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Phylogenetic Distribution of Virulence-Associated Genes among *Escherichia coli* Isolates Associated with Neonatal Bacterial Meningitis in The Netherlands

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Seventy cerebrospinal fluid *Escherichia coli* isolates from infants with neonatal bacterial meningitis (NBM), as submitted to the Netherlands Reference Laboratory for Bacterial Meningitis from 1989 through 1997, were assessed for phylogenetic background and extended virulence genotypes, in comparison with the *E. coli* reference collection, by using molecular methods. Phylogenetic group B2 significantly predominated overall (81%). The 4 major phylogenetic clusters exhibited distinctive virulence genotypes, suggesting diverse evolutionary histories for the individual genes. Many genes not previously studied in NBM, notably diarrhea-associated *cdtB* (cytolethal distending toxin [46%]) and urinary tract infection–associated *ompT* (outer membrane protease T [96%]), were as or more prevalent than traditional NBM-associated traits, such as *ibeA* (invasion of brain endothelium [33%]), *sfaS* (S fimbriae [59%]), and K1 capsule (81%). These findings provide novel insights into the phylogenetic origins of NBM-associated *E. coli* and suggest numerous new potential targets for preventive interventions against this dire disease.

Escherichia coli is one of the main causes of neonatal bacterial meningitis (NBM), a rare but potentially devastating illness that is associated with 17%–38% mortality and 58% long-term neurological sequelae [1–5]. Improved understanding of the causative *E. coli* strains is needed to guide the development of effective preventive measures.

Previous studies indicated that *E. coli* NBM isolates can be distinguished from the general *E. coli* population by their high prevalence of certain serotypes, such as O18:K1:H7, O83:K1, and O7:K1, and of certain virulence-associated factors (VFs), such as S fimbriae *(sfa),* K1 capsule, and invasion of brain endothelium *(ibeA)* [6–9]. These meningovirulent strains appear to derive predominantly from *E. coli* phylogenetic group B2 [10], which also is the source of most of the extraintestinal pathogenic *E. coli* (ExPEC) strains that cause urinary tract infections (UTIs) and bacteremia [11–16].

However, previous studies of NBM-derived *E. coli* isolates have several epidemiologic and methodologic limitations. They either have included only small numbers of definite NBM isolates or have pooled NBM isolates from diverse locales [6–10]. In addition, they have examined comparatively few extraintestinal VFs, compared with the broad range that is now recognized [15, 17]. The latter include properties such as cytolethal distending toxin (CDT) and the gene encoding it *(cdt),* which, although first described among diarrheogenic *E. coli* and in other species [18–24], are increasingly recognized as prevalent also among ExPEC, including strains causing UTI and/or septicemia in humans, dogs, cats, lambs, and calves [15, 17, 25, 26]. Finally, no previous study of NBM isolates has examined the population's clonal structure both broadly, in comparison with the *E. coli* reference (ECOR) collection, which provides a phylogenetic "benchmark" for clinical isolates [10, 27], and narrowly, according to individual clonal groups or serotypes within the broader phylogenetic divisions [7, 9].

Thus, we undertook the present study to define the phylogenetic background and VF profiles of a comparatively large population ($n = 70$) of recent consecutive NBM isolates from The Netherlands. Specifically, we sought to define the population's clonal structure and extended virulence genotypes to assess the phylogenetic distribution of VFs within the population

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and to identify associations between individual VFs, all in comparison with the ECOR strains.

Materials and Methods

Strains. Seventy anonymous cerebrospinal fluid (CSF) isolates from newborns (<28 days old) with NBM were analyzed. The isolates represented a randomly selected subset of the 87 such isolates referred to The Netherlands Reference Laboratory for Bacterial Meningitis (Amsterdam) from 1989 through 1997. This laboratory receives ~85% of meningitis isolates from children in The Netherlands [28]. These strains were characterized previously for O:K:H serotype by the National Institute of Public Health and the Environment (Bilthoven, The Netherlands). Data regarding underlying host status and clinical outcome were not available. As phylogenetic controls, members of the ECOR collection were obtained from Howard Ochman (University of Arizona, Tucson) [27] and the American Type Culture Collection. Strains were stored in 20% glycerol at −70° C until use.

Phylogenetic analysis. The 70 CSF isolates were categorized according to phylogenetic group on the basis of randomly amplified polymorphic DNA (RAPD) analysis, which provides a convenient surrogate for multilocus enzyme electrophoresis [29], as described elsewhere [16, 25, 30, 31]. Genomic profiles were generated in duplicate for each strain, using (separately) 2 arbitrary decamer primers (1281, 5′-AACGCGCAAC-3′, and 1283, 5′-GCGATCCCCA-3′) [32], to give a total of 4 profiles per strain. Amplification conditions were as described by Berg et al. [32], except that premade "Ready-to-Go" polymerase chain reaction (PCR) beads (Amersham Pharmacia Biotech) were used. These profiles were combined in a head-to-toe fashion to create a virtual composite genomic profile, 2 single profiles per composite profile and 2 composite profiles per strain. Comparable profiles were generated in parallel for 10 control strains (ECOR strains 4, 20, 69, 71, 62, 52, 39, 48, 50, and 42). These were arbitrarily selected to provide 1 (nonaligned strains), 2 (groups A, B1, and B2), or 3 (group D) representatives each from the 5 major ECOR phylogenetic groups, as defined by Herzer et al. [33], on the basis of electrophoretic mobility for 38 metabolic enzymes.

Phylogenetic analysis was done with the assistance of a computer software program (Molecular Analyst-Fingerprinting; BioRad). Pairwise Pearson correlation coefficient similarity values were generated for comparisons of each of the "unknown" strains to each of 10 ECOR control strains on the basis of analog densitometric scans of the composite profiles without subjective operator input. Unknown strains were assigned to the phylogenetic group of the most similar ECOR strain. If, during the first 2 rounds of amplifications with both primers, an unknown strain gave discrepant results with respect to which ECOR control strain it most closely resembled, it was amplified with each primer a third time, and the majority result was used. In addition, the similarity matrix from one set of composite genomic profiles was used to create a dendrogram according to the unweighted pair group method with averaging (UPGMA) [34].

Virulence genotypes. The 70 CSF isolates were tested for 25 different VFs of ExPEC, using membrane dot-blot hybridization under stringent conditions, as described elsewhere [17, 35, 36]. Probes were generated and digoxigenin-labeled, using primers described elsewhere [15, 17, 35, 37]. The VFs encompassed 5 categories: adhesins, toxins, siderophores, capsule, and miscellaneous. Adhesin genes included *papA, papG, sfa/focDE* (a consensus region in the S and F1C fimbrial operons), *sfaS* (the S fimbrial adhesin gene), *focG* (encoding a minor subunit of FIC fimbriae), *afa/draBC* (a consensus region in the afimbrial adhesin and Dr-binding adhesin operons other than *afa7* and *afa8*), *iha* (iron-regulated gene homologue adhesin, a recently described putative adhesin found in ExPEC and *E. coli* O157:H7), *bmaE* (M fimbriae), *gafD* (G fimbriae), and *nfaE* (nonfimbrial adhesin; none was detected). The toxins studied were *hly* (hemolysin), *cnf1* (cytotoxic necrotizing factor), and *cdt*. The siderophores studied were *iroN* (a novel putative catecholate siderophore) [37, 38], *fyuA* (yersiniabactin receptor) [39, 40], and *iutA* (aerobactin receptor). The capsule genes studied were *kpsMT* II (group II capsular polysaccharide synthesis) and *kpsMT* III (group III capsule synthesis; none was detected). Miscellaneous VFs included *rfc* (O4 lipopolysaccharide synthesis), *cvaC* (colicin V), *traT* (serum resistance associated), *ibeA, ompT* (outer membrane protease T), and *malX,* a marker for a pathogenicity-associated island (PAI) from archetypal ExPEC strain CFT073 (serotype O6:K2:H1) [41].

Seven VF regions (i.e., *sfa/focDE, sfaS, focG, cdtB, kpsMT* II, *kpsMT* [K1 variant], and *ompT*) also were detected by PCR, using primers and PCR conditions as described elsewhere [17, 35]. On the basis of previous findings, strains that were positive by dot-blot hybridization but negative by PCR for *kpsMT* II were considered to be K2 capsule positive [17].

Strains that were positive for any *pap* element were tested for 12 alleles of *papA* (F7-1, F7-2, F8-F16, and "F48") and for the 3 alleles of *papG* by using multiplex PCR-based assays, as described elsewhere [42]. All virulence genotypes were determined at least in duplicate, using boiled lysates prepared from 2 separate colonies of each strain. Discrepant results were investigated further, with additional determinations as needed. Virulence genotypes for the ECOR strains were previously determined in the laboratory of one of the investigators (J.R.J.), using similar methods, as reported elsewhere [15].

Phenotypic detection of CDT production. Selected *E. coli* isolates were assessed for production of CDT by exposing HeLa cells to either a sonic lysate or the supernatant of the bacterial culture

Table 1. Phylogenetic origin of 70 *Escherichia coli* bacterial meningitis (NBM) isolates from neonates in The Netherlands and of *E. coli* reference (ECOR) collection strains.

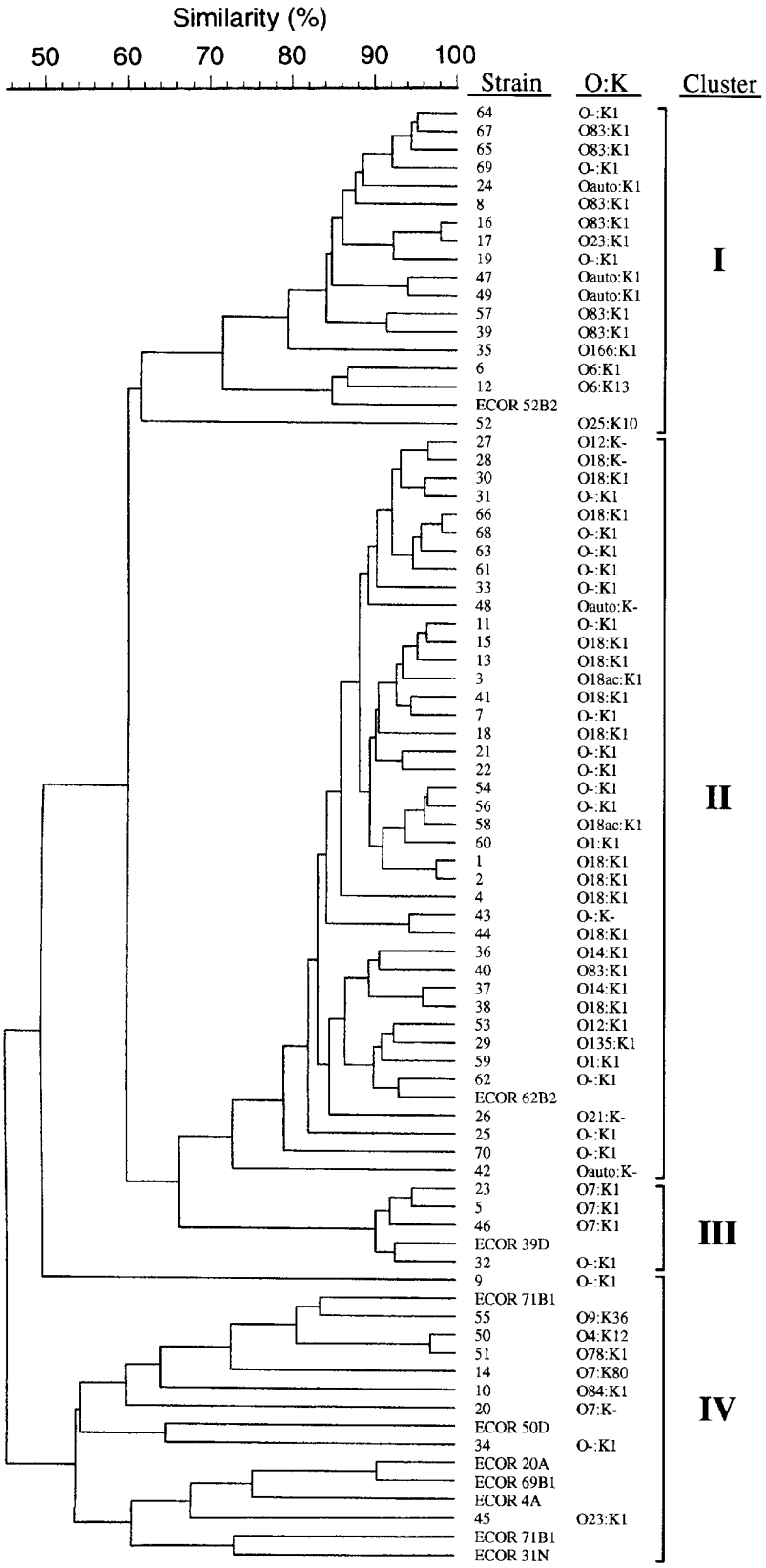
E. coli phylogenetic group[a]	Prevalence of phylogenetic group		
	NBM (n = 70)[b]	ECOR (n = 72)	P[c]
A	1 (1)	25 (35)	<.001
B1	7 (10)	16 (22)	.07
B2	57 (81)	15 (21)	<.001
D	5 (7)	12 (17)	>.10
Ungrouped	0	4 (6)	>.10

NOTE. Data are no. (%) of isolates, except where noted.

[a] Defined by Herzer et al. [33] on the basis of the electrophoretic mobility for 38 metabolic enzymes.

[b] Isolates from the present study.

[c] Fisher's exact test.


Similarity (%)
50
60
70
80
90
100
Strain
O:K
Cluster
64 O-:K1
67 O83:K1
65 O83:K1
69 O-:K1
24 Oauto:K1
8 O83:K1
16 O83:K1
17 O23:K1
19 O-:K1
47 Oauto:K1
49 Oauto:K1
57 O83:K1
39 O83:K1
35 O166:K1
6 O6:K1
12 O6:K13
ECOR 52B2
52 O25:K10
I
27 O12:K-
28 O18:K-
30 O18:K1
31 O-:K1
66 O18:K1
68 O-:K1
63 O-:K1
61 O-:K1
33 O-:K1
48 Oauto:K-
11 O-:K1
15 O18:K1
13 O18:K1
3 O18ac:K1
41 O18:K1
7 O-:K1
18 O18:K1
21 O-:K1
22 O-:K1
54 O-:K1
56 O-:K1
58 O18ac:K1
60 O1:K1
1 O18:K1
2 O18:K1
4 O18:K1
43 O-:K-
44 O18:K1
36 O14:K1
40 O83:K1
37 O14:K1
38 O18:K1
53 O12:K1
29 O135:K1
59 O1:K1
62 O-:K1
ECOR 62B2
26 O21:K-
25 O-:K1
70 O-:K1
42 Oauto:K-
II
23 O7:K1
5 O7:K1
46 O7:K1
ECOR 39D
32 O-:K1
III
9 O-:K1
ECOR 71B1
55 O9:K36
50 O4:K12
51 O78:K1
14 O7:K80
10 O84:K1
20 O7:K-
ECOR 50D
34 O-:K1
ECOR 20A
ECOR 69B1
ECOR 4A
45 O23:K1
ECOR 71B1
ECOR 31N
IV

and then by examining the HeLa cells for distension, growth arrest in G_2/M phase, nuclear swelling, and chromatin fragmentation, in comparison with appropriate positive and negative controls, as described elsewhere [24, 26].

Statistical methods. Comparisons involving the prevalence of a given trait in different populations were tested using Fisher's exact test. Comparisons involving the prevalence of different traits within the same population were tested using McNemar's test [43]. Associations between traits were assessed using Cramer's Φ coefficient [15]. Because of multiple comparisons, the threshold for statistical significance was $P < .01$, with $P < .05$ considered to reflect borderline statistical significance.

Results

Phylogenetic structure and serotypes. Composite RAPD analysis indicated that 57 (81%) of the 70 NBM isolates corresponded with *E. coli* phylogenetic group B2, 7 (10%) corresponded with group B1, 5 (7%) corresponded with group D, and 1 (1%) corresponded with group A ($P < .01$, prevalence of group B2 vs. all other groups, singly or combined, McNemar's test; table 1). Among the NBM isolates, the prevalence of groups B2 and A was significantly greater and significantly lower, respectively, than that among the members of the ECOR collection, which provided a surrogate for the *E. coli* population as a whole (table 1).

Cluster analysis by UPGMA revealed 4 major clusters among the NBM isolates (figure 1). The first 2 clusters (I and II), which were the most populous, were linked directly to one another and contained all the phylogenetic group B2 NBM isolates. Cluster I comprised predominantly O83:K1 NBM isolates (6 of 10 O-typeable isolates in this cluster). It also included a small subcluster comprising ECOR 52 (O25:[non-K1]:H1, the only ECOR strain in cluster I) and 2 O6:[non-K1] NBM isolates (figure 1). Cluster II, which included ECOR 62 (O2:K1:H4), comprised predominantly O18:K1 NBM isolates (14 of 24 O-typeable isolates). It also included 2 NBM isolates each of serotypes O1:K1 and O12:[K1 or K−], which were intermingled with the O18:K1 isolates rather than forming a separate subcluster (data not shown).

Cluster III consisted of ECOR 39 (O7:K1:H−, group D) and 4 O7:K1 NBM isolates, all putatively from group D (figure 1). Cluster IV consisted of various non–group B2 ECOR strains, 7 serologically diverse putative group B1 NBM isolates, and 1 NBM isolate each from groups A and D (figure 1).

Prevalence of VFs. In the population as a whole, the various VFs ranged in prevalence from >90% *(kpsMT* II, *ompT,* and *fyuA)* to 1% (*papG* allele III, *focG,* and *rfc*; table 2), with only *papG* allele I, *nfaE,* and *kpsMT* III not being detected in any strain. Additional VFs that occurred in >50% of the population included *sfa/focDE, sfaS, iroN, iutA,* the K1 *kpsMT* variant, *cvaC, traT,* and the PAI marker *malX* (table 2). Of note, *pap* elements and *ibeA* were present in only 29% and 33% of all isolates, respectively, whereas the diarrhea-associated toxin gene *cdtB* occurred in 46% of isolates. Because the observed prevalence of *cdtB* was so unexpectedly high, the dot-blot results were confirmed by PCR detection, which yielded highly comparable results (data not shown). In addition, all 12 *cdtB*-positive strains that were assessed phenotypically produced a CDT similar to that previously isolated from an enteropathogenic *E. coli* strain [44] (data not shown).

Phylogenetic distribution of VFs. Most of the VFs studied exhibited a significant nonrandom distribution among the 4 major phylogenetic clusters; the only exceptions involved VFs that were present in <10% or >90% of the isolates, plus *iha, iutA,* and *traT* (table 2). Conversely, each of the 4 clusters exhibited a significantly higher and/or lower prevalence of ⩾1 virulence genes than did the remainder of the study population (table 2). Traits that were significantly concentrated in cluster I included *sfa/foc, cdtB,* and *ibeA,* with similar borderline significant trends for *sfaS, focG, iroN,* and *malX* (table 2). Traits concentrated in cluster II included the F11 *papA* allele and K1 *kpsMT* variant (both with borderline significance; table 2). Cluster III was significantly associated with *papA,* the F10 *papA* allele, *papG,* and *papG* allele II, whereas cluster IV was significantly associated with *afa/dra, bmaE,* and the K2 *kpsMT* variant (table 2). As for negative associations, cluster I exhibited a decreased prevalence of *papG* allele II (borderline significance); cluster III was significantly negatively associated with *iroN, cvaC,* and *malX;* and cluster IV was significantly negatively associated with *sfa/foc, sfaS, cdtB, kpsMT* II, the K1 variant, *ibeA,* and *malX* (table 2).

When the population was stratified dichotomously as group B2 (clusters I and II combined) versus non–group B2 (clusters III and IV combined), VFs significantly associated with B2 status included the F11 *papA* allele, *sfa/foc, sfaS, cdtB, iroN, fyuA, kpsMT* II (by PCR), K1, *cvaC, ibeA,* and *malX* (table 2). In contrast, VFs associated with non–group B2 status included the F10 *papA* allele, *afa/dra, iha, bmaE,* and the K2 *kpsMT* variant (table 2).

Figure 1. Phylogenetic relationships among 70 neonatal bacterial meningitis (NBM)–associated *Escherichia coli* isolates, according to composite random amplified polymorphic DNA (RAPD) analysis. RAPD profiles for the 70 NBM isolates (strains 1–70) and 10 control strains from the *E. coli* reference (ECOR) collection, as generated using primers 1281 and 1283, were compared digitally and subjected to cluster analysis. Major phylogenetic clusters I, II, III, and IV (demarcated by brackets at right) were defined by visual inspection of the resulting dendrogram. Correspondence of these clusters with the major ECOR phylogenetic groups [33] was inferred from the placement of ECOR control strains (which are labeled as to ECOR number and phylogenetic group) within the RAPD-based dendrogram. O:K serotypes for the NBM isolates are shown adjacent to the corresponding strain name. Serotype abbreviations: Oauto, autoagglutinating (i.e., rough); O−, O-nontypeable; K−, K-nontypeable.

Table 2. Phylogenetic distribution of virulence factors (VFs) among 70 meningitis-associated *Escherichia coli* isolates from neonates.

VF	Total (n = 70)	Individual group				Combined groups		P[a]
		I (n = 16)	II (n = 41)	III (n = 4)	IV (n = 9)	I + II (n = 57)	III + IV (n = 13)	
papA	20 (29)	1 (6)[b]	12 (29)	4 (100)[c]	3 (33)	13 (23)	7 (54)	
F10	4 (6)	0	0	4 (100)[d]	0	0	4 (31)	.001
F11	9 (13)	0	9 (22)[b]	0	0	9 (16)	0	.04
papG	16 (23)	1 (6)	10 (24)	4 (100)[c]	1 (11)	11 (19)	5 (38)	
Allele II	14 (20)	0[b]	10 (24)	4 (100)[d]	0	10 (18)	4 (31)	
Allele III	1 (1)	1 (6)	0	0	0	1 (2)	0	
sfa/focDE	43 (61)	15 (94)[c]	27 (66)	1 (25)	0[d]	42 (74)	1 (8)	<.001
sfaS	41 (59)	13 (81)[b]	27 (66)	1 (25)	0[d]	40 (70)	1 (8)	<.001
focG	1 (1)	1 (6)[b]	0	0	0	1 (2)	0	
afa/draBC	18 (26)	3 (19)	7 (17)	0	8 (89)[d]	10 (18)	8 (62)	.003
iha	13 (19)	2 (13)	5 (12)	2 (50)	4 (44)	7 (12)	6 (46)	.011
bmaE	4 (6)	0	1 (2)	0	3 (33)[c]	1 (2)	3 (23)	.019
gafD	2 (3)	0	1 (2)	0	1 (11)	1 (2)	1 (8)	
hlyA	6 (9)	2 (13)	3 (7)	0	1 (11)	5 (9)	1 (8)	
cnf1	6 (9)	2 (13)	3 (7)	0	1 (11)	5 (9)	1 (8)	
cdtB	32 (46)	13 (81)[c]	19 (46)	0	0[c]	32 (56)	0	<.001
iroN	45 (64)	14 (88)[b]	28 (68)	0[b]	3 (33)	42 (74)	3 (23)	.001
fyuA	66 (94)	16 (100)	40 (98)	3 (75)	7 (78)	56 (98)	10 (77)	.019
iutA	43 (61)	10 (63)	27 (66)	2 (50)	4 (44)	37 (65)	6 (46)	
kpsMT II (b)[e]	68 (97)	16 (100)	40 (98)	4 (100)	8 (89)	56 (98)	12 (92)	
kpsMT II (P)[f]	60 (86)	16 (100)	38 (93)	4 (100)	2 (22)[d]	54 (95)	6 (46)	<.001
K1	57 (81)	14 (88)	37 (90)[b]	4 (100)	2 (22)[d]	51 (89)	6 (46)	.001
"K2"	8 (11)	0	2 (5)	0	6 (67)[d]	2 (4)	6 (46)	<.001
rfc	1 (1)	0	1 (2)	0	0	1 (2)	0	
cvaC	47 (67)	12 (75)	31 (76)	0[c]	4 (44)	43 (75)	4 (31)	.006
traT	58 (83)	12 (75)	36 (88)	2 (50)	8 (89)	48 (84)	10 (77)	
ibeA	23 (33)	10 (63)[c]	13 (32)	0	0[b]	23 (40)	0	.003
ompT	67 (96)	16 (100)	40 (98)	3 (75)	8 (89)	56 (98)	11 (85)	
malX	55 (79)	16 (100)[b]	38 (93)[d]	0[d]	1 (11)[d]	54 (95)	1 (8)	<.001

NOTE. Data are no. (%) of isolates, except where noted. *afa/draBC,* Dr-binding adhesins; *bmaE,* M fimbriae; *cdtB,* cytolethal distending toxin; *cnf1,* cytotoxic necrotizing factor 1; *cvaC,* colicin V; *focG,* F1C fimbriae; *fyuA,* yersiniabactin receptor; *gafD,* G fimbriae; *hlyA,* hemolysin; *ibeA,* invasion of brain endothelium A; *iha,* putative adhesin-siderophore; *iroN,* putative catecholate siderophore; *iutA,* aerobactin receptor; K1, K1 *kpsMT* II variant; "K2," putative K2 *kpsMT* II variant; *kpsMT* II, group II capsule synthesis; *malX,* marker for pathogenicity-associated island from strain CFT073; *ompT,* outer membrane protease T; *papA,* P fimbrial structural subunit; *papG,* P fimbrial adhesin molecule, with variants II and III; *rfc,* O4 lipopolysaccharide synthesis; *sfa/focDE,* S and F1C fimbriae; *sfaS,* S fimbrial adhesin; *traT,* serum resistance-associated outer membrane protein.

[a] P values (for comparison of combined groups [I + II] vs. [III + IV]) shown only when $P < .05$.

[b] $P < .05$, indicated group vs. all other strains.

[c] $P < .01$, indicated group vs. all other strains.

[d] $P < .001$, indicated group vs. all other strains.

[e] Detected by blot hybridization (b).

[f] Detected by polymerase chain reaction (P).

Comparisons between individual clusters with respect to the prevalence of individual VFs revealed substantial differences even within the same phylogenetic group. The 2 group B2 clusters (I and II) differed from one another by ≥20% with respect to the prevalence of several *pap* elements plus *sfa/foc, cdt, iroN,* and *ibeA* (table 2). Likewise, the 2 non–group B2 clusters (III and IV) differed from one another by ≥20% with respect to the prevalence of several *pap* elements plus *afa/dra, bmaE, iroN,* the K1 and K2 *kpsMT* variants, *cvaC,* and *traT* (table 2).

Virulence genotype comparison with the ECOR. Since many of the VFs analyzed are appreciably prevalent within *E. coli* in general, the present study population was compared with the ECOR collection, to more accurately assess which of them might be significantly associated specifically with NBM (table 3). Compared with the total ECOR, the NBM isolates exhibited a higher prevalence of multiple VFs, including the F11 *papA* allele, *sfa/foc, sfaS, afa/dra, cdtB, iroN, fyuA, iutA,* group II and K1 capsule genes, *cvaC, traT, ibeA, ompT,* and *malX.* In contrast, they exhibited a lower prevalence of *papG* allele III and *focG* (table 3).

However, these findings could simply reflect the disproportionately greater contribution of (virulence-associated) phylogenetic group B2 to the present NBM population (compared with the ECOR collection) in conjunction with the known concentration of many extraintestinal VFs in phylogenetic group B2. Consequently, a stratified analysis was done in which the 57 putative group B2 NBM isolates (clusters I and II) were compared with the 15 group B2 ECOR strains (table 2). Even with this group B2–specific analysis, the NBM isolates still differed substantially from the ECOR strains with respect to mul-

Table 3. Phylogenetic distribution of virulence factors (VFs) among 70 meningitis-associated *Escherichia coli* isolates from neonates, compared with the distribution among 72 strains from the *E. coli* reference (ECOR) collection.

	Total population			Phylogenetic group B2		
VF	Present study (n = 70)	ECOR (n = 72)	P[a]	Present study (n = 57)	ECOR (n = 15)	P[a]
papA	20 (29)	22 (31)		13 (23)	9 (60)	.01
F10	4 (6)	9 (13)		0	2 (13)	.04
F11	9 (13)	2 (3)	.03	9 (16)	1 (7)	
papG	16 (23)	19 (26)		11 (19)	9 (60)	.003
Allele II	14 (23)	11 (15)		10 (19)	3 (20)	
Allele III	1 (1)	8 (11)	.03	1 (2)	6 (40)	<.001
sfa/focDE	43 (61)	11 (15)	<.001	42 (74)	10 (67)	
sfaS	41 (59)	1 (1)	<.001	40 (70)	1 (7)	<.001
focG	1 (1)	9 (13)	.017	1 (2)	8 (53)	<.001
afa/draBC	18 (26)	3 (4)	<.001	10 (18)	1 (7)	
iha	13 (19)	23 (32)		7 (12)	6 (40)	.02
bmaE	4 (6)	0		1 (2)	0	
gafD	2 (3)	1 (1)		1 (2)	0	
hlyA	6 (9)	9 (13)		5 (9)	7 (47)	.002
cnf1	6 (9)	8 (11)		5 (9)	6 (40)	.008
cdtB	32 (46)	2 (3)	<.001	32 (56)	2 (13)	.004
iroN	45 (64)	16 (22)	<.001	42 (74)	10 (67)	
fyuA	66 (94)	46 (64)	<.001	56 (98)	15 (100)	
iutA	43 (61)	21 (29)	<.001	37 (65)	5 (33)	.04
kpsMT II (b)[b]	68 (97)	28 (39)	<.001	56 (98)	12 (80)	.03
kpsMT II (P)[c]	60 (86)	26 (36)	<.001	54 (95)	11 (73)	.03
K1	57 (81)	11 (15)	<.001	51 (89)	3 (20)	<.001
"K2"	8 (11)	2 (3)		2 (4)	1 (7)	
rfc	1 (1)	4 (6)		1 (2)	4 (27)	.006
cvaC	47 (67)	3 (4)	<.001	43 (75)	1 (7)	<.001
traT	58 (83)	22 (31)	<.001	48 (84)	1 (7)	<.001
ibeA	23 (33)	2 (3)	<.001	23 (40)	2 (13)	
ompT	67 (96)	44 (61)	<.001	56 (98)	14 (93)	
malX	55 (79)	21 (29)	<.001	54 (95)	15 (100)	

NOTE. Data are no. (%) of isolates, except where noted. *afa/draBC,* Dr-binding adhesins; *bmaE,* M fimbriae; *cdtB,* cytolethal distending toxin; *cnf1,* cytotoxic necrotizing factor 1; *cvaC,* colicin V; *focG,* F1C fimbriae; *fyuA,* yersiniabactin receptor; *gafD,* G fimbriae; *hlyA,* hemolysin; *ibeA,* invasion of brain endothelium A; *iha,* putative adhesin-siderophore; *iroN,* putative catecholate siderophore; *iutA,* aerobactin receptor; K1, K1 *kpsMT* II variant; "K2," putative K2 *kpsMT* II variant; *kpsMT* II, group II capsule synthesis; *malX,* marker for pathogenicity-associated island from strain CFT073; *ompT,* outer membrane protease T; *papA,* P fimbrial structural subunit; *papG,* P fimbrial adhesin molecule, with variants II and III; *rfc,* O4 lipopolysaccharide synthesis; *sfa/focDE,* S and F1C fimbriae; *sfaS,* S fimbrial adhesin; *traT,* serum resistance-associated outer membrane protein.

[a] P values shown only when $P < .05$.

[b] Detected by blot hybridization (b).

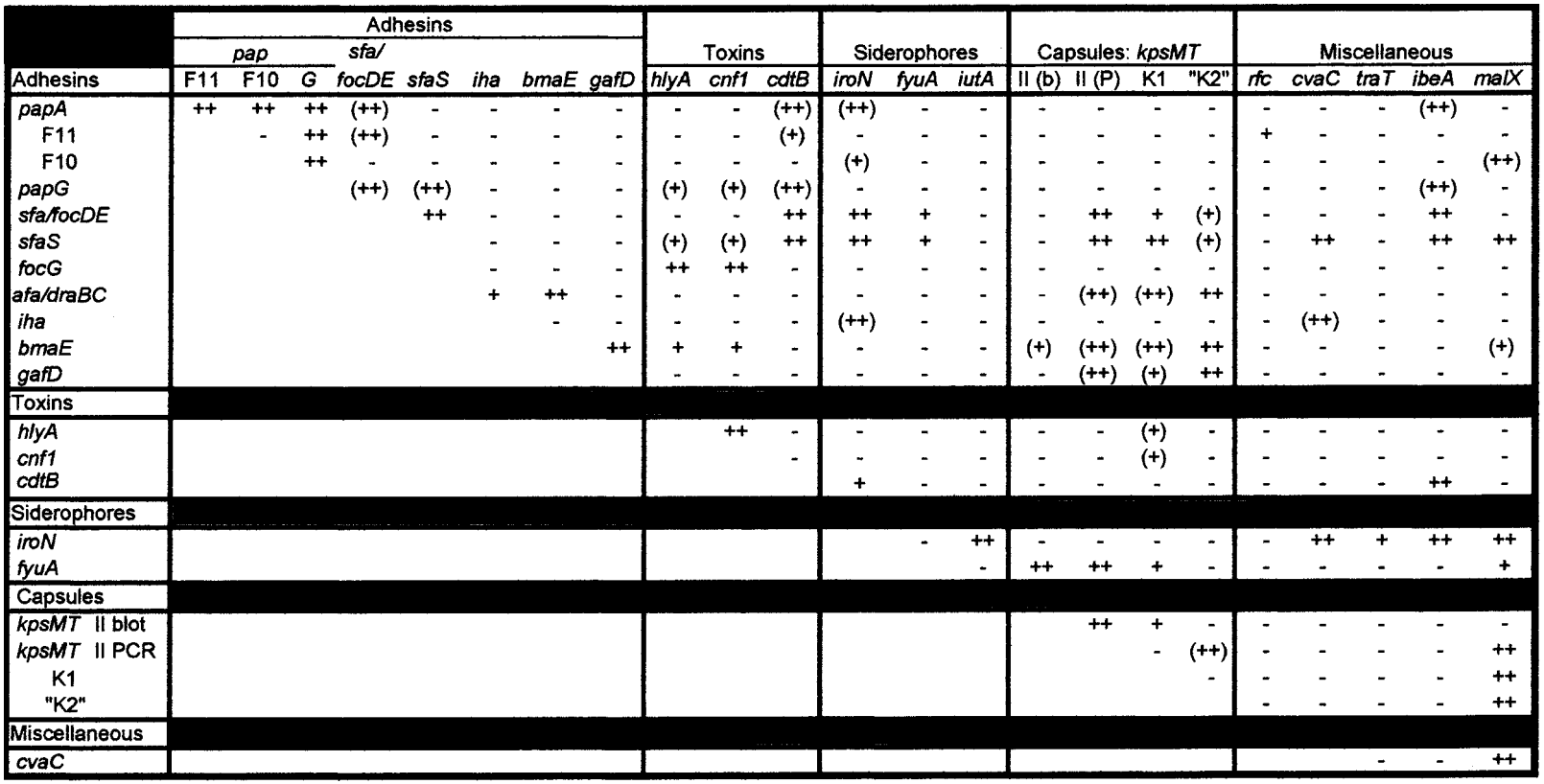
[c] Detected by polymerase chain reaction (P).

tiple VFs (table 3). Although several previously significant differences were no longer evident, the group B2 NBM isolates still exhibited a greater prevalence of *sfaS, cdtB, iutA,* group II and K1 capsule genes, *cvaC,* and *traT* than did the B2 ECOR strains (table 3). In addition, the group B2 NBM isolates now exhibited a lower prevalence than did the ECOR strains of multiple VFs other than the previously noted *papG* allele III and *focG,* including *papA, papG, iha, hlyA, cnf,* and *rfc* (table 3).

Associations between VFs. Within the total NBM population, the various VFs exhibited multiple distinctive positive and negative associations with one another, many of which contrasted with associations previously described among isolates from patients with urosepsis or cystitis or within the ECOR collection as a whole (figure 2). Of note were the negative associations of *sfa* and *foc* with *pap* elements, *hly,* and *cnf;* the negative association of *cdtB* with *pap* elements but its positive associations with *sfa* elements and *ibeA;* and the negative associations of *iroN* with *pap* elements (figure 2).

Discussion

This clonal and pathotypic analysis of 70 NBM *E. coli* isolates from The Netherlands represents the largest and the only population-based study of its type to date. Our findings confirm the previously described predominance of phylogenetic group B2 and the rarity of phylogenetic group A among NBM isolates



	Adhesins								Toxins			Siderophores			Capsules: *kpsMT*				Miscellaneous				
	pap			*sfa/*																			
Adhesins	F11	F10	*G*	*focDE*	*sfaS*	*iha*	*bmaE*	*gafD*	*hlyA*	*cnf1*	*cdtB*	*iroN*	*fyuA*	*iutA*	II (b)	II (P)	K1	"K2"	*rfc*	*cvaC*	*traT*	*ibeA*	*malX*
papA	++	++	++	(++)	-	-	-	-	-	-	(++)	(++)	-	-	-	-	-	-	-	-	-	(++)	-
F11		-	++	(++)	-	-	-	-	-	-	(+)	-	-	-	-	-	-	-	+	-	-	-	-
F10			++	-	-	-	-	-	-	-	-	(+)	-	-	-	-	-	-	-	-	-	-	(++)
papG				(++)	(++)	-	-	-	(+)	(+)	(++)	-	-	-	-	-	-	-	-	-	-	(++)	-
sfa/focDE					++	-	-	-	-	-	++	++	+	-	-	++	+	(+)	-	-	-	++	-
sfaS						-	-	-	(+)	(+)	++	++	+	-	-	++	++	(+)	-	++	-	++	++
focG						-	-	-	++	++	-	-	-	-	-	-	-	-	-	-	-	-	-
afa/draBC						+	++	-	-	-	-	-	-	-	-	(++)	(++)	++	-	-	-	-	-
iha							-	-	-	-	-	(++)	-	-	-	-	-	-	-	(++)	-	-	-
bmaE								++	+	+	-	-	-	-	(+)	(++)	(++)	++	-	-	-	-	(+)
gafD									-	-	-	-	-	-	-	(++)	(+)	++	-	-	-	-	-
Toxins																							
hlyA										++	-	-	-	-	-	-	(+)	-	-	-	-	-	-
cnf1											-	-	-	-	-	-	(+)	-	-	-	-	-	-
cdtB												+	-	-	-	-	-	-	-	-	-	++	-
Siderophores																							
iroN													-	++	-	-	-	-	-	++	+	++	++
fyuA														-	++	++	+	-	-	-	-	-	+
Capsules																							
kpsMT II blot																++	+	-	-	-	-	-	-
kpsMT II PCR																	-	(++)	-	-	-	-	++
K1																		-	-	-	-	-	++
"K2"																			-	-	-	-	++
Miscellaneous																							
cvaC																					-	-	++

Figure 2. Correlations between different virulence-associated factors (VFs) among 70 meningitis-associated *Escherichia coli* isolates from neonates. Only those VFs that yielded ⩾1 association at the $P < .01$ level are shown. Significance codes: −, $P > .01$; +, $P < .01$; ++, $P < .001$; parentheses indicate a negative association. For *kpsMT* (group II capsule synthesis), "blot" denotes detection by dot-blot, "PCR" denotes detection by polymerase chain reaction, "K1" indicates the K1 *kpsMT* variant, and "'K2'" indicates detection of putative *kpsMT* II by blot but not by PCR, consistent with the K2 *kpsMT* variant [17]. Identity of other VFs is as follows: *afa/draBC,* Dr-binding adhesins; *bmaE,* M fimbriae; *cdtB,* cytolethal distending toxin; *cnf1,* cytotoxic necrotizing factor 1; *cvaC,* colicin V; *focG,* F1C fimbriae; *fyuA,* yersiniabactin receptor; *gafD,* G fimbriae; *hlyA,* hemolysin; *ibeA,* invasion of brain endothelium A; *iha,* putative adhesin-siderophore; *iroN,* putative catecholate siderophore; *iutA,* aerobactin receptor; *malX,* marker for pathogenicity-associated island from strain CFT073; *papA,* P fimbrial structural subunit; *papG,* P fimbrial adhesin molecule, with variants II and III; *sfaS,* S fimbrial adhesin; *rfc,* O4 lipopolysaccharide synthesis; *sfa/focDE,* S and F1C fimbrial; and *traT,* serum resistance-associated outer membrane protein.

[10]. The findings provide novel insights into the prevalence of and associated VFs of specific clonal groups within the NBM population, and they greatly expand the range of VFs studied in this clinical context, including several "new" VFs (e.g., *cdt, fyuA, traT,* and *ompT*) that were as or more prevalent than certain traditional NBM-associated VFs, such as *sfaS,* the K1 capsule, and *ibeA* [6–10].

Previous studies of NBM-associated *E. coli* either have included only a small number of confirmed NBM isolates within a larger population of predominantly neonatal septicemia isolates [6, 7, 9] or have pooled NBM isolates from diverse locales [8, 10]. They also have analyzed convenience samples assembled on the basis of unspecified selection criteria, in some instances including previously published reference strains [6–10]. In contrast, the present population consisted of 70 randomly selected strains representing 80% of the CSF isolates from Dutch neonates as submitted to The Netherlands Reference Laboratory for Bacterial Meningitis between 1989 and 1997. All isolates were previously uncharacterized except for O:K serotype. Thus, this study provides the first opportunity to examine the prevalence of clonal groups and VFs within a defined *E. coli* NBM population in the absence of selection bias. However, because of the geographically circumscribed study population, the ability to more broadly generalize these results needs to be assessed in future population-based studies.

By including 24 different VFs plus the 3 *papG* alleles and the 12 *papA* alleles, the present study greatly exceeded the scope of previous studies of NBM-derived *E. coli,* which addressed no more than 8 VFs each [6–10]. In addition, to our knowledge, the present study is the first to resolve NBM isolates both into the major phylogenetic groups of *E. coli* and into constituent clonal groups and subclones thereof. Correlation of these bacterial traits with host characteristics and clinical outcomes would make a valuable addition to future such studies.

The observed striking predominance of phylogenetic group B2 among the present NBM isolates is consistent with previous findings from every analyzed collection of extraintestinal infection isolates of *E. coli,* regardless of clinical syndrome [11, 12, 14, 16, 25, 45, 46], and contrasts markedly with the minority contribution of group B2 to the general *E. coli* population [33]. This skewed distribution of phylogenetic groups among extraintestinal clinical isolates has been thought to reflect a special virulence capability of B2 strains for causing extraintestinal infections [12, 15, 47]. The 81% prevalence of group B2 is the highest noted to date in any collection of extraintestinal *E. coli* and contrasts with the 68% prevalence documented by Bingen et al. [10] among 69 NBM isolates ($P = .08$). In contrast to group B2, group A, which is associated with commensal status in humans [27], was represented by only 1 isolate in the present population, similar to the 6% prevalence found by Bingen et al. [10] among their NBM isolates.

The seemingly greater virulence of group B2 strains among human clinical isolates has been presumed to be due to the numerous extraintestinal VFs that are concentrated within this phylogenetic group [13, 48, 49]. Indeed, direct experimental assessment of extraintestinal virulence [12] showed that, although the group B2 strains were the most virulent overall, VF repertoire actually was a stronger independent predictor of virulence than was phylogenetic background [49]. In the present study, the group B2 NBM isolates had a significantly higher prevalence of many VFs than even the group B2 ECOR strains, most (73%) of which are fecal rather than clinical isolates [27]. This suggests that the present population represents primarily an extra-high virulence subset from within phylogenetic group B2. Whether the observed pathotypic differences between the group B2 NBM isolates and the group B2 ECOR strains actually reflect NBM-specific VFs (as has been inferred elsewhere [10]) or, instead, generic extraintestinal VFs, remains to be determined by direct comparisons between NBM isolates and clinical isolates from other clinical syndromes. It should be noted that the ECOR strains were not selected randomly [27]; hence, they are not an ideal substrate for epidemiologic analyses.

The 2 most prevalent clonal groups in the present population, represented by clusters I and II, correspond largely with the 2 predominant O:K serotypes that have been identified in previous studies of NBM and neonatal sepsis (i.e., O83:K1 and O18:K1). Cluster II (predominantly O18:K1) clearly corresponds with ECOR 62, a known O2:K1:H4 strain that exhibits a VF profile characteristic of the O1:K1 and O-:K1 isolates from cluster II [15]. Previous analyses have shown that NBM-derived strains of serotypes O1:K1, O2:K1, and O18:K1 are quite closely related [7, 50]. However, consistent with our findings, European isolates of *E. coli* O18:K1 typically represent the outer membrane protein pattern (OMP) 9 subclone of *E. coli* O18:K1:H7 and lack *pap,* in contrast to the typically *pap*-positive members of the OMP 6 subclone of O18:K1:H7, which predominates in the United States in both NBM and cystitis [6, 30, 51–53]. Thus, it is noteworthy that, with few exceptions [52, 54], recent molecular analyses of O18:K1:H7 NBM isolates, including those from European investigators, have focused on representatives of the OMP 6 subclone of *E. coli* [55–58].

Cluster I, which consisted predominantly of O83:K1 strains, was characterized by a high prevalence not only of classic meningitis-associated traits, such as *sfaS,* K1, and *ibeA,* but also of *cdt* (81%). CDTs constitute a family of genetically related bacterial proteinic toxins that can block the proliferation of numerous cell lines by triggering a host-cell signaling pathway that prevents the transition between the G2 and M phases of the cell cycle [26, 59, 60]. Although CDT has yet to be conclusively shown to contribute to virulence in vivo, a possible antiproliferative effect on epithelial or endothelial membranes [59] and/or inhibition of T and B cell function [61] are possible mechanisms for such an effect.

CDT was originally described among enteropathogenic *E. coli* and other diarrhea-associated *E. coli* variants [18, 19, 23], but it recently has been found to occur also among *E. coli* iso-

lates from extraintestinal infections in humans and dogs [15–17, 25, 35, 46]. Among extraintestinal isolates, *cdt* has been encountered in only 2 clonal groups (i.e., *E. coli* O2:K5/K7:H1 and *E. coli* O6:K53:H1) [15–17, 25, 31, 62]. The present study provides novel evidence of *cdt* also within the O83:K1 clonal group and, to a lesser extent, the O18:K1 clonal group. Within each of these group B2–derived clusters (i.e., clusters I and II), the prevalence of *cdtB* (81% and 68%, respectively) was actually higher than that of *ibeA* (63% and 32%, respectively).

Whether CDT contributes directly to the pathogenesis of NBM warrants empirical assessment. Of note, experimental evidence supports another "non–meningitis-associated" cytotoxin, CNF, as a contributor to invasion of brain microvascular endothelial cells by NBM isolate RS218, a member of the OMP 6 subclone of *E. coli* O18:K1:H7 [56]. Of interest, members of the American OMP 6 subclone of *E. coli* O18:K1:H7, such as archetypal NBM isolate RS218, characteristically lack *cdt* [25, 30, 63].

In comparison with the members of clusters I and II, the members of (non–group B2) clusters III and IV exhibited different rather than simply fewer VFs. This result, plus the balanced phylogenetic distribution of certain VFs, such as *iutA, traT,* and *ompT,* conflicts with previously proposed models for the acquisition of extraintestinal VFs by *E. coli*. According to the previously proposed models, phylogenetic group B2 was the original source of extraintestinal VFs within the species, with VFs later migrating horizontally from group G2 to other lineages [13, 64]. Such models are based on a limited set of VFs that indeed are concentrated within phylogenetic groups B2 and/or D. Analysis of a broader array of VFs has suggested considerably more complex evolutionary histories for the various extraintestinalVFs of *E. coli* than those posited for *pap, hly, sfa,* and *kps* [15–17].

We found that many VFs were as or more prevalent in the NBM population than were the major traditional NBM-associated VFs. For example, more prevalent than the K1 *kpsMT* variant (81%) were *fyuA* (94%), *traT* (83%), and *ompT* (96%); more prevalent than *sfaS* (59%) were *iroN* (64%), *iutA* (61%), *cvaC* (67%), and *malX* (79%); and approximating the prevalence of *ibeA* (33%) were *cdtB* (46%, as discussed above), *papA* (29%), and *afa/dra* (26%). The high prevalence of *ompT,* which encodes an outer membrane protease (OmpT) and has been epidemiologically associated with UTI [65, 66], is of interest in view of the recent evidence that OmpT may contribute to invasion of human endothelial cells, a necessary step in the pathogenesis of NBM [67]. Of note, not included in our analysis was *fimH,* which is almost universally prevalent within *E. coli* [15].

These prevalence values do not mean that the traits studied necessarily contribute to NBM. Nevertheless, they do suggest the possibility of numerous additional targets for protective interventions if a virulence role for any of these traits in NBM can be confirmed in vivo. The results of subtractive hybridization analysis of NBM isolate C5 [55, 56] and of genome mapping of strain RS218 [57, 68], together with the high prevalence of PAI marker *malX* noted here, suggest that numerous additional VFs await discovery within the PAIs of NBM-derived *E. coli*.

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