Outbreak caused by *Escherichia coli* O18:K1:H7 sequence type 95 in a neonatal intensive care unit in Barcelona, Spain

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Conflict of Interests

The authors declare that they have no conflict of interests.

ABSTRACT

Background

Escherichia coli is one of the most frequent causes of late-onset neonatal sepsis. The aim of this study was to characterize an outbreak of neonatal sepsis occurring in the neonatal intensive care unit (NICU) of the Hospital Clinic of Barcelona from April to August 2013.

Methods

After presentation of the index case, all *E. coli* isolates from previously hospitalized neonates, health care workers, and neonates admitted to the NICU from April to October 2013 were tested for K1 antigen positivity and epidemiologically compared by pulse-field gel electrophoresis. Furthermore, the *E. coli* K1 strains collected from neonates during this period were analyzed by different methods (serotyping, phylotyping, PCR of virulence factors, antimicrobial resistance, and "in vitro" assays in HMBEC).

Results

An *E. coli* O18:K1:H7 sequence type 95 and phylogenetical group B2 strain was the cause of the outbreak involving 6 preterm neonates: one with late septicemia due to a urinary focus and 5 with late-onset septicemia and meningitis, 3 of whom died. All showed the same pulsotype, full resistance to ampicillin and intermediate resistance to gentamicin. The outbreak strain carried the PAI II_{J96}-like domain that could explain the high-grade bacteremia necessary to develop meningitis.

Conclusions

All the *E. coli* isolates responsible for this outbreak belonged to a single clone suggesting a common source of infection, and it was categorized as O18:K1:H7. Despite the bacteria's pathogenicity has an important role in the severity of infection, the host-associated factors were crucial for the fatal outcomes.

INTRODUCTION

Late-onset neonatal sepsis (LONS) is acquired after the first 72 hours of life and often leads to meningitis. *Escherichia coli* frequently causes septicemia and meningitis[1]. However, at present, the pathogenesis of meningitis caused by *E. coli* is only partially understood for two reasons: i) this infectious disease is a complex process formed by multiple bacterial-host interactions, and ii) the high genetic diversity of the pathotypes among neonatal meningitis *E. coli* (NMEC) strains.

It is known that there are a few features that distinguish these strains. NMEC strains are part of the extraintestinal pathogenic *E. coli* (ExPEC) subgroup, most of which belong to phylogroup B2[2] and the sequence type (ST) 95 complex[3]. One of the most common serotypes is O18:K1:H7[4] to which two of the representative NMEC strains belong, C5 and RS218. The K1 capsular antigen has frequently been detected among isolates causing septicemia and is also presented by approximately 80% of *E. coli* strains causing neonatal meningitis[5]. Type 1 fimbriae, S fimbriae, outer membrane protein A, cytotoxic necrotizing factor 1, invasion brain endothelial cell proteins, arylsulfatase-like, and TraJ have been described as traditional virulence-associated factors involved in different stages of meningitis[6–9]. Some of these genes are usually located in clusters classified as "ectochromosomal DNA" (ECDNA)[10], which can be horizontally transferred and hence, may be easily spread.

The equilibrium between host defenses and the pathogenicity of the bacteria in terms of virulence and resistance determine the extent of bacterial infection and the outcome of the disease[1]. Prematurity, and consequently, low birth weight are risk factors for the development of septicemia and meningitis in neonates[11], allowing these infections to be caused by "low-virulent" bacteria[1]. Neonatal intensive care units (NICUs) are sites in

which infants are more prone to acquire nosocomial infections and are a focus of outbreaks, including diarrhea[12] or meningitis[13,14]. For all these reasons, the identification of these pathogens is a challenge, especially if they present multi-drug resistance leading to a therapeutic failure.

The aim of this study was to characterize an outbreak of neonatal sepsis occurring in the NICU at the Hospital Clinic of Barcelona from April to August 2013.

MATERIALS AND METHODS

Microbiologic examination

Cerebrospinal fluid, urine and blood samples from the neonates involved in the outbreak were taken for microbiologic examination. In addition, 8 stool samples from health care workers (HCWs) and 29 from neonates admitted from April to October 2013 were included to evaluate the dissemination in the NICU and to find the possible cause of the outbreak. Stool samples were inoculated on MacConkey agar and incubated at 37ºC overnight. Suspected colonies were confirmed by MALDI-TOF. All *E. coli* isolates were tested for K1 antigen by the agglutination assay using a latex KIT (PASTOREX Meningitis Kit, Bio-Rad). The isolates that were positive were confirmed using specific primers for neu-PCR, which amplify the neuraminidase locus identified as a specific K1 target[15]. Furthermore, the same PCR was performed in 42 *E. coli* isolates from pharynx and otic smears, blood cultures and one urine culture positive from neonates hospitalized from January 2011 to April 2013.

Analysis of chromosomal DNA by Pulse-Field Gel Electrophoresis (PFGE)

Bacterial suspensions were prepared and embedded in agarose following a previous protocol with slight modifications[16]. PFGE of the strains was performed using *XbaI* as

the restriction enzyme. One % agarose gel was run on a CHEF-Mapper contour-clamped homogenous electric field apparatus for 20 h at 200 V (initial switch time 5 s, final switch time 35 s). The cluster analysis was performed with InfoQuest-FP software using the Dice similarity coefficient and dendrogram type UPGMA (unweighted-pair group method with arithmetic mean using average linkages) (optimization 0.50%, position tolerance 1.50%). A value of more than 95% of band similarity was considered as the same clone.

Strains

The strains selected to perform the different assays were the strain N38 belonging to the outbreak and other four K1-positive *E. coli* strains collected during the same period: N36 and N39, which were collected from healthy colonized neonates; N40, which caused an infection in a mother leading a fetal death; and N49, which was collected from a neonate suffering late-onset sepsis.

Multilocus sequence typing (MLST)

This study used the MLST scheme for *E. coli* developed by Wirth et al.[17]. Allele sequences were analyzed with a database available online (*http://mlst.warwick.ac.uk/mlst/dbs/Ecoli*).

Phylogenetic analysis

The new phylo-typing method by Clermont at al.[18] with several modifications[19] was used to assign the *E. coli* isolates to the eight phylogroups (B2, D, B1, A, E, Non-typeable, F, C, and E clade 1).

"In vivo" killing assay with *Caenorhabditis elegans*

Killing assays were performed in *C. elegans* according to a previously described model[20] but using Luria Broth (LB) instead of brain heart infusion medium. Each strain was repeated more than 5 times using *E. coli* OP50 as the internal control. Lethal Time 50% (LT50) is the number of days required to kill 50% of the nematode population.

Mass spectrometric sequencing of protein silver-stained polyacrylamide gels

The total proteome of N36 and N38 was analyzed using 2D gel electrophoresis followed by mass spectrometric identification as previously described by Párraga et al.[21].

RNA sequencing

RNA extraction was performed as described elsewhere[22]. rRNA depletion was done with the Ribo-Zero™ Magnetic Kit for Gram-negative bacteria. The TruSeq Stranded mRNA Sample Prep Kit protocol was followed according manufacturer's instructions. Libraries were validated by qPCR with Kapa Paired end and 75 nt read length libraries were sequenced on an Illumina Miseq resulting in a total output of 38 million reads. An average Phred quality score of 37 was obtained for the average of 3.1 million reads per sample. Reads were mapped onto the reference genome (*E. coli* O7:K1 str. CE10, complete genome (NC_017646) and its associated plasmids, *E.coli* O7:K1 str. CE10 plasmid pCE10A (NC_017647), pCE10B (NC_017648), pCE10C (NC_017649) and pCE10D(NC_017650)) using the EDGEpro software[23]. Resulting count datasets were exported to DESeq2[24], where they were normalized and pair-wise differential expression was carried out. Genes below p 0.05 were considered significant and used for Gene Ontology and Pathway analysis conducted by David[25].

Real-Time PCR experiments

RNA extraction was performed as mentioned above in RNA sequencing, and the Real-time PCR reactions were carried out following the same protocol[22]. For the RT-PCR, 500 ng of each RNA sample was used to make the reverse transcription, and the cDNA template was diluted 1/5. On one hand, the genes selected were those overexpressed in N38 compared to N36 by RNA-seq. These genes included: *rfaI*, *rfaL*, *rfaP*, *rfaY*, *waaT*, *waaV*, *waaW* (all involved in lipopolysaccharide biosynthesis of *E. coli*), *papI*1 (encoding the pyelonephritis adhesin pili operon regulatory protein PapI) and, *fliD* and *fliC* (involved in flagellar assembly). On the other hand, other genes were considered relevant because of the functions of their products. These genes were: *neuC* (encoding the polysialic acid biosynthesis of the K1 capsule), *kpsC* and *kpsD* (encoding capsule polysaccharide transport proteins), *fimD* (encoding a type-1 fimbria), *ompA* (encoding an outer membrane protein), and *aslB*, *ibeB*, *ibeC* (encoding virulence factors associated with meningitis). 16S rRNA was used as the endogenous control. Primers to amplify these genes were the same as those used in previous studies or designed by Primer Express® software (see Table, Supplemental Digital Content 1). The optimal concentration of the primers was from 2 to 9 µM after several assays. Amplification was performed using a StepOne™ Real-Time PCR System using the Sybr Premix Ex Taq "Tli RNaseH Plus" kit and the Universal Thermal Cycling conditions: 2 min at 50ºC, 10 min at 95ºC followed by 40 cycles of 95 ºC for 15 s and 60 ºC for 1 min. Data were analyzed with StepOne software v2.0, and the relative expression level for each sample ($2^{-\Delta\Delta CT}$) was obtained.

Detection of virulence factor genes (VFGs)

The virulence profile was analyzed by PCR using gene-specific primers (see Table, Supplemental Digital Content 2) as described elsewhere[26]. Twenty-three genes were studied: hemolysin (*hlyA*), cytotoxic necrotizing factor (*cnf1*), autotransporter toxin

(*sat1*), P-fimbriae (*papA, -EF, -C*), type-1 fimbriae (*fimH*), type 1-C fimbria (*focG*), S-fimbriae (*sfa/foc* and *sfaS*), yersiniabactin (*fyuA*), siderophores (*iutA* and *iroN*), aerobactin (*iucC*), invasion of brain endothelium factors (*ibeA* and *ibeC*), two genes involved in meningitis (*aslA* and *traJ*) and heat-resistant agglutinin (*hra*). Additionally, a PCR was performed to detect the PAI II_{J96}-like domain.

Adherence and invasion assays in human brain microvascular endothelial cells (HBMEC)

A HMBEC line was grown in endothelial cell medium, supplemented with 5% of fetal bovine serum (FBS), 1% of endothelial cell growth factors and 1/100 dilution of penicillin/streptomycin solution (10000 units-10 mg/mL). HMBEC were seeded onto 24-well tissue culture plates at a density of 2.5 $x10^5$ cells. Bacterial cultures incubated overnight in LB at 37º without shaking were used to infect each plate at a multiplicity of infection of approximately 100. Adherence and invasion assays were performed following a previously described protocol[27]. The only modification was the use of gentamicin (100 mg/mL) or kanamycin (50 mg/ml) for the invasion assay depending on the strain's antimicrobial susceptibility. All experiments were run in duplicate on at least three different days.

Serotyping

Serotyping was performed in the Federal Institute for Risk Assessment (BfR) at the National Reference Laboratory for *E. coli* in Berlin, Germany. The Orksov[28] [28] and Ewing[29] protocols were used.

Antibiotic susceptibility testing

Minimal inhibitory concentrations were determined using E-test strips on Müeller-Hinton agar plates inoculated with 0.5 MacFarland densities. Susceptibility was tested

for the following antimicrobial agents: ampicillin (AMP), amoxicillin-clavulanic acid (AMC), piperacillin-tazobactam (TZP), cefotaxime (CTX), meropenem (MEM), tetracycline (TET), trimethoprim-sulfamethoxazole (SXT), gentamicin (GEN), amikacin (AK), kanamycin (KAN), nalidixic acid (NA), ciprofloxacin (CIP), and fosfomycin (FOF). The ATCC 25922 strain was used as a standard control and results were analyzed according to the 2014 CLSI guidelines[30].

Biofilm assay

Biofilm formation was detected using a previous protocol described by Merrit et al.[31]. The result was considered positive when absorbance was greater than 4-fold the value of the absorbance of the negative control.

RESULTS

Outbreak description

The index case was a 7-day-old female neonate with a gestational age and birth weight of 29.2 weeks and 1,000 g, respectively. The neonate presented LONS the 13th of April 2013, in addition to meningitis and intraventricular hemorrhage (IVH)-Grade II (Table 1). Five more cases were detected thereafter. All were preterm neonates: one had late septicemia from a urinary focus, and 4 presented late onset septicemia and meningitis with severe neurological sequelae leading to death in three. The gestational age and birth weight of the neonates ranged from 25.2 to 29.2 weeks and from 750 to 1,000 g, respectively. The neonates also had other pathologies such as different grades of hyaline membrane disease, necrotizing enterocolitis and central nervous system hemorrhage. Due to AMP and GEN resistance, the treatment was CTX, CTX + AK, MEM + CIP or

MEM + AK according to the case. The length of the outbreak was 5 months, from April to August 2013 (Table 2).

Characterization of the strain causing the outbreak

An *E. coli* O18:K1:H7 strain was the cause of the outbreak. In epidemiological terms, the sequence type of the outbreak strain was ST95 (ST95 complex) and it belonged to phylogroup B2. In addition, this strain carried the PAI II_{J96}-like (containing the *hly*A, *cnf*1 and *hra* genes) and the PAI III_{536}-like (containing the *sfa/foc* and *iroN* genes) domains. As for antimicrobial resistance, this strain showed full resistance to ampicillin, amoxicillin-clavulanic acid, tetracycline, and fosfomycin, and intermediate resistance to gentamicin.

Comparative study with other K1-positive *E. coli* counterparts

One (12.5%) and 5 (17.2%) isolates were found to be K1 antigen positive among 8 and 29 *E. coli* isolates from the HCWs and neonates hospitalized during the study period, respectively. The prevalence of this antigen was 23.8% (10/42) among the neonates hospitalized from January 2011 to October 2013. PFGE was performed in all K1-positive *E. coli* isolates resulting in 2 well differentiated clusters and 7 *E. coli* isolates were considered to be from the same clone due to 98.58% of band profile similarity. Six isolates were recovered from the symptomatic neonates belonging to the outbreak, while one was from an asymptomatic neonate who was hospitalized in the NICU at the time of the outbreak (Figure 1). All the *E. coli* isolates causing the outbreak belonged to phylogroup B2 as did most of the other strains analyzed. Only 2 strains belonged to the F phylogenetic group.

Besides the strain recovered from the outbreak case (N38), another K1-positive strain isolated from a healthy colonized neonate during the same period (N36) was tested in the *C. elegans* infection assay (see Figure, Supplemental Digital Content 3). Both clinical isolates showed a significant difference (p-value<0.0001) in virulence regarding the mean of survival compared with the avirulent *E. coli* OP50 control strain. The LT50 was five versus eight days. However, no significant differences were found between N36 and N38. Neither was any significant differences found in the sequencing of proteins.

RNA-seq analysis was performed in order to investigate the differences in gene expression profiles between strains N36 and N38. One hundred eight genes were differentially expressed between the strains (see Table, Supplemental Digital Content 4 for a complete list of these genes), with the expression levels of 68 genes being more than three-fold higher in strain N38 than its counterpart N36, specifically, in relevant genes related to lipopolysaccharide biosynthesis, virulence and flagellar assembly (Table 3). In addition to the previous strains (N36 and N38), 3 more K1-positive strains (N39, N40 and N49) collected during the same period (April to October 2013) were selected to confirm the expression of several genes (Table 4). In this case, only 3 genes were overexpressed in the outbreak strain compared to the others: 2 genes associated with lipopolysaccharide biosynthesis (*rfa*I and *rfa*L) and the *pap*I1 gene which encodes for a regulator protein of the pap operon, showing significant overexpression.

Regarding the VFGs, the outbreak strain carried S-fimbriae (*sfa/foc* and *sfa*S) and the PAI II_{J96}-like domain that were not present in the other K1 strains (Table 5).

Strains N38 and 40 showed a higher capacity of adhesion than the other strains (Figure 2*A)* whereas strain N39 was the most invasive of the HBMECs (Figure 2*B*).

All the isolates belonging to the outbreak showed multi-drug resistance having identical susceptibility patterns as those mentioned above (see Table, Supplemental Digital Content 5). Other strains also showed resistance to other antimicrobial agents. All the strains studied, including that causing the outbreak, had the ability to form "in vitro" biofilm.

E. coli O18:H7 strains were recovered from the outbreak cases, whereas the other four strains (N36, N39, N40 and N49) were Or:H4 (Or means "rough" lipopolysaccharide), O2:H6, O75:[H5] ([Hxx] refers to H antigens listed by E&E in brackets), and O2:[H4], respectively.

DISCUSSION

This was the first outbreak of neonatal sepsis and meningitis at the NICU of the Hospital Clinic in Barcelona. An *E. coli* O18:K1:H7 sequence type 95 and phylogenetic group B2 strain was the cause of the outbreak involving six preterm neonates, one with late septicemia from a urinary focus and five with late septicemia and meningitis leading to death in three of them. All *E. coli* isolates from the outbreak were shown to belong to a single clone, suggesting a common source of infection.

The prevalence of K1 antigen positivity among the *E. coli* isolates collected from the HCWs and neonates hospitalized at the Maternity was lower than that found among previously hospitalized neonates, a similar finding to what Sarff et al.[32] described in a study carried out among healthy individuals but very low compared with another study performed in France[33].

The reason why the K1 capsule and only a few *O*-lipopolysaccharide antigens are associated with *E. coli* meningitis is still unclear, but their resistance properties allow these microorganisms to produce high-grade bacteraemia[9]. The outbreak strain belonged

to the serotype O18:K1:H7, which is one of the strains most frequently found among NMEC strains[4,34]. In addition, none of the serogroups from the other strains analyzed has been previously reported among *E. coli* causing neonatal meningitis. All the isolates belonged to phylogroups B2 and F, considered the most virulent among ExPEC isolates. Indeed, phylogroup B2 is commonly the most representative among NMEC isolates[2,35,36]. Likewise, ST95 is also frequent among ExPEC isolates and in particular those that cause neonatal meningitis[37,38].

Regarding the prevalence of VFGs by Real-Time PCR, the *papI*1 gene was overexpressed in the outbreak strain compared to the other genes. This is a transcriptional regulatory factor of the pap operon required in the P-fimbriae phase variation, a switch between the expression (Phase-ON) and the loss of expression (Phase-OFF) of these fimbriae. This is a regulatory complex that allows the cells to phenotypically change in response to environmental factors or other signals and represents an advantage for survival in hostile environments[39]. All the isolates including those belonging to the outbreak showed the genes encoding iron acquisition systems (*fyuA*, *iutA*, *iroN*, and *iucC*) which are necessary in iron-limited environments and relevant in septicemia and other extraintestinal infections[11,40,41]. In particular, the *fyuA* gene was present in all 11 representative NMEC strains used in a study performed by Yao et al.[36]. In contrast to the other strains, *E. coli* strains belonging to the outbreak carried the PAI II_{J96}-like (*hly*A, *cnf*1 and *hra* genes positive)[42] and the PAI III_{536}-like (*sfa/foc* and *iroN* genes positive) domains[43]. These ECDNA-like domains, along with the possession of the *ibeA* gene, are very frequent among O18:K1 strains[4] and are involved in the virulence of NMEC isolates but do not explain the whole pathogenesis of meningitis[35]. The PAI I_{C5}, which is similar to the PAI II_{J96}, and is harboured by strain C5, has been directly associated with bacterial survival in blood inducing high

bacteraemia but not with the passage of the bacteria across the blood-brain barrier[44]. As opposed to their counterparts, the outbreak strain possessed the *hra* gene, the presence of which is 91% homologous in uropathogenic *E. coli* strains compared to NMEC C5 and RS218 strains[45]. Moreover, it is suspected that these two strains have developed an extraintestinal virulence specialization, such as uropathogenicity and meningitis, which has been helped by the genetic background of the clonal group O18:K1[35]. There is controversy about the role of S fimbriae and Cnf1 in HMBEC binding or/and invasion[9,44,46]. These VFGs may have contributed to the higher ability of adhesion of the outbreak strain shown compared with their counterparts and other *E. coli* strains in other studies[37,47]. The only exception was strain N40, which caused infection in a mother leading to fetal death and showed the highest attachment of HMBEC. Strain N39 showed the highest capacity to invade HMBEC, whereas strain N38 displayed the outstanding lowest frequency, which was very similar to that shown by the negative control *E. coli* K-12 HB101 in several assays[37,48].

Multi-drug resistance is a problem for the administration of adequate treatment. The outbreak strain showed full resistance to ampicillin and intermediate to gentamicin, with these antibiotics being the 1st line combination used to combat neonatal sepsis and meningitis. This results support that a change on the empiric regimen is needed in developed countries. Thus, cefotaxime could be used instead gentamicin due to the low percentages of resistance found among *E. coli* strains causing neonatal sepsis. In addition, all the strains were biofilm-producers, a feature which makes the pathogen more resistant and virulent[49], especially when medical devices are used and may facilitate the transmission of colonizing microorganisms.

One three-month-old neonate developed urinary tract infection and septicemia but not meningitis. Age has been reported to be related to the achievement of the threshold level of bacteraemia required for the development of meningitis, but not for HMBEC binding and/or invasion[50]. At the time of the development of sepsis this neonate did not have a low weight and therefore likely presented a stronger immune system than the other neonates. Hence, a high bacterial inoculation might have been required to reach the necessary level of bacteraemia to develop meningitis. Features such as multi-drug resistance, capacity of biofilm-production, virulence-associated factors (PAI II_{J96}-like and PAI III_{536}-like domains), and pertinence to the O18:K1:H7 serotype, sequence type 95, and phylogroup B2, may have been key factors for the strain to cause the outbreak. Nonetheless, the status of the immune system of the neonates and the lack of host defenses undoubtedly played a major role in the outcome of the disease.

The method of transmission remained unclear, although mothers, HCWs and, even other neonates, as in the present case, are potential reservoirs and routes of entry of pathogenic organisms associated with nosocomial infections in NICUs.

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Figure legends

Figure 1. Characterization of the strains causing the outbreak and the K1-positive *E. coli* counterparts used in this study. Abbreviation: ND, not determined. Bold letters represent the strains belonging to the outbreak.

Figure 2. Adhesion (A) and invasion (B) assays of *E. coli* isolates to HBMECs. The displayed data are the mean ± standard deviation of adhesion and invasion %, respectively, of at least three independent experiments by duplicate. The strain N38 belonged to the outbreak, whereas N36, N39, N40 and N49 are non-outbreak strains.

Supplementary figure legend

Supplementary Figure 1. *C. elegans* infection assay of strains N36 (non-outbreak) *vs.* N38 (outbreak strain).

List of Supplemental Digital Content

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Table 1

Number of strain	Date of sepsis (day/month)	Sex	Gestational age (weeks)	Birth Weight (g)	Age [a] (days)	Diagnosis	Other pathologies	Evolution	Treatment
N35	13/04/2013	Female	29.2	1,000	7 [b]	Late sepsis & meningitis	IVH[d]-Grade II	Meningitis and death	CTX + AK
N38	03/05/2013	Female	28	750	12 [b]	Late sepsis & meningitis	Hyaline membrane disease+ IVH-Grade II	Meningitis, hydrocephalus and death	CTX + MEM→ MEM + AK
N41	31/05/2013	Male	28.2	710	105	Late sepsis & UTI [b]	Hyaline membrane disease + Inguinal hernia	Good evolution	CTX
N43	09/07/2013	Female	25.2	776	96	Late sepsis & meningitis	Hyaline membrane disease+ IVH-Grade III + Necrotizing enterocolitis	Hydrocephalus	MEM + CIP
N47	08/08/2013	Male	26.3	900	11[b]	Late sepsis & meningitis	Hyaline membrane disease + central nervous system hemorrhage	Meningitis and death	MEM + CIP
N48	15/08/2013	Female	27.3	870	23	Late sepsis &	Hyaline membrane disease	Hydrocephalus	MEM + CIP

	meningitis

[a] Age when the neonate developed sepsis

[b] Patient died

Abbreviations: UTI, Urinary Tract Infection; IVH, Intraventricular hemorrhage; CTX, Cefotaxime; AK, Amikacin; MEM, Meropenem; CIP, Ciprofloxacin.

Table 1. Characteristics of the neonates belonging to the outbreak.

Table 2

		Month (Year 2013)							
Case	**Date of birth** (day/ month)	**F**	**M**	**A**	**My**	**J**	**JL**	**AG**	S
N35	7/4			7/4 [a] 13/4 29/4[b]					
N38	21/4			21/4[a]	3/5 22/5 [b]				
N41	15/2	15/2 [a]		22/4[c]		30/5 [a]	31/5	11/6[c]	
N43	4/4			4/4 [a]			9/7	26/8[c]	
N47	28/7						28/7[a]	8/8	4/9[b]
N48	22/7						22/7[a]	15/8	27/9[c]

Grey color, length of stay at the hospital. Dark grey color, date of sepsis.

[a]Date of admission to the NICU in the Hospital Clinic, Barcelona

[b]Patient died

[c]Date of discharge

Abbreviations: F, February; M, March; A, April; My, May; J, June; JL, July; AG, August; S, September.

Table 2. Temporal distribution of the cases belonging to the outbreak.

Table 3

Table 3. Transcriptional values of representative genes overexpressed in N38 (outbreak strain) comparing to N36 (non-outbreak strain). Fold change indicates the ratio between the levels of expression in N38 and N36. Only statistically significant results ($p < 0.05$) are shown.

Locus tag	Gene	Description	Fold change (N38/N36)
LIPOPOLYSACCHARIDE BIOSYNTHESIS			
CE10_4186	*rfaI*	UDP-glucose:(Glucosyl) LPS alpha1. 3-glucosyltransferase	266.39
CE10_4181	*rfaL*	O-antigen ligase	156.4
CE10_4187	*rfaP*	kinase that phosphorylates core heptose of lipopolysaccharide	5.28
CE10_4184	*rfaY*	lipopolysaccharide core biosynthesis protein	195.615
CE10_4185	*waaT*	UDP-galactose:(Glucosyl) LPS alpha1. 2-galactosyltransferase	143.05
CE10_4182	*waaV*	putative beta1.3-glucosyltransferase	59.46
CE10_4183	*waaW*	UDP-galactose:(Galactosyl) LPS alpha1. 2-galactosyltransferase	216.03
VIRULENCE			
CE10_3431	*papI*1	pap operon regulatory protein PapI	46.80
FLAGELLAR ASSEMBLY			
CE10_2209	*fliD*	flagellar filament capping protein	23.96
CE10_2208	*fliC*	flagellar filament structural protein (flagellin)	5.40

Table 4. Fold change of gene expression of N36, N39, N40, and N49 (all non-outbreak strains) versus the outbreak strain N38. NE, not expressed.

Gene	Description/function	N36	N39	N40	N49
*neu*C	UDP-N-acetylglucosamine 2-epimerase	-1.462	-1.008	1.31	2.463
*kps*C	capsule polysaccharide export protein	1.717	5.198	22.297	18.879
*kps*D	polysialic acid transport protein	1.103	-8.684*	-1.162	-5.858*
*fim*D	Type-1 fimbria	3.3112*	6.561*	-1.3	3.971*
*omp*A	Outer membrane protein A	1.001	1.652	1.791	9.247**
*asl*B	VFG associated with meningitis	-2.239	2.172	-1.187	4.867
*ibe*B	VFG associated with meningitis	2.043	15.144	2.614	5.255
*ibe*C	VFG associated with meningitis	4.238**	1.457	3.866*	2.027
***papI*1**	**Pap operon regulatory protein PapI**	**NE****	**NE****	**-8.471***	**NE****
***rfa*I**	**UDP-glucose:(Glucosyl) LPS alpha1, 3-glucosyltransferase**	**NE****	**-4.879**	**-36.744****	**-4.568**
***rfa*L**	**O-antigen ligase**	**NE****	**-1.092**	**-4.794****	**-2.16**
*rfa*P	kinase that phosphorylates core heptose of lipopolysaccharide	-1.338	1.5	-1.187	1.079
*rfa*Y	lipopolysaccharide core biosynthesis protein	NE**	180.403**	47.001**	62.175**
*waa*T	UDP-galactose:(Glucosyl) LPS alpha1, 2-galactosyltransferase	NE	2.46	-1.695	2.031
*waa*V	putative beta1,3-glucosyltransferase	NE**	168.868**	74.456**	136.712**
*waa*W	UDP-galactose:(Galactosyl) LPS alpha1, 2-galactosyltransferase	NE**	156.637**	85.067**	160.839**
*fli*D	flagellar filament capping protein	NE*	108.737	NE	NE
*fli*C	flagellar filament structural protein (flagellin)	NE*	3.764	NE**	NE

Bold letters represent genes for which the expression is higher in N38 than in all the other strains.

*p-value<0.05

**p-value<0.01

Table 5

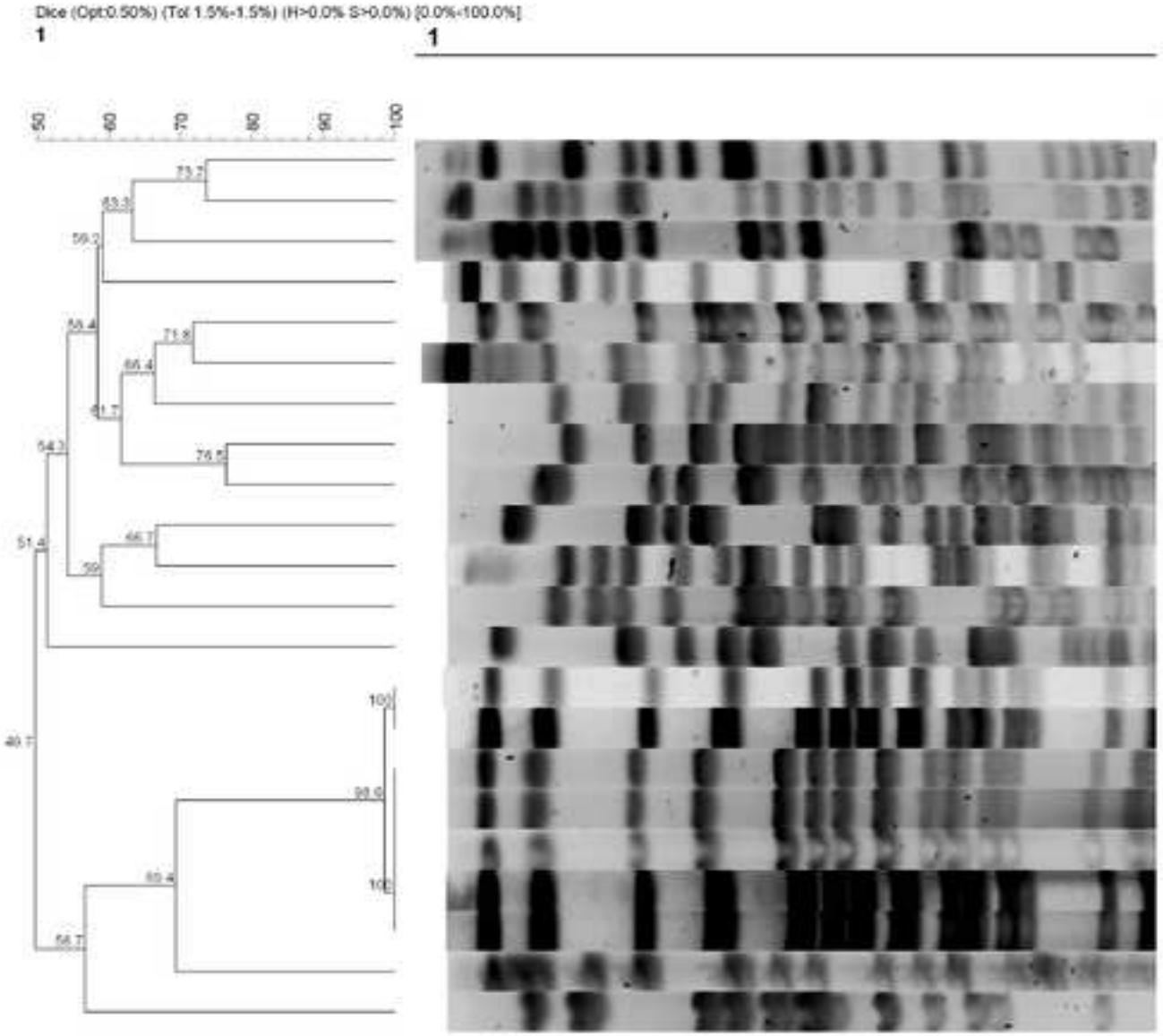
Gene	Description/function	N36	N38	N39	N40	N49
Toxins						
hlyA	hemolysin	-	+	+	-	-
cnf1*	**cytotoxic necrotizing factor**	**-**	**+**	**-**	**-**	**-**
sat1	autotransporter toxin	-	-	-	+	-
P- fimbriae						
papA		-	-	-	-	+
papEF		-	-	-	-	+
papC		-	-	+	-	-
Type-1 fimbriae						
fimH		+	+	+	+	+
Type 1-C fimbria						
focG		-	-	-	-	-
S-fimbriae						
sfa/foc*		**-**	**+**	**-**	**-**	**-**
sfa*S		**-**	**+**	**-**	**-**	**-**
Iron uptake systems						
fyuA	yersiniabactin	+	+	+	+	+
iutA	siderophore	+	+	+	+	+
iroN	siderophore	+	+	+	+	+
iucC	aerobactin	+	+	+	+	+
Other VFGs associated with virulence						
ibeA	invasion of brain endothelium factor	+	+	+	-	-
ibeC	invasion of brain endothelium factor	+	+	+	+	+
aslA	arylsulfatase-like	-	-	+	+	+
traJ	VFG involved in meningitis	+	-	+	+	+
hra*	**heat-resistant agglutinin**	**-**	**+**	**-**	**-**	**-**

*Bold letter represent the genes present in N38 but absent in the other strains.

Table 5. Prevalence of virulence factor genes (VFGs) among N38 (outbreak strain) and N36, N39, N40 and, N49 (non-outbreak strains).

Figure 1

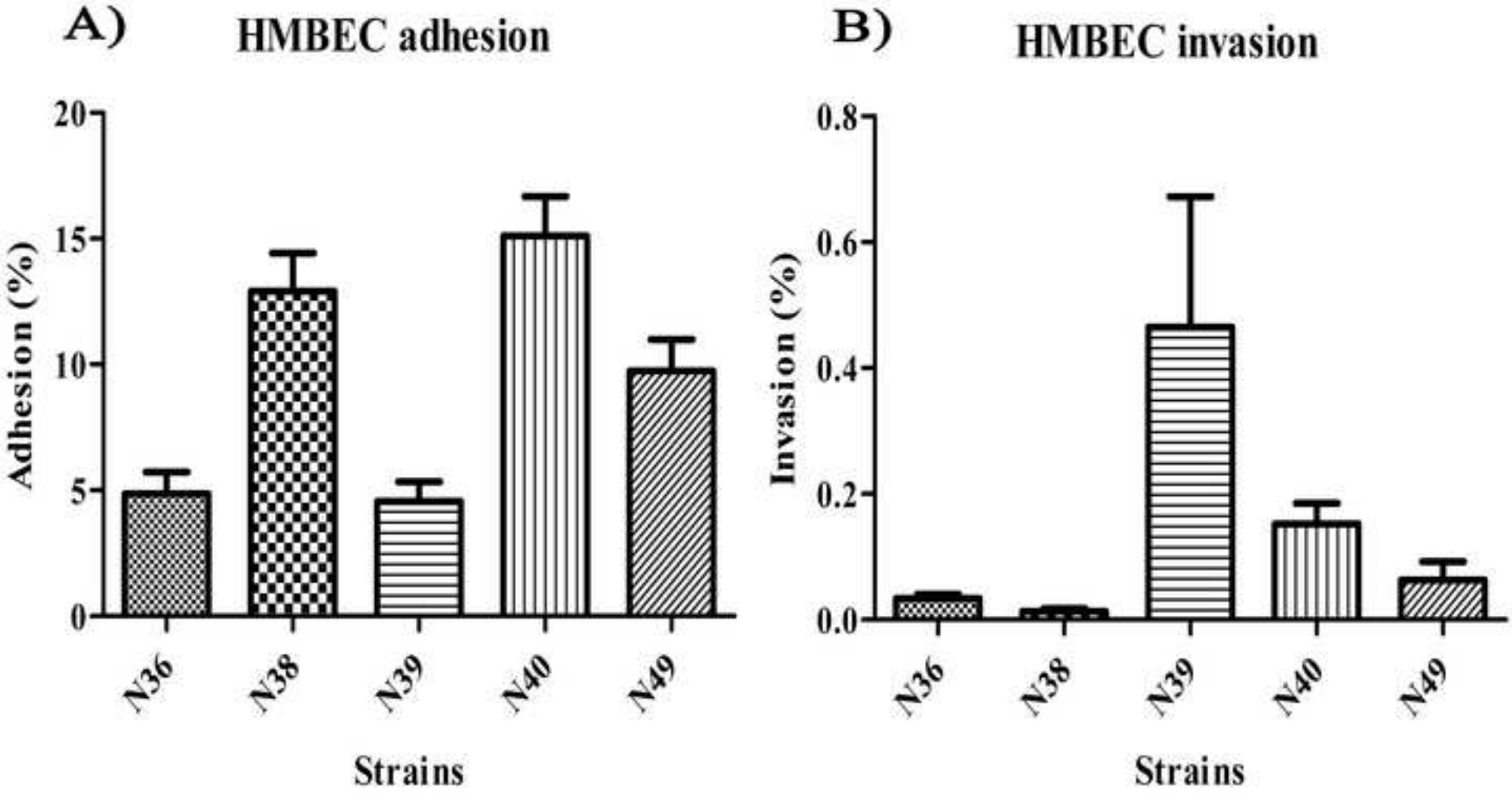
Click here to download Figure Figure_1.tif



Strain	Isolation site	PG
22	Stools	ND
34	Stools	ND
29	Stools	ND
15	Stools	ND
N10	Blood Culture	B2
N49	Otic smear	B2
N40	Pharynx smear	B2
N26	Pharynx smear	B2
N36	Otic smear	B2
N5	Otic smear	F
23	Stools	ND
N18	Pharynx and otic smear	F
N1	Blood culture	F
2	Stools	B2
N48	**Blood culture and CSF**	**B2**
N35	**Blood culture and CSF**	**B2**
N38	**Blood culture and CSF**	**B2**
N41	**Blood culture**	**B2**
N43	**Blood culture and CSF**	**B2**
N47	**Blood culture and CSF**	**B2**
N39	Pharynx smear	B2
N12	Otic smear	B2

Figure 2

Click here to download Figure Figure_2.tif



Supplemental Digital Content 1. Table. Primers used in Real-Time PCR experiments.

Gene	Primer sequence (5´-3´) (F/R)
*neu*C	AGGCAGAAAGGCCGTGTTC/CCCTCTGACGATTGCATTTTTT
*kps*C	GCCGGAAATACAGCTCTGATAAG/TCCCCGGTCACGATGGT
*kps*D	GCGAATGCAGGAAGCACAA/CCACGGTGCGTGCTTTC
*fim*D	CGCGCGTTGGGATAAAACT/CAAACGGCAGCGGCTTA
*omp*A	CAGGAGTGATCGCATACTCAACA/ACGACACCGGCGTTTCTC
*asl*B	CGCCTGGCTGATGAAACG/ATATCGCCGGGAGCATGTAG
*ibe*B	GTTAAATTACCGGCGGGCTT/GGTCAGGCTGATAGACGGGAA
*ibe*C	CCAGCGTGGACGCATGA/AGCTCCGGCGTGGTTTC
*rfa*I	CTGGGCCGGTTATCCAAGT/TCCAGGGCGATGCTTCTTT
*rfa*L	CAGCTTCCCACGCTACAACA/TTGATGCCAGTAAAGAAGGGAAA
*rfa*P	ATGCTGCGGGCATTAACC/GCAAGTGCAGCAGGAAATGA
*rfa*Y	ACGGCAGAGCGGAAAGC/CAATACCCAGGTGACGTTCCA
*waa*T	AAACGCCCCAGAGCTAAATGT/CGCCAGCACCATACAAAAAA
*waa*V	TTGCGCACGAAAGAATCTACTC/TGAATTTCTTCTTTCCGGTTACCT
*waa*W	GACGAATTATCCCTGCCAGAAG/GCCACATCATTCCAGCAAGA
*papI*_1	GGAGGGAAAACCGCAGAAA/CGTGCCTGATAATCCGTTACC
*fli*D	TGCCAGCGGCGTAGGT/GGTTGTGATGCCGGTTTTTT
*fli*K	GCGATGCTGCACAAGATTTTC/GTTGTCTCGCCTGCTAATGCT
*fli*C	CCATCGACAAATTCCGTTCA/CGCAGAATCCAGACGGTTCT

Supplemental Digital Content 2. Table. Primers used to detect the virulence factor genes (VFGs) and PAI II_{J96}-like domain.

Gene	Primer sequence (5´-3´) (F/R)	Reference
*hly*A	AACAAGGATAAGCACTGTTCTGGCT/ACCATATA AGCGGTCATTCCCGTCA	(1)
*cnf*1	AAGATGGAGTTTCCTATGCAGGAG/CATTCAGAG TCCTGCCCTCATTATT	(1)
*sat*1	ACTGGCGGACTCATGCTGT/AACCCTGTAAGAAG ACTGAGC	(1)
*pap*A	ATGGCAGTGGTGTCTTTTGGTG/CGTCCCACCATA CGTGCTCTTC	(1)
*pap*EF	GCAACAGCAACGCTGGTTGCATCAT/AGAGAGAG CCACTCTTATACGGACA	(1)
*pap*C	GACGGCTGTACTGCAGGGTGTGGCG/ATATCCTT TCTGCAGGGATGCAATA	(1)
*fim*H	CAGCGATGATTTCCAGTTTGTGTG/TGCGTACCAG CATTAGCAATGTCC	(2)
*foc*G	CAGCACAGGCAGTGGATACGA/GAATGTCGCCTG CCCATTGCT	(1)
sfa/foc	CTCCGGAGAACTGGGTGCATCTTAC/ CGGAGGAGTAATTACAAACCTGGCA	(3)
*sfa*S	AGAGAGAGCCACTCTTATACGGACA/CCGCCAGC ATTCCCTGTATTC	(1)
hra	CAGAAAACAACCGGTATCAG/ACCAAGCATGATG TCATGAC	(1)

*fyu*A	TGATTAACCCCGCGACGGGAA/CGCAGTAGGCAC GATGTTGTA	(1)
*iut*A	GGCTGGACATCATGGGAACTGG/CGTCGGGAACG GGTAGAATCG	(1)
*iro*N	AAGTCAAAGCAGGGGTTGCCCG/GACGCCGACAT TAAGACGCAG	(1)
*iuc*C	AAACCTGGCTTACGCAACTGT/ACCCGTCTGCAA ATCATGGAT	(4)
*ibe*A	AGGCAGGTGTGCGCCGCGTAC/TGGTGCTCCGGC AAACCATGC	(1)
*ibe*C	CACAGAAGTCCAGGCTAAACC/ TACCCACCTGATCGCCATAC	This study
*asl*A	CGGTGTCTGATATGTACACCG/ CATCCCTTTCCAGTAAACG	(6)
*tra*J	TCTGACGGCAGTTATTCAGG/GATGCGTGTTTCTT TGATGTGG	This study
PAI II_{J96}-like domain	GGATCCATGAAAACATGGTTAATGGG/ GATATTTTTGTTGCCATTGGTTACC	(7)

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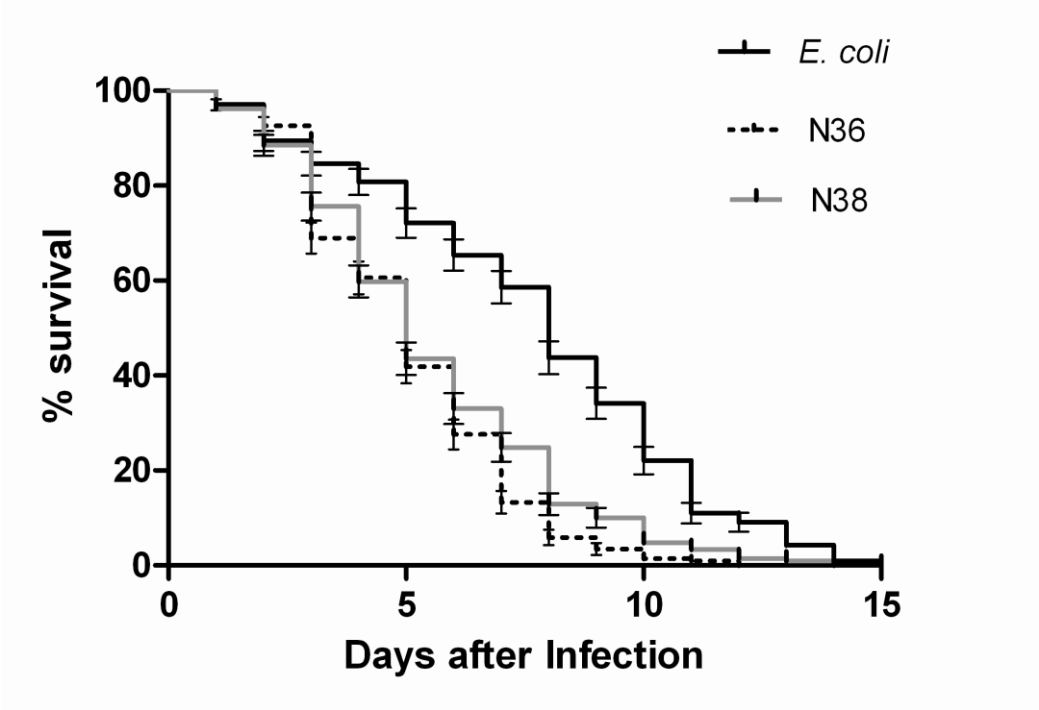
Supplemental Digital Content 3. Figure. *Caenorhabditis elegans* infection assay of strains N36 (non-outbreak) *vs.* N38 (outbreak strain).



Supplemental Digital Content 4. Table. Transcriptional values of representative genes differentially expressed in the outbreak strain N38 comparing to the non-outbreak strain N36. Fold change indicates the ratio between the levels of expression in N38 and N36. Only statistically significant results ($p < 0.05$) are shown.

Locus tag	Gene	Description	Fold change (N38/N36)
CE10_4186	*rfaI*	UDP-glucose:(Glucosyl) LPS alpha1, 3-glucosyltransferase	266.39
CE10_1685	*yddB*	putative porin protein	194.92
CE10_4183	*waaW*	UDP-galactose:(Galactosyl) LPS alpha1, 2-galactosyltransferase	216.03
CE10_3421	*papX*	HTH-type transcriptional regulator	184.27
CE10_4184	*rfaY*	lipopolysaccharide core biosynthesis protein	195.61
CE10_1687	*yddA*	ABC transporter ATP-binding protein	156.87
CE10_3206	*mazF*	mRNA interferase toxin, antitoxin is MazE	134.06
CE10_4181	*rfaL*	O-antigen ligase	156.40
CE10_2290	*ibrA*	immunoglobulin-binding regulator A	100.25
CE10_4185	*waaT*	UDP-galactose:(Glucosyl) LPS alpha1, 2-galactosyltransferase	143.05
CE10_4203	*dinD*	DNA-damage-inducible protein	86.38
CE10_3207	*mazE*	antitoxin of the ChpA-ChpR toxin-antitoxin system	58.53
CE10_4611	*tsx2*	nucleoside-specific channel-forming protein Tsx	67.25
CE10_1043	*essD2*	DLP12 prophage phage lysis protein	56.86
CE10_1762	*celA*	6-phospho-beta-glucosidase	49.48
CE10_4182	*waaV*	putative beta1,3-glucosyltransferase	59.46
CE10_3431	*papI*1	pap operon regulatory protein PapI	46.80

CE10_1496	*yciE*	putative rubrerythrin/ferritin-like metal-binding protein	48.93
CE10_1684	*pqqL*	putative peptidase	14.08
CE10_5142	*quuQ*	Qin prophage antitermination protein Q	28.62
CE10_2292	*yaiP*2	putative glucosyltransferase	26.38
CE10_2209	*fliD*	flagellar filament capping protein	23.96
CE10_1858	*ynfF*	S- and N-oxide reductase, A subunit, periplasmic	23.14
CE10_4830	*eptA*	putative metal dependent hydrolase	8.59
CE10_1608	*ynbB*	putative CDP-diglyceride synthase	20.92
CE10_0326	*yaiO*1	outer membrane protein	19.33
CE10_1610	*ynbD*	putative phosphatase inner membrane protein	18.12
CE10_1857	*ynfE*	putative selenate reductase, periplasmic	17.49
CE10_2055	*ydjK*	putative transporter	9.27
CE10_2293	*yaiX*2	putative nucleotidyl transferase	16.77
CE10_1460	*essD*3	DLP12 prophage phage lysis protein	17.38
CE10_1497	*yciF*	YciF protein	15.58
CE10_1726	*tfaE*	e14 prophage tail fiber assembly protein	15.21
CE10_2294	*yaiO*2	outer membrane protein	14.39
CE10_1605	*ydbC*	putative oxidoreductase, NAD(P)-binding protein	12.83
CE10_0327	*yaiX*1	putative transferase	13.65
CE10_3139	*ascB*	cryptic 6-phospho-beta-glucosidase	11.19
CE10_1609	*ynbC*	putative hydrolase	11.59
CE10_2053	*ydjI*	putative aldolase	6.07

CE10_2054	*ydjJ*	putative oxidoreductase, Zn-dependent and NAD(P)-binding protein	7.20
CE10_3180	*cysI*	sulfite reductase, beta subunit, NAD(P)-binding, heme-binding protein	10.07
CE10_4829	*basR*	DNA-binding response regulator in two-component regulatory system with BasS	6.10
CE10_2317	*yeeT*1	CP4-44 prophage protein	11.12
CE10_1394	*chaA*	calcium/sodium:proton antiporter	4.04
CE10_2052	*ydjH*	putative kinase	5.61
CE10_1692	*ydeP*	putative oxidoreductase	4.20
CE10_4187	*rfaP*	kinase that phosphorylates core heptose of lipopolysaccharide	5.28
CE10_3125	*srlA*	PTS system glucitol/sorbitol-specific transporter subunit IIC	6.98
CE10_3128	*srlD*	sorbitol-6-phosphate dehydrogenase	8.74
CE10_2208	*fliC*	flagellar filament structural protein (flagellin)	5.40
CE10_2636	*arnB*	uridine 5'-(beta-1-threo-pentapyranosyl-4-ulose diphosphate) aminotransferase, PLP-dependent	4.85
CE10_3936	*gntT*	gluconate transporter, high-affinity GNT I system	5.18
CE10_1045	*arrQ*1	Qin prophage lysozyme	8.20
CE10_0595	*entB*	isochorismatase	5.92
CE10_1693	*ydeQ*	putative fimbrial-like adhesin protein	4.12
CE10_4851	*cadB*	putative lysine/cadaverine transporter	5.08
CE10_4389	*asnA*	asparagine synthetase A	3.53

CE10_0917	*lolA*	chaperone for lipoproteins	4.20
CE10_2344	*ugd*	UDP-glucose 6-dehydrogenase	5.46
CE10_5067	*uxuB*	D-mannonate oxidoreductase, NAD-binding protein	4.92
CE10_1694	*ydeR*	putative fimbrial-like adhesin protein	3.17
CE10_0143	*htrE*	putative outer membrane usher protein	6.70
CE10_1435	*ompW*	outer membrane protein W	4.45
CE10_2638	*arnA*	fused UDP-L-Ara4N formyltransferase/UDP-GlcA C-4'-decarboxylase	3.68
CE10_0328	*yaiP*1	putative glucosyltransferase	6.56
CE10_4638	*yijD*	inner membrane protein	3.11
CE10_2343	*cld*	regulator of length of O-antigen component of lipopolysaccharide chains	4.55
CE10_1607	*ynbA*	inner membrane protein	6.40
CE10_3199	*scrR*	Sucrose operon repressor	-491.15
CE10_3662	*yhaV*	toxin of the SohB(PrlF)-YhaV toxin-antitoxin system	-292.77
CE10_3201	*ygcG*	hypothetical protein	-213.97
CE10_2355	*rfbC*	dTDP-4-deoxyrhamnose-3,5-epimerase	-151.44
CE10_3198	*scrB*	sucrose-6-phosphate hydrolase	-78.95
CE10_3197	*scrA*	PTS system sucrose-specific transporter subunit IIBC	-82.48
CE10_3661	*sohA*	antitoxin of the SohA(PrlF)-YhaV toxin-antitoxin system	-70.09
CE10_3196	*scrY*	sucrose porin	-66.66

CE10_4290	*yicL*	hypothetical protein	-58.63
CE10_0280	*yahA*	c-di-GMP-specific phosphodiesterase	-30.90
CE10_4540	*yihO*	putative transporter	-51.37
CE10_4548	*yihW*	putative DNA-binding transcriptional regulator	-40.77
CE10_1650	*pptA*	4-oxalocrotonate tautomerase	-33.64
CE10_4539	*ompL*	outer membrane porin L	-31.66
CE10_4555	*yiiF*	hypothetical protein	-25.18
CE10_3195	*scrK*	aminoimidazole riboside kinase	-24.33
CE10_4542	*yihQ*	alpha-glucosidase	-22.06
CE10_0154	*fhuA*	ferrichrome outer membrane transporter	-7.91
CE10_3807	*yhdZ*	putative amino-acid transporter subunit	-9.18
CE10_1709	*yneE*	hypothetical protein	-6.46
CE10_4544	*yihS*	aldose-ketose isomerase, D-mannose isomerase	-10.77
CE10_2274	*yeeN*	hypothetical protein	-8.39
CE10_4078	*bcsB*	regulator of cellulose synthase, cyclic di-GMP binding protein	-6.72
CE10_1581	*abgT*	p-aminobenzoyl-glutamate transporter, membrane protein	-6.35
CE10_4563	*frvR*	putative frv operon regulator, contains a PTS EIIA domain	-9.65
CE10_3805	*yhdX*	putative amino-acid transporter subunit	-6.39
CE10_1098	*efeO*	inactive ferrous ion transporter EfeUOB	-4.71
CE10_3178	*iap*	aminopeptidase in alkaline phosphatase isozyme conversion	-4.34
CE10_0355	*ykiA*	hypothetical protein	-5.20

CE10_1526	*osmB*	lipoprotein	-5.40
CE10_3090	*ygaC*	hypothetical protein	-5.14
CE10_0136	*panB*	3-methyl-2-oxobutanoate hydroxymethyltransferase	-4.60
CE10_2379	*wza*	lipoprotein required for capsular polysaccharide translocation through the outer membrane	-7.66
CE10_3931	*yhgA*	putative transposase	-4.15
CE10_0281	*yahB*	putative DNA-binding transcriptional regulator	-3.88
CE10_2001	*ydiY*	putative outer membrane protein, acid-inducible	-4.46
CE10_0525	*arrD*1	DLP12 prophage lysozyme	-5.63
CE10_0133	*yadD*	putative transposase	-3.35
CE10_2250	*yedZ*	inner membrane heme subunit for periplasmic YedYZ reductase	-3.76
CE10_4079	*bcsA*	cellulose synthase, catalytic subunit	-4.58

Supplemental Digital Content 5. Table. Minimum inhibitory concentrations (μg/ml)

No. of Strain	Antimicrobial agents												
	AMP	AMC	TZP	CTX	MEM	TET	SXT	GEN	AK	KAN	NA	CIP	FOF
N36	3	8	1.5	0.5	0.012	2	1	0.5	1.5	2	3	0.012	0.50
N38	>256	16	2	0.5	0.016	128	0.006	4	8	6	3	0.012	192
N39	3	6	1	0.5	0.012	2	0.094	0.5	2	3	128	0.19	0.75
N40	>256	8	1	0.5	0.012	64	>32	48	2	6	>256	>256	0.5
N49	>256	12	1	0.38	0.006	>256	>32	0.5	2	1.5	3	0.006	1

among N38 (outbreak strain) and N36, N39, N40 and, N49 (non-outbreak strains).

Abbreviations: AMP, ampicillin; AMC, amoxicillin-clavulanic acid; TZP, piperacillin-tazobactam; CTX, cefotaxime; MEM, meropenem; TET, tetracycline; SXT, trimethoprim-sulfamethoxazole; GEN,gentamicin; AK, amikacin; KAN, kanamycin; NA, nalidixic acid; CIP, ciprofloxacin, and FOF, fosfomycin.