Appl Biochem Biotechnol (2011) 165:290–299
DOI 10.1007/s12010-011-9251-x

First Detection of Shiga Toxin-Producing *Escherichia coli* in Shellfish and Coastal Environments of Morocco

Mohamed Bennani · Samira Badri · Tarik Baibai · Nadia Oubrim · Mohammed Hassar · Nozha Cohen · Hamid Amarouch

Received: 4 November 2010 / Accepted: 4 April 2011 /
Published online: 21 April 2011

Abstract Shiga toxin *Escherichia coli* (STEC), also called verotoxin-producing *E. coli*, is a major cause of food-borne illness, capable of causing hemorrhagic colitis and hemolytic–uremic syndrome (HUS). This study was carried out to evaluate the presence of (STEC) and *E. coli* O157:H7 in shellfish and Mediterranean coastal environments of Morocco. The contamination of shellfish and marine environment with Shiga toxin-producing *E. coli* (STEC) and *E. coli* O157:H7, was investigated during 2007 and 2008. A total of 619 samples were analyzed and 151 strains of *E. coli* were isolated. The presence of the *stx1, stx2,* and *eae* genes was tested in *E. coli* isolates strains using a triplex polymerase chain reaction. STEC was detected in three positives samples (1.9%), corresponding to the serotype O157:H7, the others Shiga toxin-producing *E. coli* non-O157 were also detected.

Keywords Coastal environment · *Escherichia coli* · Shiga-like toxin-producing *E. coli* · STEC · PCR triplex

Introduction

Shiga toxigenic *Escherichia coli* (STEC) strains are an important cause of gastrointestinal diseases in humans, particularly since these infections may result in life-threatening sequelae; such as hemolytic–uremic syndrome "HUS" [1–3]. The STEC family is very diverse, and strains belonging to a broad range of O:H serotypes have been associated with

M. Bennani (✉) · S. Badri · N. Oubrim · M. Hassar · N. Cohen
Laboratoire de Microbiologie et d'Hygiène des Aliments et de l'Environnement,
Institut Pasteur du Maroc, Casablanca, Morocco
e-mail: mohamed.bennani@pasteur.ma

T. Baibai
Laboratoire de Physiologie et de génétique moléculaire, Département de Biologie, Faculté des Sciences Ain Chock Casablanca, Casablanca, Morocco

H. Amarouch
Laboratoire de Microbiologie, Département de Biologie, Faculté des Sciences Ain Chok Casablanca, Université Hassan II. Morocco, Casablanca, Morocco

human diseases [1]. However, several epidemiological studies have revealed that O157 STEC strains are prevalent in the gastrointestinal tract of healthy cattle; and therefore cattle are regarded as the principal reservoir of these pathogens [4]. Other animals such as pigs and dogs can also harbor STEC strains [5, 6]. Another environmental source of STEC strains is wastewater from sewage treatment plant [7, 8]. STEC could be present in coastal areas which are particularly exposed [9]. Because of their filter-feeding activities, shellfish can concentrate and retain pathogenic microbes present in waters of their environment [10–12]. The risk to public health associated with the consumption of shellfish, traditionally consumed raw or undercooked; is well documented [13–16]. STEC strains produce one or both of two major types of Shiga toxin, designated *stx1* and *stx2*; and the production of the latter is associated with an increased risk of developing HUS [17–19]. In addition, a subset of STEC strains considered to be highly virulent for humans has the capacity to produce attaching and effacing lesions on intestinal mucosa, a property encoded on a pathogenicity island termed the locus for enterocyte effacement (*LEE*). *LEE* encodes a type III secretion system and *E. coli*-secreted proteins, which deliver effector molecules to the host cell and disrupt the host cytoskeleton [20]. *LEE* also carries *eae,* which encodes an outer membrane protein (intimin) required for intimate attachment to epithelial cells [21]; *eae* has been used as a convenient diagnostic marker for *LEE*-positive STEC strains [22, 23]. However, the presence of *eae* is not absolutely linked to human virulence, as some sporadic cases of severe STEC disease; including HUS; as well as occasional outbreaks that have been caused by *LEE*-negative strains [23, 24]. Most STEC strains isolated from humans (both *LEE* positive and *LEE* negative) also carry large (>90 kb) plasmid-encoding proteins such as the enterohemorrhagic *E. coli* enterohemolysin (*EhxA*) [23] and an extracellular serine protease (*EspP*) [25], both of which may be accessory virulence factors. The aims of this study were to determine the occurrence of Shiga toxin-producing *E. coli* O157:H7 and non-O157, in shellfish and Mediterranean coastal environments of Morocco, to serotype the STEC isolates, and to determine their prevalence.

Materials and Methods

Description of the Study Area

This study was realized in Tamouda Bay; located in the Mediterranean coast of Morocco, between Sebta at the north (35°54′ N, 5°17′10″ W) and Cap Negron at the south (35°40′ N, 5°16′40″ W). Climate is typically Mediterranean. The average annual temperature is about 18°C, while the annual rainfall average ranges between 800 and 1000 mm.

Three sites were selected in Tamouda bay: site 1, Marina kabila is located on the release of sewage; site 2, is located near of Smir's river; and site 3, Marina beach which is an area of swimming, surfing, and leisure. Every site is different in geography, population, and ecology. The locations of the sites (Fig. 1) were determined by the global positioning system.

Samples Collection and Preparation

From January 2007 to December 2008, samples were collected bimonthly in three sampling sites. For each exploration, four ecological types of samples were collected, seawater, planktons, shellfish, and sediments. Using boat, seawater samples (1 L) were collected at a depth of 1 m from the surface in sterilized plastic bottles. Planktons were collected by dragging the water horizontally, at a depth of about 1 m, with 200-μm mesh plankton net [26].

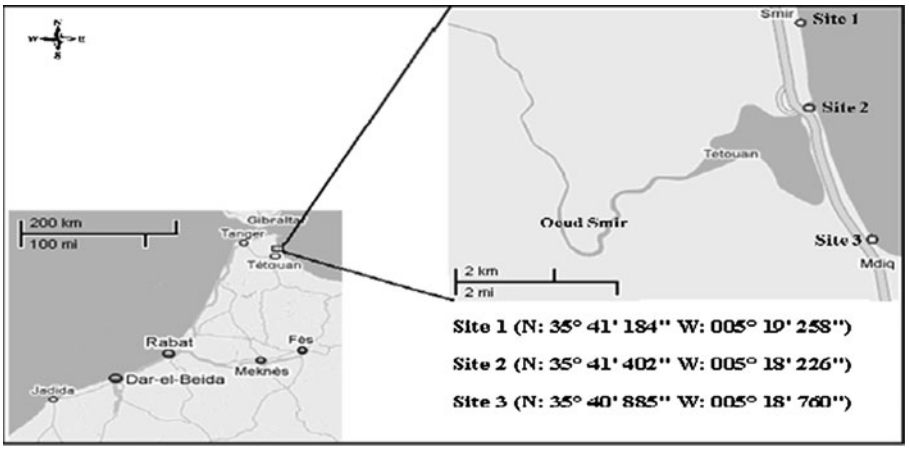


Fig. 1 Map showing the geographical location of three sampling sites

Shellfishes samples were purchased from local fisherman including Mussels (*Mytilus edulis* and *Mytilus galloprovincialis*) and cockles (*Cerastoderma edule*), while the sediments were collected from the surface of the coast using sterile plastics pots. After collection, the samples were transported immediately to the laboratory in insulated coolers with frozen gel-packs to maintain the temperature around 4°C. A total of 619 samples, composed of seawater (n=219), plankton (n=203), seafood (n=82), and sediment (n=115) were collected.

Bacterial Isolation

The fecal contamination (*E. coli*) of samples was estimated by the AFNOR standardized five-tube MPN method [27]. A portion of (25 g) from shellfish or sediments was placed into a separate sterile stomacher bag with 225 ml of buffered peptone water and then pummeled with a MIX I mixer (AES Laboratory, Combourg, France). For seawater samples, 1 L was filtered using 0.22-μm membrane filters. Planktons samples were used directly for isolation. 100 μl of each sample was streaked on Violet Red Bile Lactose Agar (Biorad) and incubated at 37°C for 24 h. Colonies showing *E. coli* characteristics were Gram-color stained and identified by standard biochemical tests: oxidase negative, indole positive, Simon's citrate negative, urease negative, and hydrogen sulfide negative [28] .The isolates were confirmed *E. coli* using the Enterobacteriaceae API 20E commercial kit (BioMérieux, Marcy l'Etoile, France). To detect *E. coli* O157, a complementary isolation procedure was performed [9, 29]. All strains were enriched in Trypticase soy broth at 42°C for 6 h, the strains were plated on Tryptone Bile X-Glucuronide (TBX) Medium cultured at 44°C for 24 h and presumed *E. coli* O157 strains were confirmed by cefixine tellurite sorbitol MacConkey (CT-SMAC) (bioMérieux), the non-sorbitol-fermenting isolates from the plates was cultured at 37°C for 24 h in Trypticase soy plate and confirmed by latex agglutination *E. coli* O157 kit (Oxoid), all strains isolated were stored at −80°C in 30% sterile glycerol.

Detection of Hemolysin

Hemolytic activity of the isolated strains was investigated by plating the bacteria on tryptic soy agar (TSA) (Biorad) plates containing 5% washed human blood cells group O. The hemolytic activity was observed after overnight incubation at 37°C.

Extraction of Bacterial DNA

Bacterial DNA was isolated using InstaGene Matrix kit (Biorad), following the instructions given by the manufacturer. Isolated DNA was used as a template for PCR with the following protocols.

Detection of Virulence Genes by Triplex PCR

The strain used as control in this study was: EDL933, the isolates of *E. coli* were subjected to triplex polymerase chain reaction for detection of *stx1*, *stx2,* and *eae* genes. The primers and the predicted lengths of PCR amplification products are listed in Table 1. PCR assays were carried out in a 25 μL volume as described by [23] with slight modifications. Samples were subjected to 35 cycles, consisted of 1.5 min of denaturation at 94°C; 1.5 min of annealing at 57°C, and 1 min of extension at 72°C, followed by final extension of 7 min at 72°C. Amplified PCR products were analyzed by gel electrophoresis in 2% agarose containing ethidium bromide (0.5 μg/ml) [30]. The products were visualized with UV illumination and imaged with gel documentation system.

Results

Bacterial Isolation Test

Of the total analyzed samples (n=619), 151 *E. coli* strains were isolated, which represent a prevalence of 24.4%. The distribution of strains according to the sampling sites and the type of samples analyzed is presented in Fig. 2. The prevalence of *E. coli* strains were high in sediment samples (40%) and shellfish samples (30%) compared to plankton samples (16%) and sea water samples (14%). The identification results of *E. coli* in each site showed the same tendency of contamination between all ecological types of samples. However, the abundance of *E. coli* in site 1 and 2 was consistently higher particularly in sediments and shellfish. This may be explained by the important flow of visitors during summer period and during cold season and rains, associated with hurricanes, which brought considerable freshwater runoff from the Smir River (Fig. 2).

Table 1 The primers used for detection of the various genes by PCR

Gene	Primers	Oligonucleotid sequence (5′–3′)	Amplicon size	References
stx1	*stx1F*	ATA AAT CGC CAT TCG TTG ACT AC	180	Paton and Paton, (1998)
	stx1R	AGA ACG CCC ACT GAG ATC ATC		
stx2	*VT2 425*	TTA ACC ACA CCC CAC CGG GCA GT	524	Pollard et al. (1990)
	VT2 952	GGA TAT TCT CCC CAC TCT GAC ACC		
eaeA	*SK1*	CCC GAA TTC GGC ACA AGC ATA AGC	864	Karch and Bielaszewska, (2001)
	SK2	CCC GGA TCC GTC TCG CCA GTA TTC G		

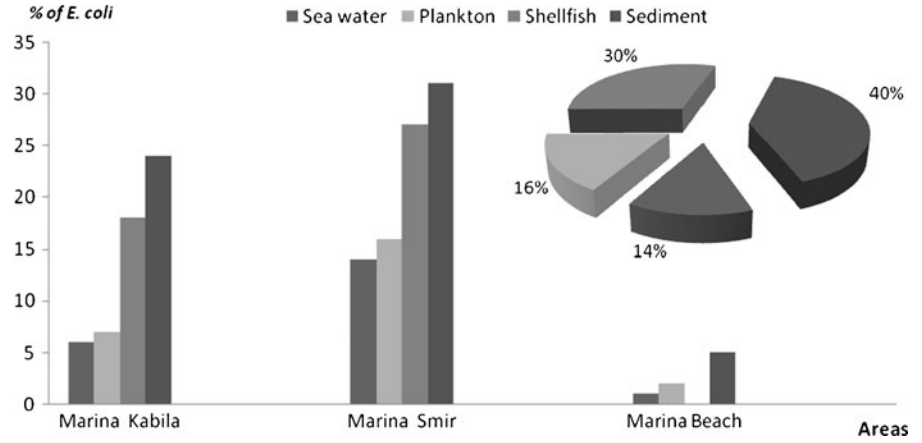


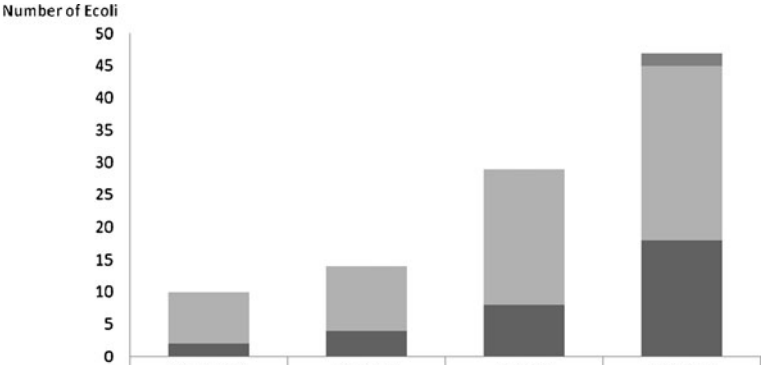
Fig. 2 Distribution and percentage of *E. coli* in differents sites and samples

Hemolysin Test

The hemolysis test showed that among the 151 *E. coli* identified, 100 are hemolytic strains, which represents a prevalence of 66.2%. The prevalence of hemolytic strains in sediment, shellfish, plankton, and seawater samples are respectively 78,3%, 64,4%, 56%, 47,6%, and the prevalence of hemolytic strains of *E. coli* by sites are high at site 2 (75%) followed by site 1 (58,1%) and site 3 (25%) these results are illustrated in (Fig. 3).

Test of β Glucuronidase

The test of β-glucuronidase and sorbitol of *E coli* strains isolated in this study demonstrated, that 13 strains were glucuronidase negative and only five strains were sorbitol negative, three strains of them were isolated from shellfish's samples and two from sediments samples. Four strains are from site 2 and one strain from site 1 and no strain



	Sea water	Plankton	Shellfish	Sediment
Marina Beach	0	0		2
Marina Smir	8	10	21	27
Marina Kabila	2	4	8	18

Fig. 3 Distribution of *E. coli* hemolytic in differents sites and samples

Table 2 The incidence of virulence genes of *E. coli* detected by the triplex PCR

Virulence genes detected by PCR triplex				
Sample type	Number of positive/tested	*stx1*	*stx2*	*eaeA*
Sea water	0/219	–	–	–
Planktons	0/203	–	–	–
Shellfishs	5/82	+ (5/5)	+ (3/5)	–
Sediments	8/115	+ (8/8)	–	–

isolated at site 3. However, no strain from seawater samples or plankton samples was Glucuronidase negative and/or sorbitol negative.

PCR Triplex

Table 2 below shows the incidence of virulence genes of *E. coli* detected by the triplex PCR. This result showed five *E. coli* strains isolated from shellfish samples that have virulence gene *stx1* and three out of five have the *stx2,* for sediments samples virulence gene are detected in eight strains corresponded to *stx1*, conversely in all samples no *eae* gene was detected. While no virulence gene was detected in *E. coli* strains isolated from seawater samples and planktons samples, (Fig. 4).

Discussion

In this study, the main objective was to determine the prevalence of *E. coli*-positive STEC isolates from variable samples of sea water, plankton, seafood, and sediments around three areas on the Mediterranean Moroccan coast.

Few works that focused on the detection of STEC or *E. coli* O157 strains were studied in shellfish [9–12, 31, 32]. The total prevalence of *E. coli*-STEC containing stx1 in this study was at the order of 2.1%. Positive samples for stx2 were found only among seafood with the prevalence of 0.48%. This shows that the prevalence of stx1 is greater than stx2. The

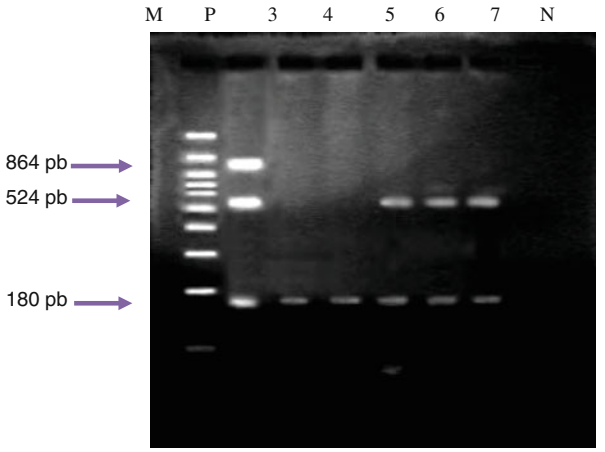


Fig. 4 Agarose gel electrophoresis in 2% showing products of PCR Triplex; *M*, lanes molecular weight size marker (100-bp DNA ladder); *P*, positive, EDL 933 (*E. coli* O157:H7) with three genes; *N*, negative control, Hb 101 (no gene); wells *3*, *4*, *5*, *6*, and *7*, strains presumes

higher prevalence of *stx1* in STEC isolates observed in the present work was in agreement with the findings of other investigators which have reported a similar trend in STEC isolates from several animals [33–35] in Australia and Spain, respectively. These results indicate that, potentially, isolates of *E. coli* species can be found ubiquitously in the Moroccan coast and are in agreement with the results of a previous study described the contamination of the environment with STEC in France [9]. In fact, the results obtained in this study show that shellfish collected in coastal environments can be positive for *stx* genes as indicator for STEC. Spatially, the prevalence of STEC in this study were higher in site 2 with the presence of fecal indicator bacteria and a number of *E. coli*, more importantly; this was well explained by [36–38] that frequency of *stx*-positive samples varied depending on the activities upstream and the weather conditions, which can be a favorable factor to obtain a fecal bacterial shellfish contamination downstream. The *stx* genes present in shellfish samples could originate from STEC or from *stx* bacteriophages. Muniesa and Jofre in 1998 [39] have evaluated the prevalence of Shiga toxin-converting phages in waste waters from two origins; the number of phages infectious for *E. coli* O157:H7 and carrying the *stx2* gene were in the range of 1–10 per mililiter of sewage. According to [40], *stx* gene-encoding bacteriophages are common in sewage from industrialized countries. In order to determine the presence of virulence genes, many classical PCR systems have been developed in simplex or in multiplex [11, 23, 41–46]. The most STEC-targeted genes had been *eae*, *stx1*, *stx2*, and *ehxA*. As shown in the Table 2, STEC was detected in 13 strains (eight positive samples in sediments all isolates carried *stx1*, five strains was observed for the sea food and only three positive strains carried *stx2*). However, all isolated STEC reveal the absence of *eae* gene that in agreement with the findings of several studies [34, 47] who also reported that STEC isolates from small ruminants rarely carried *eae* gene. This indicates that these isolates could be less virulent in humans as *eae* gene and may be required for the expression of full virulence of STEC for humans [48–50]. As this gene is considered as a potential virulence factor for humans [51–54], their presence in a high percentage of STEC isolates might increase the pathogenicity of these isolates for human beings. The occurrence of all these virulence gene combinations is summarized in Table 2 and the representative gene profiles are depicted in Fig. 4.

Conclusion

For the first time in the Mediterranean coast of Morocco, Shiga toxin *E. coli* (STEC) was detected in shellfish. The contamination of shellfish and marine environment with Shiga toxin-producing *E. coli* (STEC) and *E. coli* O157:H7, STEC was detected in three positives samples (1.9%), corresponding to the serotype O157:H7, the others Shiga toxin-producing *E. coli* non-O157 were also detected.

References

1. Karmali, M. A. (1989). Infection by verotoxin-producing *Escherichia coli*. *Clin Microbiol Rev, 2*, 15–38.
2. Nataro, J. P., & Kaper, J. B. (1998). Diarrheagenic *Escherichia coli*. *Clin Microbiol Rev, 11*, 142–201.
3. Paton, A. W., & Paton, J. C. (2005). Multiplex PCR for Direct Detection of Shiga Toxigenic *Escherichia coli* Strains Producing the Novel Subtilase Cytotoxin. *J Clin Microbiol, 43*, 2944–2947.
4. Kuhnert, P., Dubosson, C. R., Roesch, M., Homfeld, E., Doherr, M. G., & Blum, J. W. (2005). Prevalence and risk-factor analysis of Shiga toxigenic *Escherichia coli* in faecal samples of organically and conventionally farmed dairy cattle. *Vet Microbiol, 109*(1–2), 37–45.

5. Beutin, L., Geier, D., Steinruck, H., Zimmermann, S., & Scheutz, F. (1993). Prevalence and some properties of verotoxin (Shiga-like toxin)-producing *Escherichia coli* in seven different species of healthy domestic animals. *J Clin Microbiol, 31*, 2483–2488.
6. Bouvet, J., Bavai, C., Rossel, R., Le Roux, A., Montet, M. P., Ray Gueniot, S., et al. (2001). Prevalence of verotoxin-producing *Escherichia coli* and *E. coli* O157: H7 in pig carcasses from three French slaughterhouses. *Int J Food Microbiol, 71*, 249–255.
7. Vernozy-Rozand, C., Feng, P., Montet, M. P., Ray-Gueniot, S., Villard, L., Bavai, C., et al. (2000). Detection of *Escherichia coli* O157:H7 in heifers faecal samples using an automated immunoconcentration system. *Lett Appl Microbiol, 30*, 217–222.
8. Holler, C., Koschinsky, S., & Witthuhn, D. (1999). Isolation of enterohaemorrhagic *Escherichia coli* from municipal sewage. *Lancet, 353*, 2039.
9. Gourmelon, M., Montet, M. P., Lozach, S., Le Mennec, C., Pommepuy, M., Beutin, L., et al. (2006). First isolation of Shiga toxin 1 d producing *Escherichia coli* variant strains in shellfish from coastal areas in France. *J Appl Microbiol, 100*, 85–97.
10. Guyon, R., Dorey, F., Collobert, J. F., Foret, J., Goubert, C., Mariau, V., et al. (2000). Detection of Shiga toxin-producing *Escherichia coli* O157 in shellfish (*Crassostrea gigas*). *Sci Aliments, 20*, 457–465.
11. Kumar, H. S., Otta, S., & Karunasagar, I. (2001). Detection of Shiga-toxigenic *Escherichia coli* (STEC) in fresh seafood and meat marketed in Mangalore, India by PCR. *Lett Appl Microbiol, 33*, 334–338.
12. Kumar, H. S., Karunasagar, I., Karunasagar, I., Teizou, T., Shima, K., & Yamasaki, S. (2004). Characterisation of Shiga toxin-producing *Escherichia coli* (STEC) isolated from seafood and beef. *FEMS Microbiol Lett, 233*, 173–178.
13. Lipp, E. K., & Rose, J. B. (1997). The role of seafood in foodborne diseases in the United States of America. *Rev Sci Tech, 16*, 620–640.
14. Feldhusen, F. (2000). The role of seafood in bacterial foodborne diseases. *Microbes Infect, 2*, 1651–1660.
15. Potasman, I., Paz, A., & Odeh, M. (2002). Infectious outbreaks associated with bivalve shellfish consumption: a worldwide perspective. *Clin Infect Dis, 35*, 921–928.
16. Cohen, N., Karib, H., Ait saïd, J., Lemee, J., Guenole, A., & Quilici, M. L. (2007). Prévalence des vibrions potentiellement pathogènes dans les produits de la pêche commercialisés à Casablanca (Maroc). *Rev Med Vet, 158*(11), 562–568.
17. Ostroff, S. M., Tarr, P. I., Neill, M. A., Lewis, J. H., Hargrett-Bean, N., & Kobayashi, J. M. (1989). Toxin genotypes and plasmid profiles as determinants of systemic sequelae in *Escherichia coli* O157:H7 infections. *J Infect Dis, 160*, 994–999.
18. Kleanthous, H., Smith, H. R., Scotland, S. M., Gross, R. J., Rowe, B., Taylor, C. M., et al. (1990). Haemolytic uraemic syndromes in the British Isles, 1985–8: association with Verocytotoxin producing *Escherichia coli*. Part 2: microbiological aspects. *Arch Dis Child, 65*, 722–727.
19. Boerlin, P., McEwen, S. A., Boerlin-Petzold, F., Wilson, J. B., Johnson, R. P., & Gyles, C. L. (1999). Associations between virulence factors of Shiga toxin-producing *Escherichia coli* and disease in humans. *J Clin Microbiol, 37*, 497–503.
20. Robert, K., Shaw, K. S., Cleary, J., Garmendia, J., Straatman-Iwanowska, A., Frankel, G., et al. (2005). Enteropathogenic *Escherichia coli* Type III Effectors EspG and EspG2 Disrupt the Microtubule Network of Intestinal Epithelial Cells. *Infect Immun, 73*(7), 4385–4390.
21. Jerse, A. E., Yu, J., Tall, B. D., & Kaper, J. B. (1990). A genetic locus of enteropathogenic *Escherichia coli* necessary for the production of attaching and effacing lesions on tissue culture cells. *Proc Natl Acad Sci USA, 87*, 7839–7843.
22. Gannon, V. P. J., Rashed, M., King, R. K., & Thomas, E. J. G. (1993). Detection and characterization of the *eae* gene of Shiga-like toxin-producing *Escherichia coli* using polymerase chain reaction. *J Clin Microbiol, 31*, 1268–1274.
23. Paton, A. W., & Paton, J. C. (1998). Detection and characterization of Shiga toxigenic *Escherichia coli* by using multiplex PCR assays *stx1, stx2, eaeA*, enterohemorrhagic *E. coli hlyA*, *rfb* O157. *J Clin Microbiol, 36*, 598–602.
24. Keskimäki, M., Ikäheimo, R., Kärkkäinen, P., Scheutz, F., Puohiniemi, R. L., & Siitonen, A. (1997). Shiga toxin producing *Escherichia coli* serotype OX3:H21 causing hemolytic uremic syndrome. *Clin Infect Dis, 24*, 1278–1279.
25. Brunder, W., Schmidt, H., & Karch, H. (1997). *EspP*, a novel extracellular serine protease of enterohaemorrhagic *Escherichia coli* O157:H7 cleaves human coagulation factor V. *Mol Microbiol, 24*, 767–778.
26. Anon (2000). NF V 08–600. Microbiologie des aliments – Dénombrement des *Escherichia coli* présumés dans les coquillages vivants – Technique du nombre le plus propable (Microbiologie of Foods and Foodstuffs Products –Enumeration of Presumptive *Escherichia coli* in Living Shellfish – MPN Technique). AFNOR, 16 pp.

27. Signoretto, C., Burlacchini, G., Pruzzo, C., & Canepari, P. (2004). Persistence of *Enterococcus faecalis* in Aquatic Environments via Surface Interactions with Copepods. *Applied and Environmental Microbiology, 71*(5), 2756–2761. doi:10.1128/AEM.71.5.2756-2761.2005.
28. Koneman, E. W., Allen, S. D., Schrekenberger, P. C., Janda, W. M., & Winn, W. C. (1997). *Color Atlas and Textbook Microbiology* (5th ed.). Philadelphia: Lippincott Company.
29. Karch, H., & Bielaszewska, M. (2001). Sorbitol-fermenting Shiga toxin-producing *Escherichia coli* O157:H7 strains: Epidemiology, phenotypic and molecular characteristics, and microbiological diagnosis. *J Clin Microbiol, 39*, 2043–2049.
30. Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). *Molecular cloning, a laboratory manual*. New York: Cold Spring Harbor Laboratory Press.
31. MacRae M, Strachan NJC, Jacklin M, Ogden ID (2003). Methods to isolate and incidence of *Escherichia coli* O157 in UK Shellfish. In 5th International Symposium on 'Shiga toxin-producing *E. coli* infections', VTEC 2003, Edinburgh, 8 to 11 June 2003 (poster presentation).
32. Badri, S., Filliol, I., Carle, I., Hassar, M., Fassouane, A., & Cohen, N. (2009). Prevalence of virulence genes in *Escherichia coli* isolated from food in Casablanca (Morocco). *Food Control, 20*(6), 560–564.
33. Djordjevic, S. P., Hornitzky, M. A., Bailey, G., Gill, P., Vanselow, B., Walker, K., et al. (2001). Virulence properties and serotypes of Shiga toxin-producing *Escherichia coli* from healthy Australian slaughter age sheep. *J Clin Microbiol, 39*, 2017–2021.
34. Orden, J. A., Ruiz-Santa-Quiteria, J. A., Blanco, M., Blanco, J. E., Mora, A., Cid, D., et al. (2003). Prevalence and characterization of vero cytotoxin-producing *Escherichia coli* isolated from diarrhoeic and healthy sheep and goats. *Epidemiol Infect, 130*, 313–321.
35. Bhat, M. A., Nishikawa, Y., & Wani, S. A. (2008). Prevalence and virulence gene profiles of Shiga toxin-producing *Escherichia coli* and enteropathogenic *Escherichia coli* from diarrhoeic and healthy lambs in India. *Small Ruminant Res, 75*, 65–70.
36. Ackman, D., Marks, S., Mack, P., Caldwell, M., Root, T., & Birkhead, G. (1997). Swimming-associated haemorrhagic colitis due to *Escherichia coli* O157:H7 infection: Evidence of prolonged contamination of a fresh water lake. *Epidemiol Infect, 119*, 1–8.
37. Kuhnert, P., Boerlin, P., & Frey, J. (2000). Target genes for virulence assessment of *Escherichia coli* isolates from water, food and the environment. *FEMS Microbiology Rev, 24*, 107–117.
38. Lee, R. J., & Morgan, O. C. (2003). Environmental factors influencing the microbiological contamination of commercially harvested shellfish. *Water Sci Technol, 47*, 65–70.
39. Muniesa, M., & Jofre, J. (1998). Abundance in sewage of bacteriophages that infect *Escherichia coli* *Escherichia coli* O157:H7 and that carry the Shiga toxin 2 gene. *Appl Environ Microbiol, 64*, 2443–2448.
40. Muniesa, M., & Jofre, J. (2000). Occurrence of phages infecting *Escherichia coli* O157:H7 carrying the Stx 2 gene in sewage from different countries. *FEMS Microbiol Lett, 183*, 197–2000.
41. Karch, H., & Meyer, T. (1989). Single primer pair for amplifying segments of distinct Shiga-like-toxin genes by polymerase chain reaction. *J Clin Microbiol, 27*, 2751–2757.
42. Brian, M. J., Frosolono, M., Murray, B. E., Miranda, A., Lopez, E. L., Gomez, H. F., et al. (1992). Polymerase chain reaction for diagnosis of enterohemorrhagic *Escherichia coli* infection and hemolyticuremic syndrome. *J Clin Microbiol, 30*, 1801–1806.
43. Read, S. C., Clarke, R. C., Martin, A., De Grandis, S. A., Hii, J., McEwen, S., et al. (1992). Polymerase chain reaction for detection of verocytotoxigenic *Escherichia coli* isolated from animal and food sources. *Mol Cell Probes, 6*, 153–161.
44. China, B., Pirson, V., & Mainil, J. (1996). Typing of bovine attaching and effacing *Escherichia coli* by multiplex in vitro amplification of virulence-associated genes. *Appl Environ Microbiol, 62*, 3462–3465.
45. Osek, J. (2003). Development of a multiplex PCR approach for the identification of Shiga toxin-producing *Escherichia coli* strains and their major virulence factor genes. *J Appl Microbiol, 95*, 1217–1225.
46. Bischoff, C., Luthy, J., Altwegg, M., & Baggi, F. (2005). Rapid detection of diarrheagenic E. coli by realtime PCR. *J Microbiol Meth, 61*, 335–341.
47. Cortes, C., De La Fuente, R., Blanco, J., Blanco, M., Blanco, J. E., Dhabi, G., et al. (2005). Serotypes, virulence genes and intimin types of verotoxin-producing *Escherichia coli* and enteropathogenic E. coli isolated from healthy dairy goats in Spain. Veterinary. *Microbiology, 110*(1–2), 67–76.
48. Louie, M., de Azavedo, J., Clarke, R., Borczyk, A., Lior, H., Richter, M., et al. (1994). Sequence heterogeneity of the eae gene and detection of verotoxin-producing *Escherichia coli* using serotype-specific primers. *Epidemiol Infect, 112*(3), 449–461.
49. Eklund, M., Leino, K., & Siitonen, A. (2002). Clinical *Escherichia coli* strains carrying stx genes: stx variants and stx-positive virulence profiles. *J Clin Microbiol, 40*, 4585–4593.
50. Schmidt, H., Plaschke, B., Franke, S., Rüssmann, H., Schwarzkopf, A., Heesemann, J., et al. (1994). Differentiation in virulence patterns of *Escherichia coli* possessing eae genes. *Med Microbiol Immunol, 83*, 23–31.

51. Barrett, T. J., Kaper, J. B., Jerse, A. E., & Wachsmuth, I. K. (1992). Virulence factors in Shiga-like toxin-producing *Escherichia coli* isolated from humans and cattle. *J Infect Dis, 165*, 979–980.
52. McDaniel, T. K., Jarvis, K. G., Donnenberg, M. S., & Kaper, J. B. (1995). A genetic locus of enterocyte effacement conserved among diverse enterobacterial pathogens. *Proc Natl Acad Sci USA, 92*, 1664–1668.
53. Beutin, L., Geiger, D., Zimmermann, S., & Karch, H. (1995). Virulence markers of Shiga like toxin producing *Escherichia coli* isolates originating from healthy domestic animals of different species. *J Clin Microbiol, 33*, 631–635.
54. Oswald, E., Schmidt, H., Morabito, S., Karch, H., Marches, O., & Caprioli, A. (2000). Typing of intimin genes in human and animal enterohemorrhagic and enteropathogenic *Escherichia coli*: Characterization of a new intimin variant. *Infect Immun, 68*, 64–71.