



# Target genes for virulence assessment of *Escherichia coli* isolates from water, food and the environment

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## Abstract

The widespread species *Escherichia coli* includes a broad variety of different types, ranging from highly pathogenic strains causing worldwide outbreaks of severe disease to avirulent isolates which are part of the normal intestinal flora or which are well characterized and safe laboratory strains. The pathogenicity of a given *E. coli* strain is mainly determined by specific virulence factors which include adhesins, invasins, toxins and capsule. They are often organized in large genetic blocks either on the chromosome ('pathogenicity islands'), on large plasmids or on phages and can be transmitted horizontally between strains. In this review we summarize the current knowledge of the virulence attributes which determine the pathogenic potential of *E. coli* strains and the methodology available to assess the virulence of *E. coli* isolates. We also focus on a recently developed procedure based on a broad-range detection system for *E. coli*-specific virulence genes that makes it possible to determine the potential pathogenicity and its nature in *E. coli* strains from various sources. This makes it possible to determine the pathotype of *E. coli* strains in medical diagnostics, to assess the virulence and health risks of *E. coli* contaminating water, food and the environment and to study potential reservoirs of virulence genes which might contribute to the emergence of new forms of pathogenic *E. coli*. © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

**Keywords:** *Escherichia coli*; Virulence assessment; Food; Water; Gene probe; Gene array; Toxin gene; Adherence gene

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## 1. Introduction

Strains constituting the species *Escherichia coli* are of a broad variety. Beside apathogenic commensal representa-

tives found in the intestinal flora of man and animals, certain strains are highly pathogenic. Laboratories all over the world use a broad range of derivatives of a particular strain of *E. coli*, the K-12 derivatives, which are intensively used in research and industrial processes as safe biological containment strains. *E. coli* K-12 strains were shown to be non-pathogenic strains [1] and are unable to colonize or to survive in the environment [2]. They are the

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‘workhorse’ of molecular biologists and have contributed to a major extent to the modern knowledge of elementary molecular biological mechanisms and the genetic basis of life. The first comprehensive linkage map of an organism was obtained from *E. coli* K-12, long before any DNA sequence data were available (see [3] for a recent update), and led to the last milestone which was the determination of its complete genome sequence [4]. On the other hand, a single pathogenic type of *E. coli*, serotype O157:H7, has recently induced tremendous food-borne epidemics in industrialized countries worldwide causing ten thousands of hospitalizations and many deaths [5]. The thousands of derivatives of K-12 [6] used in research and production laboratories around the world can be readily identified by a single PCR method [7]. However, the identification of other strains, in particular the pathogenic ones, requires a large number of microbiological, biochemical, biological and genetic tests.

The written scientific history of *E. coli* started with its first description in 1885 by Theodor Escherich [8]. He identified a bacterium he called *Bacterium coli commune* as the cause of infantile diarrhea. Its present name *Escherichia coli* became used and was officially accepted in 1958 in honor of its discoverer. Whereas in research on basic genetics and molecular biology *E. coli* was generally known as a non-pathogenic bacterium, in medicine *E. coli* is known as an important pathogen infecting worldwide millions of humans each year both in industrialized and in developing countries. Hence, during the last few decades molecular biologists have started to work on the mechanisms of bacterial pathogenicity of *E. coli* [9–12]. The vast amount of new knowledge and genetic data on pathogenic *E. coli* indicates that up to 10–20% of the genomic information found in highly pathogenic *E. coli* is not present in *E. coli* K-12. Most of the additional genes found in pathogenic *E. coli* encode various virulence factors which directly determine their virulence and pathotype. Diagnostic methods used nowadays focus on the detection of either specific toxins and their virulence attributes or specific target genes which permit the identification of the corresponding pathotype.

In this review we give an overview of the various types of *E. coli*, their known virulence factors as well as their distribution and role in epidemiology. Moreover, we summarize characterization and typing methods, present how genetic methodology based on virulence genes allows the determination of the pathotype of clinical isolates and how to assess the potential pathogenicity of *E. coli* strains found in water, food and in the environment.

## 2. *E. coli* as a pathogen

*E. coli* is a major component of the normal intestinal flora of humans and other mammals. A great diversity of commensal non-pathogenic *E. coli* strains belonging to

many different serotypes can be isolated from the feces of healthy individuals. These strains are massively shed in the environment and may contaminate food of animal origin or other foods like vegetables, fruits and their derivatives. They may also contaminate surface and underground water, generally without any adverse effects on human health.

*E. coli* from the normal intestinal flora are usually harmless to the host and rather represent opportunistic pathogens. Only in very rare instances can they become a threat to normal individuals. This is mainly the case in patients with impaired immune defenses not able to contain these commensals in their natural habitat or after a traumatic break in the natural barriers between the gut and other normally sterile sites of the body or after surgical interventions. They can also be part of mixed infections when primary pathogens break down the local defenses of a host.

Some specific *E. coli* strains nevertheless represent primary pathogens with an enhanced potential to cause disease. These pathogens have been broadly classified into two major categories: the enteric pathogens and the extraintestinal pathogens. With the exception of Shiga toxin-producing *E. coli*, which also indirectly affect body parts other than the intestine, the enteric pathogens are agents of diarrhea in humans and animals. The extraintestinal pathogenic *E. coli* constitute a separate group mainly causing infections of the urinary tract in all age categories or sepsis and meningitis in small children and young animals. The major subcategories of these pathogens are described below. For classes of animal pathogens not mentioned here, the reader is referred to other more comprehensive textbooks [10].

Enteric pathogenic *E. coli* [11,13] have been broadly divided into enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), Shiga toxin-producing *E. coli* (STEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC).

In humans, ETEC [11] are a major cause of diarrhea in young children from developing countries and in adults from industrialized countries traveling to these regions (traveler’s diarrhea). ETEC also cause diarrhea in newborn animals, including mainly piglets [14,15], calves [16], and lambs [17]. Fecal contamination of food and drinking water is the major route of infection for humans. In animals, contamination of the environment also seems to play a role. After ingestion, ETEC colonize the small bowel and attach to its mucosa. They usually do not cause any noticeable local inflammation or histological change in the intestinal mucosa, but cause a major dysfunction of the electrolyte and water transport in enterocytes leading to the typical signs of ETEC infections (mechanisms reviewed in [11]). These signs include watery diarrhea, low-grade fever, vomiting, cramps, and nausea and are similar to those of cholera, but generally milder.

EPEC represent another major cause of diarrhea in

third world countries. Only small children below age two are generally affected by this pathogen, which does not represent a source of traveler's diarrhea. Symptoms are relatively severe and children present with symptoms of profuse watery diarrhea, vomiting and fever. EPEC belong to a limited number of serogroups (mainly O14, O55, O86, O111, O119, O125, O126, O127, O128, and O142). EPEC are usually transmitted by contaminated food and colonize the small intestine where they attach tightly to the epithelial cells of the villus tips and cause typical lesions called attaching and effacing lesions [18]. A major feature of these lesions is the reorganization of the enterocyte structure including destruction of the brush border and the formation of pedestal-like structures on top of which the bacteria sit and remain in intimate contact with the cells. EPEC usually do not invade the intestinal mucosa, do not produce toxins similar to ETEC toxins, and the exact mechanisms responsible for EPEC-associated diarrhea are still under investigation [19]. Strains similar to EPEC are also found in rabbits, where they cause severe diarrhea and histological lesions similar to those observed in humans [20].

STEC (previously called Shiga-like toxin-producing *E. coli* or verotoxin-producing *E. coli*) cause a broad range of symptoms in humans [21] including uncomplicated diarrhea, but also more severe diseases like hemorrhagic colitis and the often deadly hemorrhagic uremic syndrome. STEC infections represent a typical disease of industrialized countries and severe forms of infection are observed mainly in young children and the elderly. Cattle form the main reservoir of STEC and fecal contamination of food represents the usual source of infection for humans but due to an apparently low infectious dose, human to human transmission has also been observed in outbreaks. STEC have also been shown to be responsible for diarrhea in calves [16] and some specific serotypes are responsible for edema disease in pigs [22]. STEC belong to a very large variety of serotypes, but the majority of clinical infections registered in humans and particularly in food-borne outbreaks are associated with the serotypes and serogroups O157:H7, O157:H-, O26, O103, O111, O113, and O145. Those serotypes and strains associated with severe disease and outbreaks are also called enterohemorrhagic *E. coli* (EHEC [13]). STEC colonize the colon where they cause necrosis of villus tips but they do not invade the intestinal mucosa. The majority of EHEC have been shown to present the typical pattern of localized adherence on cell cultures also known from EPEC and to be able to cause attaching and effacing lesions also associated with the latter pathogens [11].

EIEC closely resemble *Shigella* and cause mainly watery diarrhea and dysentery in severe cases. They invade and destroy enterocytes, thus leading to a strong inflammation. The exact mechanisms leading to diarrhea have not yet been clarified [11]. EIEC are relatively rarely isolated in industrialized countries and may be more frequent in de-

veloping countries. Their epidemiology is still poorly understood, but infections due to EIEC may occur both in the form of outbreaks and in sporadic cases. Transmission seems to be mainly through contaminated food and water.

EAEC present a typical adherence pattern on cell cultures, with bacteria aligning in parallel clusters like bricks in a wall [23]. Experiments on volunteers and on animal models have demonstrated the pathogenic potential of EAEC. Association of EAEC with pediatric diarrhea with lesions similar to those observed in animal models has subsequently confirmed their virulence. EAEC infections occur as sporadic cases or outbreaks mainly in developing countries, but they have also been observed in industrialized countries [23]. The clinical signs of EAEC infections are usually watery mucoid (sometimes bloody) diarrhea with low-grade fever and little or no vomiting. Typical is the persistence of diarrhea for often more than 14 days. The epidemiology and the exact pathogenic potential of EAEC are less well understood than those of EPEC and will not be discussed here.

Extraintestinal pathogenic *E. coli* have been categorized mainly into uropathogenic *E. coli* (UPEC) and neonatal meningitis *E. coli* (NMEC). UPEC represent by far the most frequent cause of urinary tract infections in humans [24]. *E. coli* also frequently cause urinary tract infections in dogs and cats and clinical isolates from these animals seem to share some common properties with UPEC of human origin [20]. UPEC can be associated with asymptomatic bacteriuria and uncomplicated cystitis, but can also be at the origin of severe pyelonephritis following ascendant infections. UPEC belong to a relatively limited number of serotypes and present enhanced adherence to epithelial cells of the urinary tract, particularly those isolated from pyelonephritis cases.

NMEC cause neonatal meningitis and sepsis in babies [25]. NMEC most probably enter the body through the intestinal mucosa and subsequently cause sepsis or localize in the central nervous system. Transmission of NMEC to babies usually occurs through the mother or nursery staff and colonization takes place early in life.

### 3. Virulence factors of *E. coli* and their distribution

*E. coli* pathogenicity is a complex multi-factorial mechanism involving a large number of virulence factors which vary according to the pathotype. They include attachment functions, host cell surface modifying factors, invasins, and many different toxins as well as secretion systems which export toxins and other virulence factors and pilot them to the target host cells. The term 'virulence factor' is actually not precisely defined since a single component might not be sufficient to transform an *E. coli* strain into a pathogenic one but could only play a role in combination with other (virulence) determinants [26,27]. In the

present review we use the term ‘virulence factor’ for determinants that have a major contribution to a strain’s pathogenic potential. Antibiotic resistance genes, a class of factors that could also be seen as virulence attributes, are not discussed in this review. Moreover, lipopolysaccharide or endotoxin, a general virulence attribute of Gram-negative bacteria and not specific for *E. coli* in general or a particular pathotype, is not discussed further in this work. In the following, an overview of the classes of *E. coli* virulence factors and their distribution is given.

Toxins are the most obvious virulence factors found in practically all pathogenic *E. coli*. Moreover, some toxins show a strong association with specific pathotypes (Table 1). The Shiga toxins (formerly designated Shiga-like toxins or verotoxins) Stx1 and Stx2, encoded by the genes *stx1* and *stx2*, respectively, are prominent toxins and gave the name to the Shiga toxin-producing *E. coli* pathotype STEC. After internalization [28], Stx1 and Stx2 depurinate specific residues of the host cell ribosomes [29,30], thereby blocking the binding of aminoacyl tRNA to the ribosomes and inhibiting the protein synthesis. The highly pathogenic EHEC subgroup of this pathotype contains in addition the locus of enterocyte effacement with the intimin gene *eae* (see below) and generally harbors the EHEC hemolysin plasmid carrying the *ehx* gene [31]. EHEC hemolysin is strongly associated with STEC isolates from severe disease in humans [26], but its exact role in pathogenicity of STEC is unknown. The heat-labile toxins LTI (gene designation *eltIA*) and LTII (*eltIIA*) are found in ETEC [32]. They have ADP-ribosylating activity and strongly resemble the cholera toxin in structure and function. After internalization, the A1 subunit of these toxins indirectly blocks the adenylate cyclase of the enterocytes in an active state. This leads to an increased concentration of cAMP in the cells, which in turn completely deregulates the water and electrolyte secretion of the cells [11]. The two unrelated heat-stable toxins STa (*stIA*) and STb (*stIB*) are also characteristic of the ETEC pathotype. STa binds to the surface of enterocytes and causes activation of guanylate cyclase and accumulation of cGMP. Similar to the accumulation of cAMP with LT, the accumulation of cGMP indirectly leads to massive loss of electrolyte (mainly chloride ions) and water [11,33]. In contrast, STb does not increase the level of cAMP or cGMP in the cells, but leads by not yet definitively characterized mechanisms to an increased secretion of bicarbonate and water [34]. The  $\alpha$ -hemolysin (*hly*) is a pore-forming toxin of the RTX family [35,36]. It is predominantly detected in UPEC strains where it seems to be associated with virulence and it is also a major marker of most porcine ETEC and STEC causing edema disease in pigs. The cytotoxic necrotizing factor (*cnf-1*) is a second toxin strongly associated with  $\alpha$ -hemolysin and virulence in uropathogenic isolates [37]. The heat-stable toxin EAST1 (*astA*), which is unrelated to STa and STb, is not restricted to a specific pathotype but has now been found in all types of enteric *E. coli* of clinical significance

[38]. This toxin seems to have an active site and a mechanism of action similar to STa [39].

A second major class of virulence determinants represents the adhesion factors including mainly fimbriae (for a review, see [40]). Whereas type I fimbriae (*fim1pil*) are not restricted to pathogenic *E. coli* and can be found in most strains including the laboratory strain K-12, other fimbriae, in particular P fimbriae (*pap/prs*), F1C fimbriae (*foc*) and S fimbriae (*sfa*), are typical for UPEC [24]. These fimbriae are supposed to be responsible for adherence to the host mucosa at different stages of the infection. S fimbriae are also frequently found in NMEC. Colonization factor antigens CFA/I (*cfalI*) and CFA/II (*cfalII*) are typical adhesion structures of ETEC [11]. These fimbriae allow ETEC to adhere to the intestinal mucosa of the small bowel where they can efficiently deliver their toxins directly to their target. Bundle-forming pili (*bfp*) encoded by the EAF plasmid are typical for EPEC [41,42]. EAEC contain a different type of bundle-forming pilus called aggregative adherence fimbriae AAF/I (*aafI*) which is supposed to be a major factor contributing to the lesions observed with EAEC [43]. The afimbrial adhesin intimin (*eae*) responsible for intimate adherence on epithelial cells is typical for EPEC and EHEC strains. The *eae* is indicative for the presence of a ‘pathogenicity island’ called locus of enterocyte effacement (LEE) which also encodes a type III secretion system and several secreted proteins [44,45].

Invasion plasmid antigens are typically found with numerous other invasion-associated factors in the EIEC pathotype and in *Shigella*. The function of the multicopy *ipaH* gene [46], often used as a specific gene probe for the invasion plasmid, remains unknown. The high efficiency iron uptake system is mediated by the siderophore aerobactin. The gene product of *iucC* is involved in biosynthesis of aerobactin and *iucC* is therefore an indicator for the presence of the aerobactin-mediated iron transport system. It is generally found in extraintestinal *E. coli* isolates causing septicemia and occasionally in EIEC [47,48].

Capsule polysaccharides have antiphagocytic activity and protect bacteria against complement-mediated serum killing [49,50]. Polysaccharide capsules are frequently encountered in UPEC and NMEC and represent an important virulence factor for these extraintestinal pathogens. The most frequent capsular types found in UPEC and NMEC are K1 and K5. The products of the *neuA* and *neuC* genes, and the *kfiB* gene, respectively, are involved in the synthesis of these two important capsular types and can therefore be used as probes for the K1 and K5 capsules [51–53].

The genome of *E. coli* is of high plasticity allowing it to gain and lose (virulence) genes at a relatively high frequency. Adding to the plasticity is the fact that many virulence genes in *E. coli* are located on mobile elements like plasmids, phages or transposons [54]. They are often clustered on large genetic blocks, so-called pathogenicity islands (PAI) [55] which are characterized by specific

flanking sequences (mainly particular tRNA) known to be hot-spots of integration/excision. A recent review of the whole concept of pathogenicity island is given in [56]. The impact PAI have on the detection of virulence genes is that by showing the presence of a single PAI gene, in general the presence of all the additional virulence attributes encoded on the PAI is demonstrated. From an epidemiological point of view it means that virulence attributes are not exchanged as single entities but as virulence blocks or packages thereby even defining a certain pathotype. The combination of PAI with DNA elements encoding other virulence determinants might then lead to the emergence of new pathogenic types, as is seen e.g. with the newly emerged EHEC strains, where the LEE combined with a virulence plasmid and one or both of the Shiga toxins genes recently gave rise to a much feared food-borne pathogen represented by serotype O157:H7. So far five PAI have been detected in *E. coli*, most of them in UPEC [56]. The pathogenicity island PAI V for example contains the genes encoding the well known  $\alpha$ -hemolysin, the cytotoxic necrotizing factor and the P fimbriae. So far the LEE is the only PAI found in intestinal *E. coli*.

Since most virulence factors are encoded on mobile genetic elements, strains with new combinations of virulence genes might emerge in the future. Association of specific virulence genes with certain categories of *E. coli* may consequently become more and more difficult. Therefore, a system to assess the virulence patterns of *E. coli* isolates comprising the entire set of known major virulence genes will be of great help to better characterize clinical *E. coli* isolates as well as strains from food, water and the environment. It will definitely help to get a clearer picture of their pathogenic potential. It may also help to detect newly emerging clones of pathogenic *E. coli*.

## 4. Detection and typing

### 4.1. Phenotypic methods

The presence of *E. coli* and other coliform bacteria (e.g. enterococci) is generally an indication for fecal contamination of water and food. Quantification of the total number of *E. coli* present is therefore an integral part of quality assessment of food and water. This can be achieved either by enumeration of viable colonies, or by using biochemical methods, e.g. quantifying ATP using luciferase in a chemiluminescence assay [57]. In this rapid assay, the ATP measured is an indicator of the number of bacteria present in a sample. This assay is not designed to discriminate coliforms from other bacteria but gives an estimation of the global degree of bacterial contamination. It is mainly used for quality assessment in food processing. A more specific investigation of the content of *E. coli* in bacterially contaminated samples is normally done by cul-

tivating bacteria after concentration by filtration or by use of enrichment and/or selective media. Isolation of the microorganisms by cultivation on various growth media generally represents the first and basic step in more detailed investigations of the nature, the source, and the potential risk of a bacterial contamination of food, water and the environment. Further bacterial characterization at the level of species and beyond are made by biochemical, biological, immunological and genetic methods in order to determine the serotype, biotype or pathotype. Serotyping is a common method used for the characterization of clinical isolates of *E. coli* and has a broad use in epidemiology and also in medical diagnostics [58]. Serotyping is mainly based on the determination of the lipopolysaccharide O antigens, the flagellar H antigens and the capsular K antigens. Hence the existing association between serotype and pathotype makes this method a valuable tool for typing *E. coli* and also other bacterial species. The newly emerged pathogen *E. coli* O157:H7 is an example of a strong association of a specific serotype with a pathotype. It could be diagnosed before any virulence properties of this strain were known [59,60].

Functional typing of *E. coli* strains, mainly based on their toxigenic potential, requests either the use of specialized cell cultures or ultimately experimental animal infections. Even though all these tests provide the most direct evidence for the presence of biologically active toxin and/or infectious bacteria in a sample, they are laborious and not all of them can be used in a laboratory for the investigation of large numbers of samples. Moreover, ethical considerations, legislation on animal experimentation and the low public acceptance of using animals in laboratories cause these biological tests to be applied very rarely. In addition, many *E. coli* pathogens are adapted to a host species and will not induce disease in other hosts thus making animal models unsuitable for their study.

### 4.2. Genetic methods

Due to the vast knowledge of the genetic bases of *E. coli* virulence, genetic methods are more and more frequently applied in detection and typing of *E. coli*. These methods show a high resolution, a good potential for automation and under certain conditions even avoid the bacterial culture step. Many genetic methods are based on the detection of specific (pathogenicity) markers, others use epidemiological markers such as ribotyping [61]. Most specifically, the polymerase chain reaction (PCR) allows the fast and direct detection of virulence genes in a sample even without prior cultivation of the microorganisms. This is particularly useful for the detection of low-number contamination in food and also for the analysis of clinical samples. A major improvement of the PCR technique is the quantitative real-time PCR using the TaqMan<sup>®</sup> system which allows both qualitative and quantitative determinations [62,63]. The low contamination risk, high sensitivity

Table 1  
*E. coli* pathotypes and distribution of typical virulence factors

Virulence factor	ETEC	EPEC	STEC/EHEC	EIEC	EAEC	UPEC	NMEC
<i>Toxins</i>							
Shiga toxin 1 (Stx1)			++				
Shiga toxin 2 (Stx2)			++				
Heat-labile toxin I (LT1)	++						
Heat-labile toxin II (LTII)	+ <sup>a</sup>						
Heat-stable toxin I (STa)	++						
Heat-stable toxin II (STb)	+ <sup>a</sup>						
Low-MW heat-stable toxin (EAST1)	+	+	++		+		
α-Hemolysin (Hly)			++ <sup>b</sup>			++	
EHEC hemolysin (Ehx)			+ <sup>c</sup>				
Cytotoxic necrotizing factor 1 (CNF1)						+	
<i>Adhesins</i>							
P fimbriae						++	
S fimbriae						++	+
F1C fimbriae						++	
Colonization factor antigen I (CFA/I)	+						
Colonization factor antigen II (CFA/II; CS3)	++						
Bundle-forming pilus (Bfp)		++					
Aggregative adherence fimbriae I (AAF/I)					++		
Intimin (Eae)		++	++				
<i>Invasins</i>							
Invasion plasmid antigen (Ipa)				++			
<i>Iron acquisition</i>							
Aerobactin				+		++	+
<i>Capsule</i>							
K1 capsule antigen						+	++
K5 capsule antigen						+	

+ occasionally detected, ++ normally detected.

<sup>a</sup>Primarily in animal isolates.

<sup>b</sup>In porcine isolates.

<sup>c</sup>Predominantly strains of the EHEC sub-pathotype.

and high throughput including automation and computer analysis make this technique of great interest for research and for routine diagnostics. Kits for food-borne pathogens such as the EHEC pathotype of *E. coli* using the TaqMan<sup>®</sup> technique are already available and are mainly used in food hygiene. While PCR methods are highly specific, they are unable to determine certain variants of the target genes present in particular strains. Hence hybridization methods using gene probes for Shiga toxins and the heat-labile and/or heat-stable toxins are frequently used to investigate populations of toxigenic *E. coli* [11].

Sequencing DNA regions specifically amplified by PCR can also be used for detection and add to a more precise identification of *E. coli*. Whereas 16S rRNA genes when used as a target for this method are good phylogenetic markers for species identification, they cannot be used to define *E. coli* subsets or pathotypes.

While all the above described methods are most valuable tools for the identification of defined types of *E. coli* in medical diagnosis and food hygiene, they are not designed for a general assessment of the genetic potential of virulence of *E. coli* isolates. However, this latter step would be of particular interest for *E. coli* isolates found occasionally in water, in particular waste water, and the environment in

order to assess the presence of such genes in yet unassigned types of *E. coli* strains. Such strains, which per se might still be inoffensive to humans and animals, might under certain circumstances represent an important reservoir of virulence genes which might remain apparently silent until in combination with other factors they might give rise to new strains which could become of concern for humans and animals.

## 5. Broad-range screening for *E. coli* virulence genes

In order to efficiently test *E. coli* strains for the presence of known virulence genes and to get a complete picture of the virulence attributes of a given strain, we have recently developed a reverse dot-blot hybridization procedure [64]. Specific segments of all currently known major virulence genes of *E. coli* (Table 2) were designed in order to present similar hybridization parameters and were cloned on plasmids. The probes can be isolated from plasmids and subsequently be amplified by PCR as unlabelled probes in amounts sufficient to be bound to nylon membranes. The filters which represent arrays of *E. coli* virulence genes are then hybridized to labelled genomic DNA of *E. coli* strains to be tested. Known pathogenic *E. coli* isolates and

well known non-pathogenic *E. coli* laboratory strains were analyzed by this method. These hybridization results demonstrated that the ferrichrome-iron receptor gene (*fhuA*) is ubiquitous in the species *E. coli* and that the type 1 fimbria gene (*fimA*) can be present both in *E. coli* isolated from normal flora and in pathogenic isolates. These genes therefore are not considered targets for assessment of virulence, but the probes are used as controls for the method [64]. The hybridization results obtained with this macro-array assay showed that the non-pathogenic *E. coli* strains did not react with any of the virulence genes (Table 2). On the other hand, clinical isolates of pathogenic *E. coli* showed hybridization patterns of the typical virulence genes expected to be present in these pathogens, corresponding to the pathotype predicted from the clinical symptoms they caused [64].

The method is illustrated in Fig. 1, which gives examples of the results obtained with *E. coli* strains of various origins. A strain of the well characterized non-pathogenic *E. coli* K-12 derivative, which is an approved biological safety strain, only shows signals with the control probes for *fhuA* (Fig. 1, probe A1) and *fimA* (probe A2) genes but not with any virulence gene probes as expected. In contrast, a uropathogenic *E. coli* strain, UPEC, shows the characteristic signals for this pathotype with the specific fimbrial genes *pap* (Fig. 1, probe A3), *sfa* (probes A4 and A5) and *foc* (probe A6) found in UPEC as well as the toxin genes *hlyA* (probe C5), *cnf-1* (probe C7), the capsule K5 gene (probe B7) and the gene probe for aerobactin

*iucC* (probe B5). As further examples, Fig. 1 shows the hybridization patterns of an *E. coli* strain isolated from contaminated food (Fig. 1, sample a) and from an *E. coli* isolated from contaminated surface water (Fig. 1, sample b). Sample a, which originates from contaminated food and can cause severe intoxication, shows hybridization reactions for both *stx* genes (probes C1 and C2), the aerobactin-specific gene (probe B5) and the gene for EHEC hemolysin (probe C6) in addition to the control probes. This virulence gene pattern is characteristic for the shiga-toxigenic *E. coli*, STEC (see Table 1), a pathotype that is particularly pathogenic to human. Intoxication with this pathotype can cause most severe diseases which are generally transmitted by contaminated food of animal origin, in particular cattle meat and milk products [11]. Such a result hence is not only an alarming sign for the production line of the food product concerned, but also gives most valuable indications on the potential source of the contamination.

The second example, illustrated in sample b of Fig. 1, represents a strain isolated from surface water. Besides the control probes it hybridized with the gene probes for the colonization factor antigen CFA/II (Fig. 1, probe B1, sample b), the heat-stable toxin ST (probe B8) and the heat-labile toxin LTI (probe C3), characteristic for an enterotoxigenic *E. coli*, ETEC (see Table 1). The virulence gene pattern indicated that this strain is potentially toxigenic and hence indicates a water contamination which is harmful to humans and also animals. Such pathogenic *E. coli*

Table 2  
Probes used in this study, genes they derived from and references for gene sequences

Probe number	Name	Gene	Reference
A1 <sup>a</sup>	Ferrichrome-iron receptor (Fhu)	<i>fhuA</i>	[67]
A2 <sup>a</sup>	Type 1 fimbriae (Fim)	<i>fimA</i>	[68]
A3	P fimbriae (Pap)	<i>papA</i>	[69]
A4	S fimbriae (Sfa)	<i>sfaA</i>	[70]
A5	S fimbriae (Sfa)	<i>sfaS</i>	[71]
A6	F1C fimbriae	<i>foc</i>	[72]
A7	Bundle-forming pilus (Bfp)	<i>bfpA</i>	[42]
A8	Colonization factor antigen I (CFA/I)	<i>cfalI</i>	[73]
B1	Colonization factor antigen II (CFA/II; CS3)	<i>cfalII</i>	[74]
B2	Aggregative adherence fimbriae (AAF/I)	<i>aafII</i>	[75]
B3	Intimin (Eae)	<i>eae</i>	[76]
B4	Invasion plasmid antigen (Ipa)	<i>ipaH</i>	[46]
B5	Aerobactin	<i>iucC</i>	[77]
B6	K1 capsule antigen	<i>neuA+neuC</i>	[51,52]
B7	K5 capsule antigen	<i>kfiB</i>	[53]
B8	Heat-stable toxins (STa, STb)	<i>stIa/stIb</i>	[78]
C1	Shiga toxin 1 (Stx1)	<i>stxI</i>	[79]
C2	Shiga toxin 2 (Stx2)	<i>stxII</i>	[80]
C3	Heat-labile toxin I (LTI)	<i>eltI</i>	[81]
C4	Heat-labile toxin II (LTII)	<i>eltIIa</i>	[82]
C5	$\alpha$ -Hemolysin (Hly)	<i>hlyA</i>	[83]
C6	EHEC hemolysin (Ehx)	<i>ehxA</i>	[31]
C7	Cytotoxic necrotizing factor (CNF1)	<i>cnf-1</i>	[84]
C8	Low-MW heat-stable toxin (EAST1)	<i>astA</i>	[85]

<sup>a</sup>The ferrichrome-iron receptor and the type 1 fimbriae are not considered virulence factors. These gene probes are used as positive controls for *E. coli*.

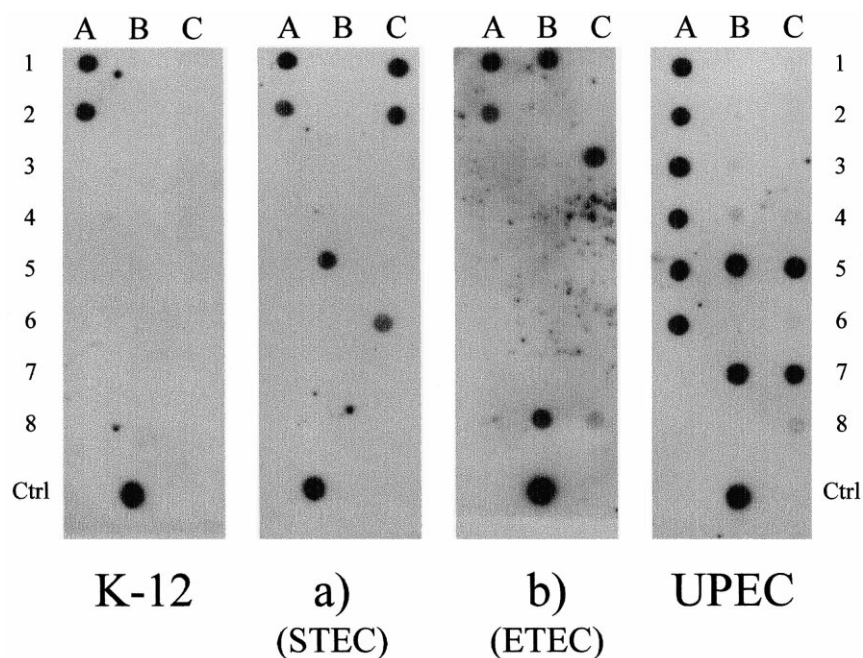


Fig. 1. Example of the described reverse dot-blot technique for virulence assessment of *E. coli* isolates. Probes A1–C8 of the gene array filters are listed in Table 2. Probe Ctrl contains part of the *E. coli* 16S rRNA gene (*rrs*) as a technical control to react with any bacterial species, while probes A1 and A2 constitute two probes of non-virulence genes found commonly in *E. coli*. Dark spots represent positive hybridization signals indicating the presence of the respective virulence gene. K-12 is an *E. coli* laboratory strain (C600) used as biological safety strain. UPEC is a uropathogenic *E. coli* (RZ475) isolated from a human patient suffering from pyelonephritis. Sample a represents an example of a strain isolated from contaminated food, sample b is a strain isolated from contaminated surface water.

are fortunately found only rarely in water samples and are mainly the result of a direct fecal contamination by the affected individual [65]. However, *E. coli* strains possessing other virulence gene patterns which resemble those of extraintestinal *E. coli* are found rather frequently in surface water samples ([65] and P. Kuhnert unpublished results). Based on these results, approximately 40% of *E. coli* strains isolated from surface water were shown to contain at least one virulence gene specific for extraintestinal *E. coli*. Nevertheless, the combinations or patterns of virulence genes found in *E. coli* which are isolated from environmental samples do vary significantly. In order to assess the potential risk which such strains constitute for humans and animals, a complete analysis for the presence or absence of all virulence genes is essential. It illustrates the power of the broad-range reverse dot-blot method for virulence assessment, which not only gives the pathotype of a given *E. coli* isolate, but directly provides information on its virulence potential and on its genetic pool of virulence genes. In order to apply this technique in routine applications for the analysis of large numbers of isolates or for the online analysis of food, water and environmental samples, the currently used technique [64] will require further development including gene array detection systems commonly named gene chips, micro-chips or bio-chips. Hence such developments are assumed to permit rapid online analysis for the presence of potentially hazardous *E. coli* and ultimately even other bacterial contaminants.

## 6. Concluding remarks

*Escherichia coli* is a bacterium which is strongly adapted to humans and many animals where it either lives in beneficial symbiosis or creates severe disease. The presence of *E. coli* in the environment, in particular in water resources and food, therefore requires the highest attention. While the presence of non-pathogenic *E. coli* in water and food is a clear sign of bad hygiene, the presence of pathogenic types of *E. coli* must be considered a direct threat to human and animal health.

During evolution, bacterial virulence genes have adapted to their particular hosts for specialized tasks during the process of infection. Being capable of transferring not only between members of a given bacterial species but also between different bacterial species, they created a large number of different types with variable pathogenicity within a species. New pathotypes seem to emerge by new combinations of different virulence genes [66]. Since virulence factors are directly involved in the mechanism of pathogenicity, their genes represent ideal targets for the molecular analysis of potential pathogenicity and typing of different pathotypes, not only in medical diagnosis but also in quality control processes of food and water, as well as for safety assessments for biotechnological applications. For the species of *E. coli* a large number of virulence genes have been identified, the most important ones being summarized in Table 1. Since many of the virulence genes of



*E. coli* are located on mobile elements such as plasmids, phages, transposons or pathogenicity islands, new pathotypes with new combinations are constantly emerging. In addition one must expect a very large number of types of *E. coli* which harbor certain virulence genes whose combination does not give them a particular pathogenic phenotype. Such strains, which are mainly not typed or analyzed in particular, might constitute a huge environmental genetic reservoir for virulence attributes and might be leading actors in the creation of new pathogens. Broad-range multi-array virulence gene detection systems such as the reverse dot-blot method used for virulence gene analysis of *E. coli* strains [64] will not only be useful for a clear distinction between non-pathogenic and pathogenic variants of *E. coli* strains and the identification of specific pathotypes of the latter group, but will also be a most useful tool for the detection and analysis of new types of *E. coli*. Moreover, they will provide the means to assess the potential reservoirs of virulence genes which are expected to play a key role as the origin of newly emerging diseases caused by *E. coli* and which are expected to be present in yet unexplored ecological niches. In combination with the rapidly developing gene array sensor devices, the virulence gene probes will also constitute a powerful tool for the molecular detection of pathogenic microorganisms in future quality control systems for safe drinking water and food.

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