathogenic *E. coli* on carcass surfaces at slaughterhouse level indicates an high risk for the public health.

The results confirm that *E.coli* harbored in sheep have different and very complex virulence profiles.

As in others similar studies concerning sheep matrices, only few strains isolated in our study allowed to O157 serogroup, while non-O157 serogroups were prevalent. Also Djordjevic et al. (2004) assert that non-O157 STEC should not be overlooked in human disease investigations, because of the following: non-O157 are more prevalent than O157 STEC in the feces of meat-producing animals, indicating that humans are more likely to become exposed to these STEC strains as contaminants in foods; O157 STEC strains are not commonly isolated from the feces of healthy sheep, as demonstrated in many studies carried out in vary parts of the world.

Blanco et al. (2003) showed that numerous ovine STEC serotypes were associated with disease in humans. Although our results, and those of other authors indicate that STEC strains from human and animal origin with the same serotype are similar regarding the presence of known virulence-associated factors, further studies are necessary to establish if animal and human strains represent the same clones or are only related subpopulations. Our examination of non-O157 STEC serotypes from sheep, revealed remarkable differences in respect to those from cattle, which could

point out an animal-host serotype specificity for some serotypes. Nevertheless, these are usually less frequently investigated, because they not allow to the gang of five (EFSA, 2009).

Indeed, the identification of O91 *E. coli* support the theory that sheep is the reservoir of different and less common serogroups. In many studies, O91 was the main serogroup detected in sheep and it was frequently associated with human disease (Beutin et al., 1993; Rey et al., 2000; Djordjevic et al., 2001; Kudva et al., 1997; Fach et al., 2001).

Concerning the virulence profile of the isolates, as previous showed, the finding of several genes coding for accessory toxins (*cd-tv*, *cif-int*, *nleA*) shall to be considered as a possible source of damage to intestinal cells in animals and humans (Marchès et al., 2003; Creuzburg and Schmidt, 2007).

VTEC isolated in our study, often harbored the *eae* gene, giving them expression of full virulence for humans. Furthermore, about a third of VTEC strains isolated in this study from healthy sheep, were *hlyA*-positive. As this gene is considered a potential virulence factor for humans (Beutin et al., 1995), their

presence in this percentage of VTEC strains from sheep might increase the pathogenicity of these strains for humans. Of particular interest is the fact that about the half part of *eae*-positive VTEC strains carried also *hlyA*-gene, and that most of them were recovered from carcasses, with an high risk of contamination for humans.

Nevertheless, others studies (Blanco et al., 2003) have underlined the strong association between carriage of the *eae* gene and the capacity of STEC isolated to cause severe human disease, especially HUS. As this important virulence gene is present in only a proportion of ovine non-O157 STEC strains, some of these ovine strains probably have low levels of virulence for humans. Nevertheless, production of intimin is not essential for pathogenesis, because a number of sporadic cases of HUS have been caused by eae-negative non-O157 STEC strains.

Finally, the obtained data are also relevant to develop and implement suitable procedures to prevent the entry of pathogenic strains of VTEC in the slaughterhouse. This is in accordance with the opinion of the Scientific Committee on Veterinary Measures relating to Public Health (SCVPH), according to which the definition of microbiological guidelines aimed at reducing the faecal contamination along the

meat producing chain can help to reduce risks to public health, including VTEC (EC Regulation 2073/2005).

The following measures can be considered:

- the control of the introduction of the carriers, including through measures at farms of origin (carrier detection and elimination in the feces, antibiotic treatment, could hardly be envisaged in sheep farms);
- the reducing of the possibility of direct cross-contamination in the meat plant, through the application of good operating procedures and hygienic handling of the process by operators (reduction of visible faecal contamination).

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SUMMARY