Whole genome analysis reveals pathogenic potential of multi-drug resistant wastewater *Escherichia coli*

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Abstract

Wastewater treatment plants play an important role in antibiotic resistance development. While it has been shown that wastewater effluents contain resistant bacteria, resistance genes, and antibiotics, there is little knowledge on the link between resistance genotype and phenotype. Here we present the first study, which combines a culture-based phenotypic screen with the analysis of whole genome sequences for the indicator species *Escherichia coli* of the inflow and outflow of a sewage treatment plant. Our analysis reveals that nearly all isolates are multi-drug resistant and many are potentially pathogenic. This holds in particular for the outflow of the treatment plant. We devise a computational approach correlating genotypic variation and resistance phenotype, which identifies known and candidate resistance genes. The identified genes stem from the pan genome, which is large and thus reflects the genomic heterogeneity of a treatment plant. Overall, the screen and analysis show that sewage treatment plants provide a favourable environment for antibiotic resistance development and that resistant bacteria do not appear to suffer from a competitive disadvantage in wastewater. These findings should find consideration in future improvements of wastewater treatment.

Introduction

In 1945, Alexander Fleming, the discoverer of Penicillin, warned of antibiotic resistance. Today, the WHO echoes this warning, calling antibiotic resistance a global threat to human health. Humans are at the center of the modern rise of resistance. The human gut (1), clinical samples (2, 3), soil (4, 5), and wastewater (6) all harbor resistant bacteria and resistance genes. At the heart of modern resistance development is a human-centered network of clinics, industry, private homes, farming, and wastewater. However, it is unclear, where antibiotic resistance emerges and in particular, there are contradictory views on the role of wastewater treatment plants (6). On the one hand, the harsh environment of a treatment plant appears unfavourable for resistant bacteria (7), but on the other hand, it forms a very rich genetic reservoir, where highly diverse bacteria mingle (6). Also, it is unclear, whether or not any bacteria with pathogenic potential emerge after treatment.

To address these questions, we collected 1178 *Escherichia coli* isolates from a waste treatment plant's inflow and outflow in the city of Dresden, Germany. We selected 20 antibiotics, which are the most prescribed ones in the area from which the wastewater inflow originates (data provided by the public health insurer AOK). We analyzed the isolates' resistance to these 20 antibiotics and selected 103 isolates for whole genome sequencing. Our analysis reveals that wastewater outflow harbors multi-drug resistant *Escherichia coli* with pathogenic potential and very flexible genomes harboring resistance genes.

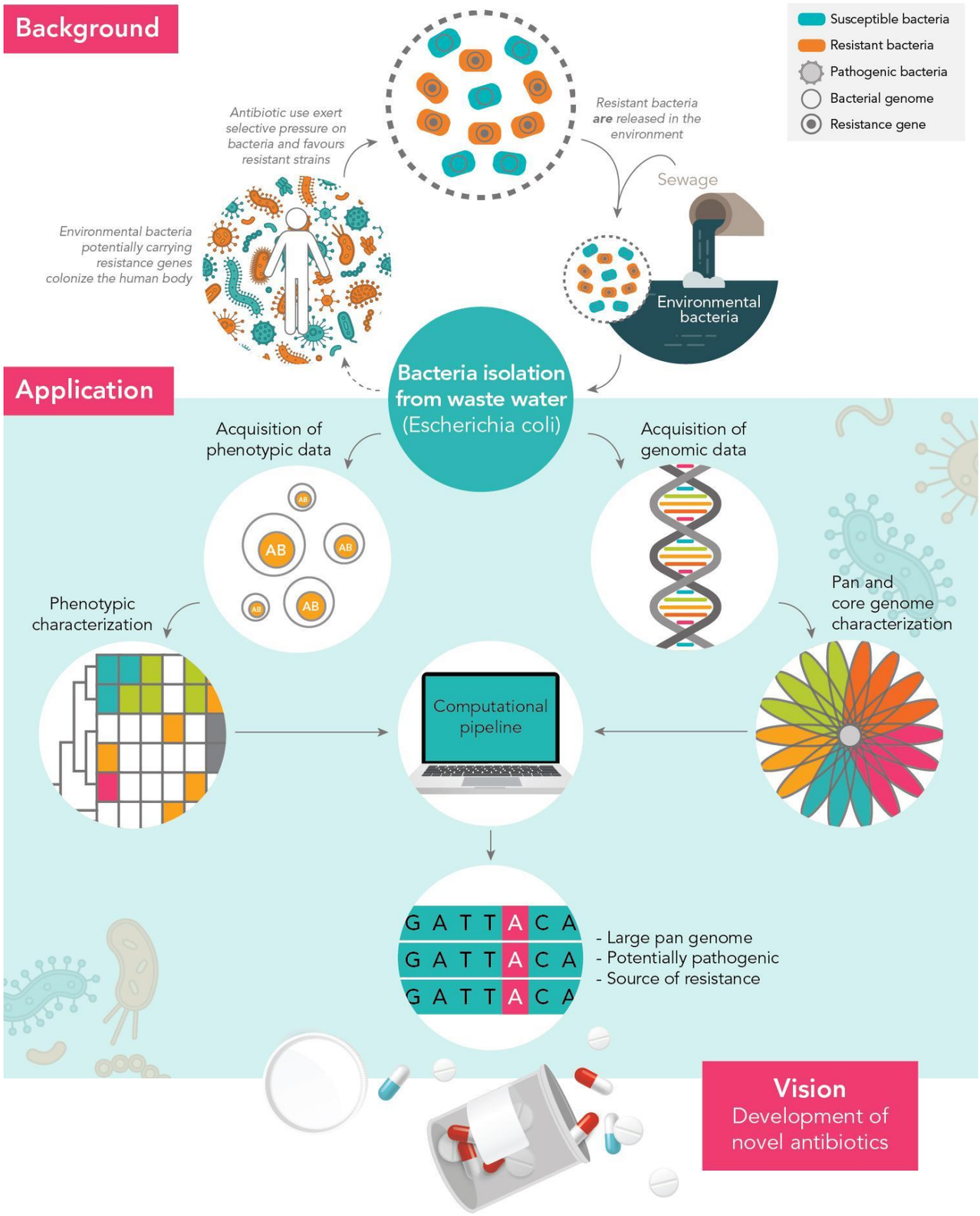

Figure 1: Wastewater plays an important role in antibiotic resistance development. Wastewater *E. coli* isolates are tested for antibiotic resistance and sequenced. Many isolates are multi-drug resistant and potentially pathogenic. Their large pan-genome is a source of potentially novel resistance genes.

Results

Pathogenic potential. *Escherichia coli* strains exhibit great variation. Many exist as harmless commensals in the human gut, but some are intra- (InPEC) or extra-intestinal pathogenic *Escherichia coli* (ExPEC). To assess the pathogenic potential without in vivo testing, clinical research has developed databases of virulence factors and genotyping schemes. The sequenced isolates contain some 700 of the 2000 *Escherichia coli* virulence factors in the virulence factor database (8), averaging to 153 and to 155 virulence factors per isolate for inflow and outflow, respectively. Hence, there is no significant difference (Welch test, CI 95%) between inflow and outflow. In particular, we found combinations of virulence factors for 16 isolates (see methods), which are indicative of ExPEC. Eight of these 16 isolates were obtained from the outflow of the treatment plant (see Fig. 2).

Besides the presence of known virulence factors, the pathogenic potential can be assessed using genotyping with multi-locus sequence types (9) and phylogroups (10). Broadly, *Escherichia coli* has, among others, four phylogroups, A, B1, B2 and D. Commensal *Escherichia coli* fall mostly into groups A and B1 and ExPEC into B2 and D (10). Fig. 2 shows a phylogenetic tree of the sequenced wastewater *Escherichia coli* isolates along with the commensal phylogroups A (red) and B1 (blue) and the pathogenicity-associated groups B2 (yellow) and D (green), as well as the finer-grained multi-locus sequence types. The tree is based on genomic variations compared to the reference genome of *Escherichia coli* K12 MG1655. Fig. 2 reveals that nearly one third of isolates belong to group B2 and D, in which ExPEC are usually found. In particular, B2 and D include 14 of the 16 potential ExPEC isolates. Remarkably, half of the B2 and D isolates are from the wastewater treatment plant's outflow.

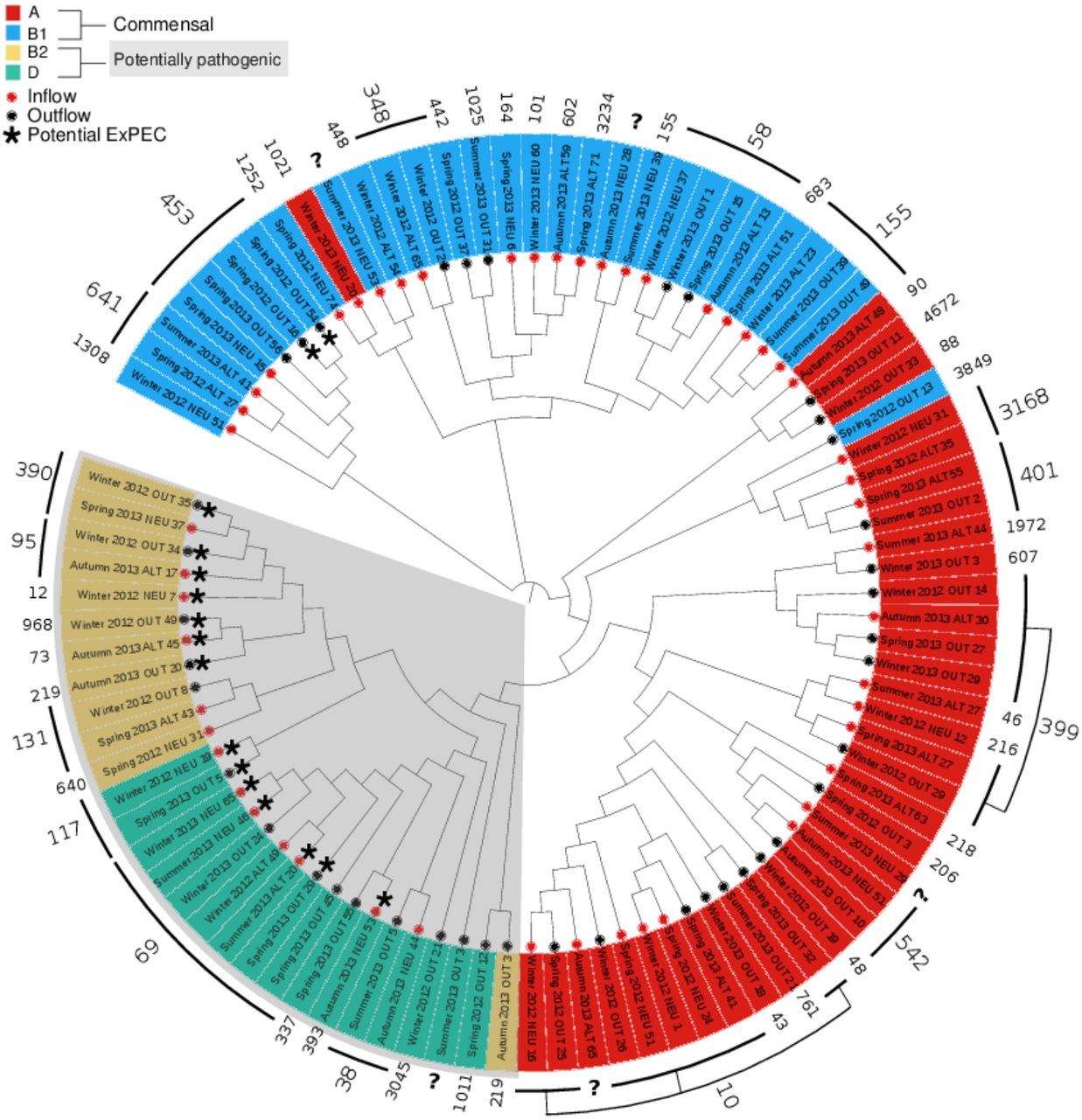

Figure 2: Phylogeny and pathogenic potential of wastewater *Escherichia coli*. Phylogenetic tree, multi-locus sequence types, and phylogroups of 92 sequenced wastewater *Escherichia coli* isolates reveal 16 potential ExPEC isolates (marked with a black star) in phylogroups B2 (yellow) and D (green), which are associated with pathogenicity. Half of the potentially pathogenic isolates stem from the outflow of the treatment plant.

The wastewater pan-genome. The concept of evolution implies that genomes of organisms of the same species differ. Differences range from small single nucleotide polymorphisms to large genome rearrangements. As a consequence, *Escherichia coli* possesses a core of genes present in all genomes, as well as genes only present in some genomes, or even just in one. The union of all of these genes is called the pan-genome. It is believed, that the *Escherichia coli* core genome comprises around 1400-1500 genes, while the pan-genome may be of infinite size (11).

To assess the degree of genomic flexibility of the wastewater isolates, we relate the wastewater pan-genome and the wastewater core genome. At 16582 genes, the wastewater pan-genome is nearly six times larger than the wastewater core genome of 2783 genes, a reservoir of some 14000 genes. Despite this large reservoir, the size difference of nearly 1000 genes between the wastewater *Escherichia coli* core genome and the whole species core genome suggests that the full diversity of *Escherichia coli* is still not covered in our wastewater sample.

The balance between maintaining the core genome and spending energy on acquisition of new genetic material can be captured by the ratio of the core genome size and the average genome size, which is 4700 genes in our sample. This means that only 1400/4700 = 30% of genes in our wastewater *Escherichia coli* are core genes. Most of the non-core genes are very unique and appear only in one or two isolates each. More precisely, 50% of the pan-genome genes appear in only one or two isolates each. This implies that the wastewater *Escherichia coli* studied are highly individual.

But do *Escherichia coli* maintain such a rich genome after wastewater treatment? Fig. 3 shows that they do. The 42 *Escherichia coli* genomes of the plant's outflow comprise nearly 12000 genes and the pan-genome growth curves between in- and outflow are nearly identical, which means that wastewater treatment does not affect the genetic diversity of *Escherichia coli*. Fig. 3 also shows a clinical dataset of ExPEC and these clinical *Escherichia coli* are more homogeneous and hence their pan-genome is smaller. In contrast, the diversity of the wastewater *Escherichia coli* match other datasets comprising mixtures of commensal and pathogenic

Escherichia coli, as well as *Shigella* genomes (see Table 1). This underlines the great diversity of genomes before and after wastewater treatment and leads to the question whether these diverse genomes harbour antibiotic resistance genes?

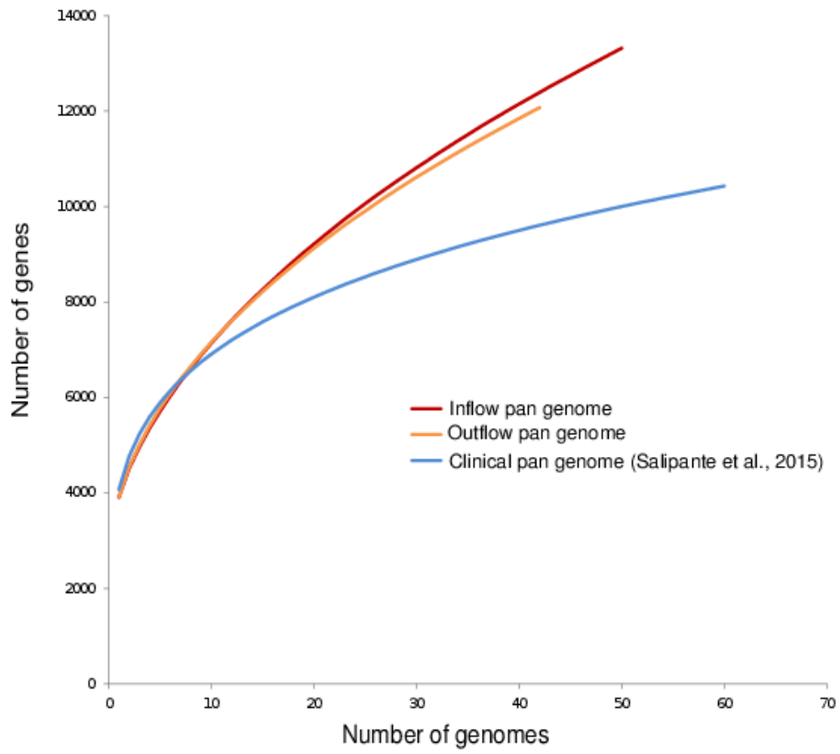

Figure 3: The pan-genome at the outflow has the same size as at the inflow, suggesting that highly flexible *Escherichia coli* emerge from a treatment plant. The wastewater pan-genome is larger than a clinical pan-genome one and of similar size to (see Table 1) highly diverse samples comprising pathogenic, commensal, and lab *Escherichia coli*, as well as *Shigella*.

Ref	Pan	Core	Strains	Path.	Comm.	Lab	*Shig.*
This study	16582	2783	92	28	62	0	0
Kaas et al., 2012[15]	16373	1702	186	171			15
Vieira et al., 2011[13]	14986	1957	29	21	8	0	6
Gordienko et al., 2013[12]	12000	2000	32	16	6	3	7
Lukjancenko et al., 2010[16]	13000	1472	53	35	11	7	0
Rasko et al., 2008 [17]	13000	2344	17	14	1	2	0
Touchon et al., 2009[14]	11432	1976	20	10	3	0	7

Table 1: Highly diverse samples comprising pathogenic, commensal, and lab *Escherichia coli*, as well as *Shigella.*

Resistance genes in the wastewater pan-genome. Wastewater *Escherichia coli* are known to host antibiotic resistance genes. While there are many known resistance genes (see e.g. CARD (12)), they fall mostly into a few groups, such as beta-lactamases. Here, we seek to confirm and expand the space for candidate resistance genes. Firstly, we measured antibiotic resistance in all 1178 isolates to the 20 antibiotics. Fig. 4 reveals a high degree of resistance and huge differences between different antibiotics, including a general trend indicating greater resistance to antibiotics that have been available for longer. Concretely, antibiotics from the 50s and 60s have a significantly different number of resistances than the more recent antibiotics (Welch test, p-value < 0.0025). However, there is no significant difference in the number of resistances between isolates from the inflow and the outflow (p-value 0.0001), suggesting that wastewater treatment is not affecting resistance at all.

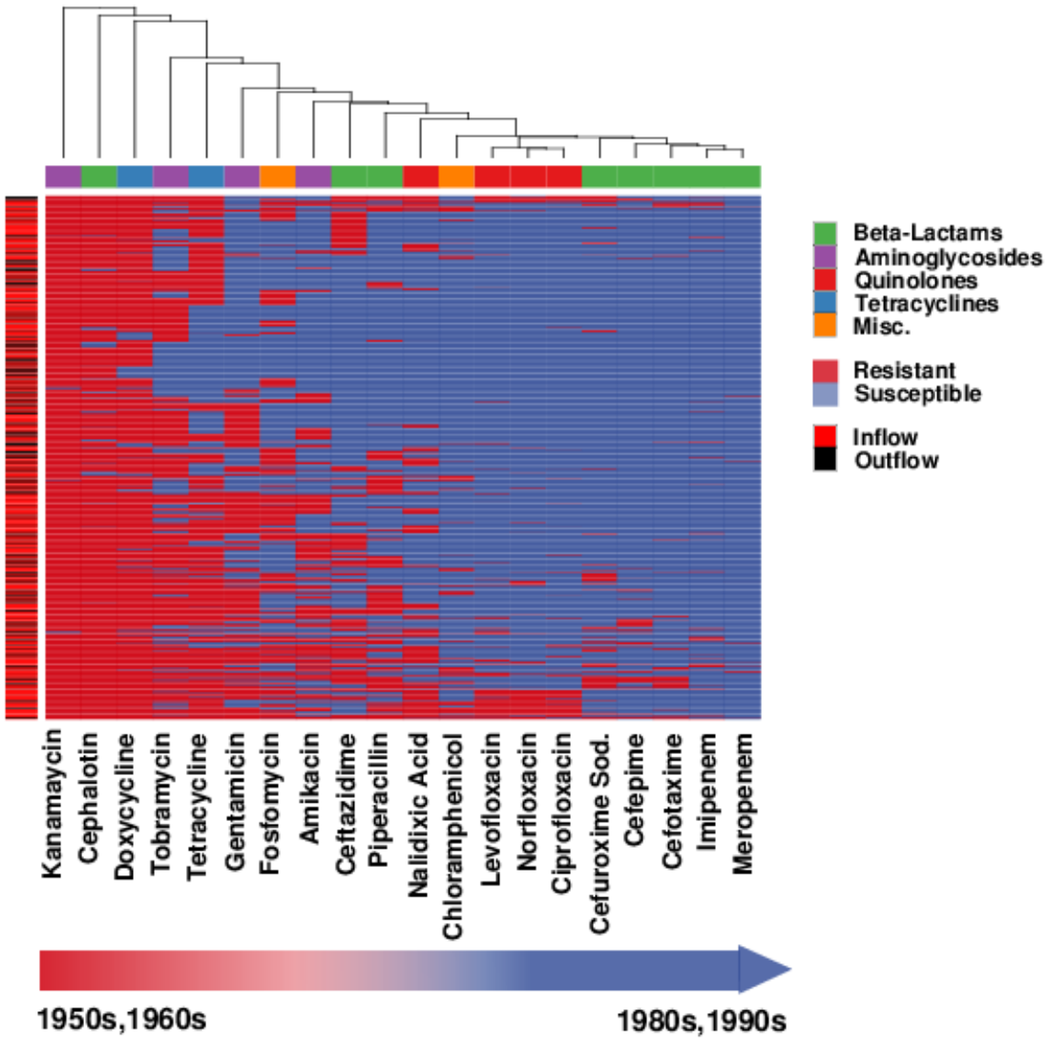

Figure 4: 1178 Wastewater *Escherichia coli* isolates are tested for antibiotic resistance to 20 antibiotics. Nearly all isolates are multi-drug resistant. Generally, isolates are more susceptible to betalactams and fluoroquinolones than to tetracyclins and aminoglycosides. Surprisingly, the outflow isolates show similar resistance as inflow (p-value 0.0001), suggesting that wastewater treatment is not reducing resistance development.

Next, we correlated the presence of each gene in the sequenced isolates with their phenotypic antibiotic resistance profiles. We excluded meropenem and imipenem, since nearly all isolates are susceptible. For each of the 18 remaining antibiotics, we list the top ten candidate resistance genes in Table 2. These 180 genes comprise 88 unique confirmed genes, including many well-known resistance genes, such as efflux pumps (MT1297 and *emr*E), membrane and transport proteins (*aida*-I*, yia*V*, yij*K*, pit*A*, ics*A, and *pag*N), tetracycline (*tet*A, *tet*R, and *tet*C), chloramphenicol (*cat*), and piperacillin (the beta lactamase *bla*2) resistance genes. However, the 180 genes also comprise a large number of open reading frames encoding hypothetical proteins (41) and genes not yet linked to antibiotic resistance (116). These genes have to be studied further to determine whether they are novel resistance genes or just correlating (e.g. because they are on the same genetic element with a resistance gene). All but three of the known and the potentially new resistance genes are present in some isolates obtained from the treatment plant's outflow.

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	Amikacin	Gentamicin	Kanamycin	Tobramycin	Doxycycline	Tetracycline	Cefepime	Cefotaxime	Ceftazidime	Cefuroxime Sod.	Cephalotin	Piperacillin	Ciprofloxacin	Levofloxacin	Nalidixic Acid	Norfloxacin	Chloramphenicol	Fosfomycin
1	Hypothetical Protein	4-hydroxyacetophenone monooxygenase **hapE**	Transposase IS200 like protein	Autotransporter precursor **aida-I**	Tetracycline resistance protein, class B **tetA**	Oxygen-dependent choline dehydrogenase **betA**	Ash protein family protein	Hypothetical Protein	cell division protein	Type-1 restriction enzyme R protein **hsdR**	GTPase **era**	Beta-lactamase TEM precursor **bla**	Virulence regulon transcriptional activator **virB**	Transposon Tn10 protein **tetD**	Mercuric resistance operon regulatory protein **merR**	Transposon Tn10 protein **tetD**	Chloramphenicol acetyltransferase **cat**	Invasin
2	Caudovirales tail fiber assembly protein	Phosphoadenosine phosphosulfate reductases	putative multidrug-efflux transporter/MT1297	putative protease **yhbU** precursor	Tetracycline repressor protein class B **tetR**	NAD/NADP-dependent betaine aldehyde dehydrogenase **betB**	Fibronectin type III protein	Hypothetical Protein	Plasmid stability protein	Type I restriction enzyme EcoKI M protein **hsdM**	Prophage CP4-57 regulatory protein **alpA**	Transposon Tn3 resolvase **tnpR**	Sporulation initiation inhibitor protein **Soj**	Tetracycline resistance protein, class B **tetA_1**	Mercuric resistance protein **merC**	Tetracycline resistance protein, class B **tetA_1**	Streptomycin 3''-adenylyltransferase **ant1**	Putative DNA-invertase Rac **pinR**
3	Swarming motility protein **ybiA**	putative multidrug-efflux transporter/MT1297	Phosphotransferase enzyme family protein	Chaperone protein **dnaK**	Transposon Tn10 TetC protein **tetC**	HTH-type transcriptional regulator **betI**	Transcriptional activator **perC**	Transcriptional activator **perC**	HTH-type transcriptional regulator **cmtR**	**mrr** restriction system protein	Hypothetical Protein	Tyrosine recombinase **xerD**	putative HTH-type transcriptional regulator	Tetracycline repressor protein class B from transposon Tn10 **tetR**	mercuric transport protein **merT**	Tetracycline repressor protein class B from transposon Tn10 **tetR**	Chromosome-partitioning ATPase **soj**	Transcriptional repressor **dicA**
4	Phospholipase **ytpA**	Phosphotransferase enzyme family protein	Hypothetical Protein	putative ABC transporter ATP-binding protein **yjjK**	HTH-type transcriptional regulator **cmtR**	Tetracycline resistance protein, class B **tetA**	Hypothetical Protein	Hypothetical Protein	Phage-related minor tail protein	Outer membrane protein **lcsA** precursor	Hypothetical Protein	Acetyltransferase (GNAT) family protein	DNA-binding transcriptional regulator **dicC**	Transposon Tn10 protein **tetC**	Mercuric transport protein periplasmic component precursor **merP**	Transposon Tn10 protein **tetC**	**parG**	Hypothetical Protein
5	Carbonic anhydrase 1 **cynT**	Hypothetical Protein	Streptomycin 3''-adenylyltransferase **ant1**	cell envelope integrity inner membrane protein **tolA**	Tetracycline resistance protein, class C **tetA**	Tetracycline repressor protein class B **tetR**	Hypothetical Protein	Hypothetical Protein	Phage tail protein E	Hypothetical Protein	Hypothetical Protein	Virulence regulon transcriptional activator **virB**	Hypothetical protein	putative HTH-type transcriptional regulator	Anti-adapter protein **iraM**	CAAX amino terminal protease self-immunity	Hypothetical Protein	Hypothetical Protein
6	Hypothetical Protein	Hypothetical Protein	Hypothetical Protein	Inner membrane protein **yiaV** precursor	putative inner membrane transporter **yedA**	Transposon Tn10 TetC protein **tetC**	Chromosome partition protein **smc**	Hypothetical Protein	Hypothetical Protein	Fibronectin type III protein	Transposon Tn10 **tetD** protein	Transposase	Lysine--tRNA ligase **lysS**	DNA-binding transcriptional regulator **dicC**	Hypothetical protein	mRNA interferase **pemK**	Hypothetical Protein	Hypothetical Protein
7	Xanthine dehydrogenase molybdenum-binding subunit **xdhA**	Hypothetical Protein	Zinc-responsive transcriptional regulator	Entericidin B membrane lipoprotein	Tetracycline repressor protein class A from transposon 1721 **tetR**	High-affinity choline transport protein **betT**	Hypothetical Protein	Invasin	Hypothetical Protein	Hypothetical Protein	putative multidrug-efflux transporter/MT1297	Tetracycline resistance protein, class B **tetA**	Transposon Tn10 protein **tetD**	Hypothetical protein	Mercuric reductase **merA_1**	Antitoxin **pemI**	Acetyltransferase (GNAT) family protein	Molybdenum cofactor biosynthesis protein A
8	Nicotinate dehydrogenase FAD-subunit **ndhF**	Hypothetical Protein	**merE** protein	Low-affinity inorganic phosphate transporter 1 **pitA**	Hypothetical Protein	Formate dehydrogenase H **fdhF**	Aldehyde-alcohol dehydrogenase **adhE**	Hypothetical Protein	Tyrosine recombinase **xerC**	Hypothetical Protein	Phosphotransferase enzyme family protein	Tetracycline repressor protein class B **tetR**	Tetracycline resistance protein, class B **tetA_1**	CAAX amino terminal protease self-immunity	Hypothetical protein	putative HTH-type transcriptional regulator	putative multidrug-efflux transporter/MT1297	ATP-dependent zinc metalloprotease **ftsH4**
9	Nicotinate dehydrogenase small FeS subunit **ndhS**	Phage polarity suppression protein **psu**	Phosphoadenosine phosphosulfate reductases	Methyl-accepting chemotaxis protein II **tar**	Transposon Tn10 **tetD** protein	S-fimbrial protein subunit **sfaH**	Aldehyde-alcohol dehydrogenase **adhE**	Hypothetical Protein	Hypothetical Protein	Hypothetical Protein	Outer membrane protein **pagN** precursor	Transposon Tn10 **tetC** protein	Tetracycline repressor protein class B transposon Tn10 **tetR**	mRNA interferase **pemK**	zinc-responsive transcriptional regulator	DNA-binding transcriptional regulator **dicC**	Phosphotransferase enzyme family protein	Molybdenum cofactor biosynthesis protein A
10	putative fimbrial-like protein ElfG precursor **elfG**	DNA primase **traC**	Caudovirales tail fiber assembly protein	Leucine-specific-binding protein precursor **livK**	putative multidrug-efflux transporter/MT1297	Beta-lactamase TEM precursor **bla**	Cob(I)yrinic acid a,c-diamide adenosyltransferase **yvqK**	Type-1 restriction enzyme R protein **hsdR**	Hypothetical Protein	Hypothetical Protein	Tetracycline resistance protein, class B **tetA**	Multidrug transporter **emrE**	Transposon Tn10 protein **TetC**	Antitoxin **PemI**	MerE protein	Caudovirales tail fiber assembly protein	Leucine-specific-binding protein precursor **livK**	Hypothetical Protein

Table 2: Known and candidate resistance genes from correlation of genomes to resistance phenotype. Top 10 genes for 18 antibiotics.

Discussion

The cost of resistance. There is a debate on whether the evolution of resistance is a competitive disadvantage. Some evidence indicates that resistant bacteria may be outcompeted by susceptible bacteria (13) and that they may be collaterally sensitive (14), i.e. resistant bacteria may become susceptible through an appropriate co-treatment. In contrast, it appears that our isolates do not suffer from evolutionary disadvantages despite their resistance and the harsh environment of wastewater treatment. The latter includes the reduction of the bacterial population as indicated by a reduction in biochemical oxygen demand, which is part of secondary wastewater treatment. Despite this bacterial reduction, wastewater outflow has similar resistance levels as the inflow. These findings support evidence (15) that bacteria can compensate for the cost of resistance.

Pathogenic potential and resistance. Ultimate proof for pathogenicity can only be obtained from in vivo studies. However, the pathogenic potential can be assessed from an analysis of a genome for virulence markers. Here we chose to consider three independent approaches: classification by phylogenetic groups, by multi-locus sequence tags, and by identification of specific virulence factors (see methods). While the three approaches showed consistent results, they are by no means proof for pathogenicity, since there can be exceptions to these classification rules. As an example, consider the strain ed1a, which belongs to the phylogenetic group B2, but it is not pathogenic in mice (16). Similarly, pathogenicity may not only arise from the acquisition of genes, but also from the loss (17). Regarding resistance there are similar confounding factors. *Escherichia coli* is inherently resistant to kanamycin and cephalotin, which is also clearly shown in Fig. 4. More generally, antibiotic resistance is ancient (18) and naturally occurring in the environment. Nonetheless, there are pronounced differences between pristine and human environments (19). This is also supported by Fig. 4, which shows that antibiotics introduced in the 50s and 60s have more resistances than those introduced later (p-value < 0.0025), which suggests, that the naturally occurring resistances do not play a major role in the emergence of observed resistances.

From clinic to river. We have shown that there are *Escherichia coli* at the wastewater outflow, which are multi-drug resistant and have pathogenic potential. But are they abundant enough to have an impact in the aquatic system they are released into? They do. The percentage of possibly pathogenic *Escherichia coli* in the outflow is considerable and may correspond to a large absolute amount. If an average of 100 *Escherichia coli* colony forming units (CFU) are released per ml, then 10^{13} CFUs per day are released (assuming a release of 10^5 m^3 per day). This is in accordance with Manaia *et al.*, who showed that 10^{10}-10^{14} CFU of ciprofloxacin-resistant bacteria are released by a mid-sized wastewater treatment plant (20). Furthermore, a study in a Japanese river shows the presence of pathogenic *Escherichia coli.* Gomi *et al.* (21) sequenced over 500 samples from the Yamato river and most of their prevalent multi-drug resistant and clinical strains are also present in our samples. In a related study, Czekalski *et al.* found that particle-associated wastewater bacteria are the responsible source for antibiotic resistance genes in the sediments of lake Geneva in Switzerland (22). Assuming that the river Elbe is comparable to these aquatic systems, it suggests, that clinic and river are connected with wastewater treatment plants in between.

Composition of phylogroups. It is interesting to compare the breakdown into phylogenetic groups of wastewater *Escherichia coli* to compare samples from human and animal environments. It is, e.g., known that the phylogenetic group B2 is more abundant among commensal *Escherichia coli* from human faeces (43%) than from farm animals (11%) (23). Therefore, the composition of wastewater *Escherichia coli* as shown in Fig. 2 resembles commensal *Escherichia coli* from farm animals more closely. Similarly, Tenaillon find that groups A and B1 make up one third in human faeces (23), whereas we find two thirds. This suggests that animal waste plays an important role for resistance of waste water bacteria.

Pan and core genome. We identified known and candidate resistance genes by correlating their presence in the genome against their resistance phenotype across isolates. By virtue of this correlation, the identified genes are not to be found in the core genome, but in the pan genome. As many authors have pointed out, *Escherichia coli* has a large and flexible pan genome. Lapierre *et al.* argue that *Escherichia coli* appears to have unlimited ability to absorb genetic material and hence its pan genome is open (11). In a recent study comprising over 2000

genomes Land *et al.* put this into numbers and arrive at a pan genome of 60000-89000 gene families for over 2000 sequenced *E. coli* genomes (24) . This upper limit shows that the wastewater pan genome of 16582 genes is still not the top. Nonetheless, it is considerably larger than a clinical pan genome. These differences indicate the heterogeneity of genomes. Clinical *Escherichia coli* genomes are not as diverse as the ones in a wastewater pool, which comprises besides human faeces also animal waste.

Random sampling and hypothesis-free analysis. The initial 1178 isolates were sampled randomly over different times of the year, from two different inflows and the outflow. In contrast, the 103 sequenced isolates were chosen in such way that all of the phenotypes encountered were represented (see methods). Within a phenotype group isolates were chosen randomly. This random, but representative choice and the subsequent link from genotype to phenotype is an example of high-throughput hypothesis-free analysis. And although, there was no pre-defined resistance mechanism, which we aimed to hit, many of the well-known resistance genes were ranked high. This supports the hope that high-throughput, hypothesis-free methods such as deep sequencing will help to uncover novel resistance mechanisms and in particular that some of the candidate resistance genes will prove to have a causal link to resistance.

Conclusion

Overall, we have shown for the first time that *Escherichia coli* isolates from a wastewater outflow have pathogenic potential and large pan-genomes, which harbor known and novel candidate resistance genes. Together with the estimates on absolute *Escherichia coli* abundance, this means that despite treatment, there is a considerable pathogenic potential at the outflow of a wastewater treatment plant. These results underline the need to include wastewater treatment plants in the combat against antibiotic resistance.

Methods

Collection. 1178 samples were collected from the municipal wastewater treatment plant Dresden, Germany. Samples were collected on 11/4/2012 (Spring 2012), 30/7/2012 (Summer 2012), 21/1/2013 (Winter 2012), 27/3/2013 (Spring 2013), 6/8/2013 (Summer 2013), 14/10/2013 (Autumn 2013), and 17/12/2013 (Winter 2013). Samples were collected either at the outflow (OUT) or at one of two inflow locations (Altstadt ALT and Neutstadt NEU), representing the area south and north of the river Elbe).

Isolation. *Escherichia coli* and total coliforms bacteria were enumerated via serial fold dilution plating of the original wastewater (triplicate samples). Wastewaters were diluted in double distilled water, until the enumeration of bacterial colonies was possible. *Escherichia coli* and coliform counts were always performed in triplicates. The *Escherichia coli* colonies were selected and picked after overnight growth at 37°C on a selective chromogenic media (OXOID Brilliance *Escherichia coli*/Coliform Selective Agar, Basingstoke, England). To minimize the risk of colony contamination, picked colonies were spiked a second time on the same selective media and pure single colonies were grown overnight on LB media at 37°C and stored on glycerol stock at -80° C.

Resistance phenotyping. Antibiotic resistance phenotypes were determined by the agar diffusion method using 20 antibiotic discs (OXOID, England) according to EUCAST (or CLSI when EUCAST was not available) (13, 18). The selected drugs belong to the most commonly prescribed antibiotics for diseases caused by bacteria according to the German health insurance AOK Plus: piperacillin (100*µg*), nalidixic acid (30*µg*), chloramphenicol (30*µg*), imipenem (10*µg*), cefotaxime (30*µg*), cephalotin (30*µg*), kanamycin (30*µg*), tetracycline (30*µg*), gentamicin (10*µg*), amikacin (30*µg*), ciprofloxacin (5*µg*), fosfomycin (50*µg*), doxycycline (30*µg*), cefepime (30*µg*), ceftazidime (10*µg*), levofloxacin (5*µg*), meropenem (10*µg*), norfloxacin (10*µg*), cefuroxime sod. (30*µg*), tobramycin (10*µg*) (25). After 24 hours of incubation at 37°C, the resistance diameters were measured. Clustering of antibiotics and of isolates was performed using the R function heatmap.2 from the R library (26) Heatplus and hierarchical clustering of matrices based on Euclidean distances between isolates and between antibiotics.

Sequencing. To select isolates representative of phenotype, we clustered isolates according to the diameters of inhibition zone against the 20 antibiotics using k-means clustering based on Euclidean distances between isolates (vectors of 20 inhibition zone diameters). The analysis and graphs were produced using R version 3.2.4 (26). As clusters may be highly skewed in number of cluster members, we tested all cluster numbers from 1 to 100 and plotted within class sum of squares against *k.* At $k = 47$, the sum of squares tails off and there is a steep local decrease, so that $k = 47$ was fixed as k-means parameter. We obtained 103 isolates, which were subsequently used for sequencing and further analysis. To further validate the choice, we plotted the average number of resistances against number of isolates and antibiotics vs. number of isolates for the total 1178 and the selected 103 isolates (see Supp Fig. 1) and concluded that both distributions are roughly similar. 3000ng DNA were extracted from each of the 103 selected isolates using MasterPure extraction kit (Epicentre) according to the manufacturer's instructions. Sequencing was performed using Illumina Flex GL.

Assembly. Genomes were assembled with Abyss (version 1.5.2) (27). In order to optimize *k* for the best assembly, k-mer values had to be empirically selected from the range of 20-48 (see Supp. Fig. 2) on a per sample basis to maximize contiguity (3). To determine the k-mer length that achieved highest contiguity, the 28 assemblies per draft genome/isolate were compared based on *N50* values. 11 assemblies with an *N50* statistic of less than 5×10^4 bp were excluded (28).

Genes. Reference gene clusters were computed from 58 complete *Escherichia coli* genomes (see Table 2) available in June 2015 from NCBI. Genes were identified in wastewater and refererence genomes using Prokka (version 1.11) (29). Genes were clustered at 80% using CD-HIT (30) (version 4.6.3, arguments -n 4 -c 0.8 -G 1 -aL 0.8 –aS 0.8 -B 1). Genes with over 90% sequence identity, but only 30% coverage, as well as genes with 80% or greater identity and covered to phage and virus sequences (31) were discarded. A gene cluster is defined to be present in an isolate if there is a Prokka gene in the genome, which is longer than 100 amino acids and has over 80% sequence identity and coverage against the gene cluster representative.

Pan- and core-genome. To generate the pan- and core-genome size graph we followed the procedure in (3, 16). We had 92 genomes available. We varied *i* from one to 92. At each subset size *i*, we randomly selected *i* genomes and computed the sizes of the union (pan) and intersection (core) of gene clusters. This random selection was carried out 2000 times in each step.

Gene clusters to rank genes by correlation to phenotype. Prokka genes were identified in all isolate genomes and then clustered with CD-HIT at 60% sequence identity and 50% coverage (arguments -n 4 -c 0.6 -G 1 -aL 0.8 -aS 0.5 -B 1). A 80% identity cutoff was also tried but dismissed, because the 60% threshold yielded 25% less clusters while adequately clustering homologous gene sequences with lower sequence similarity. This threshold value is also supported by the widespread default use of the BLOSUM62 matrix, the basis of which is sequences clustered by 62% sequence identity.

Tree. The phylogenetic tree of 92 isolates was built following the procedure of (32, 33)using FastTree version 2.1 (34). Sequence reads were aligned to *Escherichia coli* K12 MG 1665 and single nucleotide variant calling was carried out using GATK (35). Quality control for variant calling was performed; variants supported by more than ten reads or likelihood score greater than 200 were always in the range of 84 – 99% of variants called per isolate with the exception of 2

isolates where only 59% and 60% of the variants were above the threshold for quality and supporting reads. FastTree 2.1 (34) was then used to build the maximum likelihood tree based on the sequences derived from variant calling.

Phylogrouping. For phylogrouping, the classification system established by Clermont *et al.* (10) based on the genes chuA and yjaA and the DNA fragment TspE4.C2 was used. Blast was performed to check each genome assembly for presence or absence of the aforementioned elements with an identity cutoff ≥ 90%.

MLST. Concerning epidemiology and Multi-Locus Sequence Typing, we used the webserver at https://cge.cbs.dtu.dk/services/MLST/ that follows the MLST scheme in (36) for predicting MLSTs from whole genome sequence data (37) . 92 Draft genome assemblies were submitted and results were obtained; 5 isolates were unidentified demonstrating novel sequence types.

Virulence factors. Virulence factors protein sequences were downloaded from VFDB: Virulence Factors database (8, 38). 2000 sequences which were *Escherichia coli* related were chosen. Sequences were then clustered at 80% sequence identity using CD-HIT (version 4.6.3, arguments -n 4 -c 0.8 -G 1 -aL 0.8 -aS 0.8 –B 1). A virulence factor was considered present in an isolate's genome if there is a Prokka gene in the genome that has over 80% sequence identity and coverage against the virulence factor cluster representative.

ExPEC classification. There are intra- and extra-intestinal pathogenic *E.coli*, which can be classified from the presence of virulence factors (39-42). InPEC are characterised by the virulence factors stx1, stx2, escV, and bfpB. They are ExPEC if they contain over 20 of 58 virulence factors afa/draBC, bmaE, gafD, iha cds, mat, papEF, papGII, III, sfa/foc, etsB, etsC, sitD ep, sitD ch, cvaC MPIII, colV MPIX, eitA, eitC, iss, neuC, kpsMTII, ompA, ompT, traT, hlyF, GimB, malX, puvA, yqi, stx1, stx2, escV, bfp, feob, aatA, csgA, fimC, focG, nfaE, papAH, papC, sfaS, tsh, chuA, fyuA, ireA, iroN, irp2, iucD, iutA, sitA, astA, cnf1, sat, vat, hlyA, hlyC, ibeA, tia, and pic.

Data availability statement

Genome assemblies of the analyzed isolates that support the findings of the study will be made available on the NCBI upon paper publication (see Table 3).

Accession numbers of novel whole genome assemblies of analyzed isolates.

Bioproject	Biosample	Accession	strain
PRJNA380388	SAMN06641941	NBBP00000000	Escherichia coli Win2013_WWKa_OUT_3
PRJNA380388	SAMN06641940	NBBQ00000000	Escherichia coli Win2013_WWKa_OUT_29
PRJNA380388	SAMN06641933	NBBR00000000	Escherichia coli Win2013_WWKa_OUT_18
PRJNA380388	SAMN06641932	NBBS00000000	Escherichia coli Win2013_WWKa_OUT_24
PRJNA380388	SAMN06641931	NBBT00000000	Escherichia coli Win2013_WWKa_OUT_1
PRJNA380388	SAMN06641928	NBBU00000000	Escherichia coli Win2013_WWKa_NEU_65
PRJNA380388	SAMN06641927	NBBV00000000	Escherichia coli Win2013_WWKa_NEU_20
PRJNA380388	SAMN06641926	NBBW00000000	Escherichia coli Win2013_WWKa_NEU_60
PRJNA380388	SAMN06641901	NBBX00000000	Escherichia coli Win2013_WWKa_ALT_23
PRJNA380388	SAMN06641884	NBBY00000000	Escherichia coli Win2012_WWKa_OUT_49
PRJNA380388	SAMN06641883	NBBZ00000000	Escherichia coli Win2012_WWKa_OUT_8
PRJNA380388	SAMN06641882	NBCA00000000	Escherichia coli Win2012_WWKa_OUT_34
PRJNA380388	SAMN06641881	NBCB00000000	Escherichia coli Win2012_WWKa_OUT_35
PRJNA380388	SAMN06641880	NBCC00000000	Escherichia coli Win2012_WWKa_OUT_29
PRJNA380388	SAMN06641879	NBCD00000000	Escherichia coli Win2012_WWKa_OUT_26
PRJNA380388	SAMN06641878	NBCE00000000	Escherichia coli Win2012_WWKa_OUT_33
PRJNA380388	SAMN06641877	NBCF00000000	Escherichia coli Win2012_WWKa_OUT_21
PRJNA380388	SAMN06641876	NBCG00000000	Escherichia coli Win2012_WWKa_OUT_2
PRJNA380388	SAMN06641875	NBCH00000000	Escherichia coli Win2012_WWKa_NEU_7
PRJNA380388	SAMN06641874	NBCI00000000	Escherichia coli Win2012_WWKa_OUT_14
PRJNA380388	SAMN06641873	NBCJ00000000	Escherichia coli Win2012_WWKa_NEU_51
PRJNA380388	SAMN06641872	NBCK00000000	Escherichia coli Win2012_WWKa_NEU_31
PRJNA380388	SAMN06641871	NBCQ00000000	Escherichia coli Win2012_WWKa_NEU_37
PRJNA380388	SAMN06641870	NBCR00000000	Escherichia coli Win2012_WWKa_NEU_16
PRJNA380388	SAMN06641869	NBCS00000000	Escherichia coli Win2012_WWKa_NEU_19
PRJNA380388	SAMN06641868	NBCT00000000	Escherichia coli Win2012_WWKa_NEU_12
PRJNA380388	SAMN06641867	NBCU00000000	Escherichia coli Win2012_WWKa_ALT_65
PRJNA380388	SAMN06641866	NBCV00000000	Escherichia coli Win2012_WWKa_NEU_1
PRJNA380388	SAMN06641865	NBCW00000000	Escherichia coli Win2012_WWKa_ALT_49
PRJNA380388	SAMN06641864	NBCX00000000	Escherichia coli Win2012_WWKa_ALT_54
PRJNA380388	SAMN06641863	NBCY00000000	Escherichia coli Sum2013_WWKa_OUT_5
PRJNA380388	SAMN06641862	NBCZ00000000	Escherichia coli Sum2013_WWKa_OUT_39
PRJNA380388	SAMN06641861	NBDA00000000	Escherichia coli Sum2013_WWKa_OUT_49
PRJNA380388	SAMN06641860	NBDB00000000	Escherichia coli Sum2013_WWKa_OUT_3
PRJNA380388	SAMN06641859	NBDC00000000	Escherichia coli Sum2013_WWKa_OUT_31
PRJNA380388	SAMN06641858	NBDD00000000	Escherichia coli Sum2013_WWKa_OUT_2
PRJNA380388	SAMN06641857	NBDE00000000	Escherichia coli Sum2013_WWKa_OUT_21
PRJNA380388	SAMN06641856	NBDF00000000	Escherichia coli Sum2013_WWKa_NEU_53
PRJNA380388	SAMN06641855	NBDG00000000	Escherichia coli Sum2013_WWKa_NEU_46
PRJNA380388	SAMN06641854	NBDH00000000	Escherichia coli Sum2013_WWKa_NEU_39
PRJNA380388	SAMN06641853	NBDI00000000	Escherichia coli Sum2013_WWKa_ALT_44
PRJNA380388	SAMN06641852	NBDJ00000000	Escherichia coli Sum2013_WWKa_NEU_29
PRJNA380388	SAMN06641851	NBDK00000000	Escherichia coli Spr2013_WWKa_OUT_27

PRJNA380388	SAMN06641844	NBDL00000000	Escherichia coli Sum2013_WWKa_ALT_41
PRJNA380388	SAMN06641843	NBDM00000000	Escherichia coli Sum2013_WWKa_ALT_27
PRJNA380388	SAMN06641842	NBDN00000000	Escherichia coli Spr2013_WWKa_OUT_56
PRJNA380388	SAMN06641841	NBDO00000000	Escherichia coli Sum2013_WWKa_ALT_20
PRJNA380388	SAMN06641840	NBJM00000000	Escherichia coli Spr2013_WWKa_OUT_5
PRJNA380388	SAMN06641839	NBJN00000000	Escherichia coli Spr2013_WWKa_OUT_55
PRJNA380388	SAMN06641838	NBJO00000000	Escherichia coli Spr2013_WWKa_OUT_32
PRJNA380388	SAMN06641837	NBJP00000000	Escherichia coli Spr2013_WWKa_OUT_45
PRJNA380388	SAMN06641822	NBJQ00000000	Escherichia coli Spr2013_WWKa_OUT_15
PRJNA380388	SAMN06641821	NBJR00000000	Escherichia coli Spr2013_WWKa_OUT_29
PRJNA380388	SAMN06641820	NBJS00000000	Escherichia coli Spr2013_WWKa_NEU_6
PRJNA380388	SAMN06641819	NBJT00000000	Escherichia coli Spr2013_WWKa_OUT_11
PRJNA380388	SAMN06641818	NBJU00000000	Escherichia coli Spr2013_WWKa_NEU_15
PRJNA380388	SAMN06641817	NBJV00000000	Escherichia coli Spr2013_WWKa_NEU_37
PRJNA380388	SAMN06641816	NBJW00000000	Escherichia coli Spr2013_WWKa_ALT_63
PRJNA380388	SAMN06641815	NBJX00000000	Escherichia coli Spr2013_WWKa_ALT_71
PRJNA380388	SAMN06641814	NBJY00000000	Escherichia coli Spr2013_WWKa_ALT_51
PRJNA380388	SAMN06641813	NBJZ00000000	Escherichia coli Spr2013_WWKa_ALT_55
PRJNA380388	SAMN06641812	NBKA00000000	Escherichia coli Spr2013_WWKa_ALT_43
PRJNA380388	SAMN06641811	NBKB00000000	Escherichia coli Spr2013_WWKa_ALT_27
PRJNA380388	SAMN06641810	NBKC00000000	Escherichia coli Spr2013_WWKa_ALT_41
PRJNA380388	SAMN06641809	NBKD00000000	Escherichia coli Spr2012_WWKa_OUT_37
PRJNA380388	SAMN06641808	NBKE00000000	Escherichia coli Spr2012_WWKa_OUT_54
PRJNA380388	SAMN06641807	NBKF00000000	Escherichia coli Spr2012_WWKa_OUT_25
PRJNA380388	SAMN06641806	NBKG00000000	Escherichia coli Spr2012_WWKa_OUT_3
PRJNA380388	SAMN06641805	NBKH00000000	Escherichia coli Spr2012_WWKa_OUT_16
PRJNA380388	SAMN06641804	NBKI00000000	Escherichia coli Spr2012_WWKa_OUT_13
PRJNA380388	SAMN06641803	NBKJ00000000	Escherichia coli Spr2012_WWKa_NEU_74
PRJNA380388	SAMN06641802	NBKK00000000	Escherichia coli Spr2012_WWKa_OUT_12
PRJNA380388	SAMN06641801	NBKL00000000	Escherichia coli Spr2012_WWKa_NEU_31
PRJNA380388	SAMN06641800	NBKM00000000	Escherichia coli Spr2012_WWKa_NEU_51
PRJNA380388	SAMN06641799	NBKN00000000	Escherichia coli Spr2012_WWKa_NEU_24
PRJNA380388	SAMN06641798	NBKO00000000	Escherichia coli Spr2012_WWKa_ALT_27
PRJNA380388	SAMN06641797	NBKP00000000	Escherichia coli Spr2012_WWKa_ALT_35
PRJNA380388	SAMN06641796	NBKQ00000000	Escherichia coli Aut2013_WWKa_OUT_3
PRJNA380388	SAMN06641793	NBKR00000000	Escherichia coli Aut2013_WWKa_OUT_10
PRJNA380388	SAMN06641792	NBKS00000000	Escherichia coli Aut2013_WWKa_OUT_20
PRJNA380388	SAMN06641791	NBKT00000000	Escherichia coli Aut2013_WWKa_NEU_51
PRJNA380388	SAMN06641789	NBKU00000000	Escherichia coli Aut2013_WWKa_NEU_53
PRJNA380388	SAMN06641788	NBKV00000000	Escherichia coli Aut2013_WWKa_NEU_44
PRJNA380388	SAMN06641786	NBKW00000000	Escherichia coli Aut2013_WWKa_ALT_65
PRJNA380388	SAMN06641785	NBKX00000000	Escherichia coli Aut2013_WWKa_NEU_28
PRJNA380388	SAMN06641784	NBKY00000000	Escherichia coli Aut2013_WWKa_ALT_59
PRJNA380388	SAMN06641782	NBKZ00000000	Escherichia coli Aut2013_WWKa_ALT_48
PRJNA380388	SAMN06641780	NBLA00000000	Escherichia coli Aut2013_WWKa_ALT_45
PRJNA380388	SAMN06641779	NBLB00000000	Escherichia coli Aut2013_WWKa_ALT_30
PRJNA380388	SAMN06641778	NBLC00000000	Escherichia coli Aut2013_WWKa_ALT_17
PRJNA380388	SAMN06641777	NBLD00000000	Escherichia coli Aut2013_WWKa_ALT_13
PRJNA380388	SAMN06670745	NBNO00000000	Escherichia coli Win2012_WWKa_OUT_19

Table **3:** Accession numbers of 92 de novo assembled *E. coli* genomes.

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