ACTA PÆDIATRICA
NURTURING THE CHILD

Acta Pædiatrica ISSN 0803-5253

REGULAR ARTICLE

Escherichia coli virulence patterns may help to predict vesicoureteral reflux in paediatric urinary tract infections

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Keywords
Escherichia coli infection, urinary tract infection, vesicoureteral reflux

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Received
11 March 2015; accepted 16 June 2015.

DOI:10.1111/apa.13092

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ABSTRACT

Aim: Ultrasound and biological tools are used to predict high-grade vesicoureteral reflux, but other markers are needed to better select patients who need voiding cystography. Our aim was to determine whether studying *Escherichia coli* virulence factors would help to predict vesicoureteral reflux in patients with their first acute pyelonephritis.

Methods: We included children presenting with *E. coli*-related acute pyelonephritis or cystitis. Vesicoureteral reflux was assessed by voiding cystography. Virulence factors were identified by multiplex polymerase chain reaction. Statistical analysis was performed using logistic regression and the mean c-statistic test.

Results: We included 198 patients: 30 with cystitis and 168 with acute pyelonephritis, including 46 with vesicoureteral reflux. High-grade reflux was associated with acute pyelonephritis caused by the *E. coli* lacking virulence factors papGII (82% versus 47%, $p < 0.001$) or papC (85% versus 53%, $p < 0.001$) or belonging to phylogenetic group A or B1. When we added genetic data (lack of papGII, fyuA and phylogenetic groups) to classical predictors of vesicoureteral reflux (ultrasound examination, gender, age), the ability to predict high-grade reflux increased, with the c-statistic rising from 0.88 to 0.93.

Conclusion: Bacterial virulence factors and clinical factors helped to predict high-grade reflux and may help to avoid unnecessary voiding cystographies.

INTRODUCTION

Urinary tract infections (UTIs) are among the most common infections in children, and 1–2% of children will present with at least one UTI before the age of 1 year. The cumulative incidence of UTIs in children under 6 years old is estimated to be 3–7% in girls and 1–2% in boys (1). *Escherichia coli* is the most frequent bacteria involved (2). As with any infection, it is the association between the characteristics of the patient and the bacteria that results in a UTI (3). On the one hand, a patient with urological abnormalities, such as vesicoureteral reflux, megaureter, pelviureteric junction or ureteral duplication, is more likely to develop a UTI. On the other hand, the study of *E. coli* virulence factors implicated in germ adhesion to the urothelium, such as papGII adhesion, is more frequent in acute pyelonephritis than in cystitis and in acute pyelonephritis without reflux than with reflux (4).

Abbreviations

chuA, *Escherichia coli* haeme uptake A; cnf1, Cytotoxic necrotising factor-1; fyuA, Ferric yersiniabactin uptake receptor; hek/hra, Haemagglutinin and heat-resistant agglutinin.; hlyC, Haemolysin-activating lysine-acyltransferase; iroN, Salmochelin siderophore receptor; iucC, Aerobactin synthesis genes; papC, papGII, papGIII – P fimbriae; PCR, Polymerase chain reaction; sfa/foc, Fimbriae adhesin factors; UTI, Urinary tract infection.

Key notes

- Searching for high-grade vesicoureteral reflux after acute pyelonephritis remains challenging and may result in unnecessary voiding cystographies.
- Our study of 198 patients, 30 with cystitis and 168 with acute pyelonephritis, found that presenting with acute pyelonephritis caused by less virulent strains usually found in cystitis, predicted high-grade reflux.
- Considering bacterial virulence factors together with ultrasound and biological parameters may help to predict high-grade vesicoureteral reflux and reduce unnecessary voiding cystographies.

Furthermore, vesicoureteral reflux and repeated acute pyelonephritis are known to induce renal scars and to expose to renal failure and hypertension. This explains why children with acute pyelonephritis have been systematically screened for vesicoureteral reflux by voiding cystography because isolated ultrasound is not sufficient to diagnose reflux (5). However, Craig et al. (6) showed that increasing the treatment of vesicoureteral reflux did not improve the renal outcome of the patients and it has been shown that prophylactic antibiotic treatment did not decrease either the number of acute pyelonephritis episodes or the development of renal scars, at least in low-grade reflux (grade I to III) over a 1-year follow-up (7,8). Thus, systematic screening by voiding cystography of all children with acute pyelonephritis is discussed more often because of UTI secondary to voiding cystography, radiation and the cost of this strategy. As a result, guidelines have not recommend cystography after a first acute pyelonephritis (9,10). This explains why studies have focused on finding new markers to predict high-grade reflux (IV and V), which exists in about 10% of children with their first acute pyelonephritis, without performing a cystography in all children. Leroy et al. (11) demonstrated that the procalcitonin blood level was an independent predictive factor of high-grade vesicoureteral reflux with a specificity of 46%, but a greater sensitivity of 86%. The same group showed that pelvic and/or ureteral dilation was associated with high-grade reflux (12). Houdouin et al. (4) screened virulence factors of *E. coli* involved in UTI and showed that *E. coli* from the phylogenetic group A, or the lack of papGII virulence gene, predicted underlying urinary tract abnormalities with a specificity of 90%, but a sensitivity of 58%.

In our monocentric retrospective study, the primary outcome was to evaluate whether the study of nine *E. coli* virulence genes – sfa/foc, papC, papGII, papGIII, fyuA, hlyC, iucC, cnf1, iroN, chuA and hek/hra – and of *E. coli* phylogenetic groups would help to predict high-grade reflux.

The examined virulence factors can be briefly summarised in three categories:

1 *adhesins and invasins,* such as fimbriae adhesin factors (sfa/foc), P fimbria (papC, papGII, papGIII), and haemagglutinin and heat-resistant agglutinin (hek/hra).
2 *iron uptake system proteins,* such as ferric yersiniabactin uptake receptor (fyuA); haemolysin-activating lysine-acyltransferase (hlyC); aerobactin synthesis genes (iucC), a hydroxamate siderophore; salmochelin (iroN), a glucosylated siderophore; and chuA, an outer membrane protein responsible for haeme uptake. In Gram-negative bacteria, specific outer membrane receptors facilitate the import of iron-chelating siderophores and iron from host organisms. Uropathogenic *E. coli* utilises a wide range of receptors to acquire iron, an essential nutrient from within the iron-limited urinary tract.
3 *toxins* such as the cytotoxic necrotising factor-1 (cnf1), which alters host cell actin cytoskeleton and promotes bacterial invasion. The function of these virulence genes is briefly explained in Figure 1.

METHODS

Study population

We retrospectively included all children under 18 years old who consulted the emergency care unit of Armand Trousseau Hospital with an *E. coli*-related acute pyelonephritis over two consecutive years, together with all patients with *E. coli*-related cystitis over a 1-year period.

Acute pyelonephritis was defined as a positive urine sample associated with fever >38.5°C and a C-reactive protein level >25 mg/dL and/or procalcitonin >0.5 ng/mL and/or leucocytes >15 000/mm^3. Cystitis was defined as the association of urinary symptoms without fever and a positive urine sample. To be eligible for the study, we required a kidney and urinary tract ultrasound and a voiding cystography to determine the presence of reflux and its grade according to the international classification. Voiding cystography was performed in patients after a first acute pyelonephritis with urinary tract abnormalities on ultrasound or after a second one with normal ultrasound. In patients with elevated procalcitonin serum levels and

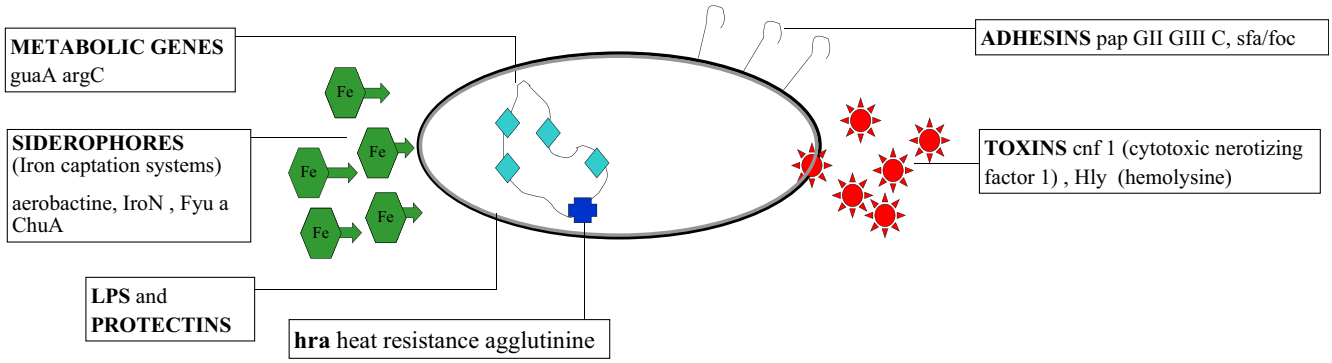

Figure 1 Function of *Escherichia coli* virulence factors (sfa/foc, papC, papGII, papGIII, fyuA, hlyC, iucC, cnf1, iroN, chuA, hek/hra) relevant for urinary tract infection.

normal ultrasound, no voiding cystography was performed after a first acute pyelonephritis.

Urine was collected upon admission in a sterile bag or with the midstream clean-catch technique if the patient was able to urinate voluntarily. Samples were cultured within 1 h of collection, and the resulting isolates were stored at −80°C until characterisation. A positive urine sample was defined as significant bacteriuria (>100 000 CFU/mL) with pyuria (>100 000 leucocytes / mL) to reduce the risk of selection bias induced by potential sample contamination.

Virulence genes study

Virulence genes (sfa/foc, papC, papGII, papGIII, fyuA, hlyC, iucC, cnf1, iroN, chuA, hek/hra) and major phylogenetic groups (A, B1, D and B2, with identification of subgroup $B2_1$) were studied by multiplex polymerase chain reaction (PCR) as previously described (13).

Statistical analysis

The association between each clinical variables, ultrasound results and virulence factor was studied by univariable logistic regression. Variables with a p-value < 0.20 were included in the multivariable models. To estimate the increase of the predictive ability induced by virulence factors determination, we tested three models. Model 1 only included clinical and ultrasound variables. Model 2 included clinical and ultrasound variables, as well as the lack of papGII or belonging to phylogenetic group A, which are the factors already known to be associated to high-grade reflux. Model 3 included all previously mentioned factors as well as the presence of fyuA. The accuracy of the model to predict high-grade reflux was assessed with the mean c-statistic that tests the discrimination ability of the model and the Hosmer–Lemeshow goodness-of-fit test that assesses the matching between the prediction of reflux by the model and its presence.

We performed a sensitivity analysis on patients who were <4 years old.

To assess the reliability of our results, we compared the distribution of *E. coli* virulence factors and phylogenetic groups between patients with acute pyelonephritis and with cystitis to check that our results were similar to those in the literature.

Statistical analysis was performed with SAS software 9.2 (SAS Institute, Inc, Cary, NC, USA).

RESULTS

We included 168 patients with acute pyelonephritis, 104 girls and 64 boys. Of these, 16 presented with high-grade reflux, 30 presented with low-grade reflux, and 122 had no such abnormalities. The patients' characteristics are presented in Table 1.

Male gender, younger age and ultrasound abnormalities were associated with high-grade reflux (p-value < 0.2) and thus were included in the multivariable models.

With regard to virulence factors, the lack of papC, papGII or chuA, the presence of fyuA and belonging to phylogenetic groups A and B1 were associated with high-grade reflux (Table 2). As papC and papGII are highly correlated, we only included papGII in the models. ChuA is used to determine phylogenetic groups, and groups A and B1 are always chuA negative, and groups B2 and D are always chuA positive. Therefore, phylogenetic groups were included, but not chuA.

Table 3 presents the results of the three multivariable models. Clinical variables and ultrasound examination alone demonstrated good discrimination with a c-statistic of 0.88 (Model 1). We found an increase in the model discrimination when adding the lack of papGII and belonging to the *E. coli* phylogenetic group A (Model 2: c-statistic 0.90). When adding the presence of fyuA and the phylogenetic group in five groups, the c-statistic increased

Table 1 Patients' characteristics

	High-Grade VUR		No High-Grade VUR		
	N	%	N	%	p-value
Gender (male)	9	56.25	55	36.18	0.12
US abnormality	12	92.31	37	25.34	<0.0001
	Median	IQ	Median	IQ	
Age (months)	8.98	(4.21–21.09)	4.28	(1.98–9.93)	0.09
Total number of patient		16		152	168

Table 2 Virulence factor prevalence in acute pyelonephritis strains from patients without versus with high-grade vesicoureteral reflux

	APN without HG VUR		APN with HG VUR		
Virulence factor	N	%	N	%	p
sfa/foc	42	27.6	5	31.3	0.76
papC	127	84.1	9	56.3	0.01
papGIII	13	8.6	2	12.5	0.6
papGII	123	80.9	8	50.0	<0.01
iroN	81	53.3	10	62.5	0.48
fyuA	144	95.4	14	87.5	0.2
iucC	147	96.7	15	93.8	0.3
chuA	146	96.1	10	62.5	<0.001
hlyC	55	36.2	6	37.5	0.92
cnf1	37	24.3	3	18.8	0.62
hek/hra	41	27.2	5	31.3	0.73
Phylogenetic groups					
B2	67	43.5	6	37.5	0.002
$B2_1$	33	21.4	1	6.3	
D	48	31.2	3	18.8	
B1	1	0.7	2	12.5	
A	5	3.3	4	25.0	
Total	152		16		

Table 3 Prediction of reflux by multivariable models

	Variables included	Mean c-statistics
Model 1	Gender, US abnormality, age	0.88
Model 2	Model 1 + lack *papGII*+Group A	0.90
Model 3	Model 2 + Groups + *fyuA*	0.93

to 0.93 (Model 3). All three models showed no significance with the goodness-of-fit test, indicating that the prediction of vesicoureteral reflux by those three models fitted well with what was found in our patients by voiding cystography. The sensitivity analysis on patients under the age of four showed similar results with a c-statistic that increased from 0.86 to 0.92. These patients are supposed to have more high-grade reflux than those with a first acute pyelonephritis later in life.

We analysed patients with cystitis to assess whether the bacterial profile in our population was comparable with what has been previously published in other populations. The analysis included 30 cases of cystitis, 26 girls and four boys, with a median age of 7.4 years and IQR of 3.9–8.4. Univariable analysis found that the presence of papC, papGII, iroN, fyuA, iucC, chuA and hlyC was associated with an increased risk of acute pyelonephritis, whereas belonging to phylogenetic group A was associated with cystitis (Table 4). The multivariate analysis only revealed that *E. coli* with papGII was associated with acute pyelonephritis (OR 3.32, CI 95% 1.12–9.88).

Table 4 Virulence factor prevalence in cystitis strains versus acute pyelonephritis strains

	Cystitis		APN		
Virulence factor	N	%	N	%	p
sfa/foc	5	16.7	47	28.0	0.2
papC	19	63.3	136	81.4	0.03
papGIII	4	13.3	15	8.9	0.45
papGII	12	40.0	131	78.0	<0.0001
iroN	8	26.7	91	54.2	0.007
fyuA	25	83.3	158	94.6	0.04
iucC	20	66.67	151	89.88	0.001
chuA	23	76.7	156	92.86	0.009
hlyC	6	20.0	61	36.31	0.09
cnf1	4	13.3	40	23.8	0.21
hek/hra	10	33.3	46	27.5	0.52
Phylogenetic groups					
B2	9	30.0	71	42.3	0.02
$B2_1$	1	3.3	34	20.2	
D	13	43.3	51	30.4	
B1	1	3.3	3	1.8	
A	6	20.0	9	5.4	
Total	30		168		

DISCUSSION

Phylogenetic analysis has shown that *E. coli* is composed of four main phylogenetic groups (A, B1, B2 and D) and that virulent extra-intestinal strains mainly belong to groups B2 and D. *Escherichia coli* strains that cause extra-intestinal *E. coli* infections represent a highly specialised subset of the total *E. coli* population. The enhanced virulence of those strains is caused by the association of multiple virulence factors (14). Many studies have investigated the relation between the virulence factors and the type of UTI in adults (15,16) or in renal transplant recipients (17), but few have investigated this association and the impact of reflux in children. Houdouin et al. (4) found that acute pyelonephritis with *E. coli* lacking papGII and belonging to phylogenetic group A were significantly more frequent in patients with urinary tracts abnormalities. This finding is consistent with other published data (16,18,19).

The studied pap genes code for adherence via fimbria, which have been given names such as P fimbria, gal-gal adhering fimbria and mannose-resistant haemagglutination. In the past, other researchers have studied phenotypic features of *E. coli* and phenotypic characteristics of infected patients (20–23) and found that bacterial attachment can be a predictor of renal abnormalities (24).

Phenotypic analysis of *E. coli* is possible in a clinical setting at the time of culture as reported previously with either a P fimbriae kit or haemagglutination. That study found significantly more virulent *E. coli* strains in acute pyelonephritis, compared to cystitis, and in patients with high-grade reflux, compared to those without high-grade reflux (24). These phenotypic data on virulence factors are consistent with our genetic analysis.

Urine flow is the most important host defence factor in the urinary tract, and adhesion to the urothelium, to overcome the effects of urine flow, is therefore the most important virulence factor (25). Other bacterial species than *E. coli* are more common in children with urological abnormalities because they lack the ability to adhere to the urothelium. Marcus et al. (26,27) showed that urological abnormalities and reflux were more frequent in patients with UTI due to enterococcal species and pseudomonas aeruginosa, respectively, compared to *E. coli*.

We found similar results since presenting an acute pyelonephritis caused by *E. coli* strains lacking papGII or belonging to phylogenetic group A were statistically associated with high-grade reflux. As expected, we found similar results for the lack of papC, which is highly correlated with papGII or chuA and which is used to define phylogenetic groups. We also found an association between the presence of fyuA, virulence factor from the iron-binding subgroup and high-grade reflux. The importance of iron binding is explained by the use of oxygen as an electron acceptor in bacteria. This requires the protection of the cell membranes against peroxide radicals. Several enzymes that destroy the harmful radicals, such as peroxidases and catalases, contain iron, and therefore, iron plays an important role in the maintenance of the cell.

We did not find any significant association in multivariable statistical analysis between acute pyelonephritis and the presence of haemolysin (hlyC) or cytotoxic necrotising factor-1 (cnf1), whereas studies have found such an association on phenotypic levels (28,29). Apart from this difference, the results of the multivariable analysis of the phenotypic virulence factors (28) were similar to our genetic study.

The inverse association between virulence factors and reflux has already been reported, but fewer studies have investigated what a study of *E. coli* virulence factors would add to classical strategies to diagnose high-grade reflux. Our results underline the importance of clinical examinations and ultrasound examinations as those criteria allowed a good discrimination of patients with high-grade reflux, showing a c-statistic of 0.88 when 0.7 is usually considered as an acceptable discrimination power. This reinforces current guidelines.

However, our results showed that studying *E. coli* virulence genes, especially papGII, fyuA and phylogenetic groups, may improve our capacity to predict high-grade reflux as the discrimination of our model increased after adding those variables (c-statistic 0.93). As the study of *E. coli* virulence genes using multiplex PCR is nowadays both convenient and inexpensive, it may be a valuable tool to improve the rate of high-grade reflux diagnosis while carrying on the current top-down strategy to decrease the number of voiding cystographies.

The main strength of our study is that we have shown both the inverse association between virulence genes and high-grade reflux and the added value – to biological parameters and ultrasound – in predicting high-grade reflux. We also performed a secondary analysis of the distribution of virulence factors among our patients with acute pyelonephritis and a group of patients with cystitis to ascertain that our findings were similar to those in the literature.

However, our study has several limitations. For some patients, either C-reactive protein or procalcitonin was not available, which prevented us from studying the association between those factors and high-grade reflux. Leroy et al. (30) demonstrated the good sensitivity (86%) of procalcitonin, with an external validation study showing a sensitivity of only 64%.

Despite the reduced sensitivity in the validation study, many protocols of acute pyelonephritis management are still based on the association between ultrasound and procalcitonin blood levels. However, the low specificity of procalcitonin induces a high number of voiding cystographies in children without reflux. Thus, studies are needed to assess the value of procalcitonin together with clinical examination and ultrasound examination to predict high-grade reflux and to evaluate its association with *E. coli* virulence genes.

The other limitations include some methodological considerations that should be taken into account while evaluating these findings. Firstly, as we selected our patients on clinical and biological criteria, and not based on a DMSA scan, there might have been a selection bias that led us to consider that some patients with cystitis had acute pyelonephritis. However, this effect would decrease the associations, not modify them.

Finally, because of the relatively small size of our sample, we were unable to perform an internal validation study. As our sample was used to create the model and evaluate its discrimination ability, other studies on different samples would be required to assess the reproducibility of our results.

CONCLUSION

In conclusion, acute pyelonephritis in patients with high-grade reflux is induced by less virulent strains than acute pyelonephritis in patients without high-grade reflux. In our study, strains from phylogenetic group A, usually found in cystitis, could cause acute pyelonephritis in association with a high-grade reflux and strains lacking papGII (and papC) or presenting the fyuA virulence gene.

Clinical factors together with bacterial virulence factors can help in deciding which children are at risk of high-grade reflux. The most important virulence factor is adhesion with P fimbria, which could be very rapidly tested for in all *E. coli* strains. Further studies are needed to examine models that combine clinical parameters, ultrasound, virulence genes and procalcitonin to predict high-grade reflux and assess the cost-effectiveness of such a strategy.

ACKNOWLEDGEMENTS

This study did not receive any external funding.

CONFLICTS OF INTEREST

The authors have no conflict of interest to declare.

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