FEMS Microbiology Reviews, fuw005, 40, 2016, 437–463

doi: 10.1093/femsre/fuw005
Advance Access Publication Date: 8 March 2016
Review Article

REVIEW ARTICLE

Escherichia coli: an old friend with new tidings

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One sentence summary: New knowledgements about pathogenesis, antimicrobial resistance and clinical aspects of *Escherichia coli*.

Editor: Kenn Gerdes

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ABSTRACT

Escherichia coli is one of the most-studied microorganisms worldwide but its characteristics are continually changing. Extraintestinal *E. coli* infections, such as urinary tract infections and neonatal sepsis, represent a huge public health problem. They are caused mainly by specialized extraintestinal pathogenic *E. coli* (ExPEC) strains that can innocuously colonize human hosts but can also cause disease upon entering a normally sterile body site. The virulence capability of such strains is determined by a combination of distinctive accessory traits, called virulence factors, in conjunction with their distinctive phylogenetic background. It is conceivable that by developing interventions against the most successful ExPEC lineages or their key virulence/colonization factors the associated burden of disease and health care costs could foreseeably be reduced in the future. On the other hand, one important problem worldwide is the increase of antimicrobial resistance shown by bacteria. As underscored in the last WHO global report, within a wide range of infectious agents including *E. coli*, antimicrobial resistance has reached an extremely worrisome situation that 'threatens the achievements of modern medicine'. In the present review, an update of the knowledge about the pathogenicity, antimicrobial resistance and clinical aspects of this 'old friend' was presented.

Keywords: *Escherichia coli*; pathogenesis; antimicrobial resistance; clinical; epidemiology; evolution

Received: 23 September 2015; **Accepted:** 4 February 2016

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INTRODUCTION

Escherichia coli is the most-studied microorganism. It is both a common commensal inhabitant of the gastrointestinal tract and one of the most important pathogens in humans. Thus, the most frequent cause of bloodstream infection and urinary tract infections (UTIs) among Gram-negative bacteria (GNB) is *E. coli*. Such isolates possess specialized virulence factors such as adhesins, toxins, iron-acquisition systems, polysaccharide coats and invasins that are not present in commensal and intestinal pathogenic strains (Sannes *et al*. 2004). In addition, *E. coli* are the enteric Gram-negative bacilli most frequently found in the genital tract of women, causing vaginal and/or endocervical colonization as well as different infections in pregnant women, such as intra-amniotic and puerperal infection, and neonatal infections, such as early and late neonatal sepsis (Guiral *et al*. 2011).

Antimicrobial resistance in *E. coli* is consistently highest for antimicrobial agents that have been in use the longest time in human and veterinary medicine, such as ampicillin. However, in the past two decades increases have been observed in the emergence and spread of multidrug-resistant bacteria, including strains resistant to newer antibiotics such as fluoroquinolones and extended-spectrum cephalosporins (Levy and Marshall 2004).

An update of the clinical aspects, pathogenesis and antimicrobial resistance of *E. coli* is compiled in this review.

PATHOGENESIS OF *E. coli*

Extraintestinal *E. coli*

Escherichia coli is an extremely common and complex human pathogen. It is the most frequently isolated species in clinical microbiology laboratories and is the leading cause of UTIs, with millions of cases and billions of dollars in associated health care costs annually in the USA (Russo and Johnson 2003). Although *E. coli* is most closely linked to UTI, it can infect any extraintestinal site, causing meningitis, skin structure infections, myositis, osteomyelitis and epididymo-orchitis. Severe *E. coli* infections, which often involve bloodstream infection (of which *E. coli* is a leading cause), frequently result in the systemic inflammatory response syndrome (SIRS), contributing to an estimated 40 000 deaths annually in the USA (Russo and Johnson 2003). This major burden of morbidity, mortality and costs greatly exceeds that associated with diarrheagenic *E. coli*, which in contrast receive much more attention from the press, public health system and lay public.

Escherichia coli is a diverse species which, from a human health perspective, can be viewed as comprising three main subsets (Johnson and Russo 2002; Touchon *et al*. 2009). Commensal strains innocuously colonize the colon of healthy hosts, causing extraintestinal disease only in the presence of a large inoculum (e.g., with penetrating abdominal trauma) and/or significant host compromise. Diarrhoeagenic strains cause diarrhoea syndromes that vary in clinical presentation and pathogenesis according to the strain's distinctive virulence traits. These differences serve as the basis for the classifying the strains into sub-pathotypes, e.g., enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC) and enteropathogenic *E. coli* (EPEC) and enteroaggregative *E. coli* (EAEC). Such strains almost never cause extraintestinal infection and, outside the developing world, rarely colonize healthy hosts. Finally, like commensal strains, extraintestinal pathogenic *E. coli* (ExPEC) often innocuously colonize the human gut. However, they have a unique ability to enter and survive within normally sterile extraintestinal body sites, and to cause disease when they do so. ExPEC strains are the main cause of human extraintestinal *E. coli* infections. Although traditionally designated as uropathogenic *E. coli* (UPEC) because of their association with UTI, such strains are now recognized as being more broadly pathogenic, leading to widespread use of the more inclusive term ExPEC (Russo and Johnson 2000).

The three main *E. coli* clinical subsets, or pathotypes (commensal, diarrhoeagenic and ExPEC), can be distinguished by comparative genome analysis. Although sharing a set of genes that is common to all *E. coli*, i.e., the *E. coli* core genome, the three pathotypes differ according to the presence/absence of multiple so-called accessory traits, which are dispensable for vegetative growth but determine the strains' distinctive clinical behaviors. The three pathotypes also tend to segregate within the phylogenetic tree of *E. coli*, with ExPEC occurring mainly in groups B2 and D and commensal and diarrhoeagenic strains in other groups (Touchon *et al*. 2009). However, certain non-group B2/D strains and clonal groups have acquired sufficient relevant accessory traits (probably via horizontal gene transfer) to become ExPEC (Johnson *et al*. 2001). Conversely, some group B2/D strains that exhibit ExPEC-like characteristics also exhibit characteristics that define the diarrheagenic pathotypes known as adherent and invasive *E. coli* (AIEC) and enteroaggregative *E. coli* (EAEC) (Nash *et al*. 2010; Vejborg *et al*. 2011; Olesen *et al*. 2014), which are discussed in greater detail in separate sections below. Despite the many genetic similarities between AIEC and ExPEC, the latter do not exhibit the adherent-invasive phenotype of AIEC (Martinez-Medina *et al*. 2009b). This fact indicates that additional 'virulence' factors conferring better fitness for colonization, and resistance to unfavorable conditions, may be implicated in the AIEC phenotype. It is still unknown whether particular unidentified genes or mutations of certain genes present in all AIEC are responsible for the AIEC phenotype, or to the contrary, if changes in different genes may lead to the same phenotype, and thus, AIEC might have emerged several times from different phylotypes. Genome sequencing of four AIEC has revealed some specific genes for AIEC; however, these strains belong to the same B2 phylogroup (Clarke *et al*. 2011). Given the high diversity of seropathotypes among AIEC, it is crucial to analyze several isolates of different phylogenetic origin in order to unravel the genetic basis of the AIEC phenotype. Additionally, it would be of interest to analyze for differences between AIEC and non-AIEC at the level of gene expression or to reveal the presence of point mutations, since isolates that are genomically very similar may differ in the AIEC phenotype (Martinez-Medina *et al*. 2009a).

The specialized traits that distinguish ExPEC strains from other *E. coli* subserve functions that promote survival on or in the host, including through attachment, nutrient acquisition, competition with other bacteria, and avoidance or subversion of host defense mechanisms, and/or lead to host cellular or tissue injury and, hence, disease (Johnson and Russo 2005). Although such traits traditionally have been regarded as 'virulence factors', many of them are increasingly considered as being 'fitness factors' that can promote colonization (including of the intestine) without leading to disease.

ExPEC strains tend to have multiple virulence factors, including multiple representatives of a given functional category (Johnson and Russo 2005; see UPEC section as example). Many different combinations of virulence factors occur in different ExPEC strains, evidence that there are many pathways to extraintestinal virulence in *E. coli*. Although virulence factor profiles tend to be fairly lineage-specific, consistent with vertical inheritance, variations in profiles are common even with a given

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lineage, consistent with horizontal gene transfer or deletions. Horizontal transfer involving virulence factor-encoding genes can occur via plasmids, phage-mediated transduction or other forms of recombination, and often involves blocks of DNA called 'fitness islands' or 'pathogenicity-associated islands' (PAIs) that contain multiple known or suspected virulence genes (Hacker and Carniel 2001; see specific section in this review). Analysis of genes of unknown function that are encountered adjacent to known virulence genes within such islands can lead to the discovery of novel virulence genes (Swenson *et al.* 1996).

Three main approaches can be used to determine whether a given *E. coli* isolate is ExPEC. The simplest and most widely used, although least reliable, uses clinical context and source of isolation to indicate the strain's virulence potential. Here, all clinical *E. coli* isolates from sites of extraintestinal infection are regarded as ExPEC and all colonizing isolates (e.g., in feces) are regarded as non-ExPEC (Sannes *et al.* 2004). However, this logic is flawed, since compromised hosts can develop serious infections due to low-virulence organisms (Johnson 1998), and the gut is an important reservoir for the *E. coli* strains that cause serious extraintestinal infections in otherwise healthy hosts (i.e., presumptive ExPEC) (Yamamoto *et al.* 1997).

A more reliable approach is to characterize the isolate for phylogenetic background and virulence factor profile, which allows inferences regarding the isolate's likely virulence potential. This approach also has limitations, since the correspondence between these bacterial characteristics and actual virulence is incompletely defined, and not all relevant traits have been identified or can be readily tested for.

A more direct approach, which requires no prior knowledge of the isolate's source or molecular characteristics, is to challenge animals with the isolate in an experimental infection model, and to observe disease outcomes (Hagberg *et al.* 1983; Johnson *et al.* 2006). Although this approach also has limitations, including being unsuitable for routine or large-scale use and the uncertain reliability of animal models for mimicking human disease, it provides the most direct assessment of an isolate's virulence potential, and is useful for testing specific bacterial traits as possible virulence factors (Falkow 1988).

Despite the considerable diversity of ExPEC strains, they are actually much less diverse than is the *E. coli* species as a whole, and they tend to be dominated by certain high-prevalence (i.e., frequently encountered) lineages, which traditionally have been called 'virulent clones' (Achtman 1986; Johnson and Russo 2002; Bonacorsi *et al.* 2003). The basis for the epidemiological success of these dominant ExPEC lineages, which implies enhanced fitness in some niche compared to other *E. coli*, is an area of active investigation. Explanations proposed for these lineages' success include enhanced host-to-host transmission, gut colonization, virulence and antimicrobial resistance (Banerjee and Johnson 2014). Notably, certain observational and experimental data suggest that specific bacterial traits may enhance fitness in multiple such domains. For example, in household colonization studies the most widely shared strains (such as intestinal or vaginal colonizers) tend to be those with the most virulence genes, those from phylogenetic group B2, and those that caused UTI in one or more household members (Johnson, Clabots and Kuskowski 2008). Likewise, in experimental systems certain traits classically regarded as virulence factors (e.g., P fimbriae and group 2 capsule) contribute to gut colonization, suggesting that they may also be colonization factors (Herías *et al.* 1995, 1997), which is most likely the true evolutionary basis for their selection and retention.

Pathogenesis of neonatal sepsis by *E. coli*

Neonatal sepsis is an important but underestimated problem around the world. Neonatal sepsis is defined as disease affecting newborns ≤ 1 month of age with clinical symptoms and positive blood cultures. The incidence of this disease is 1/1000 in normal term neonates and 4/1000 in preterm neonates, increasing up to 300/1000 in low weight neonates. Sepsis by bacterial infection is one of the most important causes of morbidity and mortality in newborns in both developed and developing countries (Stoll *et al.* 2011).

Many of these bacterial pathogens can frequently be found colonizing the vagina, cervix or rectum of pregnant adult women. Such pathogens may reach the fetus during the late stages of pregnancy by crossing the amniotic membrane or by directly infecting the baby during childbirth (Romero *et al.* 1988). In the case of early neonatal sepsis (by intrauterine or congenital transmission through the placenta) secondary to bacteria, these microorganisms could arise from a prematurely ruptured amniotic membrane which becomes infected, genuinely infected amniotic liquid (chorioamnionitis), or preterm delivery in a mother colonized by such bacteria, who may have a much higher risk of infecting her offspring due to the immaturity of the neonate's immune system. Preterm delivery constitutes 5%–10% of pregnancies and its occurrence has previously been linked to infection in the genital tract of women during pregnancy (McGregor and French 1996; Gravett *et al.* 2000; Stoll *et al.* 2002). On the other hand, late-onset neonatal sepsis is defined as infection presented four or more days after birth and is commonly caused by microorganisms acquired from the environment rather than from the mother.

Ascending infection can be described in four main steps: (i) the presence of bacteria in the vagina/cervix; (ii) the bacteria resides in the decidua; (iii) the bacteria colonizes the amnion or the chorion, going through the fetal vessels or crossing the amnion, reaching the amniotic cavity; and (iv) the bacteria enters the fetus through the infected amniotic liquid and reaches the blood.

Neonatal sepsis can be subdivided into the following.

(i) Early-onset neonatal sepsis (EONS): the microorganisms most frequently found to cause of EONS are group B streptococcus (GBS) and *E. coli*, followed by coagulase-negative staphylococcus, *Listeria monocytogenes* and *Haemophilus influenzae*. Maternal GBS colonization, premature and/or prolonged rupture of membranes, prematurity, maternal UTI and chorioamnionitis, among others, are considered as risk factors associated with EONS.

(ii) Late-onset neonatal sepsis (LONS): in contrast to EONS, there is a wide variety of microorganisms associated with LONS, including coagulase-negative *Staphylococcus*, *Staphylococcus aureus, E. coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Enterobacter cloacae, Candida* spp. and GBS. The risk factors associated with LONS are also different from those associated with EONS and include prematurity, central venous catheterization (more than 10 days), nasal cannula or continuous positive airway pressure use, gastrointestinal tract pathology, exposure to antibiotics and prolonged hospitalization, among others.

Treatment of neonatal sepsis consists in ampicillin plus an aminoglycoside, usually gentamicin. Other antimicrobials used are cefotaxime, vancomycin, metronidazole and piperacillin. The choice of the antibiotic depends on the microorganism associated with sepsis, the susceptibility of the bacterial pathogen, and the prevailing nosocomial infection trends in the nursery.

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Table 1. Studies about GBS and *E. coli* prevalence before and after intra-amniotic prophylaxis.

	Before IAP[a]		After IAP	
Reference	GBS	*Escherichia coli*	GBS	*Escherichia coli*
Levine *et al.* (1999)	1.7/1000	0.29/1000	0	1.3/1000
Stoll *et al.* (2002)	5.9/1000	3.2/1000	1.7/1000	6.8/1000
Daley and Garland (2004)	1.43/1000	0.32/1000	0.25/1000	No change
López Sastre *et al.* (2005)	1.10/1000	0.17/1000	0.7/1000	0.38/1000
Schrag and Stoll (2006)	1.7/1000	3.2/1000	0.34/1000	6.8/1000
van den Hoogen *et al.* (2010)	1.8%	1%	0.7%	0.3%
Lin *et al.* (2011)	45.4%	40.9%	20%	70%

[a]IAP, intra-amniotic prophylaxis.

To avoid enhanced risk of vertical transmission, several diagnostic and prophylactic protocols have been proposed. In 2002 the Center of Disease Control (CDC) recommended taking vaginal and rectal samples from pregnant women in their last antenatal visit and administering a prophylactic antibiotic such as penicillin G or ampicillin during pregnancy or at the time of delivery in women found to be colonized by GBS in antenatal screenings (Raymond *et al.* 2008). The use of these prophylactic measures resulted in a decrease in the incidence of infection by GBS (cdc.gov). A good example of this success was a study carried out in 10 hospitals of Barcelona (Spain) in which it was found that the incidence of GBS as a cause of neonatal sepsis was reduced from 1.92/1000 newborns in 1994 to 0.26/1000 newborns in 2001 ($P < 0.001$). However, several studies have suggested that these prophylactic measures could lead to a possible increase in the presence of other microorganisms such as *E. coli* (Schrag and Stoll 2006; Lin *et al.* 2011; Sgro *et al.* 2011), and an increase in antimicrobial resistance, and the selection of resistant microorganisms.

Examples of the increase of *E. coli* after the implementation of the intrapartum prophylaxis are shown in Table 1.

Several studies have also related this increase of *E. coli* to preterm labor, the prophylaxis given, maternal UTI by *E. coli* and active in-utero infection associated with the rupture of membranes and concluded that the antimicrobial treatment increases the risk of maternal colonization with antibiotic-resistant organisms (Jones *et al.* 2004; Bizzarro *et al.* 2008).

High rates of resistance to ampicillin and gentamicin among *E. coli* causing neonatal sepsis have been reported worldwide (Thaver, Ali and Zaidi 2009; Amaya *et al.* 2010; Heideking *et al.* 2013). A good example of the increase of these resistances has been described in Spain from 1998 to 2008 by Guiral *et al.* (2012), who reported a rise in ampicillin and gentamicin resistance from 56% to 78% and 0% to 26%, respectively.

The increase observed in the resistance to these antibiotics selected as empirical treatment could be caused by the selection of resistant strains due to exposure to antibiotics, making a change in the treatment of neonates necessary. However, further studies are needed to elucidate the role of intrapartum antibiotic prophylaxis in the emergence of resistant strains.

Another important feature of *E. coli* is its virulence. This microorganism presents several virulence factors that favor their capacity to cause sepsis. Among these factors, capsule, LPS, fimbriae, toxins, etc. may have a role in this type of infection.

Studies on the virulence of *E. coli* causing neonatal sepsis are scarce (Watt *et al.* 2003). In this sense, IbeA has been proposed as a virulence factor that could play an important role in the translocation of *E. coli* through the amniotic membrane (Soto *et al.* 2008). The gene encoding this protein is located in the pathogenicity island GimA that contributes to the invasion of the blood-brain barrier through a carbon-regulated process. Studies carried out in our laboratory have shown that two *E. coli* toxins could also be involved in the translocation through the amniotic membrane and in the development of neonatal sepsis (Sáez-López *et al.*, unpublished data).

However, when a high degree of bacteremia occurs, *E. coli* can successfully crossing the blood–brain barrier previous binding and invasion of the human brain microvascular endothelial cells (HBMECs) (Kim 2003), causing central nervous system (CNS) inflammation resulting in neonatal meningitis.

Escherichia coli is the second cause of neonatal meningitis leading high rates of mortality (20%–29%) and morbidity among neonates, being its incidence about 0.1/1000 live births among industrialized countries (Bonacorsi and Bingen 2005). The high prevalence of these infections occurs in the neonatal period (<28 days of life), and only about 10% occurs between 1 and 3 months of age (Unhanand *et al.* 1993; Okike *et al.* 2014). As consequence of this infection, an important of neonates develops neurological damages being more frequent when the meningitis is accompanied by ventriculitis or intracerebral abscess (Dellagrammaticas *et al.* 2000).

Development of neonatal meningitis by *E. coli* comprises three steps: (i) translocation from the intestine lumen to the bloodstream (but also from urinary tract or the utero); (ii) intravascular survival and multiplication; and (iii) passage of the bacteria through the blood–cerebrospinal-fluid barrier (CSF) and invasion of the arachnoidal space (Bonacorsi and Bingen 2005; Kim 2012).

About 80% of the *E. coli* strains causing meningitis belong to capsular serotype K1, being the serotypes O18:K1 and O7:K1 the most frequently found (about 50% of all K1) (Achtman *et al.* 1983; Moulin-Schouleur *et al.* 2006). The VFs found to be associated with the ability of these *E. coli* strains to cause neonatal meningitis are related to outer membrane proteins (capsular antigen K1, OmpA protein), siderophores (*iroN, fyuA* and *iucC/iutA* genes), adhesins (P-fimbriae, S-fimbriae and type-1-fimbriae), and invasion (*ibeA*, *asl* and *cnf1* genes) (Xie, Kim and Kim 2004). In addition, successful invasion of HBMEC also requires HBMEC actin cytoskeleton rearrangement and activation of several host factors or related signalling molecules such as focal adhesion kinase (FAK), paxillin and phosphatidylinositol 3-kinase (PI3-K), PhoGTPases and cytosolic phospholipase A2 ($cPLA_2$) (Khan *et al.* 2002).

In summary, *E. coli* has replaced GBS as a cause of neonatal sepsis and meningitis in many countries in which the CDC guidelines have been implemented. More than 90% of these

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E. coli strains are resistant to ampicillin possibly due to antibiotic prophylaxis. Several virulence genes, including toxins and invasins, seem to play an important role in the pathogenesis of these infections (adhesion, invasion and translocation).

For these reasons, we think that epidemiological surveillance, in terms of resistance and etiology, of *E. coli* causing neonatal infections is needed.

Biofilm formation of *E. coli* isolates

Biofilm formation is the capability of the microorganisms to self-organize into multicellular communities embedded in a mainly self-produced, extracellular matrix directed through distinct genetic programs (Costerton *et al*. 1987; Römling 2012). Besides being the major life style of most microorganisms, biofilm formation is a medical problem of wide significance. Indeed, biofilm formation is considered both a major virulence factor of chronic infections due to the inability of the immune system to eradicate the microorganisms and a clinical problem due to the failure of antibiotics to successfully eliminate them. In addition, biofilm formation can also play a role in certain stages of acute infections and is important for transmission of pathogens. The medical problem of biofilm formation, though, goes far beyond chronic infections as biofilm associated diseases promote the extensive use of antibiotics and biofilm formation promotes the spread of antimicrobial resistance (Sørensen *et al*. 2005). The spectrum of UTIs, catheter-associated UTIs, acute and recurrent UTIs, cystitis and pyelonephritis, but also inflammatory bowel diseases (IBD) such as Crohn's disease (CD) are classical examples of diseases in which biofilm formation of *E. coli* to disease contribution was suspected and investigated early (Anderson *et al*. 2003; Darfeuille-Michaud *et al*. 2004; Wang *et al*. 2010; see specific section in this review).

Biofilm formation of commensal strains and pathovars of *E. coli* is largely unexplored considering the vast number of mainly functionally uncharacterized fimbriae as well as confirmed and potential exopolysaccharide operons (Korea *et al*. 2010) as well as the vast number of horizontally transfered genomic information adding additional proven and suggested adherence and biofilm factors (see below, Pathogenicity islands) (Ghigo 2001; Ong *et al*. 2009).

Rdar biofilm formation, a biofilm displayed by a distinct colony morphology type, was characterized in commensal and uropathogenic strains of *E. coli* has been characterized (Bokranz *et al*. 2005; Kai-Larsen *et al*. 2010). Rdar biofilm is a multicellular biofilm behavior characterized by the expression of the extracellular matrix components curli fimbriae and cellulose (Bokranz *et al*. 2005). The major hub of rdar biofilm regulation is the transcriptional regulator CsgD. Commensal isolates and *E. coli* pathovars have been reported to express the rdar morphotype under laboratory conditions, but a clear association of an rdar expression pattern with a certain *E. coli* pathovar or disease condition has not yet crystallized. Interestingly, however, a recent study demonstrated that most *E. coli* O157:H7 strains show impaired rdar biofilm formation due to impairment of major transcriptional regulators RpoS and/or MlrA of the rdar morphotype (Uhlich *et al*. 2013). Another noteworthy observation is that isolates of the probiotic *E. coli* strain Nissle 1917 have maintained their conserved rdar morphotype regulatory pattern with CsgD independent cellulose expression at 37°C for over almost 100 years in nature (Fig. 1) (Kleta *et al*. 2006; Monteiro *et al*. 2009).

Rdar morphotype expression significantly changes the interaction of *E. coli* with the host. Remarkably therefore, the extracellular matrix components curli fimbriae and the exopolysaccharide cellulose often have the opposite effect on microbial host interaction in not only a cell culture model, but also in an animal model of UTI (Wang *et al*. 2006; Monteiro *et al*. 2009; Kai-Larsen *et al*. 2010; Lamprokostopoulou *et al*. 2010). For example, while the amyloid curli fimbriae promote adhesion and invasion of commensal and pathogenic *E. coli* strains into host cells and stimulate secretion of the proinflammatory cytokine interleukin-8, (co-)expression of cellulose inhibits this process (Wang *et al*. 2006; Saldaña *et al*. 2009; Kai-Larsen *et al*. 2010). Again *E. coli* strain Nissle 1917 is an exception as cellulose promotes adherence and IL-8 production, and only slightly inhibits invasion (Monteiro *et al*. 2009).

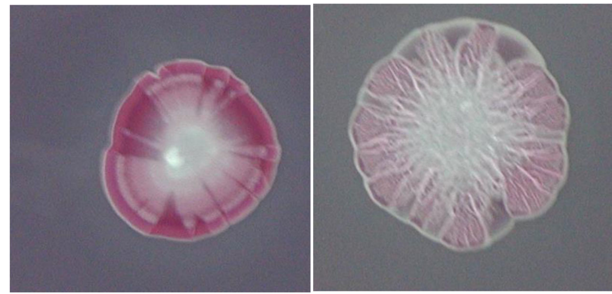

Figure 1. Rdar colony morphology type of *E. coli* Nissle 1917 on Yesca medium agar plates at 28°C (left) and 37°C (right).

Interestingly, rdar morphotype components also have an impact on an animal model of UTI since expression of curli fimbriae, but not cellulose or both components, enhances the recovery from the kidney in the early infection phase (Kai-Larsen *et al*. 2010). One reason might be the enhanced resistance of curli-expressing cells against the antimicrobial peptide LL-37, a phenotype that extends the survival of curli-expressing cells of the bladder epithelial cell line T24. In addition, expression of the macrophage inflammatory protein 2 (MIP-2) chemokine is highly enhanced upon infection of curli-producing bacteria, while cellulose-expressing strains virtually show no MIP-2 induction compared to uninocculated controls. Later in infection, however, only curli fimbriae-expressing cells are eliminated faster. This elimination is mediated by neutrophils as in neutrophil depleted animals curli fimbriae-expressing cells are maintained at equal numbers to those of the wild type strain. Although curli have been found to be expressed in human urine suggesting a contribution of the rdar biofilm to human UTI infection (Kai-Larsen *et al*. 2010), initial adherence of *E. coli* to catheter surfaces seems to be independent of *csgD* (Fig. 2) (Wang *et al*., unpublished results). Adding to the complexity of biofilm mediated bacterial host phenotypes, cellulose has recently also been identified as an inflammation inducing component of an adherent-invasive *E. coli* strain in a mouse model of colitis (Ellermann *et al*. 2015). Interesting, when investigating the interaction of AIEC strain NC 101 with macrophages, an opposite effect of cellulose production in interaction with macrophages with respect to phagocytosis and production of the proinflammatory cytokine IL12 p40 dependent on iron availability was observed.

Nevertheless, expression of the rdar morphotype is highly regulated by *E. coli* 'ex vivo' and '*in vivo*'. A major regulator of rdar biofilm formation in *E. coli*, the closely related *S.* Typhimurium and other bacteria is the ubiquitous bacterial secondary messenger cyclic di-GMP (Schirmer and Jenal 2009; Sondermann, Shikuma and Yildiz 2012; Römling, Galperin and Gomelsky 2013; Hengge *et al*. 2015). Cyclic di-GMP, originally identified as the allosteric regulator of cellulose synthase in 1987 (Ross *et al*. 1987),

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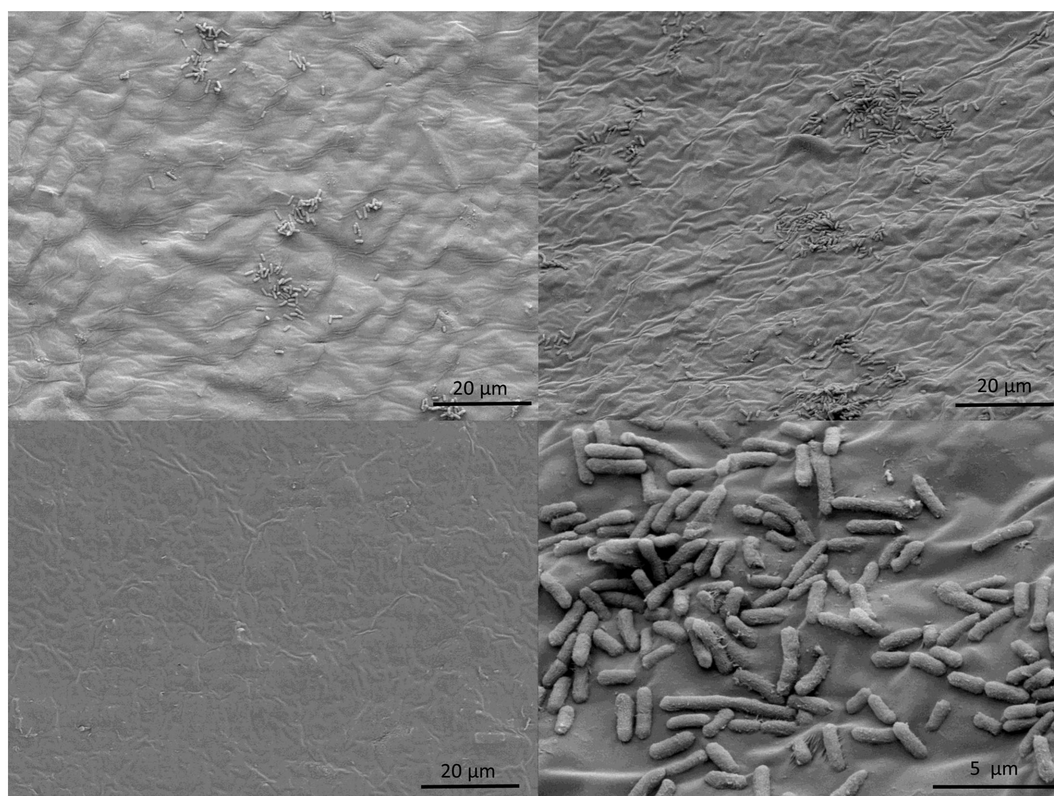

Figure 2. Adherence of *E. coli* UEB1r and its *csgD* deletion mutant to silicon-elastomer coated foley catheter surface. Photo: Heinrich Lünsdorf; experiment Xiaoda Wang, PhD thesis.

was rediscovered in 2004 as an ubiquitous secondary messenger regulating the transition between the motile and sessile life style of microbes (Paul *et al.* 2004; Simm *et al.* 2004; Tischler and Camilli 2004). A short time thereafter, transition between chronic infection and the acute virulence phenotype was also identified as a ubiquitous characteristic regulatory pattern of the cyclic di-GMP signaling system.

The cyclic di-GMP signaling system is the most complex secondary messenger network of bacteria. In *E. coli*, this signaling system is of moderate complexity with about 31 cyclic di-GMP turnover proteins (Römling 2005; Sommerfeldt *et al.* 2009). In line with the overall phenotype of biofilm producing *E. coli*, adherence, invasion and production of proinflammatory cytokines have been demonstrated to be regulated by the cyclic di-GMP signaling system by turnover proteins with unique or partially redundant function (Hu *et al.* 2009; Spurbeck *et al.* 2013). In the uropathogenic strain *E. coli* CFT073 dysregulation of the di-guanylate cyclase YfiN by deletion of the periplasmic negative regulator YfiR upregulated the rdar morphotype (Raterman *et al.* 2013). Concomitant with upregulation of the rdar morphotype, the cell number in the bladder and kidney was reduced. Reduction could be relieved by deletion of the cellulose synthase and the biofilm regulator CsgD. Although preliminary, these studies point to a central role of the cyclic di-GMP signaling system in the transition between virulence and biofilm formation (Römling, Galperin and Gomelsky 2013).

In summary, we can conclude that biofilm formation in *E. coli* is highly prevalent, but also variable. In spite of the diverse studies on *E. coli* biofilms even beyond the rdar morphotype (Rendón *et al.* 2007; Itoh *et al.* 2008; Lasaro *et al.* 2009; Steiner *et al.* 2013; Yakovenko, Tchesnokova and Sokurenko 2015; Stærk *et al.* 2016), several major questions remain unanswered. What are the evolutionary forces in commensalism and disease, and also outside the human body, which shape its regulation? We have discovered very few biofilm phenotypes so far; can we define additional ones and the circumstances, under which they are required? Cyclic di-GMP signaling is a major regulator of biofilm-motility and biofilm-virulence transition in *E. coli*; can we define specific cyclic di-GMP metabolizing enzymes involved in biofilm and virulence regulation? Very few phenotypes mediated by cyclic di-GMP signaling have been identified; can we define additional phenotypes regulated by cyclic di-GMP signaling? Biofilm formation and cyclic di-GMP signaling generally inhibit acute virulence phenotypes; we know very little about the contribution of biofilm formation and cyclic di-GMP signaling to chronic infection phenotypes. And last, but not least, what is the contribution of biofilm formation and cyclic di-GMP signaling to antimicrobial resistance phenotypes in *E. coli*?

PAIs: implications for the pathophysiology, epidemiology and diagnosis of *E. coli*

The species *E. coli* are characterized by a remarkable genomic diversity. Comparative genomics has furthered our understanding of bacterial diversity, the characteristics of populations and has demonstrated that *E. coli* genomes consist of a conserved, so-called core genome and a flexible part. The flexible gene pool codes for properties that contribute to the bacterial adaptability and fitness. It is represented by (former) mobile genetic elements including bacteriophages, plasmids, insertion sequence (IS) elements, (conjugative) transposons and integrons as well as non-functional fragments of these genetic elements (Ochman

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and Jones 2000; Touchon *et al.* 2009; Chaudhuri and Henderson 2012; Toussaint and Chandler 2012). The term pathogenicity island (PAI) defines large genomic regions that are present in pathogenic but are absent in non-pathogenic strains and harbor pathogenicity-related genes. Such elements are commonly inserted into tRNA genes; their G+C content and codon usage is distinct from that of the core genome, and they are often unstable (Hacker and Carniel 2001; Schmidt and Hensel 2004; Gal-Mor and Finlay 2006). The ever increasing amount of DNA sequencing data resulted in a more generalized view of such genetic entities belonging to the flexible gene pool and denoted PAIs as a subtype of 'genomic islands' (GEIs) (Blum *et al.* 1994; Hacker and Kaper 2002; Hacker, Hentschel and Dobrindt 2003; Dobrindt *et al.* 2004). GEIs generally represent a characteristic feature of *E. coli* and they play an important role in the evolution of different variants or pathotypes. A considerable fraction (up to 30%) of the entire *E. coli* genome is represented by GEIs and PAIs. Accordingly, pathogenicity-associated genes located on PAIs or even complete islands, e.g. the 'locus of enterocyte effacement' (LEE), frequently serves as biomarkers for individual *E. coli* pathotypes.

Comparative analysis of PAIs and GEIs revealed the dynamics of genomes within a single species, including INDELs (INsertion/DELetions), acquisition of genes and gene clusters through horizontal gene transfer, and genomic rearrangements, in addition to sequence variation within single genes, differences in regulatory sites, and SNPs that may lead to functional diversity.

The comparison of PAIs in different *E. coli* strains indicated that identical or almost identical PAIs can exist in different *E. coli* pathotypes or strains (Dobrindt *et al.* 2004; Brzuszkiewicz *et al.* 2009; Chaudhuri and Henderson 2012; Leimbach, Hacker and Dobrindt 2013). Typical examples are the 'high pathogenicity island' (HPI) or the LEE PAI. Nevertheless, many PAIs exhibit a mosaic-like, modular structure, i.e. they do not consist of a contiguous stretch of horizontally acquired DNA, but comprise DNA fragments of different origin. These different mobile genetic elements and horizontally transferred DNA regions have been acquired by independent events in the course of evolution (Hacker and Kaper 2000; Schubert *et al.* 2009; Touchon *et al.* 2009). Although these PAIs exhibit an overall similar genetic structure and gene content, there is a great variability regarding their composition and chromosomal localization even within one particular patho- or serotype. Basically functional and structurally unrelated islands can include a multitude of redundant DNA regions, which have been accumulated by repeated integration and partial deletion of mobile and accessory elements. These multiple copies of identical or very similar DNA sequences can promote homologous recombination within an island or between different horizontally acquired DNA elements and thus cause DNA rearrangements, deletions or the incorporation of foreign DNA. Accordingly, a multitude of functionally identical, but structurally variable PAIs can be found in the genomes of pathogenic *E. coli* isolates. In order to characterize or identify PAIs in clinical isolates, many laboratories screen for a few selected marker genes from known islands. Against the background described above, this approach may cause the problem that the detection of a limited set of virulence markers may not indicate the presence of one particular entire PAI of a reference strain.

The modular composition of functionally related PAIs in different *E. coli* strains can be exemplified by related PAIs of ExPEC carrying the salmochelin determinant (*iro*). The *iro* gene cluster is often associated with genes coding for the *Salmonella* iron transport (*sit*) determinant as well as with colicin and adhesin determinants (Dobrindt *et al.* 2002; Starcic Erjavec *et al.* 2003; Brzuszkiewicz *et al.* 2009). Variable regions between these genes often represent repetitive sequences or IS elements. Interestingly, many of these determinants located on this type of ExPEC PAI can also be found on islands and large virulence plasmids present in related species, e.g. *Salmonella* spp., *Shigella* spp. or *K. pneumoniae* (Brzuszkiewicz *et al.* 2009), corroborating the transfer and exchange of individual modules of these PAIs or plasmids. The interrelation between PAIs and integrative and conjugative elements (ICEs) and plasmids is clearly visible when, for example, the HPI in association with the colibactin-encoding PAIs of uropathogenic *E. coli* and *Citrobacter koseri* BAA-895 are compared with ICEEc1, ICEKp1 and the multiresistance plasmid pMET1 of *K. pneumoniae* and the *Yersinia pestis* plasmid pCRY (Schubert *et al.* 2004; Lin *et al.* 2008; Soler Bistué *et al.* 2008; Putze *et al.* 2009).

Many IS elements, genes encoding integrases or non-functional gene fragments are part of PAIs and GEIs (Brzuszkiewicz *et al.* 2006; Putze *et al.* 2009). The fact that several pathogenicity-related determinants can be localized on different types of mobile genetic elements underlines the involvement of conjugative transposons, ICEs, bacteriophages and plasmids in the evolution and ongoing further shaping of PAIs and GEIs (Dobrindt *et al.* 2004; Ahmed *et al.* 2008).

Horizontal gene transfer is important for the rapid introduction and spread of new genetic determinants, including virulence genes. PAIs, bacteriophages, conjugative plasmids, conjugative transposons, ICEs and natural transformation are well-known vehicles that can promote interspecies gene transfer, but the conditions for and the frequency of transfer events in the natural environment are not well understood. PAIs or GEIs can be transferred to suitable recipients either by helper phage or as integrative and conjugative elements (ICEs) (Schubert *et al.* 2004; Schneider *et al.* 2011; Soto *et al.* 2011). They can also have the intrinsic ability to be horizontally transferred (Lesic *et al.* 2004).

The presence of a functional bacteriophage-like integrase gene and flanking repeat structures is an important requirement for the excision of PAIs; a prerequisite for their dissemination by horizontal transfer (Hacker and Kaper 2000; Schmidt and Hensel 2004). PAI-encoded integrases are required for site-specific recombination and the excision of their cognate PAI. In addition, RecA-dependent homologous recombination can be responsible for an alternative method of PAI excision due to the presence of multiple copies of similar IS elements and other repetitive DNA sequences. As these accessory genetic elements can insert into the bacterial chromosome without extensive sequence homology at different genomic localizations, homologous recombination between them accidentally results in complete or partial excision of PAIs.

Deletion of PAIs can occur in different enterobacterial isolates including *E. coli* with frequencies ranging from 10^{-4} to 10^{-6} (Rajakumar, Sasakawa and Adler 1997; Tauschek, Strugnell and Robins-Browne 2002; Lesic *et al.* 2004; Middendorf *et al.* 2004). Interestingly, the deletion frequency depends in part on individual growth conditions. There are also indications that the PAI excision rate is directly correlated with the length and integrity of the flanking repeat structures. Furthermore, the availability of nucleoid-associated proteins and the chromosomal localization of PAIs can affect site-specific recombination as well as the expression of PAI-encoded integrases (Hochhut *et al.* 2006). Nucleoid-associated protein variants are often encoded on mobile genetic elements including PAIs, thus increasing the possibilities of differential regulation of genes located on these horizontally transferred genetic elements (Williamson and Free 2005; Müller *et al.* 2010; Levine *et al.* 2014).

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Transfer of excised *E. coli* PAIs *in vitro* by conjugation has been shown (Schneider *et al.* 2011). If and under which conditions the acquisition or deletion of PAIs or fragments thereof is induced or repressed remains to be investigated more in depth. One important aspect of PAI instability is the mobilization of PAIs, which allows rapid dissemination of determinants coding for complex traits. Furthermore, *E. coli* pathogens can quickly alter their phenotypes by adapting, with the excision or incorporation of PAIs during pathogen-host interaction. This might be advantageous for example during the transition from acute to chronic infections by preventing strong activation of the host immune response. The loss of PAI fragments during pathogen-host interaction has also been observed in enterohemorrhagic *E. coli* (EHEC), in which, the spontaneous deletion of internal regions of two GEIs in *E. coli* O157:H7 occurred *in vitro* and *in vivo*. These deletions led to the loss of tellurite resistance (*ter*) and *iha* adhesin genes, and the resulting deletion mutants exhibited a reduced colony size (Bielaszewska *et al.* 2011). Similar to the gain or loss of Shiga toxin phages in EHEC during infection (Bielaszewska *et al.* 2008; Mellmann, Bielaszewska and Karch 2009), the loss of complete or partial PAIs can result a rapid alteration of the genomic architecture and bacterial phenotype. This underlines that PAI instability during infection can not only result in different clinical outcomes, but may also interfere with proper diagnosis and epidemiology.

ANTIMICROBIAL RESISTANCE IN *E. coli*

Resistance and multidrug resistance of *E. coli* worldwide

Antibiotics are a mainstay of public health and play a key role in improving the health and well being of people all over the world. However, while antibiotics have been successful in limiting infectious diseases, their use has exponentially increased leading to the emergence and spread of antibiotic resistance (AMR). GNB, including *E. coli*, have emerged as major players in resistance, with multidrug resistance (MDR) now being relatively common (Nordmann, Naas and Poirel 2011; Dortet, Cuzon and Nordmann 2014). AMR results in reduced efficacy of antibacterials, making the treatment of patients costly and difficult, or even impossible (Tzouvelekis *et al.* 2014). In some cases, resistance extends to the entire repertoire of the therapeutic agents available (the so-called pan-drug resistant phenotypes), posing a formidable challenge to the antimicrobial therapy and turning back the clock to the pre-antibiotic era (Nordmann, Naas and Poirel 2011; WHO 2014). This is particularly worrisome in view of the current dearth of new compounds active against MDR-GNB (Theuretzbacher 2012; Tzouvelekis *et al.* 2014).

Despite the interest of public health, authorities there are currently increasing difficulties to obtain sequential data of resistance and multidrug-resistance from prospective surveillance studies. Most of the surveillance studies available (e.g. SMART, SENTRY, TEST or MYSTIC) are sponsored by private pharmaceutical companies and only involve isolates from specific infections or anatomic locations. They are mostly focused on the study of specific antibiotics, normally those under marketing promotion (Hawser *et al.* 2013; Renteria *et al.* 2014; Sader *et al.* 2014). Moreover, data are partially and fractionally published by geographic areas, period of time or resistance problems, making it difficult to obtain a broader picture of the current epidemiological situation. Nonetheless, the general perception of several publications and reports is that resistant and multidrug resistant (MDR) *E. coli* isolates have greatly increased during the last

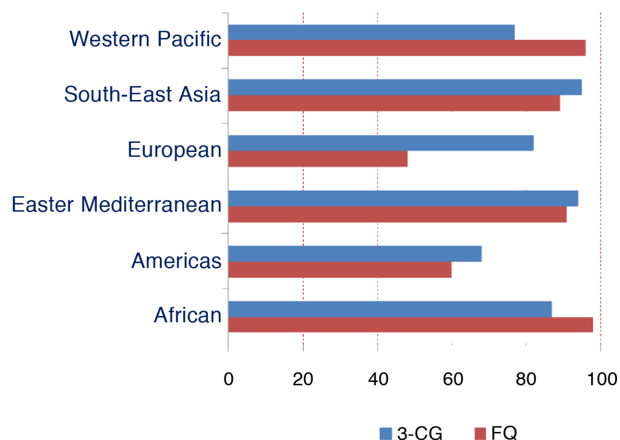

Figure 3. Resistance rates in *E. coli* isolates reported until April 2013 in different geographic areas. 3-CG, third-generation cephalosporin; FQ, fluoroquinolone.

decades and that these surveillance programs can also be used to detect emerging resistance mechanisms, not only in developed but also in developing countries (Hawser 2012, WHO 2014). Different examples of these mechanisms are the detection of plasmid AmpC β-lactamase or carbapenemase producing *E. coli* in geographic areas with unknown resistance trends (Sheng *et al.* 2013; WHO 2014).

Due to its particular ecology, *E. coli* can be considered as a sensor of the current situation of antimicrobial resistance. This organism has consolidated resistance traits that appeared many years ago and include TEM-1 β-lactamase or the more recent extended-spectrum β-lactamases (ESBLs), carbapenemases or plasmid-mediated quinolone resistance (PMQR) mechanisms. Other mechanisms such as ribosomal methylases affecting aminoglycoside or plasmid mediated fosfomycin resistance have yet to be consolidated (Cantón and Coque 2006; Rodríguez-Martínez *et al.* 2011; Wachino and Arakawa 2012; D'Andrea *et al.* 2013), that might occur in association with ESBL and/or carbapenemase production.

Some of the newer resistance mechanisms have emerged in the so-called high-risk clones, which facilitate persistence and further dissemination of resistance traits around the world. This is the case of the B2 O25:H4 ST131 clone harboring IncFII plasmids. It has been responsible for the dissemination of CTX-M-15 ESBL and is also emerging as a MDR microorganism (Coque *et al.* 2008; Banerjee and Johnson 2014). Its presence is not only confined to the human compartments but also to the environmental and animal compartment, both in pets and livestock (Liebana *et al.* 2013; Rubin and Pitout 2014). This clone is characterized for its resistance to fluoroquinolones and expanded-spectrum cephalosporins but has also been able to recruit carbapenemase genes (Banerjee and Johnson 2014; Cai *et al.* 2014).

In a recent report from the WHO, *E. coli* has been included in a list of the top nine microorganisms of international concern causing the most common infections in different settings: in the community, in hospitals or transmitted through the food chain (WHO 2014). This report highlighted third-generation cephalosporin and/or fluoroquinolone resistance in urinary tract and blood stream infections that limit empiric treatments. Figure 3 indicates the highest resistance rates in *E. coli* isolates published or reported up to April 2013 in different geographical areas. These data probably reflect outbreak situations and are much higher than those obtained in antimicrobial surveillance programs with systematic data collection. As

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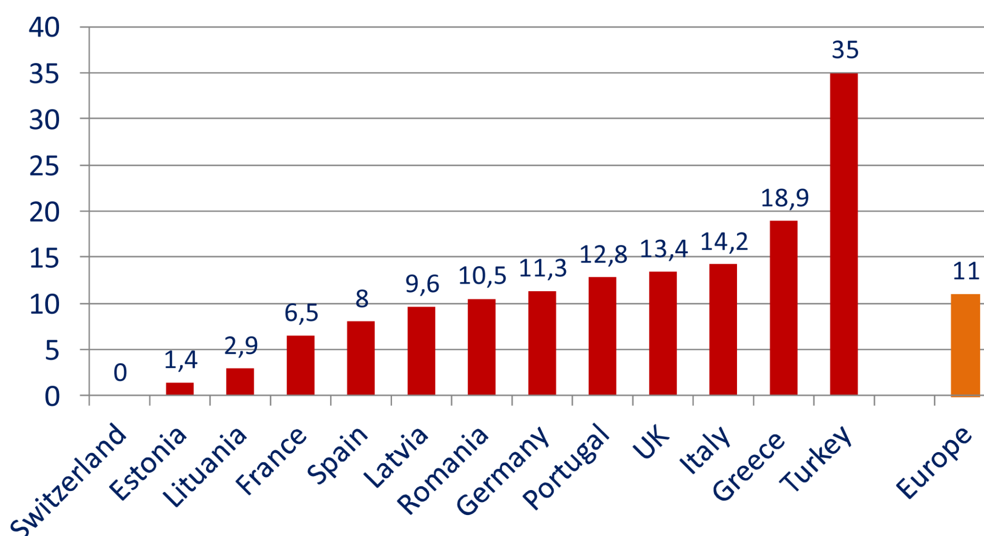

Figure 4. Third-generation cephalosporin resistance found in the SMART study.

an example, according to the last report of the European Center for Disease Control (ECDC) on antimicrobial resistance obtained through the EARS-Net database (EARS-Net), in Europe, resistance to third-generation cephalosporins in invasive isolates, mostly due to ESBL production has a mean value of 12.6% and ranged from 5.0% in Iceland to nearly 40% in Bulgaria. Most of these isolates were also resistant to aminoglycosides and fluoroquinolones and the mean percentage for combined resistance of third-generation cephalosporins, aminoglycosides and fluoroquinolones was nearly 5%. In general, this percentage was higher in those countries with higher resistance rates when considering different antibiotics individually. Interestingly, resistance to carbapenems in Europe was still only 0.2% in 2013, being higher in Italy (0.6%), Greece (1.4%) and Bulgaria (2.8%) (EARS-Net). Similar figures for third-generation cephalosporin resistance were found in the SMART study (Fig. 4) (Hawser *et al.* 2012).

In other geographic areas, such as in China, India or in the Middle East antimicrobial resistance in *E. coli* seems to be higher. A recent report of a study characterizing antimicrobial resistance mechanisms in *E. coli* in a hospital in China demonstrated the presence of isolates with multiple resistant genes. These include carbapenemase (bla_{KPC-2}), ESBL ($bla_{CTX-M-3}$, $bla_{CTX-M-14}$, $bla_{CTX-M-55}$), aminoglycoside (*aac(6′)-Ib*, *armA* and *rmtB*) and quinolone (*qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS* and *aac(6′)-Ib-cr*) resistance genes (Cai *et al.* 2014). More importantly, in another study from this country, KPC-2 production was found to be associated with the high-risk ST131 clone in 40% of the cases (Zhang *et al.* 2014). Moreover in India, co-production of the carbapenemases NDM-1 and OXA-48 was shown in a large proportion (55%) of the *E. coli* isolates recovered from UTIs (Khajuria *et al.* 2014). This co-production is also common in the Middle East countries (Zowawi *et al.* 2014).

In other countries, the emergence and spread of other resistance mechanisms in *E. coli* has also been observed. This is the case of fosfomycin resistance which has been largely described in Korea associated with transferable resistance plasmid harboring *fosA* genes. In Hong Kong, the dissemination of *fosA3* genes has been observed in diverse *E. coli* clones on multiple bla_{CTX-M}-carrying plasmid types. Fosfomycin resistance has also been particularly associated with the production of carbapenemases in European countries (Kaase *et al.* 2014).

All these data illustrate the increasing prevalence of *E. coli* in different parts of the world and the acquisition of resistance mechanisms in isolates already resistant to other antimicrobial agents. Very recently WHO has taken the initiative to organize 'The Global Antimicrobial Resistance Surveillance System (GLASS)' that will support the Global Action Plan on Antimicrobial Resistance with the aims not only to collect antimicrobial resistance data but also to standardize, compare and validate them. This initiative will give a worldwide figure of antimicrobial resistance, including that in *E. coli*.

Fluoroquinolone resistance

Quinolones are a powerful group of antibacterial agents. The first quinolone described was nalidixic acid, which was discovered by serendipity when chloroquine was being synthetized. Nalidixic acid was a secondary compound in the synthesis of this antimalarial drug. It showed a narrow spectrum of activity, being active mainly against *Enterobacteriaceae*, and was therefore not used much in the clinical setting. However, it was the initial drug from which other generations of quinolones were developed. The basic structure of almost all quinolones is based on the 1,4-dihydro-4-oxo-pyridine molecule. All quinolones have a carboxylic substituent at position 3, which together with the carbonyl group at position 4, appears to be essential for the activity of the quinolones. The second generation represented by ciprofloxacin and norfloxacin presented two main changes with respect to nalidixic acid, the first was a positively charged group at position 7 (piperazine group in ciprofloxacin) and a fluoride at position 6, therefore, since then the new quinolones have been have been called fluoroquinolones. This generation of quinolones was active against all aerobe GNB and showed a moderate activity against some Gram-positive bacteria. The third generation constituted mainly by levofloxacin presented a better activity against Gram-positive bacteria and finally, in addition to the activity presented by levofloxacin, the fourth generation showed activity against anaerobes. The representative of this generation is moxifloxacin (Madurga *et al.* 2008).

Quinolones inhibit the activity of the DNA gyrase and topoisomerase IV, which belong to type II topoisomerases and are involved in the topology of DNA. The main function of the DNA gyrase is to catalyse negative supercoiling of the DNA, thereby

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Table 2. Mechanisms of resistance to quinolone in *E. coli*.

1. Chromosomal-mediated
   - –Changes in protein targets
     - ∘ Mutations in the *gyrA* gene (amino acid codon Ser-83 and Asp-87)
     - ∘ Mutations in the *parC* gene (amino acid codon Ser-80 and Glu-84)
   - –Reduction in the accumulation of the quinolone[a]
     - ∘ Decrease in permeability – Decreased expression of OmpF
     - ∘ Increase in active efflux systems:
       - ■ AcrAB, AcrEF
       - ■ MdfA
       - ■ YdhE
2. Plasmid-mediated
   - –DNA gyrase and topoisomerase IV protection from quinolone inhibition - Qnr
   - –Aminoglycoside-acetyltransferase – AAC(6′)-Ib-cr
   - –Efflux pumps:
     - ■ QepA
     - ■ OqxAB

[a]Transcriptional factors such as MarA can concomitantly regulate the expression of OmpF and AcrAB-TolC.

playing an important role in DNA replication and transcription. Meanwhile, the function of topoisomerase IV is to decatenate interlinked DNA, thereby playing an important role in the decatenation of the two daughter molecules of DNA at the end of replication. Both enzymes show a similar structure consisting in two A subunits and two B subunits. The A subunit of the DNA gyrase is encoded by the *gyrA* gene and the B subunit by the *gyrB* gene. To catalyse negative supercoiling, the DNA gyrase performs a transient double strand break with a passage of another segment of DNA through the break and later resealing the broken strands. This reaction is catalysed by the A subunit, whereas the B subunit has ATPase activity and thus hydrolyses ATP to obtain enough energy to perform the reaction of breakage. Topoisomerase IV also has a structure constituted by two A subunits and two B subunits. The A subunit is encoded in the *parC* gene and the B subunit in the *parE* gene (Madurga *et al.* 2008).

Resistance to quinolones has steadily risen over the last decades. Currently, two general mechanisms of resistance can be found in *E. coli*; those associated with mutations in the chromosome and those related to plasmids (Table 2). In this microorganism, mutations in the *gyrA* and *parC* genes are the most important mechanisms of resistance to quinolones. Mutations associated with resistance have been mapped in a region called the quinolone resistance determining region (QRDR). Among these mutations the most frequently found are those located in the amino acid codons Ser-83 and Asp-87 of the GyrA and at the amino acid codons Ser-80 and Glu-84 of the ParC. Nonetheless, mutations in the *gyrB* and *parE* genes play a minor role in the acquisition of resistance to quinolones in *E. coli* (Vila *et al.* 1994, 1996; Ruiz *et al.* 1997; Vila 2005; Fàbrega *et al.* 2009).

Another mechanism of resistance to quinolones linked to mutations in the chromosome is the reduction in the intracellular accumulation of quinolones, which may be related to a decrease in the permeability of the outer membrane or to an increased efflux of the drug out of the cell. The penetration of a quinolone through the outer membrane seems to take place in two ways, one dependent and another independent of porins. It has been suggested that the more hydrophilic quinolones, such as ciprofloxacin, cross the outer membrane only through the porins, whereas the more hydrophobic quinolones, such as nalidixic acid penetrate through the porins and non-porin pathways. Mutants of *E. coli* with decreased OmpF expression have a 2- to 4-fold decrease in susceptibility to quinolones. Several efflux pumps which affect quinolones have been described in *E. coli* (Vila *et al.* 2011) (Table 2). However, the most important efflux pump is that encoded in the *acrAB* operon, in which the *acrB* gene encodes an inner membrane protein (AcrB), which is an efflux transporter across the inner membrane, and the *acrA* gene, which encodes a membrane fusion protein (AcrA). The third protein in this tripartite efflux pump is TolC which is an outer membrane protein. It has been shown that the overexpression of this efflux pump leads to a multidrug resistant phenotype in different bacteria, including *E. coli* (Blair, Richmond and Piddock 2014). A chromosomal locus, called *mar* (multiple antibiotic resistance), encodes a transcriptional factor which increases *micF* expression, a regulatory antisense RNA, which causes a post-transcriptional decrease of OmpF RNA and reduces the amount of OmpF. In addition, MarA can also regulate the expression of AcrAB-TolC which is increased when MarA is overexpressed. This interplay between the decreased expression of OmpF and the increased expression of ArcAB-TolC generates a multidrug-resistant phenotype in *E. coli* with increased resistance to quinolones, chloramphenicol and tetracyclines. Besides MarA, other transcriptional activator such as SoxS has been reported in *E. coli* (Oethinger *et al.* 1998). In addition of these global regulators MarA, SoxS and Rob in the regulation of the expression of the *acrAB* operon, other mechanisms of regulation have been reported in *E. coli,* such as: 1. Mutations in the local repressor gene *acrR* (Webber, Talukder and Piddock 2005) and 2. Insertion elements upstream of the *acrAB* operon, for instance in the *acrR* gene (Webber and Piddock 2001).

Up to now, three plasmid-mediated mechanisms of resistance to quinolones have been described (Table 2), which are: (i) the expression of an aminoglycoside-modifying enzyme (AAC(6′)Ib-cr) which has the ability to acetylate an amino group located in the piperazine ring of the quinolone structure. This enzyme affects all quinolones with the exception of those with a blocked amino group, such as levofloxacin (Cattoir and Nordmann 2009); (ii) the *qnr* gene family, which encode a peptide able to protect the DNA gyrase or DNA-topoisomerase IV complexes to be bound by the quinolones (Fàbrega *et al.* 2009); and (iii) two efflux pumps, OqxAB and QepA (Cattoir and Nordmann 2009). All of these genes affect relatively small increases in the MICs of quinolones, but these changes are sufficient to facilitate the selection of mutants with higher levels of resistance, mainly in the *gyrA* gene (Nordmann, Cattoir and Poirel 2008).

Plasmid-mediated AmpC-producing *E. coli*

Plasmid-mediated AmpC (pAmpC) enzymes can be grouped into six major groups, ACC, FOX, MOX, DHA, CIT and EBC (Pérez-Pérez and Hanson 2002). These enzymes are derived from the chromosomes of certain species in the *Enterobacteriaceae* family, but have been mobilized on plasmids that can transfer horizontally in medically important species such as *E. coli*, *K. pneumoniae* and *P. mirabilis*. The prevalence of these enzymes in different populations varies substantially and is also difficult to ascertain since they are not always actively searched for.

Despite these limitations, some recent reports consider the prevalence of acquired AmpCs to be similar to that of extended-spectrum beta-lactamases (ESBLs) in *E. coli*. In the CANWARD study it was shown that ESBLs were present in 7% of *E. coli* isolated in 2011, whereas AmpC was detected in 3% of the cases; one third of these being sequence type 131 (ST131) (Denisuik

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et al. 2013). CMY-2 was by far the most common pAmpC found in this study (56%). In the SMART study of intraabdominal infection in the Asia Pacific region it was found that the ratio of ESBL to AmpC was 7:2, and more than half of the pAmpC enzymes were CMY (Sheng et al. 2013). The SENTRY study from the USA published in 2010, comprising more than 3000 isolates of *Enterobacteriaceae* showed that 0.5% of the isolates had pAmpC, and also in this case CMY-2 was also the most commonly detected. Finally, a recent Swedish investigation of a national collection of *E. coli* with resistance to extended-spectrum cephalosporins ($n = 409$) showed that 7% of the isolates had pAmpC enzymes of the CMY-type, whereas the remaining 93% were ESBL-producing (Brolund et al. 2014).

CMY-2 is by far the most commonly reported pAmpC-variant and is frequently associated with IncA/C broad range plasmids. Such plasmids have been isolated from both from humans and animals, and in both in *E. coli* and *Salmonella* spp (Carattoli et al. 2012). The second most common replicon type associated with CMY-2 is I1, whereas other replicon types have been reported sporadically (Carattoli 2009). Compared to other pAmpC variants the success of CMY-2 is probably associated with its relationship with insertion sequence IS*Ecp1* (Naseer et al. 2010) which provides the promotor regions that drives high-level expression of bla_{CMY} (Nakano et al. 2007), similar to what has been observed for $bla_{CTX\text{-}M\text{-}15}$, a highly successful ESBL.

It is of note that pAmpCs are now been frequently observed both in food-producing and companion animals. Recent data from The Netherlands have shown a high occurrence in the broiler production (Dierikx et al. 2013). This finding was corroborated by recent Swedish data showing frequent occurrence of pAmpCs in Swedish broilers, which could be explained by vertical transmission in the production pyramid through imported grandparent poultry (Nilsson et al. 2014). However, Swedish data do not support transmission between animals and humans (Börjesson et al. 2013). The presence of pAmpC has also been seen in both healthy and diarrheic dogs and cats (Hordijk et al. 2013).

It has only been recently that EUCAST published the first international guidelines for the detection of pAmpC (Leclercq et al. 2013). It has been shown that pAmpCs are more often associated with a multidrug-resistant phenotype than isolates with chromosomal hyper-production of AmpC, but in some areas, isolates producing pAmpC can be relatively susceptible to other drug classes (Edquist et al. 2013). Phenotypic methods for the detection of pAmpC have relatively high sensitivities but cannot differentiate between AmpC of chromosomal- or plasmidic-origin. For this purpose, several molecular methods have been described including both in-house and commercial assays (Bogaerts 2011).

CMY-2 has all the hallmarks of a successful enzyme, including a link to ISEcp1, broad-range plasmids, and to ST131. Monitoring the occurrence of pAmpC in *E. coli* could therefore be warranted for infection control purposes and surveillance, at least in highly clinically important sample types like blood cultures.

Carbapenemase-producing *E. coli*

The safety, reliable killing properties and clinical efficacy of β-lactams place these antibiotics among those most frequently prescribed for the treatment of bacterial infections. Among them, carbapenems have the broadest spectrum of activity and are the drugs of choice to treat serious infections caused by extended-spectrum ß-lactamase (ESBL)-producing *Enterobacteriaceae*. However, their utility is being threatened by the proliferation of carbapenem-resistant *Enterobacteriaceae* (CRE) worldwide. Several mechanisms of carbapenem resistance have been reported: (i) the presence of ESBLs or AmpC enzymes in combination with porin mutations, and (ii) the production of carbapenemases (Cuzon et al. 2010a; Nordmann, Naas and Poirel 2011). Clinically relevant carbapenemases encountered in *E. coli* belong to Ambler class A enzymes, such as KPC and GES, to class D enzymes, such as OXA-48, or to metallo-ß-lactamases (MBLs) such as IMP, VIM or NDM (Nordmann, Naas and Poirel 2011). The dissemination of these enzymes among *E. coli* is a matter of great clinical concern given the major role of this pathogen as a cause of nosocomial as well as community-acquired infections (Glasner et al. 2013; Tzouvelekis et al. 2014). Carbapenemase-producing (CP) *E. coli* isolates are often resistant to most classes of antibiotics, leaving physicians with very limited antibiotic choices, if any, for treating infected patients (Falagas et al. 2014; Tzouvelekis et al. 2014). In general, infections due to CP-*E. coli* isolates were previously limited to frail and debilitated people in hospital settings. However, CP-*E. coli* isolates are now spreading in the community and represent a rising threat to the general population (Nordmann, Naas and Poirel 2011; Cantón et al. 2012; Falagas et al. 2014).

In early 2012, carbapenem-resistant *K. pneumoniae* were already endemic in countries such as India, Morocco, USA, Israel and Greece, and their presence had started to rise in many European countries (Nordmann, Naas and Poirel 2011; Cantón et al. 2012). This drastic increase is best exemplified by the Italian situation, in which the prevalence of carbapenem resistance among *K. pneumoniae* isolated from blood cultures rose from 1% in 2009 to 30% in 2012. In countries with a high prevalence of CP- *K. pneumoniae* isolates, significant increases have recently been witnessed in the prevalence of CP-*E. coli* in Greece, Italy, the Czech Republic, Slovakia, Hungary, Bulgaria, with a prevalence of 1.6%, 0.3%, 0.7%, 1.3%, 0.1%, 2.6% respectively. CP-*E. coli* isolates are now increasingly reported in many countries worldwide (Nordmann, Naas and Poirel 2011; Cantón et al. 2012). France has been previously a country with low prevalence of CP-Enterobacteriaceae (http://www.invs.sante.fr/Dossiers-thematiques/Maladiesinfectieuses/Infections-associees-auxsoins/Surveillance-des-infections-associees-aux-soins-AS/Enterobacteriesproductrices-decarbapenemases-EPC/Episodes-impliquantdes-enterobacteries-productrices-de-carbapenemasesen-France.-Situation-epidemiologique-; Dortet, Cuzon and Nordmann 2014); however, the number of episodes per year significantly rose from 1 to 415 episodes/year from 2008 to 2013 (one episode being either a single case or an outbreak of CP-Enterobactericeae), and *E. coli* now represents 30% of these episodes (http://www.invs.sante.fr/Dossiers-thematiques/Maladiesinfectieuses/Infections-associees-auxsoins/Surveillance-des-infections-associees-aux-soins-AS/Enterobacteriesproductrices-decarbapenemases-EPC/Episodes-impliquantdes-enterobacteries-productrices-de-carbapenemasesen-France.-Situation-epidemiologique-; Dortet, Cuzon and Nordmann 2014). While 2/3 of the cases were colonisations, life-threatening bacteraemia has also been described in association with high mortality rates (http://www.invs.sante.fr/Dossiers-thematiques/Maladiesinfectieuses/Infections-associees-auxsoins/Surveillance-des-infections-associees-aux-soins-AS/Enterobacteriesproductrices-decarbapenemases-EPC/Episodes-impliquantdes-enterobacteries-productrices-de-carbapenemasesen-France.-Situation-epidemiologique-).

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CP-*E. coli* isolates have now been isolated worldwide; however, the distribution of the different carbapenemases varies from continent to continent and even from country to country (Nordmann, Naas and Poirel 2011; Cantón *et al.* 2012). KPC enzymes are more prevalent in the Americas, Greece, Italy, Israel and China (Nordmann, Cuzon and Naas 2009; Cantón *et al.* 2012; Glasner *et al.* 2013). MBLs of the IMP-type seem mainly restricted to the Asian continent (especially Japan) and are only rarely identified in Europe among enterobacterial isolates (Nordmann, Cuzon and Naas 2009; Cantón *et al.* 2012; http://www.invs.sante.fr/Dossiers-thematiques/Maladiesinfectieuses/Infections-associees-auxsoins/Surveillance-des-infections-associees-aux-soins-AS/Enterobacteriesproductrices-decarbapenemases-EPC/Episodes-impliquantdes-enterobacteries-productrices-de-carbapenemasesen-France.-Situation-epidemiologique-). MBLs of the VIM-type have been identified worldwide in enterobacterial isolates responsible for large hospital outbreaks. In Europe, Greece has observed a massive spread in these isolates, and major outbreaks have been reported in Spain (Cantón *et al.* 2012; Kazmierczak *et al.* 2015). The latest MBL described, NDM-1 (New Delhi Metallo-ß-lactamase), is highly prevalent in the Indian sub-continent but has also now been identified in many countries worldwide underlining its rapid diffusion (Nordmann, Naas and Poirel 2011; Walsh *et al.* 2011; Cantón *et al.* 2012). OXA-48 producers are spreading in many countries in Europe, in the southern and eastern part of the Mediterranean Sea, North Africa and India, but identification on all continents have been reported (Poirel, Potron and Nordmann 2012a). In France, OXA-48 is the most prevalent carbapenemase and is isolated in 85% of the CP-*E. coli* isolates, suggesting a likely community spread (http://www.invs.sante.fr/Dossiers-thematiques/Maladiesinfectieuses/Infections-associees-auxsoins/Surveillance-des-infections-associees-aux-soins-AS/Enterobacteriesproductrices-decarbapenemases-EPC/Episodes-impliquantdes-enterobacteries-productrices-de-carbapenemasesen-France.-Situation-epidemiologique-; Dortet, Cuzon and Nordmann 2014).

The emergence and rapid spread of these carbapenemases is, in part, the consequence of mobile genetic elements (Tn*4401* for KPC-2, Tn*1999* for OXA-48, Tn*125* for NDM-1 and Tn*21* associated with integrons for VIM/IMP) as well as the existence of highly efficient conjugative plasmids (pOXA-48 of IncL/M-type) and epidemic clones at the origin of their diffusion worldwide (*K. pneumoniae* ST258 for KPC) (Aubert *et al.* 2006; Naas *et al.* 2008; Cuzon *et al.* 2010b; Poirel *et al.* 2012b; Potron, Poirel and Nordmann 2014; Zhao and Hu 2015; Mathers, Peirano and Pitout 2015b). MLST analysis of a worldwide collection of OXA-48 producing *E. coli* isolates has revealed a high degree of diversity among the isolates, suggesting the spread of a single epidemic plasmid pOXA-48 in different clones (Potron *et al.* 2013). However, carbapenemases such as OXA-48, NDM-1, KPC-2 or VIM-2 have also now been reported in successful epidemic *E. coli* clones; for example in the widespread *E. coli* ST131 responsible for the spread of the CTX-M-15 ESBL (Naas *et al.* 2011; Woodford, Turton and Livermore 2011; Bonnin *et al.* 2012; Morris *et al.* 2012).

As underscored in the last WHO global report (WHO 2014), antimicrobial resistance within a wide range of infectious agents including *E. coli* has reached an extremely worrying situation, that 'threatens the achievements of modern medicine' (WHO 2014). While it is difficult to anticipate the development of novel antimicrobial agents, most efforts must be focused on the prevention of the spread of carbapenemase producers by early detection and reinforced hygiene measures (Delory *et al.* 2015). Detection of CP-*E. coli* isolates is often difficult due to low carbapenem MICs that may remain within the susceptibility range. Reduced susceptibility to carbapenems should alert microbiologists to perform confirmatory testing. The last years have witnessed the development of several efficient confirmatory tests (biochemical-, spectrometric- and molecular-tests) (Dortet *et al.* 2016; Viau *et al.* 2016). In hospital settings, screened patients are kept in strict isolation until screening results are available, thereby requiring screening techniques to be sensitive, specific and rapid. Since the reservoir of carbapenemase producers continues to be the intestinal flora, stools and rectal swabs are adequate samples for performing this screening. Several selective chromogenic media and molecular tests are now available to improve the screening of carriers (Viau *et al.* 2016).

Genetic elements carrying resistance determinants

The dissemination of antimicrobial resistance in *E. coli* has been largely attributed to inter- and intra-specific DNA exchange, with horizontal transfer of plasmid-located genes being the prevalent mechanism at the origin of the acquisition of resistance. Plasmids are members of the prokaryotic family of mobile genetic elements, which play a central role in mobilizing and reorganizing genes within the genome (intracellular mobility) or between different bacterial cells (intercellular mobility) (Thomas and Nielsen 2005). Plasmids promote the horizontal transfer of resistance determinants among bacteria of different species, genera and kingdoms, depending on their narrow or broad host range, conjugative properties and efficiency of conjugation (Lawley, Wilkins and Frost 2004). Natural plasmids have systems guaranteeing their autonomous replication as well as mechanisms controlling the copy-number and ensuring stable inheritance during cell division (Sykora 1992; Thomas and Nielsen 2005). Plasmids acquire mobile genetic elements (ISs, transposons) that mobilize the antimicrobial resistance genes. In many plasmids, mobile genetic determinants are integrated in clusters, conferring resistance to multiple classes of antibiotics. These plasmids may grant a selective advantage to the bacterial host when several antimicrobials are simultaneously administered (Miriagou, Carattoli and Fanning 2006).

Particular plasmid types are more frequently detected in *E. coli* and play a major role in the diffusion of specific resistance genes. For instance, IncFII, IncN and IncI1 plasmids carrying extended-spectrum beta-lactamase genes are currently considered as 'epidemic resistance plasmids' in this species, being detected worldwide in *E. coli* of different origins and sources (Carattoli 2013).

The IncI1 resistance plasmids are one of the most diffused plasmid families in *E. coli*, and they largely contributed to the dissemination of ESBLs, in particular of the CTX-M type (Carattoli 2013). IncI1 plasmids are characterized by the presence of a cluster encoding the type IV pili, contributing to adhesion and invasion of Shiga-toxigenic *E. coli* (STEC) (Kim and Komano 1997). These peculiar pili are considered a virulence factor which, in association with resistance determinants, may support their successful dissemination. More than 400 IncI1 plasmids are currently included in the database of plasmid MultiLocus Sequence Typing (pMLST). This method allows the classification of the plasmids of the most frequent families in homology groups named Sequence Types (STs; http://pubmlst.org/plasmid/). Interestingly, IncI1 plasmids assigned to ST7 represent 56% (75/136) of all the $bla_{\text{CTX-M-1}}$ carrying plasmids from *E. coli* submitted to the pMLST database, suggesting that the spread of the CTX-M-1 ESBL is mainly due to one

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single plasmid circulating in different *E. coli* strains. This variant has been identified in *E. coli* strains from poultry meat samples and human sources in different European countries, demonstrating that these plasmids are capable of spreading very efficiently and might have a reservoir in the food chain (Leverstein-van Hall *et al.* 2011).

The $bla_{CTX\text{-}M\text{-}1}$ was also identified on plasmids belonging to the IncN group in human clinical strains of *E. coli* in pigs and farm personnel from Denmark, and it was demonstrated that these plasmids were transmitted within the farm among pigs and the farm workers, across multiple *E. coli* lineages (Moodley and Guardabassi 2009). In Greece and Italy, IncN plasmids originated the dissemination of the metallo beta-lactamase VIM-1. These plasmids have frequently been identified in *K. pneumoniae* as well as *E. coli* of nosocomial origin, persisting for a long time in different hospitals, and also acquiring Plasmid Mediated Quinolone Resistance (PMQR) genes and other additional resistance determinants. These plasmids encode the EcoRII endonucleases/methylase restriction system and demonstrate the integration of a variable region consisting of multiple integrons and transposons within the *fipA* target site, a dispensable plasmid gene that probably creates a region prone to acquiring transposon- and ISs (Carattoli *et al.* 2010; Miriagou *et al.* 2010).

Since 2000, CTX-M-15 has become the most common CTX-M variant, having been mainly identified in the pandemic, multiresistant, virulent *E. coli* O25:H4-ST131 clone (Cantón and Coque 2006). The $bla_{CTX\text{-}M\text{-}15}$ gene has been associated with multireplicon plasmids belonging to the IncF group. An interesting study performed in *Enterobacteriaceae*, not pre-selected for antimicrobial resistance demonstrated that IncF plasmids are unevenly distributed between the different species, being more prevalent in *E. coli* than in other genera (Sherley, Gordon and Collignon 2003). These data suggest that the $bla_{CTX\text{-}M\text{-}15}$ gene has been acquired on a plasmid type that frequently occurs in the *E. coli* species. In many *E. coli* strains the $bla_{CTX\text{-}M\text{-}15}$ gene has been associated with additional resistance genes on the same IncF plasmid such as the $bla_{OXA\text{-}1}$ and $bla_{TEM\text{-}1}$ beta-lactamases, the *aac(6′)-Ib-cr* and *aac(3)-II* aminoglycoside- and fluoroquinolone-resistance genes, the tetracycline resistance operon, conferring altogether a multidrug resistance phenotype to these strains (Karisik *et al.* 2006; Woodford *et al.* 2009). IncF plasmids likely contribute not only to the fitness of the bacterial host by providing virulence and antimicrobial resistance determinants but also encoding several addiction systems that guarantee their maintenance and stability in the host cell independently by the positive selective pressure exerted by the antimicrobials.

The $bla_{NDM\text{-}1}$ gene is mostly plasmid-located and several different plasmid types, including IncL/M, IncA/C, IncF, IncHI1 and novel plasmid variants of the IncN and IncHI1 type were at the origin of the dissemination of the *bla*NDM-1 gene in non-clonally-related enterobacterial isolates. IncA/C plasmids are particularly important since they show a very broad host range, being able to replicate not only in Enterobacteriaceae, but also in *Pseudomonas* (Carattoli *et al.* 2012). Plasmids of this group carry multiple resistance determinants, conferring aminoglycoside, chloramphenicol, trimethoprim, sulphonamides, tetracycline and the mercuric ion and encode restriction enzymes, antirestriction DNA methylases, and partitioning systems that promote their maintenance and persistence. In the last decades, IncA/C plasmids have been associated with the spread of the AmpC beta lactamase CMY-2 in *E. coli* (Lindsey *et al.* 2009). The NDM-1-IncA/C plasmids derive from those carrying the $bla_{CMY\text{-}2}$ gene, since a conserved genetic environment of the $bla_{CMY\text{-}2}$ gene and the same integration site within the plasmid have been observed. It is plausible that the IncA/C plasmids evolved by sequential acquisition of the $bla_{CMY\text{-}2}$ gene, followed by the acquisition of the $bla_{NDM\text{-}1}$ gene and also gained further additional resistance determinants encoding the ArmA or RmtB 16S RNA methylases, conferring resistance to all aminoglycosides (Carattoli *et al.* 2012) (Fig. 5).

CLINICAL ASPECTS OF *E. coli*

Escherichia coli and IBDs

IBD comprises a group of chronic remitting-relapsing inflammatory bowel disorders of unknown etiology that predominantly affect industrialized countries. CD and ulcerative colitis (UC) are the main IBDs and, although they share similar clinical symptoms, they are histopathologically distinct and are suspected to have different etiologies (Sartor 2006). The intestinal microbiota is one of the main factors implicated in IBD; however, it is still unclear which specific bacterial communities are responsible for the unbalanced host-microbe relationships in these diseases. A large number of studies agree that dysbiosis in CD is characterized by an increase in *E. coli* and a decrease in *Faecalibacterium prausnitzii* (Martinez-Medina *et al.* 2006; Willing *et al.* 2009; Lopez-Siles *et al.* 2014), whereas no such consensus has been reached for dysbiosis in UC patients (Martinez-Medina *et al.* 2014). Regarding the role of *E. coli*, substantial evidence indicates that *E. coli* is involved in CD, especially ileal CD, and growing data suggest that this species is also a contributing factor in the pathogenesis of UC.

Escherichia coli is a natural colonizer of the human intestinal tract and some pathogenic strains, classified into six pathovars and collectively named diarrhoeagenic *E. coli*, can lead to intestinal disease (Kaper, Nataro and Mobley 2004). However, diarrhoeagenic *E. coli* has rarely been detected in IBD patients (Baumgart *et al.* 2007). Instead, strains with features characteristic of ExPEC have frequently been associated with the intestinal mucosa of these patients (Baumgart *et al.* 2007; Schippa *et al.* 2009; Vejborg *et al.* 2011). It is of note that ExPEC-like strains with virulence genes such as adhesins, siderophores, capsule or toxins are also common in the mucosa of healthy subjects and are reportedly necessary for an effective colonization of the intestinal tract (Nowrouzian, Adlerberth and Wold 2001). Despite the common features between *E. coli* populations from IBD and healthy subjects, differences in terms of abundance and pathogenic behavior have been described.

In patients with CD, especially those with ileal and active disease, there is an overgrowth of *E. coli* (Baumgart *et al.* 2007; Willing *et al.* 2009; Schwiertz *et al.* 2010; Lopez-Siles *et al.* 2014), and increased abundance of this species has been correlated with lower time until relapse (Lopez-Siles *et al.* 2014). *Escherichia coli* can be frequently found in the mucosa inside intestinal epithelial cells and translocated in the lamina propia or deeper in the mucosa and submucosa, as well as in germinal centers of lymph follicles and within granulomas (Mylonaki *et al.* 2005). Molecular characterization of isolated strains has demonstrated that *E. coli* from CD patients are ExPEC-like and mainly belong to B2 and D phylogroups (Baumgart *et al.* 2007; Schippa *et al.* 2009), but studies conducted by Darfeuille-Michaud and collaborators revealed that many of CD isolates shared phenotypic pathogenic features that were not consistent with their genetic basis. Thus, a new *E. coli* pathotype associated with CD named Adherent-Invasive *E. coli* (AIEC) was defined (Darfeuille-Michaud *et al.* 2004).

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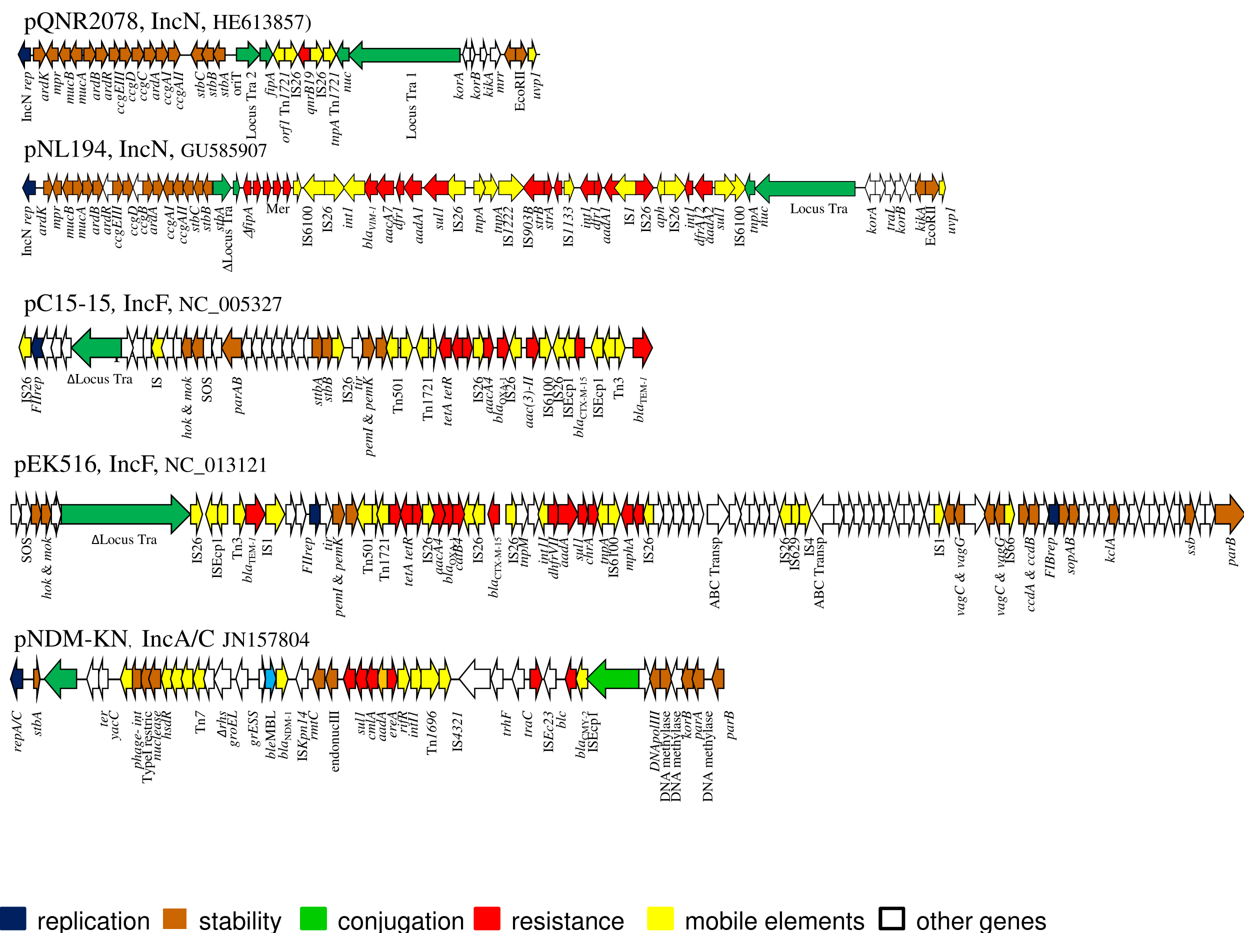

Figure 5. Major structural features of the IncN plasmids, pQNR2078 and pNL194, the IncF plasmids, pC15-15 and pEK516, and the IncA/C plasmid, pNDM-KN. The *tra* locus is indicated by green arrows. Resistance genes are indicated by red boxes, except for the *bla*NDM-1 gene that is indicated by a blue box. Transposon-related genes [tnpA, tnpR, tnpM] and insertion sequences are indicated by yellow boxes. Other genes are indicated by colored boxes as follows: violet, replicase gene *repA*; orange, restriction enzyme and DNA methylase genes; white, other plasmid genes.

In patients with UC, there is some controversy regarding the abundance of *E. coli*, its localisation in the mucosa and the pathogenic features of the strains, and thus, further research is needed to clarify these aspects (Martinez-Medina *et al.* 2014). However, recent studies support that *E. coli* abundance is increased in patients with active disease (Pilarczyk-Zurek *et al.* 2013), and some reports indicate that *E. coli* can be found inside intestinal epithelial cells and in the lamina propia of UC patients (Mylonaki *et al.* 2005). As for CD, *E. coli* isolated to date are mainly ExPEC-like and belong to the B2 and D phylogroups (Schippa *et al.* 2009). However, recent studies have shown that genes encoding for hemolysin, cytotoxic necrotizing factor 1, polyketide synthase gene complex (*pks*) and other virulence factors are more frequent in UC than in CD *E. coli* (Curova *et al.* 2009; Pilarczyk-Zurek *et al.* 2013). Altogether, this suggests that specific *E. coli* subtypes with cytotoxic and genotoxic properties may be the responsible for, or contribute to the mucosal inflammation and tissue damage in patients with UC.

The Adherent-Invasive *E. coli* (AIEC) pathotype was defined based on phenotypic traits as strains that adhere to and invade intestinal epithelial cells involving host cell actin polymerization and microtubule recruitment, and with the ability to survive and replicate within macrophages without inducing host cell death (Darfeuille-Michaud *et al.* 2004).

In the last decade, several independent studies have reported a higher prevalence of AIEC in CD, ranging from 25% to 66% in CD patients and from 0% to 18% in healthy subjects (Baumgart *et al.* 2007; Dogan *et al.* 2013). Moreover, an increased abundance and richness was especially evident for patients with ileal CD. Few studies have sought to investigate the presence of AIEC in UC and prevalence reported to date ranges from 0% to 37.5% (Darfeuille-Michaud *et al.* 2004; Curova *et al.* 2009; Negroni *et al.* 2012). However, the whole AIEC phenotype was not analyzed in all the studies. Further studies are needed to confirm the disease specificity of AIEC, not only focused on UC but also on colorectal cancer or coeliac disease, since particular subsets of *E. coli* with ExPEC features have also been detected in these disorders (Sanchez *et al.* 2008). Concerning host specificity, two studies demonstrate that the AIEC pathotype may also play a role in intestinal disease in dogs, cats and swine, suggesting a certain risk of zoonosis (Martinez-Medina *et al.* 2011). Moreover, recent studies have suggested that high fat–high sugar diets as well as food emulsifiers and stabilizers often found in the food of industrialized countries can favor AIEC gut colonization (Martinez-Medina and Garcia-Gil 2014).

The molecular mechanisms of pathogenicity have principally been studied in one AIEC reference strain, named LF82. Adhesion to intestinal epithelial cells is mainly mediated by

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the interaction between FimH adhesin of type 1 fimbriae and host CEACAM6 glycoprotein, which is overexpressed in CD (Barnich *et al.* 2007). Moreover, mutations of a recent evolutionary origin that confer a higher ability to adhere to CEACAM6 have frequently been found in AIEC FimH (Dreux *et al.* 2013). Reduced amounts of the bacterial second messenger c-di-GMP stimulate the expression of flagella and type 1 pili in strain LF82, promoting adhesion and invasion (Claret *et al.* 2007; see biofilm specific section for more information about c-di-GMP). Genomic analyses have shown mutations in some phosphodiesterases and diguanylate cyclases that may affect c-di-GMP levels in three AIEC strains (Povolotsky and Hengge 2015). Decreased concentration of c-di-GMP is associated with motile phenotypes whereas high cellular levels promote biofilm formation. Nonetheless, biofilm formation is a feature of not only the LF82 strain (Chassaing and Darfeuille-Michaud 2013) but also of a substantial number of AIEC strains (Martinez-Medina *et al.* 2009a,c). Further studies focused on the implications of c-di-GMP turnover in AIEC pathogenesis and in the whole gut microbiota are of interest, especially because the immunosuppressive drug azathioprine used in IBD inhibits the biosynthesis of c-di-GMP (Antoniani *et al.* 2013). Apart from type 1 pili mediated adhesion, an interaction between chitinases of bacterial and human origin has been elucidated (Low *et al.* 2013). Particularly, specific polymorphisms in two *chiA* chitin binding domains characteristic of LF82 and other pathogenic *E. coli* are required to interact with human chitinase CHI3L1, which is upregulated during intestinal inflammation. Invasion of intestinal epithelial cells by LF82 is in part mediated by outer membrane vesicles that interact with host receptor Gp96 via the OmpA. These vesicles are thought to contain bacterial effectors that are released into the host cell and can induce the actin polymerization and microtubule recruitment occurring during bacterial uptake (Rolhion *et al.* 2010). Intracellular replication is possible since LF82 is able to abrogate autophagy by inducing the overexpression of MIR30C and MIR130A, which, in turn, reduce the expression of ATG5 and ATG16L1 autophagy-related molecules (Nguyen *et al.* 2013). However, polymorphisms in genes related to autophagy have been associated with CD, therefore host susceptibility may influence the success of AIEC to survive inside human cells as well (Lapaquette *et al.* 2010). LF82 can also easily translocate the intestinal barrier via M cells by the interaction of type 1 pili and long polar fimbriae with the GP2 M cell receptor (Chassaing *et al.* 2011) and also intercellularly since LF82 can lead to a loss of gut barrier function by inducing the expression of the pore-forming protein claudin-2 and by displacing ZO-1 and E-cadherin from apical tight junctions (Denizot *et al.* 2012). In macrophages, intracellular AIEC replicate within the phagolysosome, thus indicating that they have the ability to resist and replicate in acidic environments, under oxidative stress and in the presence of antimicrobial peptides (Bringer *et al.* 2006). It has been demonstrated that the protease HtrA, the thiol-disulfide oxidoreductase DsbA (Bringer *et al.* 2007) and unknown target genes of the RNA-binding protein Hfq play a role in LF82 intramacrophagic survival (Simonsen *et al.* 2011). In turn, infected macrophages secrete high amounts of TNFα, which contributes to intestinal inflammation and CEACAM6 expression, thus facilitating AIEC adhesion to intestinal epithelial cells. A direct role for LF82 in delaying apoptosis of macrophages and dendritic cells has recently been reported (Dunne *et al.* 2013). Interestingly, this trait can be linked to granuloma formation, a hallmark in the pathogenesis of CD. Survival and replication within neutrophils has also been reported for LF82, but in this cell line type LF82 induces apoptosis (Chargui *et al.* 2012). Infected neutrophils secrete IL-8 also contributing to mucosal inflammation.

Despite all these mechanisms of pathogenicity, AIEC are considered pathobionts rather than true pathogens. This notion is supported by the fact that AIEC is also found in the intestinal mucosa of healthy subjects, although with a lower abundance than in CD, and that host genetic susceptibility and/or environmental risk factors may have an impact on the success of AIEC to colonize the gut and cause disease. As evidenced in animal models, an alteration of the endogenous microbiota or the induction of a low-grade inflammation is necessary for effective colonization of AIEC (Carvalho *et al.* 2009).

In summary, gut bacterial dysbiosis in IBD has led to the study of *E. coli* populations in CD and UC, which is still an active area of research with many unresolved questions. Distinct *E. coli* pathosubtypes have been suggested to be implicated in the pathogenesis of CD and UC. CD associated *E. coli* are frequently classified as AIEC and recently, *E. coli* with toxigenic features have been associated with UC. Significant efforts have been dedicated to defining the molecular mechanisms of the pathogenicity of the AIEC pathotype. However, these studies have mainly been focused on the LF82 strain and no specific genes for the pathotype have been identified to date. Genomic and transcriptomic analyses of a wider AIEC collection including different phylotypes will facilitate the identification of biological markers for the molecular identification of the pathotype as well as to shed light on the molecular basis of its pathogenicity.

UTI caused by *E. coli*

E. coli is the principal cause of UTI which can present as asymptomatic bacteriuria, cystitis and pyelonephritis, as well as prostatitis in men. Uropathogenic *E. coli* (UPEC) is the etiological agent in 75% of uncomplicated UTI and 65% of complicated UTI (Flores-Mireles *et al.* 2015).

UPEC have diverse virulence factors (Lüthje and Brauner 2014; Subashchandrabose and Mobley 2015), some of them related to pathogenicity islands, regions of DNA that are acquired by horizontal gene transfer. These virulence factors are the following.

(1) Adhesins: fimbriae or pili as type 1 fimbriae, P fimbriae (related with renal cells adherence), curli fimbriae, F1c/s fimbriae, F9 and type 3 fimbriae; and non-fimbrial adhesins (Afa/Dr adhesins, related with diarrheagic diseases) and autotransporter proteins (Ag43 adhesin and Upa uropathogenic autotransporter protein). These adhesins are responsible for adhesion to both urinary tract epithelial cells and urinary catheters, and promote biofilm formation.
(2) Toxins: endotoxin lipopolysaccharide (LPS), α-haemolysin (HlyA), CNF1 (cytotoxic necrotizing factor 1) and SPATEs (serine protease autotransporters of the Enterobacteriaceae) as Sat (Secreted Autotransported Toxin), Pic (Protease Involved in Colonization) or Vat (Vacuolating Autotrasported Protein). These toxins are related to dissemination in tissues, inflammatory response, cytotoxicity and resistance to neutrophils.
(3) Iron acquisition mechanisms: Haem receptors (iron Haem uptake regulated by ChuA and Hma) and siderophores (iron chelating molecules as enterobactin, aerobactin, salmochelin and yersiniabactin). Both mechanisms promote the availability of iron in the urinary tract and contribute to survival and persistence in the urinary tract. Zinc acquisition mechanisms are also important.

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(4) Immune evasion mechanisms: suppression of induction of cytokines and chemokines (due to O antigens of LPS), serum resistance and protection against phagocytes (due to O antigens of LPS and K antigens of capsular polysaccharides) and motility (due to flagella with F antigens).
(5) Formation of biofilm: extracellular matrix that provides protection against antimicrobial treatment and host defense mechanisms and adherence to both epithelial cells and urinary catheters, and is responsible of persistence and recurrence of UTI.

E. coli may be classified in phylogroups A, B1, B2, D and E; UPEC belong usually to phylogroup B2 and less frequently to phylogroup D. Classification is also possible by O (somatic), H (flagellar) or K (capsular) antigens (Lüthje and Brauner 2014; Dale and Woodford 2015). Commonly used molecular classification systems include multilocus sequence typing (MLST), pulsed field gel electrophoresis (PFGE) and, recently, whole genome sequencing (WGS) (Dale and Woodford 2015).

Treatment of uncomplicated cystitis include nitrofurantoin, trimethoprim-sulfametoxazol, fosfomycin or, less frequently, oral fluoroquinolones or β-lactam antibiotics such as amoxicillin-clavulanate or second or third-generation cephalosporins. In pyelonephritis, recommended treatment includes intravenous ceftriaxone, aminoglycoside, fluoroquinolones or carbapenems (Gupta *et al.* 2011).

In Europe (European Centre for Disease Prevention and Control 2015) the prevalence in *E. coli* of resistance to aminopenicillins (ampicillin or amoxicillin) is 57.1%, to fluoroquinolones is 22.4%, to third-generation cephalosporins is 12.0% (due to the production of ESBLs) and to aminoglycosides (gentamycin or tobramycin) is 9.%. Moreover, 4.8% of strains are co-resistant to third-generation cephalosporines, fluoroquinolones and aminoglycosides, thereby demonstrating multidrug resistance.

Only 0.1% of *E. coli* are resistant to carbapenems, and only 7.8% of gentamicin and/or tobramycin-resistant strains are amikacin-resistant. These data suggest that recommended empiric treatment of UTI may be adapted to the changing antimicrobial susceptibility.

E. coli sequence type 131 (ST131) is a clonal group that has spread in diverse countries in recent years (Banerjee and Johnson 2014; Mathers, Peirano and Pitout 2015a). This clone belongs to phylogenetic group B2 and is composed by different subclones: the original H30 subclone susceptible to antibiotics has evolved to the H30R subclone, resistant to fluoroquinolones (with mutant *gyrA* and *parC* alleles), and these to the H30Rx subclone, resistant also to third-generation cephalosporins (due to ESBL CTX-M-15). The most important clusters are O25b:H4 (dominated by H30 strains) and, less frequently, O16:H5. *E. coli* O16:H5 is more resistant to gentamycin and trimethoprim-sulfametoxazol, but less resistant to fluoroquinolones and third-generation cephalosporins than *E. coli* O25b:H4.

E. coli ST-131 is a problem from public health due to its global distribution, increased transmissibility, ability to colonize and persist in intestine and urinary tract, enhanced virulence (great number of virulence genes and high fitness), and extensive antibiotic resistance. This clone has been described more frequently in patients older than 50 years, in previous antibiotics consumption, healthcare-associated UTI and recurrent and persistent UTI and sepsis.

Prevention of recurrent and/or persist UTI, which is especially frequent in women, is an important question to resolve. The use of *Lactobacillus,* both in oral formulation or in vaginally inserted capsules, is controverted. *Lactobacillus* maintains acid vaginal pH (less than 4.5), produces H_2O_2 and bacteriocins for bacterial lysis and biosurfactants that reduce adhesion, and can co-agglutinate with uropathogens. Despite these characteristics, the utility of *Lactobacillus* for preventing UTI has not been proven (Barrons and Tassone 2008).

The use of cranberries for prevention of recurrent and/or persist UTI had also interesting. Cranberries (*Vaccinium macrocarpon*) contain proanthocyanidins (PACs) with antiadhesion activity (Howell 2007), specifically against P fimbriae, reducing fimbrial length and density, and producing morphological changes. But the most recent studies of the use of cranberry juice, concentrate, capsules or tablets have not demonstrated utility in reducing occurrence of UTI (Jepson, Williams and Craig 2012).

The final promising approach for preventing these infections is the use different kinds of vaccines and small molecules that are directed toward virulence factors. Candidate vaccines include those that target bacterial adhesins (e.g., PapD-PapG or FimC-FimH chaperone adhesion complexes), toxins (e.g., haemolysin HlyA) and proteases or siderophores (e.g., FyuA, Hma, IutA and IrcA). Small molecules can target bacterial adhesion by inhibiting pili function (e.g., mannosides) or assembly (e.g., pilicides) (Flores-Mireles *et al.* 2015).

Treatment of infections caused by multidrug-resistant *E. coli*

E. coli is the most frequent cause of community and healthcare-associated UTIs, and is frequently involved in intraabdominal infections. Therefore, antimicrobial resistance in *E. coli* has an important impact on the empirical regimens appropriate for these syndromes and, consequently, on the use of specific antimicrobial agents.

There are plentiful of options for the treatment of severe infections caused by susceptible *E. coli*, including penicillins, β-lactam/ β-lactamase inhibitor combinations (BLBLI), cephalosporins, monobactams, carbapenems, fluoroquinolones, aminoglycosides, and trimethoprim-sulfamethoxazole (TMP-SMX), among others. However, the spread of antimicrobial resistance in *E. coli* due to specific successful clones or transmissible resistance mechanisms significantly limits the options in real life. In fact, according to the ECDC definitions (Magiorakos *et al.* 2012), multidrug-resistance is frequent in *E. coli*. During the last decades, fluoroquinolone resistance and the production of extended-spectrum β-lactamase (ESBL) or plasmid-mediated AmpC (pAmpC) have increased dramatically worldwide, although with important regional variations.

Therapy of ESBL- and pAmpC-producers

These isolates are usually resistant to penicillins, fluoroquinolones, TMP-SMX and some aminoglycosides. Carbapenems are usually considered the drug of choice for the treatment of severe infections caused by isolates producing ESBLs (Rodríguez-Baño and Pascual 2008) because their activity is unaffected by these enzymes. In addition, the results of some observational studies have suggested that carbapenems are associated with better outcomes than cephalosporins or fluoroquinolones (Vardakas *et al.* 2012). The same is assumed for pAmpC-producers despite the scarcity of data. There is greater experience with imipenem and meropenem, and more recently with ertapenem (Wu *et al.* 2012), which may have the advantage of not providing selective pressure on *P. aeruginosa* (Nicolau

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et al. 2012). The presence of these enzymes in community isolates has led to an increase in the consumption of carbapenems (Laxminarayan et al. 2013), which is worrisome in the present context of emergence of carbapenemase-producers. Therefore, the search for alternatives to carbapenems for ESBL-producers is necessary.

ESBL (but not AmpC) are inhibited by β-lactamase inhibitors, and thus BLBLI such as amoxicillin-clavulanic acid or piperacillin-tazobactam are active against some ESBL-producers, again with regional variations. Whether BLBLI are as effective as carbapenems is controversial. Piperacillin-tazobactam but not amoxicillin-clavulanic acid has shown reduced activity when tested against high inoculum of *E. coli* (producing or not producing ESBLs) both *in vitro* (Docobo-Pérez et al. 2013) and *in vivo* (López-Cerero et al. 2010). However, the results of a recent post-hoc analysis of several prospective cohorts of patients with bloodstream infections (BSI) due to ESBL-producing *E. coli* suggested that BLBLI are not inferior to carbapenems in terms of mortality and hospital stay (Rodríguez-Baño et al. 2012). However, since the most frequent sources of BSI in this latter study were the urinary and biliary tract, these results should not be applied to other sources until more data are available (Retamar et al. 2013). A recent meta-analysis did not find that carbapenems were superior to BLBLI, but the results were limited by the quality of most of the studies included. A randomized trial comparing piperacillin-tazobactam and meropenem is currently ongoing in Australia and New Zealand (MERINO trial).

Temocillin, a β-lactam that is stable against ESBL and pAmpC enzymes, but is commercialized in only a few countries, is a very interesting agent that has shown good results in uncontrolled studies (Balakrishnan et al. 2011) and would certainly be worthy of investigation in a randomized trial. Some cephalosporins, particularly ceftazidime and cefepime, are active against ESBL-producers according to current EUCAST breakpoints (Leclercq et al. 2013), but clinical data in severe infections are lacking. UTIs may be treated with aminoglycosides when active *in vitro*. There is limited specific experience with tigecycline for ESBL-producers (Vasilev et al. 2008), but this drug has been associated with worse outcomes than comparators in different types of infections (Tasina et al. 2011). Colistin is usually reserved for extensively drug resistant isolates. Finally, fosfomycin is now being compared to meropenem for the treatment of bacteremic UTIs in an ongoing randomized controlled trial (FOREST trial).

From a practical point of view, empirical therapy of infections potentially caused by *E. coli* should include coverage against ESBL-producers according to the local epidemiology, individual risk factors, and the severity of the infection. Some predictive scores have been defined including age, a Charlson score >4, recent hospitalization or hospital transfer, recent use of fluoroquinolones or cephalosporins, and recent urinary catheterization (Tumbarello et al. 2011; Johnson et al. 2013). According to local susceptibility profiles, empirical therapy may be administered with a carbapenem, or with the combination of a cephalosporin, BLBLI or fluoroquinoline plus an aminoglycoside. According to data currently available, de-escalation to a BLBLI may be possible particularly in urinary or biliary tract infections.

Oral fosfomycin trometamol (Pullukcu et al. 2007; Rodríguez-Baño and Pascual 2008), amoxicillin-clavulanic acid (Rodríguez-Baño and Pascual 2008) and pivmecillinam (Jansåker et al. 2014) have shown good results for the treatment of cystitis in observational studies. Nitrofuraintoin is also frequently active against ESBL-producers and is, therefore, another option.

Therapy of carbapenemase-producers

The production of carbapenemases dramatically reduces the therapeutic options. Carbapenemases are more frequently found in *K. pneumoniae*, but may also be found in *E. coli*. The potential therapeutic options depend on the type of carbapenemase; thus, aztreonam are usually active against metallo-β-lactamases (MBL), and cephalosporins are usually active against OXA-48-producers unless an ESBL or AmpC is co-produced (which unfortunately is frequent). KPC-producers are frequently resistant to all β-lactams. The most frequently active options against carbapenemase-producers are colistin, tigecyline and fosfomycin, and in some cases, aminoglycosides (Tzouvelekis et al. 2012).

The evidence available to provide recommendations for therapy is limited to some observational studies mainly including KPC (Zarkotou et al. 2011; Tumbarello et al. 2012; González-Padilla et al. 2014) or OXA-48 (Navarro-San Francisco et al. 2013; Balkan et al. 2014) producing *K. pneumoniae*. Data with aztreonam for MBL-producers or cephalosporins for OXA-48 producers are lacking. The results of some of the previous studies (Tumbarello et al. 2012) have suggested that combination therapy in severe infections may be superior to monotherapy probably because the most active drugs are less effective, 'second-line' antibiotics. In addition, pharmacokinetic and pharmacodynamics data and some observational studies have suggested that a carbapenem should be part of the combination regimen if the MIC of the isolate is $\leq$8 (or even 16) mg/L and the carbapenem should be used at optimized doses so that sufficient concentrations can be reached (e.g., meropenem 2 g every 8 hours in prolonged infusion) (Tumbarello et al. 2012). However, the superiority of combination therapy is controversial because of the limitations of the previous studies and the results of a meta-analysis of studies on carbapenem-resistant GNB (Paul et al. 2014). Since the use of combination therapy is not without problems, decisions must be made on an individual basis considering the options available, the source, and the severity of the infection. It is our opinion that combination therapy is not needed in most cases of mild to moderate infections, in UTIs or when the source can be readily removed.

Plasticity of the *E. coli* genome: can we define specific pathotypes?

The term bacterial 'pathogenicity' is usually understood as the capability of certain bacterial organisms to produce diseases, in general infections, in humans or animals. Along the last decades, the predominant idea supporting research on the pathogenicity of *E. coli* (the same applies to other microbes) is that a number of basically innocent commensal *E. coli* lineages have acquired a number of genetic traits which convert them into nasty, pathogenic organisms. The microecological features shaping this conversion should be characterized to understand how a useful commensal can become a harmful pathogen (Tenaillon et al. 2010). 'Specific' genetic traits of pathogens are generally known as 'pathogenicity factors' and have been identified because of their association with the production of acquired genetic elements encoding toxins, and in more recent times, as the products encoded by genes that when disturbed (for instance by random transposition) strongly reduce the pathogenicity in suitable animal models. In the first case, 'pathogenicity' is clearly acquired; in the second, we address the 'intrinsic' (or acquired a long time ago) pathogenic factors. When we refer to 'specific pathotypes' we focus on the *E. coli* groups known as EPEC, EHEC, STEC, ETEC, EAEC, DAEC and EIEC. In

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most or all of these cases, intestinal pathogenicity is addressed, and depends on particular, well-defined acquired traits. What is much less clear is whether *E. coli* 'specific pathotypes' can be considered: UPEC (uropathogenic) NMEC (neonatal meningitis) and ExPEC (extraintestinal pathogenic). In fact, the variety of *E. coli* producing UTIs or bloodstream infections is extremely large. It is true that some particular clones are (currently) frequent in these conditions, but whether they constitute a specific 'pathotype group' is highly debatable. In fact, it is difficult to maintain the hypothesis of a sustained genetic adaptation of *E. coli* organisms to non-permanent stressful niches such as urine, deep tissues or blood which are far from those that have shaped their lifestyle and evolutionary history.

We have recently reconsidered the phylogenetic structure of *E. coli* in a well-pondered collection of 128 strains of different origins, using a 5384 bp concatenated sequence of full MLST genes (*adk, fumC, icd, mdh, purA, recA* and *gyrB*). This technique provides highly comparable results to those obtained by considering all core genes in the *E. coli* genome (data not shown) and allowed the clear identification of the recently recognized phylogenetic structure in seven lineages (A, B1, B2, C, D, E, F). Using the Neighbour Net algorithm in SplitsTree v.4., and overprinting the concatenated trees of each of the individual MLST genes to the basic phylogenetic tree, genetic interactions between lineages (recombinatorial events) were easily detected. The B2 phylogroup appeared distantly located from the other phylogroups and showed the lowest intergroup recombination frequencies (1.6%), while B1 phylogroup was highly recombinant (17.7%). Groups A (9.7%), and D (10.2%) recombination frequencies were lower than those of groups C (12.4) and F (28.6%). Groups C, E and F have probably emerged in relatively modern times by recombination between other phylogenetic lineages; for instance, phylogroup C arises from groups A and B1 and phylogroup F maintain promiscuous interactions with group D, suggesting a common ancestor. In summary, phylogroup B2 has almost exclusively intracladed recombination, whereas strains of the phylogroups A, B1 and C show the highest rate of homoplasy (Turrientes *et al*. 2014).

How are 'pathogenic lineages' (pathotypes, pathovars) distributed in these main branches of the phylogenetic tree? Current data suggests that those associated with milder and chronic diarrhea, such as EAEC and DAEC, are found throughout the tree, while those producing more severe intestinal diseases, such as EHEC, ETEC and Shigella/EIEC, are more frequently found dispersed in the A, B1, C and E 'commensal' phylogroups, and EPEC is more frequent in the B1 and B2 phylogroups (Chaudhuri and Henderson 2012). In general, it is difficult to trace long-term 'pathogenic lineages', in the same way that it is difficult to trace long-term 'extended-spectrum beta-lactamase producers'. The higher prevalence in some lineages might exclusively reflect the bias produced by epidemic events.

As far as bacteremic strains are concerned, can any extraintestinal pathogenic *E. coli* (ExPEC) 'pathotype' be recognized among these isolates? In the first publications about ExPEC, these strains were considered as 'specialized strains' possessing a unique ability to cause disease outside the host intestinal tract (Johnson *et al*. 2002). Does ExPEC belong to specific phylogenetic lineages? In a series of 528 blood isolates recovered at Ramón y Cajal Hospital along the last 17 years, these 'ExPEC' were predominantly phylogroup B2 (54%), followed by phylogroups D/E/F (21%), A/C (16%) and B1 (9%).This means that a variety of phylogroups are also associated with bacteremia, including those that are considered to group commensal intestinal strains. We postulate that any kind of *E. coli* might produce bacteremia as a result of stochastic events (like translocation), probably facilitated by a large intestinal population abundance (colonization density), and host factors (such as underlying diseases, and ageing), so that there is a thin line between commensalism and pathogenicity (Leimbach, Hacker and Dobrindt 2013).

In general, we agree with the concept that so-called ExPEC are facultative pathogens which belong to the normal gut flora of a certain fraction of the healthy population where they live as commensals (Köhler and Dobrindt 2011). However, it remains plausible that some particular *E. coli* clones, more frequently in phylogroup B2, are more prone than others to cause bacteremia, but of course, they cannot be considered as having a deterministic causation for extraintestinal diseases, that is, they cannot be considered as belonging to a specific pathotype. The successful spread of these clones (and the clones derived from them) might increase the incidence of bacteremic episodes. Pathogenicity is frequently associated with comparatively smaller population sizes but higher transmission abilities (smaller propagulum). In our hospital, a progressive but significant increase in the number of bacteremic isolates has been observed along the last decade, from 450–500 isolates per year in 1996–2002, to 600–750 isolates per year in 2002–2012. This increase can be partially attributed to a group of clones of the phylogroup B2, most being ST131 or their derived variants. In fact, the B2 non-ST131 bacteremic strains have remained fairly constant along the last 17 years.

The reason why the phylogroup B2 (at large) is more frequent than other phylogroups among bacteremic isolates is an interesting case for evolutionary biologists. Is there a link between evolutionary history of *E. coli* and its propensity for invasive infections? Human *E. coli* strains have evolved from those colonizing mammalian ancestors. Probably the first *E. coli* strains in these early lineages, before the divergence of the great apes (≥30 million of years ago) were from the B2 and D phylogroups, probably derived from avian pathogenic *E. coli* (APEC). Species-to-species transition is a hard venture. Novel organisms should compete with the already established microbial populations filling all the available niches, but the 'more pathogenic clones' (such as those of phylogroups B2 and D) have the possibility of opening new niches by obtaining resources from the host. As is frequent in the evolution of pathogenesis, less pathogenic variants tend to emerge from the more aggressive variants, assuring long-term coexistence with the host, presumptively leading to phylogroups A, B1, C and E probably 20–25 million years ago (Escobar-Páramo *et al*. 2003; Parsot and Sansonetti 2008; Touchon *et al*. 2009). The success of these phylogroups as commensals (in different animals!) maintained the old phylogroups B2 and D in their more 'pathogenic' niches. In bacterial evolution, the ancestor lineages are not necessarily replaced by the derived lineages, probably because of the frequent multiplicity of available hosts and microniches. The emergence of the highly pathogenic *E. coli* group known as *Shigella* occurred accidentally less than 5 million years ago (30 000 years ago?) by the acquisition of foreign genetic material (via mobile genetic elements) in commensal *E. coli* groups. Note that inside phylogroup B2 some clones are particularly prone to cause extraintestinal infections, such as O25-ST131 *E. coli*. These highly transmissible clones have recently spread, increased their exposure to antimicrobial drugs, acquired antibiotic resistance mechanisms, and selected by the use of antibiotics in therapy. Host-to-host transmission has produced a diversifying selection effect, so that currently at least three clusters of different PFGE-types can be recognized among our blood isolates. One of the drivers for such diversification is probably the sequential acquisition of fluoroquinolone, and then extended-spectrum beta-lactamases, so that the originally

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susceptible population was reduced in frequency, and the overall proportion of O25-ST131 was increased in blood isolates. Another diversifying driver is the high 'internal recombination' among phylogroup B2 clones, constantly providing genetic combinations offered to natural selection.

CONCLUDING REMARKS

Extraintestinal *E. coli* infections, which represent a major public health problem, are caused mainly by specialized ExPEC strains that can innocuously colonize human hosts but also cause disease upon entering a normally sterile body site. The virulence capability of such strains is determined by their combination of distinctive accessory traits, called virulence factors, in conjunction with their distinctive phylogenetic background. It is conceivable that by developing interventions against the most successful ExPEC lineages or their key virulence/colonization factors in the future it will be possible to reduce the associated burden of disease and health care costs.

The evolution of resistance plasmids clearly shows that these mobile elements accumulate a series of antibiotic resistance determinants, thus making these plasmids extremely threatening. Not only do they encode multidrug resistance, but indeed, they can be easily selected by many different selective agents. It is important to mention that successful plasmids possess great versatility to intracellular adaptation by the presence of addiction systems (toxin-antitoxin, restriction enzymes) and partitioning proteins that promote plasmid maintenance during vertical transmission in the daughter cells. These plasmids are very stable in their bacterial host and this characteristic certainly influences their success, independently by antimicrobial resistance and other factors that can positively select these molecules.

Therapy of multidrug-resistant *E. coli* is increasingly challenging. Therapeutic decisions are important not only for the individual patient but also for avoiding further selection pressure. Randomized controlled trials and high-quality observational studies are urgently needed for therapeutic decision making, which must now be undertaken on an individual basis. Some promising new drugs are being investigated, including the β-lactamase inhibitor avibactam (in combination with ceftaroline and aztreonam), ceftolozone-tazobactam, plazomicin and omadacyclin.

In conclusion, the definition of specific extraintestinal pathotypes in *E. coli* remains obscure. Organisms of particular phylogroups are more or less prone to evolve by acquisition of particular virulence traits, often in a highly unpredictable way (Baquero and Tobes 2013). However, this probabilistic view is insufficient to make any strong causal association of specific lineages (beyond specific clones) with particular infectious diseases.

ACKNOWLEDGEMENTS

Thanks to the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) for its support. Thanks to Birgitta Agerberth and Annelie Brauner for fruitful collaboration.

FUNDING

This work was supported by the 'Instituto de Salud Carlos III' of Spain [Grants PI10/01579, PI13/00137-FEDER Cofunding] co-financed by European Development Regional Fund 'A way to achieve Europe' ERDF; by Ministerio de Economía y Competitividad; and the Spanish Network for the Research in Infectious Diseases [Grant REIPI RD12/0015]. Ute Römling research on *E. coli* biofilm formation was previously financed by Karolinska Institutet's 'Elitforskartjänst' in Molecular Microbiology. U. Dobrindt's research on *E. coli* genome plasticity and pathogenicity islands was supported by the German Federal Ministry for Education and Research (grant no. 0315904A) in the frame of the ERA-NET Pathogenomics III consortium 'MobileGenomics'.

Conflict of interest. None declared.

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