university of groningen

University Medical Center Groningen

University of Groningen

Fast identification of Escherichia coli in urinary tract infections using a virulence gene based PCR approach in a novel thermal cycler

Brons, Jolanda; Vink, Stefanie N; de Vos, Marjon; Reuter, Stefan; Dobrindt, Ulrich; van Elsas, Jan Dirk

Published in:
Journal of microbiological methods

DOI:
10.1016/j.mimet.2019.105799

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2020

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Brons, J., Vink, S. N., de Vos, M., Reuter, S., Dobrindt, U., & van Elsas, J. D. (2020). Fast identification of *Escherichia coli* in urinary tract infections using a virulence gene based PCR approach in a novel thermal cycler. *Journal of microbiological methods*, *169*, [105799]. https://doi.org/10.1016/j.mimet.2019.105799

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

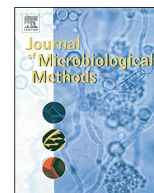
Download date: 26-12-2020

Journal of Microbiological Methods 169 (2020) 105799

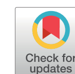
Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth



Fast identification of *Escherichia coli* in urinary tract infections using a virulence gene based PCR approach in a novel thermal cycler



Jolanda K. Brons[a,*], Stefanie N. Vink[a], Marjon G.J. de Vos[a], Stefan Reuter[b], Ulrich Dobrindt[c], Jan Dirk van Elsas[a,*]

[a] Groningen Institute for Evolutionary Life Sciences, University of Groningen, Groningen, The Netherlands
[b] Department of Medicine D, Division of General Internal Medicine, Nephrology and Rheumatology, University Hospital of Münster, Münster, Germany
[c] Institute of Hygiene, University of Münster, Münster, Germany

A R T I C L E I N F O

Keywords:
Uropathogenic *Escherichia coli* (UPEC)
(Fast) PCR (identification)
Virulence genes
Urinary tract infection (UTI)
Novel fast thermal cycler (Nextgen PCR thermal cycler)

A B S T R A C T

Uropathogenic *Escherichia coli* (UPEC) is the most common causal agent of urinary tract infections (UTIs) in humans. Currently, clinical detection methods take hours (dipsticks) to days (culturing methods), limiting rapid intervention. As an alternative, the use of molecular methods could improve speed and accuracy, but their applicability is complicated by high genomic variability within UPEC strains. Here, we describe a novel PCR-based method for the identification of *E. coli* in urine. Based on *in silico* screening of UPEC genomes, we selected three UPEC-specific genes predicted to be involved in pathogenesis (*c3509*, *c3686* (*yrbH*) and *chuA*), and one *E. coli*-specific marker gene (*uidA*). We validated the method on 128 clinical (UTI) strains. Despite differential occurrences of these genes in uropathogenic *E. coli*, the method, when using multi-gene combinations, specifically detected the target organism across all samples. The lower detection limit, assessed with model UPEC strains, was approximately 10^4 CFU/ml. Additionally, the use of this method in a novel ultrafast PCR thermal cycler (Nextgen PCR) allowed a detection time from urine sampling to identification of only 52 min. This is the first study that uses such defined sets of marker genes for the detection of *E. coli* in UTIs. In addition, we are the first to demonstrate the potential of the Nextgen thermal cycler. Our *E. coli* identification method has the potential to be a rapid, reliable and inexpensive alternative for traditional methods.

1. Introduction

Urinary tract infections (UTIs) affect up to 150 million people worldwide each year (Harding and Ronald, 1994). UTIs are caused by both Gram-negative and Gram-positive bacteria, as well as yeasts such as *Candida* spp. Uropathogenic *Escherichia coli* (UPEC) is the most common etiologic agent, which is currently responsible for approximately 90% of all globally occurring UTIs (Zhang and Foxman, 2003). Clinically, a UTI is defined as a bacteriuria with $\geq 10^5$ bacterial (and/or yeast) colony forming units per ml (CFU/ml) midstream urine, in combination with clinical symptoms (Sheffield and Cunningham, 2005). UTIs are classified as either "uncomplicated" or "complicated". Uncomplicated UTIs affect individuals (mostly females) that have no structural or neurological urinary tract (UT) abnormalities, while complicated UTIs affect individuals with underlying problems, such as UT abnormalities, kidney transplantation and/or catheterization (Flores-Mireles et al., 2015). In terms of clinical presentation, UTIs are classified as either cystitis (CY, infection of the bladder/lower urinary tract), pyelonephritis (PY, infection of the kidney/upper urinary tract) and/or urosepsis (US, UTI with sepsis). Asymptomatic bacteriuria (ASB) is not considered as an infection, but represents a risk factor in certain circumstances, e.g. in pregnancy and before traumatizing interventions of the urinary tract (Johansen et al., 2011).

Commensal *E. coli* strains are part of the normal gut microbiome and rarely cause disease in the gut or in the UT. However, particular *E. coli* strains have evolved, presumably via horizontal gene transfer (HGT), into pathogens that cause various diseases, including UTIs (Jacobsen et al., 2008). Based on its ecology, *E. coli* can be classified into three groups: (i) commensal (encompassing beneficial colonizers of the gastrointestinal tract), (ii) intestinal pathogenic (enteric disease or diarrheagenic) and (iii) extra-intestinal pathogenic (ExPEC, among them UPEC) strains (Russo and Johnson, 2000). Eight phylogenetic groups of *E. coli* are recognized, seven (A, B1, B2, C, D, E, F) belonging to *E. coli sensu stricto*, and the eighth constituting the so-called *Escherichia* cryptic clade I (Clermont et al., 2013). Most UPEC strains are members of the B2 group, although uropathogens have also been found within the other

* Corresponding authors at: Groningen Institute for Evolutionary Life Sciences, University of Groningen, Nijenborgh 7, 9747 AG, Groningen, The Netherlands.
E-mail addresses: j.k.brons@rug.nl (J.K. Brons), j.d.van.elsas@rug.nl (J.D. van Elsas).

https://doi.org/10.1016/j.mimet.2019.105799
Received 25 October 2019; Received in revised form 29 November 2019; Accepted 29 November 2019
Available online 30 November 2019
0167-7012/ © 2019 Elsevier B.V. All rights reserved.

phylogenetic groups. Human commensal strains mainly belong to *E. coli* phylogenetic group A (Johnson and Stell, 2000).

Genes involved in the colonization by *E. coli* of the UT, and subsequent pathogenesis, may provide the key to detect UPECs. Pathogenesis is thought to commence with colonization of the urethra, followed by ascension of bacteria into the bladder and growth in the urine. UPEC cells then increasingly adhere to the bladder surface, interacting with the epithelial defense system. Eventually, biofilms are formed. Then, invasion (through replication and formation of intracellular bacterial communities) occurs and UPEC cells may travel upwards to colonize the kidneys, where they may cause tissue damage with increased risk of sepsis (Terlizzi et al., 2017).

The infective process is known to depend on a suite of bacterial traits ("virulence factors"), which - together - determine the sequence of events leading to progressive disease. Most of these traits have been found to be encoded on pathogenicity islands (PAIs) within the *E. coli* chromosome or, alternatively, on plasmids. Given their continuous acquisition (and occasional loss) of genetic material, the genomes of UPECs are generally larger than those of commensal *E. coli* strains (Hacker et al., 1997; Ahmed et al., 2008; Touchon et al., 2009). For example, the genomes of three well-known UPEC strains (CFT073, 536 and UTI89, all belonging to phylogenetic group B2), that constitute currently accepted reference strains for UT infections, contain 8–22% more open reading frames (and are, consequently, 6–13% larger) than the genome of the (commensal) K-12 reference strain MG1655 (Welch et al., 2002; Brzuszkiewicz et al., 2006; Chen et al., 2006).

The distribution of genes for virulence traits in the genomes of UPEC strains is a key issue that will define the criteria for developing a system for identification that is based on their roles in disease development (Ahmed et al., 2008). UPECs exhibit a high degree of genetic diversity (primarily caused by the differential presence or absence of specific genes on PAIs) (Oelschlaeger et al., 2002). Key candidate genes for an identification system can be divided into two groups: (1) genes encoding factors associated with the bacterial cell surface, and (2) genes encoding factors that are secreted and transported to the site of interaction with the host cell (Emody et al., 2003). Both groups include genes encoding: (i) type-1 and −P, next to -S/F1C, fimbrial adhesins, (ii) toxins, i.e. cytotoxic necrotizing factor 1, α-hemolysin and secreted autotransporter toxins, (iii) host defense avoidance mechanisms, i.e. capsule or O-specific antigen, and (iv) iron acquisition systems, i.e. aerobactin, enterobactin, salmochelin and yersiniabactin (Lloyd et al., 2007).

The current "gold standard" for diagnosis of a UTI is detection of the pathogen concomitantly with the presence of clinical symptoms. Dipsticks that detect leukocyte esterase (LE) activity as an indicator of pyuria (condition of urine containing white blood cells or pus) and urinary nitrite (NIT) production as an indicator of bacteriuria are frequently used for presumptive diagnosis of UTIs (Semeniuk and Church, 1999). When the dipstick test is positive and clinical symptoms are present, broad-spectrum antibiotics active against the presumed pathogen are prescribed until culture results are available. Pathogen identification typically takes 18–30 h, with antimicrobial susceptibility testing (AST) adding another 24–48 h. AST is performed as a phenotypic assay that measures bacterial growth in the presence of specific antimicrobial agents (Davenport et al., 2017). Treatments with broad-spectrum antibiotics lead to alterations of the gastrointestinal tract microbiome, and can promote the undesirable development of antibiotic-resistant microorganisms (Kostakioti et al., 2012). Thus, a rapid method for detection of UTI causal agents is required.

In a recent overview, Davenport et al. (2017) described current (new and developing) diagnostic technologies for UTIs, i.e. urinalysis and microscopy, MALDI-TOF mass spectrometry, fluorescent in situ hybridization (FISH), microfluidics, PCR, immunology-based and forward light scattering assays. However, most of these approaches have limiting factors such as: no pathogen identification and/or antimicrobial susceptibility testing, expensive, poor data from low concentrations of cells and poor sensitivities and specificities (Davenport et al., 2017).

PCR enables the detection of target genes with great speed, specificity and sensitivity (Nissen and Sloots, 2002). A number of (multiplex) PCR methods have thus far been described for the identification of UPECs. However, these approaches, based on genes such as *fimH* (Johnson and Stell, 2000; Padmavathy et al., 2012; Ren et al., 2016) and *rfaH* (Van der Zee et al., 2016), are not specific for UPECs, as commensal strains also harbour these genes. Other studies, based on the genes *ecp*, *fyuA*, *sfa/ focDE* (Blackburn et al., 2009; López-Banda et al., 2014) were found to suffer from similar drawbacks. Hence, identification of UPECs on the basis of PCR with genetic markers clearly requires improvement. To identify such UPEC-specific genetic markers, we examined a suite of currently available *E. coli* genomes for (1) the presence of virulence genes in UPECs and (2) the absence of these genes in commensal *E. coli* and other uropathogenic organisms. Based on this screening, we developed a PCR assay for the rapid identification of UPECs. We selected three UPEC-specific genes as targets, in addition to a generic *E. coli* marker gene.

2. Material and methods

2.1. Bacterial strains and reagents

Three reference UPEC strains, i.e. XPKO359, CASC874 (obtained from Prof. dr. J. Degener, University Medical Center Groningen, the Netherlands) and CFT073 (DSMZ 103538), and one commensal *E. coli* K-12 strain, i.e. MG1655 (DSMZ 18039) were used. All strains were grown in 20 ml Luria-Bertani [LB] broth (tryptone 10 g, yeast extract 5 g, NaCl 5 g, distilled water 1 l; pH 7.2 at 37 °C for 18–24 h). Following growth, cultures were centrifuged and the resulting pellets washed twice with Artificial Urine Medium (AUM) (Brooks and Keevil, 1997), after which the final pellets were dissolved in 1 ml AUM. In addition, AUM (19 ml) was spiked separately with 1 ml of the overnight-grown UPEC strains (10^9 CFU/ml) XPKO359, CASC874 and CFT073.

2.2. In silico selection of UPEC/ E. coli marker genes and primer design

To select UPEC/*E. coli* marker genes, we determined the presence of a suite of 131 genes potentially involved in virulence (exclusively present in UPEC strain CFT073 compared to commensal and fecal strains (Lloyd et al., 2007)), in the genomes of 11 UPEC strains. The latter included seven group B2 strains, as well as one strain each of groups D, F, B1 and A, as follows: CFT073 (phylogenetic group B2), 536 (B2), UTI89 (B2), 26–1 (B2), NU14 (B2), ST131 strain EC958 (B2), NA114 (B2), UMN026 (D), IAI39 (F), CI5 (B1) and VR50 (A) (Table 1). Blastn was used, using a 95% identity cut-off (Altschul et al., 1990). To ensure *E. coli* specificity, and using the same Blastn method, we checked the occurrence of the same genes in three commensal *E. coli* strains [HS (A), K-12 MG1655 (A), K-12 W3110(A)] (Table 1), as well as in the uropathogens *Klebsiella pneumoniae* (taxid:573), *Staphylococcus saprophyticus* (taxid:29385), *Enterococcus* (taxid:1350), group B *Streptococcus* (taxid:1319), *Proteus mirabilis* (taxid:584), *Pseudomonas aeruginosa* (taxid:287), *Staphylococcus aureus* (taxid:1280), and *Candida* (taxid:1535326).

Genes that occurred in only one or two of the UPEC phylogenetic group(s) and in either commensal or non-*E. coli* species were excluded from further analyses. From the overall screening, three genes (potentially involved in the ecology of virulence) were chosen, that met the criteria to serve as targets in the detection system: *c3509*, *c3686* (*yrbH*) and *chuA*. The *E.coli* specific gene, *uidA*, was added as a control/reference gene (Kiel et al., 2018). Thus, a four-gene detection method was created *in silico*. We designed primers based on all available genetic information for each gene in Clone Manager 6, using as criteria: target region of 190–260 bp, primer length of 19/ 20 bp, and calculated annealing temperature of 60 °C (Table 2). This ensured specificity for

Table 1
E. coli strains of which the genomic information was used in this study, with the disorder caused by them, their phylogenetic group and GenBank accession numbers.

Strain	Disorder/type	Phylogenetic group	GenBank accession number
CFT073	UPEC (pyelonephritis)	B2	AE014075.1
536	UPEC (pyelonephritis)	B2	CP000247.1
UTI89	UPEC (cystitis)	B2	NC_007946.1
UPEC 26–1	UPEC (UTI)	B2	CP016497.1
NU14	UPEC (cystitis)	B2	CP019777.1
ST131 strain EC958	UPEC (UTI)	B2	NZ_HG941718.1
NA114	UPEC	B2	CP002797.2
UMN026	UPEC (cystitis)	D	NC_011751.1
IAI39	UPEC (pyelonephritis)	F	CU928164.2
CI5	UPEC (pyelonephritis)	B1	CP011018.1
VR50	UPEC (asymptomatic)	A	CP011134.1
K-12 strain MG1655	Lab-adapted human commensal *E. coli*	A	U00096.3
HS	Human commensal *E. coli*	A	NC_009800.1
K-12 strain W3110	Human commensal *E. coli*	A	AP009048.1

UPECs (based on the genomes of the reference UPEC strains), as well as amplification at the selected annealing temperature.

2.3. *E. coli* virulence gene PCR optimization

PCR mixtures were composed as follows: 8 µl of 5× KAPA2G Buffer A (KAPA Biosystems, Wilmington, United States), 60 nmol $MgCl_2$ (KAPA Biosystems), 12 nmol of each deoxyribonucleoside triphosphate (Sigma-Aldrich), 40 pmol of each primer, 10% dimethylsulfoxide (DMSO) and 1 U of (5 U/µL) KAPA2G Fast HotStart DNA polymerase (KAPA Biosystems), were combined with Ambion nuclease free water (Thermo Fisher Scientific, Waltham, United States) to 40 µl in a 0.2-ml microfuge tube. The DNA template was 1 µl of either: the spiked AUM samples, DNA extracted from these spiked AUM samples or the clinical UPEC DNA. The mixture was split in two (20ul each), one mixture was incubated in a Mastercycler Nexus PCR thermal cycler (Eppendorf, Hamburg, Germany), and the other in a Nextgen PCR thermal cycler (Molecular Biology Systems, Goes, the Netherlands), programmed for the four marker genes as follows: initial denaturation of double-stranded DNA for 30 s. at 95 °C; 30 cycles consisting of 7 s. at 95 °C, 12 s. at 60 °C, and 12 s. at 75 °C; and extension for 30 s. at 75 °C. Cycling conditions were similar for the two thermal cyclers. The PCR program in the regular thermal cycler takes 51 min, while the Nextgen PCR thermal cycler can perform this PCR in 16 min. All amplification products were analyzed by electrophoresis in 1.0% (wt/vol) agarose gels, followed by ethidium bromide staining (1.2 mg/ l ethidium bromide in 1× Tris-acetate-EDTA) (Mullis, 1990; Sambrook and Fritsch, 1989), destaining (1× Tris-acetate) and visualization under UV.

2.4. DNA extraction using the Qiagen DNeasy ultraclean microbial kit

DNA extraction using the Qiagen DNeasy ultraclean microbial kit (Qiagen, Hilden, Germany) was done according to the manufacturer's protocol. Genomic DNA was isolated from 1.8 ml of microbial culture by starting with a centrifugation step, followed by bead-beating, cell lysis, binding to silica filter and washing. The resulting DNA was recovered in 50 µl DNA-free Tris buffer.

2.5. Development of a fast DNA extraction method from urine

Deelman et al. (2002) described a fast DNA extraction method from urine (based on the Qiagen PCR purification kit), to which we made the following modifications: two ml of spiked AUM (with cells of UPEC strains grown overnight in LB at 37 °C and resuspended in AUM) were centrifuged at 10,000 x*g* for 2.5 min. The supernatant was discarded and the pellet dissolved in 100 µl sterile dH_2O; then, 500 µl of PB buffer was added and the mixture was vortexed for 30 s. The total volume was transferred to a column and centrifuged for 30 s. at 10,000 x g. The flow through was discarded and the column was washed with 600 µl PE buffer. The DNA was eluted in 50 µl EB buffer (10,000 x g for 30 s.). The total time of extraction was 4.5 min.

2.6. Adaptation of the fast DNA extraction method

Strain CFT073 was grown overnight in LB medium at 37 °C, after which the cells were centrifuged and washed two times with AUM to remove any LB residue. Dilution series were made with AUM, after which PCR (using primer set *c3686*) was performed on samples processed as follows: (i) Deelman DNA extraction method (3 min), (ii) modified Deelman DNA extraction method (4.5 min), (iii) Qiagen DNeasy ultraclean microbial kit (21 min), and (iv) no DNA extraction (cells in urine, 0 min). Dilution plating was performed to establish the number of CFU/ml in the systems (Hoben and Somasegaran, 1982).

2.7. Determining the lower detection limit of target genes in reference strain samples

The overnight LB-grown *E. coli* reference strains (XPKO359, CASC874, CFT073) were centrifuged and washed two times with AUM to remove any LB medium residue, after which dilution series (10^9–10^1 CFU/ml) were made in sterile AUM. Dilution plating was performed to determine the CFU densities in the starting culture (Hoben

Table 2
List of primers used for the *E. coli* detection in urinary tract infections. * F: forward; R: reverse.

Genes	Direction*	Primer (5′-3′)	Amplicon size (bp)	Specificity	Reference
c3509	F	ACAATCCGCCACCATCCAG	208	UPEC	This study
c3509	R	CTCTCCACCGGAGAGTGTT			
c3686	F	TTGCACCAACAACGTCTACC	259	UPEC	This study
c3686	R	TCTGCGTCTTCTACCATCAC			
chuA	F	GCTACCGCGATAACTGTCAT	221	UPEC	This study
chuA	R	TGGAGAACCGTTCCACTCTA			
uidA	F	CGCCGATGCAGATATTCGTA	259	*E. coli*	This study
uidA	R	CTGCCAGTTCAGTTCRTTGT			

and Somasegaran, 1982). PCR was performed with DNA extracted from these dilutions (10^9–10^1 CFU/ml) using the fast DNA extraction method, in both the regular PCR thermal cycler and the fast PCR thermal cycler (Nextgen PCR thermal cycler). Here, all four primer sets (*c3509*, *c3686*, *chuA*, *uidA*) were examined to determine the lower detection limits of the *E. coli* PCR.

2.8. Origin of clinical UPEC samples

The novel *E. coli* PCR-based detection system was validated on genomic DNA isolated from clinical UPEC isolates collected at the University Hospital of Münster, Münster, Germany. These clinical samples were subcultured and confirmed as *E. coli* by MALDI-TOF (Caprioli et al., 1997). The DNA was isolated, from pure cultures grown overnight in LB medium, by using the MagAttract HMW kit from Qiagen according to the manufacturer's recommendations (performed in Münster, Germany). A total of 128 UPEC strains isolated from female patients (age 19–89 years, median age 54.4) with an uncomplicated UTI ($n = 70$) and patients with a complicated UTI (kidney transplantation, $n = 58$) was tested.

2.9. Novel fast PCR thermal cycler (Nextgen PCR thermal cycler)

The detection method was tested in both a regular PCR thermal cycler (Mastercycler Nexus PCR thermal cycler, Eppendorf, Germany) and the novel fast PCR thermal cycler (Nextgen PCR thermal cycler, Molecular Biology Systems (MBS), Goes, the Netherlands). The Nextgen PCR thermal cycler operates with six aluminum heat blocks, in three zones, and is programmable to the desired temperature zone for denaturing, annealing and extension. A thin (50 μM) polypropylene PCR plate (96 or 384 wells) is transferred to the desired temperature zone in 0.1 s, and clamped between the two aluminum blocks in that zone. Due to this set-up, there is no ramping time and thus the time it takes to complete a PCR is strongly reduced.

3. Results

3.1. In silico selection of UPEC/ E. coli marker genes

The genomes of 11 selected UPEC strains were examined with respect to their distinction from those of three commensal *E. coli* strains. We specifically screened for the presence/absence of 131 genes that had been reported to be exclusively present in a set of UPEC strains as compared to commensal and fecal *E. coli* strains (Lloyd et al., 2007). In addition, we screened for the absence of these 131 genes in the other causal agents of UTIs: *Klebsiella pneumoniae*, *Proteus mirabilis, Pseudomonas aeruginosa, Enterococcus sp.*, group B *Streptococcus* (GBS), *Staphylococcus aureus*, *S. saprophyticus* and *Candida sp.* (Fig. 1).

We used three criteria to select for UPEC-specific genes from the set of 131 genes identified by Lloyd et al. (2007): 1) they should not occur in commensal *E. coli* strains and/or non-*E. coli* uropathogens, 2) they should occur in phylogenetic group B2 and in at least two out of four additional phylogenetic groups (among groups D, F, B1 and A), and 3) the genes should not be co-localized in the same genomic region. This third criterion was used in order to prevent covariant behavior of the marker genes. Thirty-four genes were discarded based on the first criterion, and an additional 80 based on criterion 2. Thus, 17 genes were identified that were present in at least three phylogenetic groups, including group B2. These 17 genes were found to be distributed over eight different regions within the *CFT073* reference genome. Based on this scattered occurrence, three of these 17 genes, i.e. *c3509*, *c3686* (*yrbH*) and *chuA* (Lloyd et al., 2007) were selected to serve as target genes for the UPEC identification system. By predicted function, all three genes could be tentatively linked to processes associated with bacterial virulence, as outlined hereunder.

Gene *c3509,* located on pathogenicity island PAI-CFT073-*metV,* was predicted to encode a putative ATP-binding protein of an ABC transport system. It was present in the genomes of all UPECs from the phylogenetic groups screened, i.e. B2, D, F, B1 and A. The second gene, *c3686,* located on pathogenicity island PAI-CFT073-*pheV,* was predicted to encode a D-arabinose 5-phosphate isomerase (API). It was present in UPECs of phylogenetic groups B2, D, F and A, but absent from the B1 group *E. coli* CI5 strain. The third gene, *chuA,* was predicted to encode an outer membrane heme/ hemoglobin receptor. It was present in the genomes of UPECs from phylogenetic groups B2, D and F. However, it was absent from *E. coli* strains CI5 (group B1) and VR50 (group A). Based on these findings, we hypothesized that these three genes, in combination, would cover key virulence determinants across a large majority of clinically relevant UPECs. An *E. coli*-specific marker gene, *uidA*, was included in order to confirm the identity of the UTI agent, as well as to serve as an internal control (Kiel et al., 2018).

3.2. Development of a PCR-based system for identification of uropathogenic E. coli in UTIs

Primers were designed to amplify the target genes in *E. coli* of all phylogenetic groups where these were present. Table 2 gives an overview of the selected primers and their amplification specificities. All selected primer combinations were tested, and PCR conditions optimized, using DNA of reference B2 group UPEC strain CFT073. As a negative control, DNA from the commensal *E. coli* K-12 strain MG1655 (phylogenetic group A) was used. On the basis of DNA from strain CFT073, the PCR amplicons were of the expected sizes for all four primer sets used. Thus, amplicons of sizes of, respectively, 208 (*c3509*), 259 (*c3686*), 221 (*chuA*) and 259 bp (*uidA*) were detected, and no side products were observed (Supplementary Fig. S1A). The *E. coli* K-12 strain MG1655 was negative for all three UPEC-specific virulence genes, and, as intended, did yield amplicons using the *uidA* primers.

3.3. Adaptation of a DNA extraction method from urine

Theoretically, a PCR is productive if at least a single copy of a target gene region is present, provided this region is sufficiently available for primers to anneal and kick-start the chain reaction. Since the urea and/ or other compounds that are present in urine can inhibit polymerases (Schrader et al., 2012), we tested the PCR in the presence of AUM (Brooks and Keevil, 1997). We used primer set *c3686* on DNA from strain CFT073 obtained using three different DNA extraction/purification methods (Deelman DNA extraction method, modified Deelman DNA extraction method, and reference Qiagen DNeasy ultraclean microbial kit), versus direct detection on a cell culture in AUM (Brooks and Keevil, 1997). The Deelman DNA extraction method enabled detection of the target from preparations down to 1.1×10^5 CFU/ml (Fig. 2A), whereas the modified Deelman DNA extraction method was more sensitive, giving a detection limit of 1.1×10^3 CFU/ml (Fig. 2B). The reference Qiagen DNeasy ultraclean microbial kit required an input of 1.1×10^4 CFU/ml for positive detection (Fig. 2C). In contrast, PCR amplification directly from cells (no DNA extraction) only allowed detection of cell numbers of 1.1×10^5 CFU/ml and up (Fig. 2D). The modification of the Deelman DNA extraction method thus enhanced the sensitivity approximately 100-fold, as compared to detection from cells. An additional advantage of the modification was that the extraction could be performed in 4.5 min (see Materials and Methods).

3.4. Lower detection limits of target genes in the genomes of reference strains

To examine the breadth of the multi-gene *E. coli* identification system, we tested the four primer sets (*c3509*, *c3686*, *chuA*, and *uidA*) on DNA from dilution series of three selected UPEC strains CASC874, CFT073 and XPKO359 in AUM (10^9–10^2 CFU/ml), using the modified Deelman DNA extraction method. All PCR systems consistently yielded

	Commensal			UPEC										
E. coli strain	HS	K-12 strain MG1655	K-12 strain W3110	CFT073	536	UTI89	26-1	Nu14	ST131 strain EC958	NA114	UMN026	IAI39	CI5	VR50
GenBank accession numbers	NC_009800.1	U00096.3	AP009048.1	AE014075.1	CP000247.1	NC_007946.1	CP016497.1	CP019777.1	NZ_HG941718.1	CP002797.2	NC_011751.1	CU928164.2	CP011018.1	CP011134.1
Phylogenetic group	A	A	A	B2	B2	B2	B2	B2	B2	B2	D	F	B1	A

gene	size	Function	Pathogenicity island	Other uropathogenic strains[a]
c0287	348	Hypothetical protein	PAI-CFT073-*aspV* (PAI III$_{cft073}$)	*Klebsiella pneumoniae*
c0294	2166	Hypothetical protein	PAI-CFT073-*aspV* (PAI III$_{cft073}$)	–
c0295	993	Hypothetical protein	PAI-CFT073-*aspV* (PAI III$_{cft073}$)	–
c0296	1059	Hypothetical protein	PAI-CFT073-*aspV* (PAI III$_{cft073}$)	–
c0297	768	Hypothetical protein	PAI-CFT073-*aspV* (PAI III$_{cft073}$)	–
c0375	138	Hypothetical protein	–	–
c0638	330	Hypothetical protein	–	–
c0646	198	Hypothetical protein	–	–
ybdO (c0691)	939	Hypothetical transcriptional regulator ybdO	–	–
c0758	471	Hypothetical protein	–	–
c0761	888	Putative dihydrodipicolinate synthase	–	*Klebsiella pneumoniae*
c0763	1131	Putative inner membrane protein	–	–
c0765	762	Putative transcriptional regulator	–	*Klebsiella pneumoniae*
c0807	960	Hypothetical protein	–	–
modD (c1647)	855	Putative pyrophosphorylase modD	–	–
c1650	759	Putative iron compound ABC transporter, ATP-binding protein	–	–
fecD (c1651)	897	Iron(III) dicitrate transport system permease protein fecD	–	–
c1761	1155	Acriflavine resistance protein A precursor	–	–
c1762	144	Hypothetical protein	–	–
c1766	1164	Putative membrane transport protein	–	–
c1810	816	Hypothetical protein	–	–
c1811	319	Hypothetical protein	–	–
c1812	399	Hypothetical protein	–	–
c1813	129	Hypothetical protein	–	–
c2418	1044	Prophage P4 integrase	PAI_CFT073-*asnT* (HPI$_{cft073}$)	*Klebsiella pneumoniae*
c2419	1305	Putative anthranilate synthase	PAI_CFT073-*asnT* (HPI$_{cft073}$)	*Klebsiella pneumoniae*
c2420	1404	Putative cytoplasmic transmembrane protein	PAI_CFT073-*asnT* (HPI$_{cft073}$)	*Klebsiella pneumoniae*
c2421	1830	Putative ABC transporter protein	PAI_CFT073-*asnT* (HPI$_{cft073}$)	*Klebsiella pneumoniae*
c2422	1803	Putative inner membrane ABC-transporter	PAI_CFT073-*asnT* (HPI$_{cft073}$)	*Klebsiella pneumoniae*
c2423	960	Putatative AraC type regulator	PAI_CFT073-*asnT* (HPI$_{cft073}$)	*Klebsiella pneumoniae*
c2429	4710	Hypothetical protein	PAI_CFT073-*asnT* (HPI$_{cft073}$)	*Klebsiella pneumoniae*
c2430	1101	Hypothetical protein	PAI_CFT073-*asnT* (HPI$_{cft073}$)	*Klebsiella pneumoniae*
c2431	135	Hypothetical protein	PAI_CFT073-*asnT* (HPI$_{cft073}$)	*Klebsiella pneumoniae*
c2432	657	Putative thioesterase	PAI_CFT073-*asnT* (HPI$_{cft073}$)	*Klebsiella pneumoniae*
c2434	285	Putative salicyl-AMP ligase	PAI_CFT073-*asnT* (HPI$_{cft073}$)	*Klebsiella pneumoniae*
c2435	270	Hypothetical protein	PAI_CFT073-*asnT* (HPI$_{cft073}$)	*Klebsiella pneumoniae*
c2436	2022	Putative pesticin receptor precursor	PAI_CFT073-*asnT* (HPI$_{cft073}$)	*Klebsiella pneumoniae*
c2437	807	Hypothetical protein	PAI_CFT073-*asnT* (HPI$_{cft073}$)	partial match *Klebsiella pneumoniae*
c2482	2148	Putative outer membrane receptor for iron compound or colicin	GI-CFT073-*cobU*	–
c2484	645	Hypothetical protein ybdM	GI-CFT073-*cobU*	*Klebsiella pneumoniae*
c2485	1290	Hypothetical protein ybdN	GI-CFT073-*cobU*	*Klebsiella pneumoniae*
c2504	138	Hypothetical protein	GI-CFT073-*cobU*	–
c2515	768	Putative ABC transporter	GI-CFT073-*cobU*	–
c2516	1059	ABC transporter, FecCD transport family	GI-CFT073-*cobU*	–
c2520	2385	Conserved hypothetical protein	GI-CFT073-*cobU*	*Klebsiella pneumoniae*
c2521	177	Hypothetical protein	GI-CFT073-*cobU*	*Klebsiella pneumoniae*
c2522	906	Conserved hypothetical protein	GI-CFT073-*cobU*	*Klebsiella pneumoniae*
c2523	1071	Conserved hypothetical protein	GI-CFT073-*cobU*	*Klebsiella pneumoniae*
c2524	786	Hypothetical protein	GI-CFT073-*cobU*	–
c2525	411	Hypothetical protein	GI-CFT073-*cobU*	–
yfaL (c2775)	3765	Hypothetical protein yfaL precursor	–	–
c3147	294	Hypothetical protein	φ-CFT073-*smpB*	–
c3292	468	Potential molybdenum-pterin-binding-protein	–	–
c3304	552	Hypothetical protein	–	–
c3390	603	Hypothetical protein	PAI-CFT073-*metV*	*Klebsiella pneumoniae*
c3405	957	2-hydroxyacid dehydrogenase	PAI-CFT073-*metV*	–
c3408	1581	PTS system, maltose and glucose-specific IIABC component	PAI-CFT073-*metV*	–
c3509	537	Putative ATP-binding protein of ABC transport system	PAI-CFT073-*metV*	–
c3686	1020	Hypothetical protein yrbH	PAI-CFT073-*pheV* (PAI I$_{cft073}$)	–
c3750	750	Putative regulator	–	–
c3753	1011	Ureidoglycolate dehydrogenase	–	–
c3755	483	Hypothetical protein	–	–
ygiK (c3756)	1347	c4-dicarboxylate permease	–	–
c3770	888	Hypothetical protein	–	–
c3771	1011	Putative iron compound-binding protein of ABC transporter family	–	–
c3772	1050	Putative iron compound permease protein of ABC transporter family	–	–
c3773	984	Putative iron compound permease protein of ABC transporter family	–	–
c3774	810	Ferric enterobactin transport ATP-binding protein fepC	–	–
assT (c3785)	1797	Arylsulfate sulfotransferase	–	–
c4016	1527	Ribose transport ATP-binding protein rbsA	–	–
c4017	897	Putative ribose ABC transporter	–	–
c4018	855	Tagatose-bisphosphate aldolase gatY	–	–
c4205	768	Hypothetical protein	–	–
chuS (c4307)	1029	Putative heme/hemoglobin transport protein	–	–
chuA (c4308)	1947	Outer membrane heme/hemoglobin receptor	–	–
chuT (c4313)	993	Putative periplasmic binding protein	–	–
chuW (c4314)	1338	Putative oxygen-independent coproporphyrinogen III oxidase	–	–
chuX (c4315)	495	Hypothetical protein	–	–
chuY (c4316)	624	Hypothetical protein	–	–
chuU (c4317)	993	Putative permease of iron compound ABC transport system	–	–
yiaN (c4400)	1278	Hypothetical protein yiaN	–	–
c4481	720	Conserved hypothetical protein	–	–
c4482	966	Hypothetical protein yajF	–	–
c4483	861	Putative aldolase	–	–
c4484	852	Putative aldolase	–	–
c4485	1092	Putative PTS enzyme-II fructose	–	–
c4487	471	Putative phosphotransferase system (PTS), fructose-specific	–	partial match *Klebsiella pneumoniae*
c4488	1599	Putative transcriptional antiterminator	–	–
c4580	222	Hypothetical protein	–	–
c4735	903	Conserved hypothetical protein	–	–
c4757	570	Hypothetical protein	–	–
c4758	1509	PTS system, glucose-specific IIBC component	–	–
c4759	1998	Transketolase 1	–	*Klebsiella pneumoniae*
c4760	846	Hypothetical protein	–	–
c4761	906	Putative conserved protein	–	–
c4762	1224	Putative permease	–	–
c4763	390	Hypothetical protein	–	–
c4764	957	Carbamate kinase-like protein yahI	–	–
c4765	1476	Hypothetical protein yahG	–	–
c4766	141	Hypothetical protein	–	–
c4767	1560	Hypothetical protein yahF	–	–
c4768	861	Hypothetical protein	–	–
c4774	1491	Hypothetical protein	–	–
c4775	510	Hypothetical protein	–	–
c4776	975	Hypothetical protein	–	–
c4777	630	Putative conserved protein	–	–
c4778	1005	Putative conserved protein	–	–
c4924	1167	Putative hippuricase	–	–
c4925	1317	Putative citrate permease	–	–
c4981	1230	Putative oxidoreductase	–	–
c4982	855	PTS system, mannose-specific IID component	–	partial match *Klebsiella pneumoniae*
c4983	798	PTS system, mannose-specific IIC component	–	*Klebsiella pneumoniae*
c4984	495	Putative sorbose PTS component	–	–
c4986	807	D-Glucitol-6-phosphate dehydrogenase	–	*Klebsiella pneumoniae*
c4987	1032	Putative transcriptional regulator of sorbose uptake and utilization genes	–	*Klebsiella pneumoniae*
c5032	2820	2-Oxoglutarate dehydrogenase E1 component	–	–
c5033	201	Hypothetical protein	–	–
c5034	1056	Dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex	–	–
c5035	1419	Putative 2-oxoglutarate dehydrogenase	–	–
c5036	1170	Succinyl-CoA synthetase beta chain	–	–
c5037	873	Succinyl-CoA synthetase alpha chain	–	–
c5038	1506	Putative membrane-bound protein	–	–
c5039	1110	Putative lactate dehydrogenase	–	–
c5040	1359	Putative C4-dicarboxylate transport transcriptional regulatory protein	–	–
c5060	405	Hypothetical protein	–	–
c5061	690	Hypothetical protein	–	–
yddO (c5077)	705	Hypothetical ABC transporter ATP-binding protein yddO	–	–
c5078	897	Putative oligopeptide ABC transporter	–	–
yddQ (c5079)	834	Hypothetical ABC transporter permease protein yddQ	–	–
yddR (c5080)	1143	Hypothetical ABC transporter permease protein yddR	–	–
c5081	1569	Putative conserved protein	–	–

[a] NCBI BLASTn against *Klebsiella pneumoniae*, *Staphylococcus saprophyticus*, *Enterococcus sp.*, group B *Streptococcus* (GBS), *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Candida* sp.

* partial match

\# no taxonomy ID in NCBI, manually checked using Mega 5.2

Gene absent

Gene present

Fig. 1. Presence/ absence analysis and potential function of 131 selected genes across *E. coli* and non-*E. coli* (uropathogenic) bacterial strains. Black: gene present, white: gene absent. Strains were: 3 commensal *E. coli* (HS, K-12 MG1655, K-12 W3110), 11 UPEC (CFT073, 536, UTI89, 26–1, NU14, ST131 strain EC958, NA114, UMN026, IAI39, CI5, and VR50) and 8 non-*E.coli* uropathogenic strains (*Klebsiella pneumoniae*, *Staphylococcus saprophyticus*, *Enterococcus sp.*, group B *Streptococcus* (GBS), *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Candida sp.*). Genes were detected using Blastn with a 95% identity cut-off.

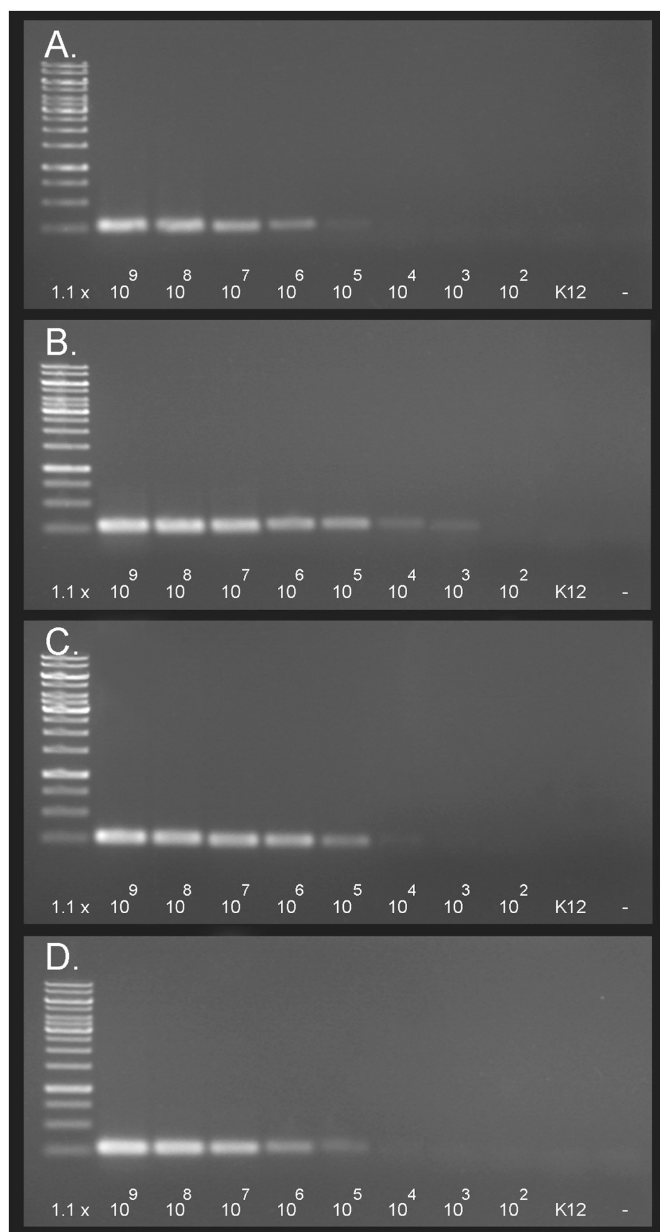


Fig. 2. A–D. Comparison of sensitivity of the different DNA extraction methods. DNA was extracted from strain CFT073 (dilutions 10^9–10^2 CFU/ml) using: Deelman DNA extraction method (A), modified Deelman DNA extraction method (B), Qiagen DNeasy ultraclean microbial kit (C), and cells in AUM, no DNA extraction (D). PCR was performed using primer set *c3686* on a general thermal cycler. DNA extraction time for each test is 3 (A), 4.5 (B), 21 (C), and 0 (D) minutes. Left: GeneRuler™ 1 kb DNA ladder (Thermo Fisher Scientific).

amplicons of the expected sizes, as evidenced from gel electrophoresis. The sole exception was the *chuA* gene in strain XPKO359, which yielded no amplicons. Across all four target gene sources, the *c3509* primer set had a lower detection limit in the 10^4 CFU/ml range, whereas the other three primer sets had lower detection limits ranging from about 1.1×10^3 to 7.7×10^4 CFU/ml (Fig. 3A and Supplementary Table 1A).

3.5. Validation on clinical strains

To validate the identification method, we screened the DNA produced from 128 recently isolated *E. coli* clinical strains from female patients with diagnosed UTIs for the presence of the selected genes. All strains were classified as belonging to the *E. coli* phylogenetic groups A, B1, B2, D, F and clade V. Specifically, 47.7% (61/128) belonged to group B2, 21.9% (28/128) to A, 11.7% (15/128) to D, 10.2% (13/128) to B1, 7.0% (9/128) to F and 1.6% (2/128) to clade V. Moreover, the strains originated from 54.7% (70/128) uncomplicated versus 45.3% (58/128) complicated UTIs (kidney transplantation). The clinical pictures were diverse, i.e. ASB (39.8%, 51/128), CY (45.3%, 58/128), PY (12.5%, 16/128), and US (2.3%, 3/128) (Fig. 4).

The *E. coli* marker gene *uidA* was present in 97.7% of all strains. The remaining 2.3% all belonged to phylogenetic group F, which is known to be quite divergent from all other *E. coli* groups (Johnson et al., 2017). An analysis of the occurrence of the *c3509*, *c3686* and *chuA* genes separately across the 128 samples revealed the following order of prevalence: *c3509* (76.6%), *c3686* (75.0%) and *chuA* (72.7%) (Table 3).

The prevalence of each gene as well as the combination of genes within the strain set was then analyzed with respect to the phylogenetic group of the strains (Table 3). All group B1 and B2 strains possessed the *c3509* gene (13/13 and 61/61, respectively), while its prevalence was lower in groups D (66.7%; 10/15), F (55.6%; 5/9), A (32.1%; 9/28), and clade V (0%; 0/2). The *c3686* gene was found in the clade V samples (100%; 2/2), followed by group B2 (91.8%; 56/61), F (88.9%; 8/9), D (86.7%; 13/15), A (42.9%; 12/28) and B1 (38.5%; 5/13). The *chuA* gene was present in all group B2, D and F samples (61/61, 15/15, 9/9), with lower occurrence in clade V (50%; 1/2) and groups A (21.4%; 6/28) and B1 (7.7%; 1/13). Group A, B1, B2, clade V and D samples all possessed the *uidA* gene. In group F, it occurred in 66.7% of the samples (6/9).

We used the presence of any one of the three marker genes in a given sample as the criterion for positive identification of UPEC. This assessment enabled positive UPEC identification in 94.5% (121/128) of the *E. coli* samples (Table 3). The seven (5.5%) samples that did not produce amplicons with any of the three target genes were all part of phylogenetic group A (which encompasses mainly commensal strains). Inclusion of the *uidA* marker gene (Table 3) resulted in a positive *E. coli* detection in 100% (128/128) of the cases.

3.6. Operational improvement of the identification method using a novel (Nextgen) PCR thermal cycler

Using DNA of reference strain CFT073, we compared the detection method on the fast Nextgen PCR thermal cycler versus the previously used regular PCR cycler. A first, side-by-side, test performed with pure DNA extracted from strain CFT073 versus strain MG1655 revealed that the detection was similar between the two machines, given that all three primer sets yielded the expected amplicons at similar target gene levels (Supplementary Fig. S1B).

We then tested the limits of detection of the novel method in the fast Nextgen PCR thermal cycler on DNA from UPEC strains CASC874, CFT073 and XPKO359 in AUM (10^9 down to 10^1 CFU/ml, using the modified Deelman DNA extraction method) (Deelman et al., 2012) (Fig. 3B and Supplementary Table 1B). Gene *c3509* had a detection limit in the 10^5 CFU/ml range, while genes *c3686*, *chuA*, and *uidA* had limits of detection ranging from about 1.1×10^3 to 7.7×10^4 CFU/ml. The detection limits of the amplification systems were grossly similar across the machines, except for primer set *c3509*, in which the regular PCR thermal cycler gave 10-fold higher sensitivity. When tested on all 128 clinical samples, all target gene regions were amplified similarly across the two machines (Fig. 4). Clearly, the main difference between the two machines was in the amplification time, with the Nextgen thermal cycler being considerably faster (16 min.) than the regular thermal cycler (51 min.).

4. Discussion

To develop a fast *E. coli* identification system, we initially performed an *in silico* screening of the genomes of a suite of reference UPEC strains. This revealed the presence, in a mosaic fashion, of potential target

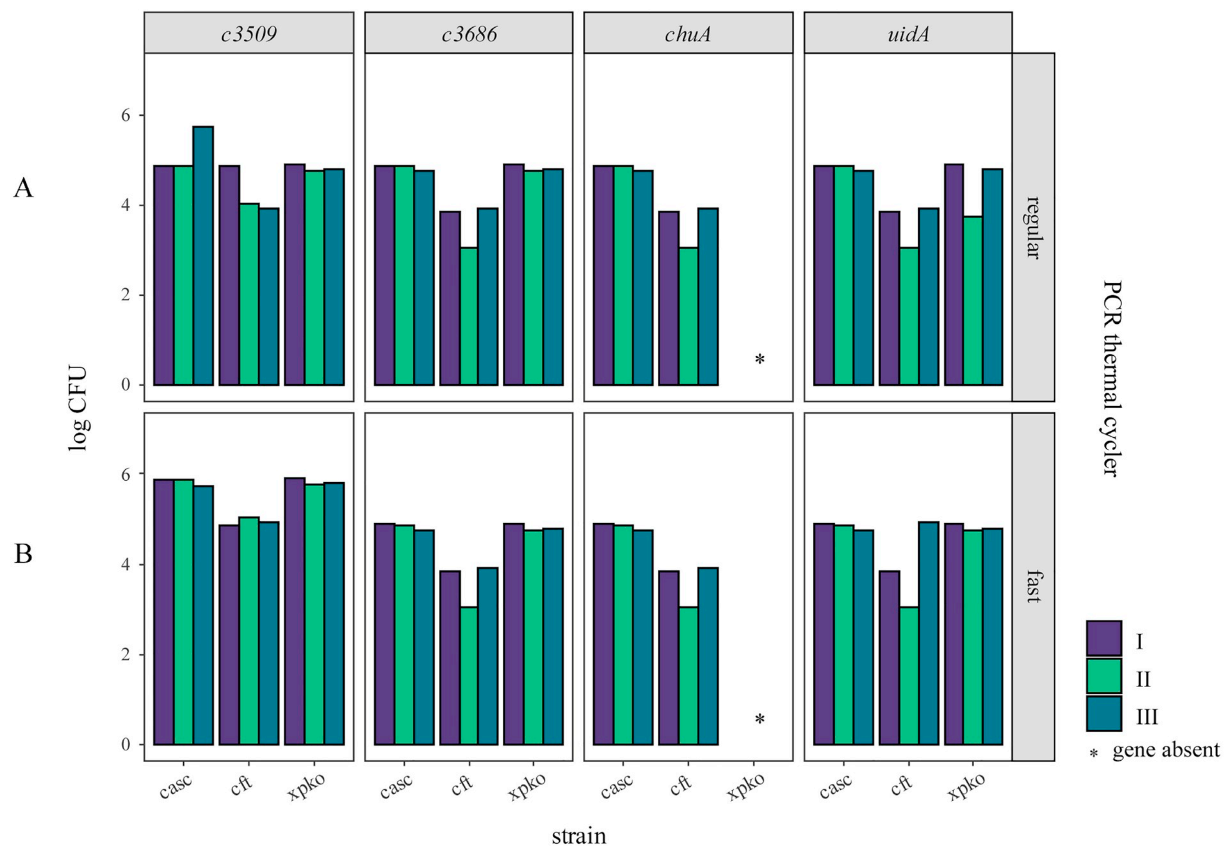


Fig. 3. A–B. Lower detection limits of each gene used in this study for three different *E. coli* reference strains (CASC874 [casc], CFT073 [cft], and XPKO359 [xpko]). PCR was performed in three biological replicates (I, II, III) on DNA extracted using the modified Deelman DNA extraction method on cells in AUM, giving 10^9–10^1 CFU/ml. PCR thermal cyclers were either the regular (A) or the fast Nextgen thermal cycler (B). Genes that were absent from a particular strain are marked with an *. Values were determined by the initial cell densities of the strains.

genes across these strains. Such mosaic occurrence is a token of the complexity of gene movements that have shaped the emerged UPECs over time, and highlights the necessity of dealing with this variability across the genomes of the target organisms when developing a detection method like the one proposed here. The result of the screening led to selection of three UPEC-specific genes (*c3509*, *c3686*, and *chuA*) that are potentially associated with virulence. In fact, all three may even be crucial in UTI pathogenesis (Lloyd et al., 2007). In addition, we included gene *uidA*, to allow for generic *E. coli* identification (Kiel et al., 2018). Optimization was performed using DNA from the UPEC reference strain CFT073 (phylogenetic group B2), versus that of the commensal K-12 strain MG1655 (group A). Indeed, the selected genes *c3509*, *c3686* and *chuA* were present in the UPEC strain, but absent from the commensal strain. This constituted a first token of evidence for their specificity for UPECs, which was confirmed in the extended comparative screens.

A fast *E. coli* identification method requires efficient DNA extraction and purification from urine. Urea is a very critical PCR inhibitor, as it incites degradation of, or damage to, the DNA polymerase used in PCR (Schrader et al., 2012). We tested our DNA analysis method with similar concentrations of urea as present in urine (10 g/L, in urine 9.3–23.3 g/L (Rose et al., 2015). Our modified DNA extraction method enabled a very fast removal of these critical compounds, resulting in 100-fold improvement of detection of targets, corresponding to 10^3 CFU/ml (based on primer set *c3686* on strain CFT073 DNA), compared to 10^5 CFU/ml for the original method (Deelman et al., 2012). Moreover, our optimized method enabled DNA extraction in just 4.5 min and allowed identification of all target genes at 10^3/10^4 CFU/ml.

To examine the potential association of selected genes to virulence in UTIs, we considered the predicted function of the proteins encoded by these genes. The *c3509* gene encodes a putative ATP-binding protein of an ABC transport system, involved in the transport of sugars, metals, peptides, amino acids and/or other metabolites, across the membrane. The system is driven by ATP binding and hydrolysis, empowering the translocation of substrates across the membrane (Tanaka et al., 2018). In CFT073, the *c3509* gene is located only five genes upstream of a mannitol phosphotransferase system gene, and so it might be involved in mannitol uptake. Mannitol is present in carrots, apples, pineapples and asparagus, and is commonly found in human urine because of its poor absorption by the human gastrointestinal tract (Bouatra et al., 2013). Since gene *c3509* was present across all 11 screened UPEC genomes as well as in a majority of the clinical samples (76.6%), it may be important for UPEC metabolism in the UT and thus fitness.

The *c3686* (*yrbH*) gene is predicted to encode a D-arabinose 5-phosphate isomerase (API) (Meredith and Woodard, 2003). API is the first enzyme in the biosynthesis of 3-deoxy-D-manno octulosonate (KDO), a sugar moiety located in the lipopolysaccharide (LPS) layer of Gram-negative bacteria. LPS in UPECs is important in the activation of proinflammatory responses in UTIs (Bien et al., 2012). Invasion of bladder epithelial cells by UPEC stimulates a response via an LPS-modulated mechanism (Schilling et al., 2001). Interestingly, LPS released by UPECs can also subvert host defenses, the invader escaping into the host cell cytoplasm, forming intracellular bacterial communities (Flores-Mireles et al., 2015). It might be that the *c3686* gene has a role in the (over) production and eventual release of LPS, thus helping to overcome the host defense in UPEC; *c3686* could therefore encode an important virulence factor.

The *chuA* gene encodes an outer membrane receptor protein, which may be involved in the uptake of compounds like heme. This gene is

Sample	Uncomplicated UTI	Complicated UTI	Age	Clinical	PCR detection				Phylogenetic
					Genes				
Nr.	No kidney transplant	Kidney transplant		picture	*c3509*	*c3686*	*chuA*	*uidA*	group
1		x	69	0					A
2	x		42	0					A
3		x	60	0					A
4		x	26	0					A
5		x	61	0					A
6		x	63	0					A
7	x		71	0					A
8	x		71	0					A
9	x		76	0					A
10	x		48	0					A
11	x		78	1					A
12	x		78	1					A
13		x	66	1					A
14	x		22	1					A
15	x		78	1					A
16		x	73	1					A
17		x	26	1					A
18		x	39	1					A
19		x	55	1					A
20		x	53	1					A
21		x	26	1					A
22	x		25	1					A
23		x	26	1					A
24	x		23	1					A
25		x	58	1					A
26		x	73	1					A
27		x	61	2					A
28		x	77	2					A
29		x	58	0					B1
30	x		86	0					B1
31	x		31	0					B1
32	x		79	0					B1
33	x		82	0					B1
34	x		58	0					B1
35		x	32	1					B1
36		x	76	1					B1
37	x		23	1					B1
38	x		73	1					B1
39		x	48	2					B1
40	x		33	3					B1
41		x	49	3					B1
42	x		28	0					B2
43		x	58	0					B2
44		x	68	0					B2
45	x		35	0					B2
46	x		82	0					B2
47		x	78	0					B2
48	x		89	0					B2
49	x		73	0					B2
50		x	72	0					B2
51		x	77	0					B2
52	x		49	0					B2
53	x		63	0					B2
54	x		79	0					B2
55	x		28	0					B2
56	x		81	0					B2
57		x	66	0					B2
58	x		79	0					B2
59		x	80	0					B2
60		x	53	0					B2
61	x		53	0					B2
62		x	46	0					B2
63	x		73	0					B2
64	x		40	1					B2
65	x		26	1					B2
66	x		59	1					B2
67		x	46	1					B2
68	x		45	1					B2
69		x	26	1					B2
70		x	73	1					B2
71	x		32	1					B2
72	x		29	1					B2
73	x		76	1					B2
74		x	42	1					B2
75	x		40	1					B2
76	x		76	1					B2
77	x		89	1					B2
78	x		27	1					B2
79		x	57	1					B2
80	x		37	1					B2
81		x	49	1					B2
82	x		82	1					B2
83	x		71	1					B2
84		x	31	1					B2
85	x		75	1					B2
86	x		78	1					B2
87		x	59	1					B2
88		x	68	1					B2
89	x		23	1					B2
90	x		73	1					B2
91		x	80	1					B2
92	x		26	1					B2
93	x		30	2					B2
94	x		25	2					B2
95	x		19	2					B2
96	x		31	2					B2
97	x		55	2					B2
98		x	31	2					B2
99		x	31	2					B2
100	x		19	2					B2
101		x	67	2					B2
102		x	68	2					B2
103		x	52	0					Clade V
104	x		70	0					Clade V
105		x	23	0					D
106		x	68	0					D
107	x		23	0					D
108		x	81	0					D
109	x		22	0					D
110	x		29	0					D
111		x	59	1					D
112		x	69	1					D
113	x		86	1					D
114	x		25	1					D
115	x		86	1					D
116		x	70	1					D
117	x		81	2					D
118		x	28	2					D
119	x		77	3					D
120		x	69	0					F
121	x		61	0					F
122	x		59	0					F
123	x		71	0					F
124		x	26	0					F
125		x	42	1					F
126	x		20	1					F
127		x	61	1					F
128		x	69	2					F
					98	96	93	125	
					c3509	*c3686*	*chuA*	*uidA*	

Clinical picture	
0	Asymptomatic UTIs (ASB)
1	Cystitis (CY)
2	Pyelonephritis (PY)
3	Urosepsis (US)

PCR detection	
	Gene present
	Gene absent

Fig. 4. *E. coli* PCR identification using the four primers, *c3509*, *c3686* (*yrbH*), *chuA*, and *uidA* on 128 clinical *E. coli* samples (regular and fast thermal cycler). Black: gene present, white: gene absent. Clinical sample metadata: uncomplicated = UTI samples without kidney transplantation, complicated = samples with kidney transplantation. Clinical picture is presented as 0 (asymptomatic UTIs, ASB), 1 (cystitis, CY), 2 (pyelonephritis, PY), and 3 (urosepsis, US).

part of the genetic locus encoding heme transport (potentially importing iron), which appears to be widely distributed among pathogenic *E. coli* strains (Torres and Payne, 1997; Wyckoff et al., 1998; Nagy et al., 2001). The *chuA* gene also appears to be important during intracellular bacterial community formation by UPECs, with many biofilm-like properties. These intracellular biofilms allow establishment of a reservoir of dormant pathogen cells inside bladder epithelial cells, which helps these to outlast a host immune response (Anderson et al., 2004; Reigstad et al., 2007). Use of *chuA* in a detection system is thus warranted given its presumed role in the bladder during urinary tract infections.

As an internal amplification control, the *E. coli* specific beta-D-glucuronidase-encoding gene *uidA* was used. This gene encodes an enzyme specific to *E. coli* and is therefore widely used in identification kits and as a specific marker for *E. coli* (Cleuziat and Robert-Baudouy, 1990). It was present in 97.7% of the clinical samples; the three samples that were negative for gene *uidA* all occurred in phylogenetic group F. Although *uidA* is globally used as an *E. coli* marker, consistent with our findings, Johnson et al. (2017) showed that it is typically absent from strains of the *E. coli* sequence type 648 complex, which belongs to phylogroup F.

The premise behind our multi-gene identification method was that detection of any one of the three marker genes would indicate the presence of a UPEC. Thus, on the basis of only three selected genes, we were able to identify UPECs in 94.5% of the samples within the clinical dataset. The remaining 5.5% were samples that all contained strains belonging to phylogenetic group A. This phylogenetic group is strongly associated with commensal strains (Johnson and Stell, 2000), which generally have smaller genomes than the UPEC strains (Hacker et al., 1997; Ahmed et al., 2008; Touchon et al., 2009). Hence, it does not come as a surprise that these samples were negative for all three marker genes. We hypothesize that these group-A strains escape detection by (1) being inadvertent 'passengers' in the UT habitat, or (2) by virtue of possessing different pathogenicity gene sets. We deem it unlikely that such strains possess mutations in all three marker genes that prevent PCR-based detection. Clearly, they do belong to the species *E. coli*, since they were positive for the *E. coli*-specific *uidA* gene. Further research into their potential pathogenicity is warranted. Inclusion of the *uidA* marker gene in the identification method resulted in a 100% positive *E. coli* detection.

The lower detection limits of the amplification systems run on the Nextgen PCR thermal cycler were, overall, similar to those obtained with the regular PCR thermal cycler. The Nextgen PCR thermal cycler operates with three different heat blocks, eliminating ramping time and therefore total PCR time. The amplification data obtained with the clinical samples were similar across the two thermal cyclers. In both machines, the detection limit for primer set *c3509* was slightly higher than that obtained with the other three target genes. Therefore, further optimization of the system based on primer set *c3509* might result in binding that is more specific, consequently raising PCR efficiency and lowering the detection limit. Since this higher detection limit was only observed with primer set *c3509*, which was always detected in combination with one of the other genes (*c3686*, *chuA*, and *uidA*), the overall detection limit for the method was in reality at 10^4 CFU/ml. We conclude that, for the purpose of detecting UPECs in clinical samples, the novel fast PCR thermal cycler meets the standards of a regular thermal cycler, both in terms of specificity and sensitivity, with a total

Table 3
Percentage of the single genes and gene combinations present in the clinical samples, based on the phylogenetic groups. * At least one of these genes is present.

Phylogenetic group	Single genes present				Gene combinations present	
					UPEC specific	*E. coli* specific
	c3509	*c3686*	*chuA*	*uidA*	*c3509-c3686-chuA**	*c3509-c3686-chuA-uidA**
A (n = 28)	32.1% (9/28)	42.9% (12/28)	21.4% (6/28)	100% (28/28)	75% (21/28)	100% (28/28)
B1 (n = 13)	100% (13/13)	38.5% (5/13)	7.7% (1/13)	100% (13/13)	100% (13/13)	100% (13/13)
B2 (n = 61)	100% (61/61)	91.8% (56/61)	100% (61/61)	100% (61/61)	100% (61/61)	100% (61/61)
Clade V (n = 2)	0% (0/2)	100% (2/2)	50% (1/2)	100% (2/2)	100% (2/2)	100% (2/2)
D (n = 15)	66.7% (10/15)	86.7% (13/15)	100% (15/15)	100% (15/15)	100% (15/15)	100% (15/15)
F (n = 9)	55.6% (5/9)	88.9% (8/9)	100% (9/9)	66.7% (6/9)	100% (9/9)	100% (9/9)
Total	76.6% (98/128)	75.0% (96/128)	72.7% (93/128)	97.7% (125/128)	94.5% (121/128)	100% (128/128)

time-to-detection of 52 min, which indeed enhances the speed of detection.

At a detection limit of 10^4 CFU/ml, we are now able to detect the *E. coli* pathogen in all of the UTIs according to the $\geq 10^5$ CFU/ml threshold described by Sheffield and Cunningham (2005). However, using the thresholds defined by Orenstein and Wong (1999), the method would not allow identification of UPECs in cases with lower CFU densities (acute cystitis in young men ($< 10^4$ CFU/ml), recurrent cystitis in young women (10^2 CFU/ml) and in catheter-associated early UTIs (10^2 CFU/ml). In those cases, a short (2-h) culturing step in LB, prior to extraction, might need to be performed.

Our novel method was able to detect *E. coli* of phylogenetic groups A, B1, B2, D, F, and clade V across diverse sample types, including a vast majority of uropathogenic *E. coli*. Moreover, the novel (Nextgen) PCR thermal cycler allowed ultrafast PCR amplification, in 16 min (total *E. coli* identification in 52 min, including DNA extraction, PCR, electrophoresis, staining, destaining and visualization under UV). The method even constitutes a basis for extension to other uropathogens. The Nextgen PCR thermal cycler clearly meets the standards of a regular PCR thermal cycler, greatly reducing the total time-to-detection. Hence, the here developed rapid molecular UTI detection method is a great asset to clinical labs worldwide.

Declaration of Competing Interest

There is no conflict of interest to declare.

Acknowledgments

We thank Prof. J. Degener (University Medical Center Groningen, the Netherlands) for providing the UPEC strains. Dr. Patricia Stevens is thanked for her preliminary work on *E. coli* pathogens.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2019.105799.

References

Ahmed, N., Dobrindt, U., Hacker, J., Hasmain, S.E., 2008. Genomic fluidity and pathogenic bacteria: applications in diagnostics, epidemiology and intervention. Nat. Rev. Microbiol. 6 (5), 387–394. https://doi.org/10.1038/nrmicro1889.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215 (3), 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2.

Anderson, G.G., Dodson, K.W., Hooton, T.M., Hultgren, S.J., 2004. Intracellular bacterial communities of uropathogenic *Escherichia coli* in urinary tract pathogenesis. Trends Microbiol. 12 (9), 424–430. https://doi.org/10.1016/j.tim.2004.07.005.

Bien, J., Sokolova, O., Bozko, P., 2012. Role of uropathogenic *Escherichia coli* virulence factors in development of urinary tract infection and kidney damage. Int. J. Nephrol. 2012, 681473. https://doi.org/10.1155/2012/681473.

Blackburn, D., Husband, A., Saldaña, Z., Nada, R.A., Klena, J., Qadri, F., et al., 2009. Distribution of *Escherichia coli* common pilus among diverse strains of human enterotoxigenic *E. coli*. J. Clin. Microbiol. 47 (6), 1781–1784. https://doi.org/10.1128/JCM.00260-09.

Bouatra, S., Aziat, F., Mandal, R., Guo, A.C., Wilson, M.R., Knox, C., et al., 2013. The human urine metabolome. PLoS One 8 (9), e73076. https://doi.org/10.1371/journal.pone.0073076.

Brooks, T., Keevil, C.W., 1997. A simple artificial urine for the growth of urinary pathogens. Lett. Appl. Microbiol. 24 (3), 203–206. https://doi.org/10.1046/j.1472-765X.1997.00378.x.

Brzuszkiewicz, E., Brüggemann, H., Liesegang, H., Emmerth, M., Olschläger, T., Nagy, G., et al., 2006. How to become a uropathogen: comparative genomic analysis of extraintestinal pathogenic *Escherichia coli* strains. Proc. Natl. Acad. Sci. U. S. A. 103 (34), 12879–12884. https://doi.org/10.1073/pnas.0603038103.

Caprioli, R.M., Farmer, T.B., Gile, J., 1997. Molecular imaging of biological samples: localization of peptides and proteins using MALDI-TOF MS. Anal. Chem. 69 (23), 4751–4760. https://doi.org/10.1021/ac970888i.

Chen, S.L., Hung, C.S., Xu, J., Reigstad, C.S., Magrini, V., Sabo, A., et al., 2006. Identification of genes subject to positive selection in uropathogenic strains of *Escherichia coli*: a comparative genomics approach. Proc. Natl. Acad. Sci. U. S. A. 103 (15), 5977–5982. https://doi.org/10.1073/pnas.0600938103.

Clermont, O., Christenson, J.K., Denamur, E., Gordon, D.M., 2013. The Clermont *Escherichia coli* phylo-typing method revisited: improvement of specificity and detection of new phylo-groups. Environ. Microbiol. Rep. 5 (1), 58–65. https://doi.org/10.1111/1758-2229.12019.

Cleuziat, P., Robert-Baudouy, J., 1990. Specific detection of *Escherichia coli* and *Shigella* species using fragments of genes coding for beta-glucuronidase. FEMS Microbiol. Lett. 60 (3), 315–322. https://doi.org/10.1111/j.1574-6968.1990.tb03909.x.

Davenport, M., Mach, K.E., Shortliffe, L.M.D., Banaei, N., Wang, T.H., Liao, J.C., 2017. New and developing diagnostic technologies for urinary tract infections. Nat. Rev. Urol. 14 (5), 296–310. https://doi.org/10.1038/nrurol.2017.20.

Deelman, L.E., van der Wouden, E.A., Duin, M., Henning, R.H., 2002. A method for the ultrarapid isolation of PCR-ready DNA from urine and buccal swabs. Mol. Biol. Today 3, 51–54.

Emody, L., Kerényi, M., Nagy, G., 2003. Virulence factors of uropathogenic *Escherichia coli*. Int. J. Antimicrob. Agents 22 (Suppl. 2), 29–33. https://doi.org/10.1016/S0924-8579(03)00236-X.

Flores-Mireles, A.L., Walker, J.N., Caparon, M., Hultgren, S.J., 2015. Urinary tract infections: epidemiology, mechanisms of infection and treatment options. Nat. Rev. Microbiol. 13 (5), 269–284. https://doi.org/10.1038/nrmicro3432.

Hacker, J., Blum-Oehler, G., Mühldorfer, I., Tschäpe, H., 1997. Pathogenicity islands of virulent bacteria: structure, function and impact on microbial evolution. Mol. Microbiol. 23 (6), 1089–1097. https://doi.org/10.1046/j.1365-2958.1997.3101672.x.

Harding, G.K.M., Ronald, A.R., 1994. The management of urinary infections: what have we learned in the past decade? Int. J. Antimicrob. Agents 4 (2), 83–88. https://doi.org/10.1016/0924-8579(94)90038-8.

Hoben, H.J., Somasegaran, P., 1982. Comparison of the pour, spread, and drop plate methods for enumeration of *Rhizobium* spp. in inoculants made from presterilized peat. Appl. Environ. Microbiol. 44 (5), 1246–1247.

Jacobsen, S.M., Stickler, D.J., Mobley, H.L., Shirtliff, M.E., 2008. Complicated catheter-associated urinary tract infections due to *Escherichia coli* and *Proteus mirabilis*. Clin. Microbiol. Rev. 21 (1), 26–59. https://doi.org/10.1128/CMR.00019-07.

Johansen, T.E., Botto, H., Cek, M., Grabe, M., Tenke, P., Wagenlehner, F.M., et al., 2011. Critical review of current definitions of urinary tract infections and proposal of an EAU/ESIU classification system. Int. J. Antimicrob. Agents 38 (Suppl), 64–70. https://doi.org/10.1016/j.ijantimicag.2011.09.009.

Johnson, J.R., Stell, A.L., 2000. Extended virulence genotypes of *Escherichia coli* strains from patients with urosepsis in relation to phylogeny and host compromise. J. Infect. Dis. 181 (1), 261–272. https://doi.org/10.1086/315217.

Johnson, J.R., Johnston, B.D., Gordon, D.M., 2017. Rapid and specific detection of the *Escherichia coli* sequence type 648 complex within phylogroup F. J. Clin. Microbiol. 55 (4), 1116–1121. https://doi.org/10.1128/JCM.01949-16.

Kiel, M., Sagory-Zalkind, P., Miganeh, C., Stork, C., Leimbach, A., Sekse, C., et al., 2018. Identification of novel biomarkers for priority serotypes of Shiga toxin-producing *Escherichia coli* and the development of multiplex PCR for their detection. Front. Microbiol. 9, 1321. https://doi.org/10.3389/fmicb.2018.01321.

Kostakioti, M., Hultgren, S.J., Hadjifrangiskou, M., 2012. Molecular blueprint of

uropathogenic *Escherichia coli* virulence provides clues toward the development of anti-virulence therapeutics. Virulence 3 (7), 592–594. https://doi.org/10.4161/viru.22364.

Lloyd, A.L., Rasko, D.A., Mobley, H.L., 2007. Defining genomic islands and uropathogen-specific genes in uropathogenic *Escherichia coli*. J. Bacteriol. 189 (9), 3532–3546. https://doi.org/10.1128/JB.01744-06.

López-Banda, D.A., Carrillo-Casas, E.M., Leyva-Leyva, M., Orozco-Hoyuela, G., Manjarrez-Hernández, Á., Arroyo-Escalante, S., 2014. Identification of virulence factors in *Escherichia coli* isolates from women with urinary tract infection in Mexico. Biomed. Res. Int. 2014, 959206. https://doi.org/10.1155/2014/959206.

Meredith, T.C., Woodard, R.W., 2003. *Escherichia coli* YrbH is a D-arabinose 5-phosphate isomerase. J. Biol. Chem. 278 (35), 32771–32777. https://doi.org/10.1074/jbc.M303661200.

Mullis, K.B., 1990. The unusual origin of the polymerase chain reaction. Sci. Am. 262 (4), 56–61. 64-5. https://doi.org/10.1038/scientificamerican0490-56.

Nagy, G., Dobrindt, U., Kupfer, M., Emödy, L., Karch, H., Hacker, J., 2001. Expression of hemin receptor molecule *chuA* is influenced by *RfaH* in uropathogenic *Escherichia coli* strain 536. Infect. Immun. 69 (3), 1924–1928. https://doi.org/10.1128/IAI.69.3.1924-1928.2001.

Nissen, M.D., Sloots, T.P., 2002. Rapid diagnosis in pediatric infectious diseases: the past, the present and the future. Pediatr. Infect. Dis. J. 21 (6), 605–612. https://doi.org/10.1097/00006454-200206000-00037.

Oelschlaeger, T.A., Dobrindt, U., Hacker, J., 2002. Pathogenicity islands of uropathogenic *E. coli* and the evolution of virulence. Int. J. Antimicrob. Agents 19 (6), 517–521. https://doi.org/10.1016/s0924-8579(02)00092-4.

Orenstein, R., Wong, E.S., 1999. Urinary tract infections in adults. Am. Fam. Physician 59 (5), 1225–1234 1237.

Padmavathy, B., Vinoth Kumar, R., Patel, A., Deepika Swarnam, S., Vaidehi, T., Jaffar Ali, B.M., 2012. Rapid and sensitive detection of major uropathogens in a single-pot multiplex PCR assay. Curr. Microbiol. 65 (1), 44–53. https://doi.org/10.1007/s00284-012-0126-3.

Reigstad, C.S., Hultgren, S.J., Gordon, J.I., 2007. Functional genomic studies of uropathogenic *Escherichia coli* and host urothelial cells when intracellular bacterial communities are assembled. J. Biol. Chem. 282 (29), 21259–21267. https://doi.org/10.1074/jbc.M611502200.

Ren, Y., Palusiak, A., Wang, W., Wang, Y., Li, X., Wei, H., et al., 2016. A high-resolution typing assay for uropathogenic *Escherichia coli* based on fimbrial diversity. Front. Microbiol. 7, 623. https://doi.org/10.3389/fmicb.2016.00623.

Rose, C., Parker, A., Jefferson, B., Cartmell, E., 2015. The characterization of feces and urine: a review of the literature to inform advanced treatment technology. Crit. Rev. Environ. Sci. Technol. 45 (17), 1827–1879. https://doi.org/10.1080/10643389.2014.1000761.

Russo, T.A., Johnson, J.R., 2000. Proposal for a new inclusive designation for extraintestinal pathogenic isolates of *Escherichia coli*: ExPEC. J. Infect. Dis. 181 (5), 1753–1754. https://doi.org/10.1086/315418.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: a Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schilling, J.D., Mulvey, M.A., Vincent, C.D., Lorenz, R.G., Hultgren, S.J., 2001. Bacterial invasion augments epithelial cytokine responses to *Escherichia coli* through a lipopolysaccharide-dependent mechanism. J. Immunol. 166 (2), 1148–1155. https://doi.org/10.4049/jimmunol.166.2.1148.

Schrader, C., Schielke, A., Ellerbroek, L., Johne, R., 2012. PCR inhibitors – occurrence, properties and removal. J. Appl. Microbiol. 113 (5), 1014–1026. https://doi.org/10.1111/j.1365-2672.2012.05384.x.

Semeniuk, H., Church, D., 1999. Evaluation of the leukocyte esterase and nitrate urine dipstick screening tests for detection of bacteriuria in women with suspected uncomplicated urinary tract infections. J. Clin. Microbiol. 37 (9), 3051–3052.

Sheffield, J.S., Cunningham, F.G., 2005. Urinary tract infection in women. Obstet. Gynecol. 106 (5 Pt 1), 1085–1092. https://doi.org/10.1097/01.AOG.0000185257.52328.a2.

Tanaka, K.J., Song, S., Mason, K., Pinkett, H.W., 2018. Selective substrate uptake: the role of ATP-binding cassette (ABC) importers in pathogenesis. Biochim. Biophys. Acta Biomembr. 1860 (4), 868–877. https://doi.org/10.1016/j.bbamem.2017.08.011.

Terlizzi, M.E., Gribaudo, G., Maffei, M.E., 2017. Uropathogenic *Escherichia coli* (UPEC) infections: virulence factors, bladder responses, antibiotic, and non-antibiotic antimicrobial strategies. Front. Microbiol. 8, 1566. https://doi.org/10.3389/fmicb.2017.01566.

Torres, A.G., Payne, S.M., 1997. Haem iron-transport system in enterohaemorrhagic *Escherichia coli* O157:H7. Mol. Microbiol. 23 (4), 825–833. https://doi.org/10.1046/j.1365-2958.1997.2641628.x.

Touchon, M., Hoede, C., Tenaillon, O., Barbe, V., Baeriswyl, S., Bidet, P., et al., 2009. Organised genome dynamics in the *Escherichia coli* species results in highly diverse adaptive paths. PLoS Genet. 5 (1), e1000344. https://doi.org/10.1371/journal.pgen.1000344.

Van der Zee, A., Roorda, L., Bosman, G., Ossewaarde, J.M., 2016. Molecular diagnosis of urinary tract infections by semi-quantitative detection of uropathogens in a routine clinical hospital setting. PLoS One 11 (3), e0150755. https://doi.org/10.1371/journal.pone.0150755.

Welch, R.A., Burland, V., Plunkett 3rd, G., Redford, P., Roesch, P., Rasko, D., 2002. Extensive mosaic structure revealed by the complete genome sequence of uropathogenic *Escherichia coli*. Proc. Natl. Acad. Sci. U. S. A. 99 (26), 17020–17024. https://doi.org/10.1073/pnas.252529799.

Wyckoff, E.E., Duncan, D., Torres, A.G., Mills, M., Maase, K., Payne, S.M., 1998. Structure of the *Shigella dysenteriae* haem transport locus and its phylogenetic distribution in enteric bacteria. Mol. Microbiol. 28 (6), 1139–1152. https://doi.org/10.1046/j.1365-2958.1998.00873.x.

Zhang, L., Foxman, B., 2003. Molecular epidemiology of *Escherichia coli* mediated urinary tract infections. Front. Biosci. 8, e235–e244. https://doi.org/10.2741/1007.