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High Prevalence of Virulence Genes and *in Vitro* Biofilm Production in Clinical Multidrug-Resistant *Escherichia coli* in Dakar Senegal

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Abstract: Bacterial virulence is a key factor determining the outcome of each bacterial infection, and virulent bacteria are often associated with high-risk infections. Thus, this study aimed to screen for virulence genes and evaluate the *in vitro* biofilm formation capacity of multidrug-resistant *Escherichia coli* isolated in Dakar. For the 16 virulence genes identified by standard polymerase chain reaction (PCR), all 78 ExPEC isolates carried at least four virulence genes. The prevalence of virulence genes was as follows: adhesin genes *fimH* (98.7%), *mrkD* (98.7%), *papC* (46.2%), *afaC* (9%), and *sfa/focDE* (1.3%); iron acquisition system genes *entB* (98.7%), *fepA* (98.7%), *ybtS* (93.6%), *fyuA* (91%), *iucA* (91%), *iucB* (91%), *iutA* (34.6), *iroB* (6.4%), *iroN* (6.4%), and toxin genes *hlyA* (10.3%) and *cnf (1 & 2)* (10.3%). Seventy-five of the 78 isolates (96.2%) carried at least two adhesin genes and two iron capture system genes. Evaluation of the biofilm formation capacity revealed that all (29/29) hospital-acquired isolates were biofilm producers with (6/29; 20.7%) strong biofilm producers, (15/29; 51.7%) moderate biofilm producers and (8/29; 27.6%) weak biofilm producers. Hospital-acquired isolates carrying *papC* had a greater biofilm formation capacity than those lacking *papC* ($p < 0.001$). The deepening of this type of study on bacterial virulence and hospital bacterial biofilms could lead to improvements in infection investigation, prevention, and therapeutic protocols.

Keywords: Virulence Genes, Virulence Factors, Extraintestinal Pathogenic *Escherichia coli*, Biofilms, Biofilm-Associated Infections

1. Introduction

Bacterial infections are the second leading cause of death in developing countries [1-3]. In addition to host risk factors such as immune status, age, diet, and stress, the two main factors determining the outcome of each bacterial infection are the virulence of the involved bacteria and their resistance to antibiotics. In fact, the virulence of a bacterium is defined as its ability to colonize, invade, and cause damage to the host [4, 5], and virulent bacteria are often associated with infections with a high risk of death [5, 6].

Escherichia coli is the most implicated pathogen in human bacterial infections, and the extraintestinal pathotype (ExPEC) is the leading cause of urinary tract infections (UTI), nosocomial pneumonia, bacterial septicemia, and neonatal meningitis [7-10]. The incidence of ExPEC infections is constantly increasing [11].

Most ExPECs isolated from humans are multidrug-resistant (MDR) organisms [12, 13], which express a wide variety of virulence factors [14-16] and are responsible for high mortality rates, such as up to 18% for bacteremia [17, 18].

Virulence factors of ExPECs encoded by plasmid or chromosomal genes are classified into five categories: adhesins, toxins, iron capture systems, protectins or protective systems against the immune system, and invasins [19, 20] (Table 1). ExPECs usually operate through a combination of virulence factors [21].

Bacterial biofilms are structured clusters of bacteria coated with a polymeric matrix and attached to a surface. Similar or different bacterial species coexist within a biofilm and often communicate with each other. This makes the bacterial biofilm a favorable platform for the exchange of intra- and interspecific genetic materials (virulence and drug resistance genes). Bacteria use quorum sensing (QS) to regulate gene expression in response to fluctuations in bacterial population density in biofilms. Biofilm formation generally occurs in four main steps: bacterial attachment to a surface, formation of microcolonies, maturation of the biofilm, and detachment and dispersal of bacteria to colonize new areas. Surface attachment is considered to be the most important step among the four [22], and some virulence factors such as type-1 fimbriae (*fimH*), type-3 fimbriae (*mrkD*), P fimbriae (*papC*), F1C fimbriae (*foc*), enterobactin (*entB*, *fepA*), yersiniabactin (*ybtS*, *fyuA*), and hemolysin (*hlyA*) are known to play key roles in bacterial surface attachment. In biofilms, bacteria display exceptional resistance to environmental stresses such that they can be up to 1000-fold more resistant to antibiotics and disinfectants than planktonic bacteria [23, 24]. Bacteria growing in biofilms cause approximately 60-80% of human microbial infections such as osteomyelitis, dental caries, chronic bacterial prostatitis, native valve endocarditis, otitis, cystic fibrosis, and periodontitis. Moreover, bacterial biofilms are the main cause of hospital-acquired infections [25–27]. The most common hospital biofilm-associated infections are those that develop in central venous catheters, mechanical heart valves, urinary catheters, contact lenses, intrauterine devices, beds, benches, doors, electronic devices, and equipment. Biofilms are also associated with recurrent UTIs [28]. Biofilms are a major public health problem that should be studied, monitored, and eradicated, particularly in hospital settings [29–33].

In Senegal and West Africa, several studies have focused on the phenotypic aspects of drug resistance, resistance genes, and mobile genetic support. To the best of our knowledge, few studies have focused on the virulence of circulating bacterial pathogens. Therefore, our study aimed to investigate virulence genes in MDR ESBL-producing ExPEC strains isolated in the bacteriology laboratory of the Aristide le Dantec University Teaching Hospital in Dakar (HALD). Moreover, according to the prevalence of virulence genes found, we sought to compare community-acquired strains to hospital-acquired strains and ExPECs isolated from UTI (UPEC) to non-uropathogenic ExPECs. Finally, we evaluated the biofilm-forming capacity of the hospital-acquired isolates.

Table 1. *ExPEC virulence factors, functions and their genetic markers.*

Virulence factors categories	Virulence factors	genes or operons	Functions
Adhesins	Type 1 fimbriae	*fim*	Binding to the epithelial cells, internalization factor, colonization factor, biofilm formation. Immune evasion factor [34]
	Type 3 fimbriae	*mrkD*	Binding to host cells and extracellular matrix proteins. Promote biofilm formation on biotic and abiotic surfaces [35]
	P fimbriae (Pilus associated with pyelonephritis)	*pap*	Responsible for adhesion to mucosal and tissue matrix and for the production of cytokines. Promote biofilm formation on biotic and abiotic surfaces [36]
	S fimbriae	*sfa*	Adhesion to bladder, kidney and brain endothelium. helps bacteria to better penetrate tissues [37]
	F1C fimbriae	*foc*	Important for adherence to epithelial and endothelial cells of bladder and kidney. Required for biofilm formation on an inert surface [38]
	Afimbrial adhesin	*afa*	Agglutinate human erythrocytes. Recognize decay-accelerating factor (DAF) as a receptor, which is a complement regulatory protein present on the surface of many human epithelial cells including epithelial cells of the urinary tract [37, 39]
	Dr fimbriae	*dra*	Recognize decay-accelerating factor (DAF) and Dr blood group antigen like receptors and agglutinate human erythrocytes [37, 39]

Virulence factors categories	Virulence factors	genes or operons	Functions
Toxins	Hemolysin A	*hlyA*	Stimulate acute inflammatory response + recruitment of polymorphonuclear neutrophils and cause tissue damage [19]
	Cytotoxic necrotizing factor	*cnf (1&2)*	Lead to cell cycle alterations, formation of megalocytic, multinucleated cells and then to cell necrosis [40]
	Secreted autotransporter toxin	*sat*	Serine protease and cytopathic activity on kidney, bladder and other cell lines [41]
	Protease involved in intestinal colonization	*pic*	Degrades mucins, facilitates epithelium colonization and damages of the cell membrane [42]
Iron uptake systems	Yersiniabactin (Iron repressible protein + Ferric yersiniabactin uptake receptor)	*ybtS, fyuA*	Enhance ferric iron uptake and decreasing iron sequestration in cells, siderophore uptake transmembrane transporter activity. Signaling receptor activity. Biofilm formation [43, 44]
	Aerobactin (aerobactin biosynthesis and ferric aerobactin receptor)	*iuc, iut*	Siderophore, acquisition of $Fe^{2+/3+}$ in the host system, Ferric aerobactin receptor [45]
	Salmochelin	*iro*	Traps intracellular iron [46]
	Enterobactin (biosynthesis and ferric-enterobactin receptor)	*ent* *fepA*	Enhance ferric iron uptake, reduce reactive oxygen species and promote biofilm formation [47, 48]
Invasins	invasion of endothelial brain protein	*ibeA, B, C*	Cell invasion into the host tissues [49]
	associated outer membrane protein	*traT*	Inhibition of the classical pathway of complement activity and serum survival [20]
Protectins / serum resistance	Outer membrane protein	*omp*	mediates bacterial biofilm formation, antibiotic resistance and immunomodulation. Enable intracellular survival, evasion from the body's defense [50]
	Capsula antigens	*KpsMI-neuA, KpsMII*	Promote resistance immunological tolerence and protect from phagocytocis. Enhance intracellular survival favoring bacteremia and meningitis [20]
	Increased serum survival	*iss*	Resistance to complement and serum survival, protection factor against phagocytosis [20]

2. Materials and Methods

2.1. Bacterial Isolates

In the present study, we used 78 non-repetitive isolates from a previously published study [51]. Antibiotic susceptibility patterns obtained in this previous study showed that all 78 isolates were MDR and extended-spectrum beta-lactamase (ESBL)-producers. These strains were isolated in the bacteriology laboratory of Aristide le Dantec University Teaching Hospital between January 2018 and March 2020. The details (sex and age) of patients from which bacterial strains were isolated were not available to us. Among these isolates, 51 were isolated from UTIs (UPEC) and 27 from pus, sputum, bronchial fluids, and vaginal secretions (non-uropathogenic ExPEC), whereas 49 were community-acquired (CA) and 29 were hospital-acquired (HA). Among the 29 hospital-acquired isolates, 14 were isolated from urine (HA UPEC) and 15 from pus and bronchial fluid (HA No-UPEC). Bacterial isolation and identification were performed using Eosin Methylene Blue (EMB) agar (Merck KGaA, Darmstadt, Germany) and Api 20E for *Enterobacteriaceae* (bioMérieux, Lyon, France).

2.2. Extraction of Bacterial DNA

Bacterial DNA was extracted from pure bacterial cultures using the thermolysis method. For each strain, a few pure and well-separated colonies were diluted in 1 ml of sterile distilled water, vortexed, boiled for 15 min at 100°C, and centrifuged at 13,200 rpm for 10 min. The supernatant was carefully collected, aliquoted, and stored at -20°C until use. DNA quantification was performed using an Invitrogen™ Qubit™ 3 Fluorometer (Thermo Fisher Scientific Inc., Strasbourg, France).

2.3. Virulence Genes Amplification

Each DNA sample was subjected to simplex end-point PCR on a Thermocycler 2720 Applied Biosystems (Lincoln Centre Drive, Foster City, California, USA) using specific primers (Table 2). The virulence genes identified were *fimH, mrkD, afaC, papC, sfa/focDE, entB, fepA, ybtS, fyuA, iucA, iucB, iutA, iroB, iroN, cnf (1 & 2)*, and *hlyA*. Each round of amplification included both the positive and negative controls. J96 strain was used as a positive control for amplification of *papC, sfa/focDE, hlyA*, and *cnf (1 & 2)*. A30 was used as the positive control for *afaC* and *fimH*. Strains previously positive for *entB, fepA, iucA, iucB, iutA, iroB, iroN, ybtS, fyuA*, and *mrkD* in our laboratory were used as positive controls for the amplification of these genes during this study. The primer sequences, hybridization temperatures, and amplicon sizes are listed in Table 2. The total reaction volume (2.5 µl DNA + 17.5 µl Master Mix FIREPol®). Amplification programs consisted of initial denaturation at 95.0°C for 3 min, 35 PCR cycles (denaturation: 94.0°C, 30s; hybridization; elongation: 72°C, 60s) and final elongation at 72.0°C for 7 min. Ten microliters of each amplicon was separated on a 2% agarose gel in 1X TAE buffer for 35 min at 135 V, and amplified fragments were visualized by UV using a GelDoc imager (BioRad).

2.4. Biofilm Formation Assay

Biofilm formation (adhesion capacity) of only the

hospital-acquired strains was assessed, using the method described by [52]. Briefly, we prepared bacterial suspensions with densities adjusted to 0.5 standard McFarland. Then 250 µl of brain-heart broth was distributed in each well of a sterile 96-well flat-bottomed plastic tissue culture plate (Thermo Fisher Scientific Inc., Göteborg, Sweden). To these wells, 20 µl of the initially prepared bacterial suspensions was added. The negative control wells contained only brain-heart broth, and we used previously known biofilm-forming capacity strains as positive controls. Negative controls, positive controls, and isolates were tested in triplicate. The plates were incubated under aerobic conditions for 24 h at 35°C. The contents of each well were aspirated, and the wells were washed three times with 300 µl sterile distilled water. Bacteria attached to the wells were fixed with 200 µl of methanol in each well. After 15 min, the plates were emptied and allowed to air-dry. The plates were then stained for 5 min with 160 µl of crystal violet per well. Excess stain was rinsed with running water. After air-drying, the stain bound to the adherent cells was extracted with 160 µl of 33% (v/v) glacial acetic acid (Merck KGaA, Darmstadt, Germany) per well. The optical density of each well was measured at 570 nm using an ICN Flow Titertek Multiscan Plus automated reader (SPW Industrial, Laguna Hills, California, USA).

Optical densities (OD) were interpreted according to [53], and isolates were classified into the following categories: non-biofilm producers, weak biofilm producers, moderate biofilm producers, or strong biofilm producers. We defined the cut-off OD (ODc) for the microtiter plate test as three standard deviations above the mean OD of the negative control. Strains were classified as follows: OD ≤ ODc (non-biofilm producers), ODc < OD ≤ 2 x ODc (weak biofilm producers), 2 x ODc < OD ≤ 4 x ODc (moderate biofilm producers), and 4 x ODc ≤ OD (strong biofilm producers).

2.5. Statistical Analysis

Data analysis was performed using Microsoft-Excel. We used Chi-square at 5% risk and p-values are obtained from the proportion comparison test. Statistical significance was established at $p < 0.05$.

Table 2. *Primers used to detect virulence genes.*

Target genes	Sequences genes	Sizes (bp)	Annealing Temp (°C)	References
fimH	F: 5' - TGCAGAACGGATAAGCCGTGG - 3' R: 5' - GCAGTCACCTGCCCTCCGGTA - 3'	508	56	[54]
mrkD	5'- CCACCAACTATTCCCTCGAA - 3' 5'- ATGGAACCCACATCGACATT - 3'	240	52	[55]
afaC	F: 5' - CGGCTTTTCTGCTGAACTGGCAGGC - 3' R: 5' - CCGTCAGCCCCCACGGCAGACC - 3'	672	65	[56]
papC	1: 5' - GACGGCTGTACTGCAGGGTGTGGCG - 3' 2: 5' - ATATCCTTTCTGCAGGGATGCAATA - 3'	331	65	[56]
sfa/focDE	1: 5' - CTCCGGAGAACTGGGTGCATCTTAC - 3' 2: 5' - CGGAGGAGTAATTACAAACCTGGCA - 3'	410	65	[56]
fyuA	F: 5' - TGATTAACCCCGCGACGGGAA - 3' R: 5' - CGCAGTAGGCACGATGTTGTA - 3'	880	58	[54]
ybtS	F: 5' – AGTGGTGCGTTCTGCGTC - 3' R: 5' - ATTTCTACATCTGGCGTTA - 3'	447	50	[57]
iucA	F: 5' – ATAAGGGAAATAGCGCAGCA - 3' R: 5' – TTACGGCTGAAGCGGATTAC - 3'	212	60	[58]
iucB	F: 5' – CCACGAATAGTGACGACCAA - 3' R: 5' – GTTTTTGATGCAGAGCGTGA - 3'	339	60	[58]
iutA	5'- GGCTGGACATCATGGGAACTGG - 3' 5'- CGTCGGGAACGGGTAGAATCG - 3'	300	58	[59]
iroB	5'- CAACCATCGGTTTGACAGTG - 3' 5'- GACGTAACACCGCCGAGTAT - 3'	166	55	[58]
iroN	5'- CTTCCTCTACCAGCCTGACG - 3' 5'- GCTCCGAAGTGATCATCCAT - 3'	648	55	[58]
entB	5'- GCGACTACTGCAAACAGCAC – 3' 5' – TTCAGCGACATCAAATGCTC – 3'	382	55	[58]
fepA	5' – TTTGTCGAGGTTGCCATACA – 3' 5' – CACGCTGATTTTGATTGACG – 3'	349	55	[58]
cnf (1&2)	F: 5' - TTATATAGTCGTCAAGATGGA - 3' R: 5' - CACTAAGCTTTACAATATTGAC - 3'	636	50	[60]
hlyA	s: 5' - AGATTCTTGGGCATGTATCCT - 3' as: 5' - TTGCTTTGCAGACTGTAGTGT - 3'	556	55	[15]

3. Results

3.1. Virulence Genes

All the 78 isolates carried at least 4 virulence genes out of 16 sought with an average of 9 out of 16. Following prevalence of virulence genes were reported: adhesins genes *fimH* (98.7%), *mrkD* (98.7%), *papC* (46.2%), *afaC* (9%), *sfa / focDE* (1.3%); iron acquisition systems genes *entB* (98.7%), *fepA* (98.7%), *ybtS* (93.6%), *fyuA* (91%), *iucA* (91%), *iucB*

(91%), *iutA* (34.6), *iroB* (6.4%), *iroN* (6.4%) and toxins genes *hlyA* (10.3%), *cnf (1 & 2)* (10.3%) (Table 3). We noticed that UPEC isolates carried more *papC* than non-uropathogenic strains (p = 0.03). When comparing CA to HA, no significant difference was noted (Table 3). *fimH* and *mrkD* was present in all UPEC isolates (51/51) and *fimH* was detected in all hospital-acquired strains (29/29). Out of the 16 virulence genes sought, the number of genes per strain varied from 4 to 15. The hospital-acquired strains carried on average 9 virulence genes out of 16 while Community-acquired strains carried 8. UPEC isolates on their part carried on average 9 virulence genes out 16 while non-uropathogenic ExPEC isolates carried 8. The accumulation of virulence genes is reported in table 4. 97.4% (76/78) carried simultaneously (*fimH* + *mrkD*) while (64; 82.1%) carried the combination of (enterobactin + aerobactin + yersiniabactin) iron uptake system genes. 97.4% (76/78) carried simultaneously siderophore genes and their siderophore-ferric iron receptor genes. Eight out of 78 (10.3%) harbored (adhesins + iron acquisition systems genes + toxins genes). Seventy-five out of 78 (96.2%) of isolates carried at least the combination of two adhesins genes and two iron capture system genes while (8/78; 10.3%) of isolates carried at least the combination of two adhesins genes and three iron capture systems genes. Among the 78 strains studied, one hospital-acquired strain isolated from urine harbored 15 virulence genes out of 16 sought.

Table 3. *Prevalence of virulence genes in total strains, CA and HA strains, UPEC and non-UPEC strains.*

Virulence factors		Virulence genes	Total strains	Pathogenicity			Origin		
			N (%)	UPEC N (%)	Non-UPEC N (%)	p	CA N (%)	HA N (%)	p
Adhesins		*fimH*	77 (98.7)	51 (100)	26 (96.3)	0.17	48 (98)	29 (100)	0.44
		mrkD	77 (98.7)	51 (100)	26 (96.3)	0.17	49 (100)	28 (96.6)	0.19
		papC	36 (46.2)	28 (54.9)	8 (29.6)	0.03*	22 (44.9)	14 (48.3)	0.77
		sfa / focDE	1 (1.3)	1 (2)	0	0.46	0	1 (3.4)	0.19
		afaC	7 (9)	3 (5.9)	4 (14.8)	0.19	4 (8.2)	3 (10.3)	0.74
Toxins		*hlyA*	8 (10.3)	6 (11.8)	2 (7.4)	0.54	4 (8.2)	4 (13.8)	0.43
		cnf (1&2)	8 (10.3)	6 (11.8)	2 (7.4)	0.54	4 (8.2)	4 (13.8)	0.43
Iron uptake systems	yersiniabactin	*ybtS*	73 (93.6)	48 (94.1)	25 (92.6)	0.79	46 (93.9)	27 (93.1)	0.89
		fyuA	71 (91)	46 (90.2)	25 (92.3)	0.72	45 (91.8)	26 (89.7)	0.91
	aerobactin	*iucA*	71 (91)	47 (92.2)	24 (88.9)	0.63	46 (93.9)	25 (86.2)	0.25
		iucB	71 (91)	47 (92.2)	24 (88.9)	0.63	46 (93.9)	25 (86.2)	0.25
	salmochelin	*iutA*	27 (34.6)	16 (31.4)	11 (40.7)	0.41	17 (34.7)	10 (34.5)	0.98
		iroB	5 (6.4)	3 (5.9)	2 (7.4)	0.79	3 (6.1)	2 (6.9)	0.89
		iroN	5 (6.4)	3 (5.9)	2 (7.4)	0.79	3 (6.1)	2 (6.9)	0.89
	enterobactin	*entB*	77 (98.7)	51 (100)	26 (96.3)	0.17	49 (100)	28 (96.6)	0.19
		fepA	77 (98.7)	51 (100)	26 (96.3)	0.17	49 (100)	28 (96.6)	0.19

UPEC, Uropathogenic *E. coli*; CA, Community-acquired; HA, Hospital-acquired; *, significant p-value (p < 0.05).

3.2. Biofilm Formation Assays

The evaluation of biofilm formation capacity of the 29 hospital-acquired strains revealed that all isolates were biofilm producers with (6/29; 20.7%) strong biofilm producers, (15/29; 51.7%) moderate biofilm producers and (8/29; 27.6%) weak biofilm producers (Figure 1). We noticed that hospital-acquired isolates from urine had greater biofilm formation capacity than hospital-acquired isolates from pus and bronchial fluids. We noticed also that isolates carrying *papC* had greater biofilm formation capacity than those lacking *papC* (p = 0.0001) (Table 5).

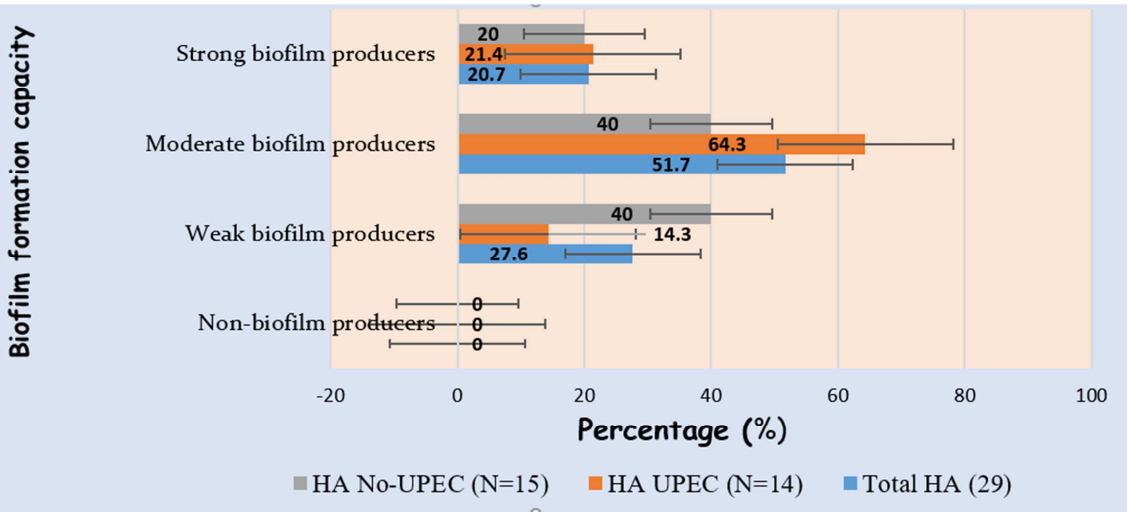

Figure 1. *biofilm formation capacity of hospital-acquired strains.*

Table 4. *Distribution of virulence genes combinations in total strains, CA and HA strains, UPEC and non-UPEC strains.*

Combination of virulence genes	Total strains	Pathogenicity		Origin	
	N (%)	UPEC N (%)	No-UPEC N (%)	CA N (%)	HA N (%)
fimH + mrkD + papC + entB + fepA + ybtS + fyuA + iucA + iucB + iutA + iroB + iroN + hlyA + cnf (1&2) + sfa / foc DE	1 (1.3)	1 (2)	0	0	1 (3.4)
fimH + mrkD +papC + entB + fepA + iucA + iucB + iutA + ybtS + fyuA + hlyA + cnf (1&2)	2 (2.6)	2 (3.9)	0	1 (2)	1 (3.4)
fimH + mrkD + papC + entB + fepA + iucA + iucB + iutA + iroB + iroN + ybtS + fyuA	1 (1.3)	0	1 (3.7)	1 (2)	0
fimH + mrkD + papC + entB + fepA + ybtS + fyuA + iucA + iucB + hlyA + cnf (1&2)	4 (5.1)	2 (3.9)	2 (7.4)	2 (4.1)	2 (6.9)
fimH + mrkD + papC + entB + fepA + iucA + iucB + iroB + iroN + ybtS + fyuA	2 (2.6)	2 (3.9)	0	1 (2)	1 (3.4)
fimH + mrkD + entB + fepA + iucA + iucB + iutA + iroB + iroN + ybtS + fyuA	1 (1.3)	0	1 (3.7)	1 (2)	0
fimH + mrkD + papC + entB + fepA + iucA + iucB + iutA + ybtS + fyuA	7 (9)	6 (11.8)	1 (3.7)	5 ()	2 (6.9)
fimH + mrkD + afaC + entB + fepA + iucA + iucB + iutA + ybtS + fyuA	2 (2.6)	1 (2)	1 (3.7)	1 (2)	1 (3.4)
fimH + mrkD + entB + fepA + ybtS + fyuA + hlyA + cnf (1&2)	1 (1.3)	1 (2)	0	1 (2)	0
fimH + mrkD + papC + entB + fepA + iucA + iucB + ybtS + fyuA	15 (19.2)	13 (12.3)	2 (7.4)	10 (20.4)	5 (17.2)
fimH + mrkD + entB + fepA + iucA + iucB + iutA + ybtS + fyuA	9 (11.5)	4 (7.8)	5 (18.5)	5	4 (13.8)
fimH + mrkD + afaC + entB + fepA + iucA + iucB + ybtS + fyuA	5 (6.4)	2 (3.9)	3 (11.1)	3 (6.1)	2 (6.9)
fimH + mrkD + papC + entB + fepA + iucA + iucB + iutA + ybtS	2 (2.6)	1 (2)	1 (3.7)	1 (2)	1 (3.4)
fimH + mrkD + entB + fepA + iucA + iucB + ybtS + fyuA	13 (16.7)	9 (17.6)	4 (14.8)	11 (22.5)	2 (6.9)
fimH + papC + entB + fepA + iucA + iucB + ybtS + fyuA	1 (1.3)	0	1 (3.7)	0	1 (3.4)
mrkD +entB + fepA + iucA + iucB + iutA + ybtS + fyuA	1 (1.3)	0	1 (3.7)	1 (2)	0
fimH + mrkD + papC + entB + fepA + iucA + iucB	1 (1.3)	1 (2)	0	1 (2)	0
fimH + mrkD + entB + fepA + iucA + iucB + iutA	1 (1.3)	1 (2)	0	1 (2)	0
fimH + mrkD + entB + fepA + iucA + iucB + ybtS	1 (1.3)	1 (2)	0	0	1
fimH + mrkD + entB + fepA + ybtS + fyuA	5 (6.4)	3 (5.9)	2 (7.4)	2 (4.1)	3 (10.3)
fimH + mrkD + entB + fepA + iucA + iucB	1 (1.3)	1 (2)	0	1 (2)	0
fimH + mrkD + iucA + iucB + fyuA	1 (1.3)	0	1 (3.7)	0	1 (3.4)
fimH + mrkD + entB + fepA	1 (1.3)	0	1 (3.7)	0	1 (3.4)

UPEC, Uropathogenic *E. coli*; CA, community-acquired; HA, hospital-acquired %, percentage; N, number of isolates.

Table 5. *Distribution of adhesin genes coupled with isolates biofilm formation capacity.*

Isolate ID	Sample	Hospital departement	biofilm-formation capacity	Adhesins genes				
				fimH	*mrkD*	*papC**	*afaC*	*sfa/focDE*
305	Pus	Surgery	Moderate biofilm	+	+	-	+	-
555		Surgery	Moderate biofilm	+	-	+	-	-
621		Internal medicine	Strong biofilm	+	+	+	-	-
712		Surgery	Moderate biofilm	+	+	-	-	-
728		Internal medicine	Weak biofilm	+	+	-	-	-
733		Internal medicine	Moderate biofilm	+	+	+	-	-
1637		Surgery	Moderate biofilm	+	+	-	-	-
1892		Anesthesiology and reanimation	Weak biofilm	+	+	-	-	-
6528		Surgery	Weak biofilm	+	+	-	+	-
6863	Bronchial fluid	Anesthesiology and reanimation	Weak biofilm	+	+	+	-	-
10247	Pus	Surgery	Moderate biofilm	+	+	+	-	-
10728	Bronchial fluid	Anesthesiology and reanimation	Weak biofilm	+	+	-	+	-
12198		Anesthesiology and reanimation	Weak biofilm	+	+	-	-	-
12983	Pus	Surgery	Strong biofilm	+	+	+	-	-
11899B		Surgery	Strong biofilm	+	+	+	-	-
496	Urine	Pediatrics	Moderate biofilm	+	+	+	-	-
778		Surgery	Moderate biofilm	+	+	+	-	-
1007		Internal medicine	Moderate biofilm	+	+	+	-	-
1437		Internal medicine	Moderate biofilm	+	+	-	-	-
1545		Internal medicine	Moderate biofilm	+	+	-	-	-
2069		Surgery	Strong biofilm	+	+	-	-	-
7254		Surgery	Moderate biofilm	+	+	+	-	-
9408		Internal medicine	Weak biofilm	+	+	+	-	-
10079		Anesthesiology and reanimation	Moderate biofilm	+	+	-	-	-
10894		Pediatrics	Strong biofilm	+	+	-	-	-
11829		Pediatrics	Weak biofilm	+	+	-	-	-
11932		Internal medicine	Moderate biofilm	+	+	+	-	+
12158		Surgery	Moderate biofilm	+	+	-	-	-
12825		Anesthesiology and reanimation	Strong biofilm	+	+	+	-	-

+, presence of gene; -, absence of gene; *, induction of moderate or strong biofilm-formation capacity

4. Discussions

Investigation of bacterial virulence factors is useful for guiding and improving patient management to avoid fatal complications [61]. We aimed to detect the distribution of five adhesins, four iron capture systems, and two toxin genes, and evaluate the biofilm formation capacity of MDR ESBL-producing ExPEC isolates.

Among adhesin genes, *fimH* had an average prevalence of 98.7%. The *fimH* gene detected in all the UPEC isolates confirmed the importance of type 1 fimbriae in UTIs. Similar studies [62–64] have also reported very high *fimH* prevalence rates of 92.7%, 98.2%, and 100%, respectively, in China, Brazil, and Mexico. However, few studies have shown a low prevalence of *fimH* in ExPECs, such as 19.2% in China [65]. The very high prevalence of 98.7% of the *mrkD* gene in *E. coli* strains is of interest because type 3 fimbriae (*mrkD*) is known as key fimbriae of *K. pneumoniae* [66]. This suggests the possible localization of *mrkD* on mobile genetic elements and the possible transfer of *mrkD* from *K. pneumoniae* to *E. coli.* Of the few previous studies in which the *mrkD* gene has been detected in *E. coli*, [67] reported a very low prevalence of 2% of the *mrkD* gene in UPEC. In the present study, the prevalence of *papC* was in strong agreement with the 31.3%, 55%, and 70% rates obtained in Brazil, Egypt, and the Republic of Korea [63, 68, 69]. These results confirmed that P fimbriae is a key virulence factor in UPEC. Several previous studies [65, 75] paradoxically obtained very low prevalence rates of 6.7% and 8% of the *papC* gene in UPEC isolates. The very low prevalence of *afaC* (9%) and *sfa/focDE* (1.3%) in our ExPEC isolates confirmed the *sfa/focDE* prevalence of 6.3% and 17%, respectively, in Saudi Arabia [16] and Brazil [63]. Studies, [14, 63] reported *afaC* prevalence of 15.9% and 4.5%, respectively. These results confirm that S fimbriae, F1C fimbriae, and afimbrial adhesin are minor adhesins in ExPEC infections. However, [70, 71] reported prevalence rates of 74.1% and 53%, respectively.

Iron is a crucial nutrient for bacterial growth, replication, and metabolism, and over hundred active enzymes in primary and secondary metabolism have iron-containing cofactors [72, 73]. In our study, we showed a high prevalence and combinations of iron-uptake system genes, with the most prevalent being enterobactin, yersiniabactin, aerobactin, and salmochelin. Similar results have been reported worldwide. For enterobactin biosynthesis gene *entB* and its receptor *fepA* [74, 75] have been reported to have a prevalence of 100%. For the aerobactin system genes, *iucA*, *iucB*, and *iutA* [74, 76] reported 90% and 48%, respectively, in Finland and Hong Kong. Abroshan and Shaheli, [77] had recently reported a 95% and 60% prevalence of *iucA* and *iucB*, respectively. Studies carried out in Iran, Korea, and Australia [14, 69, 75] showed a prevalence of 96%, 76%, and 60.1% for *ybtS* and *fyuA*, respectively. Nevertheless, [74] evoked the absence of yersiniabactin genes *ybtS* and the receptor *fyuA*. Nevertheless, these non-yersiniabactin-producing ExPEC strains are rare. Other studies had reported 11.3%, 4.5% and 3.3% for salmochelin gene and receptor *iroB*, *iroN* in Hong Kong, Brazil and Finland [63, 74, 76]. The enterobactin, yersiniabactin, and aerobactin systems constitute key iron acquisition systems in ExPECs.

If our studied strains expressed all the detected iron capture system genes, we could draw some conclusions. First, our isolates were likely hypervirulent *E. coli* isolates. Indeed, hypervirulent strains of ExPEC are known to often combine multiple iron uptake system genes coupled with adhesins and toxin genes [78]. Whole-genome sequencing can provide additional information. Second, it is advisable to detect and correct possible anemia in all patients consulting for UTI. Third, siderophore-based drugs would be excellent candidates for antibiotic therapy in our country. Indeed, several antibiotics with siderophores as vehicles or iron capture systems as targets have proven to be highly efficient in the treatment of MDR or extensively drug-resistant (XDR) strains in many countries. For example, cefiderocol, formerly S-649266, is a new injectable catechol-substituted siderophore cephalosporin that uses active bacterial iron transport channels to penetrate the outer membrane and enter the periplasmic space [79, 80]. Khasheii et al., [81] reported an enterobactin-beta-lactam antibiotic (ampicillin, amoxicillin) with 1000-fold higher antibacterial property against *E. coli*. However, larger-scale studies are needed on the iron acquisition systems used by the bacterial strains isolated in our region. The last possible conclusion that we draw is the possible usefulness of siderophore-based vaccines in the prevention of UTIs in Dakar. Indeed several studies [82–85], have shown that blocking yersiniabactin import attenuates ExPEC in cystitis and pyelonephritis, making *fyuA* a novel target to prevent UTI.

Similar to the low rate of toxin genes observed by [14], we obtained a low prevalence of *hlyA* and *cnf (1 & 2)* toxin genes of 10.3%. For *hlyA*, [69, 75] reported prevalence rates of 28% and 3 %, respectively. The low prevalence of *hlyA* and *cnf (1 & 2)* genes during our study gives us two hypotheses: the first is that there might be other key toxin genes that we must look for and the second hypothesis is that effectively, MDR ExPECs rarely express toxins, as mentioned [86, 87]. In this study, we also noticed that all isolates positive for a toxin gene simultaneously harbored *hlyA* and *cnf (1 & 2)* genes. The genes encoding *hlyA* and *cnf (1 & 2)* could be harbored on the same mobile genetic support. However, [15] found significant differences in the prevalence of *hlyA* and *cnf (1 & 2)*.

Out of the 16 virulence genes sought, our strains carried an average of nine virulence genes. Although the presence and accumulation of virulence genes do not automatically indicate the expression of these virulence factors, the accumulation of several virulence genes per strain in this study would contradict [6], who claimed that MDR strains are often not very virulent. Several studies [64, 74, 88, 89] have also reported high prevalence and accumulation of several virulence genes in MDR ExPECs. These virulent

MDR strains, whose incidence is increasing [90, 91], must be monitored and studied to limit mortality, sequelae, and healthcare costs due to bacterial infections by adjusting disease investigation and therapeutic protocols.

All (29/29) of our hospital-acquired isolates showed positive biofilm formation capacity. The fact that all hospital-acquired strains carried at least two adhesin genes could be one of the main reasons why all 29 strains were biofilm producers. However, the accumulation of adhesin genes did not influence biofilm formation. In fact, some of the isolates carrying three adhesin genes out of five had a weak adherence capacity, while strains with a strong biofilm formation capacity carried only two adhesin genes out of the five sought. This may be because of the following three reasons. The first is the expression levels of these adhesin genes. The second reason is the possible involvement of other critical adhesins, apart from the five we sought. Finally, the third possible reason is that there are other parameters and factors from adhesins that significantly influence bacterial adhesion capacity.

Several similar studies [22, 23, 92, 93] reported biofilm producer rates of (100%, 100%, 45.8 %, 43.3%) with (73%, 40%, 5.2%, 2%) strong biofilm producers; (38%, 24%, 18.3%, 0%) moderate biofilm producers; and (38.7%, 25.5%, 22%, 3%) weak biofilm producers in Libya, Pakistan, Thailand, and Nepal. Additionally, [94] in Uganda reported that 64% of UPEC strains had the ability to produce biofilms. Considering these aforementioned studies, we notice that ExPEC strains worldwide are biofilm producers with an almost homogeneous distribution in the biofilm categories they form.

Indeed, *papC* is known to play a key role in abiotic and biotic bacterial adhesion [95, 96]. In our study, we noticed that the presence of *papC* in isolates confers greater adhesion capacity to bacteria than those lacking *papC*, as reported elsewhere [92, 96, 97]. Thus, we can understand why our uropathogenic isolates tend to have a greater biofilm formation capacity than non-UPEC isolates, as *papC* was more prevalent in the UPEC isolates during our study.

Due to the high drug resistance that biofilms confer on bacteria, the usual treatments are not effective against biofilm-associated infections. Given the high prevalence of biofilm infections in developing countries, as well as their potentially lethal issues, it is necessary to start using anti-biofilm therapies. In fact, several molecules with mechanisms of action different from conventional antibiotics have been investigated as antibiofilm agents. For example, ginkgolic acid, eugenol, phenolic-free carbohydrate fraction purified and proanthocyanidins from cranberry inhibit the attachment of *E. coli* to uroepithelial cells and human red blood cells [98, 99]. Other biofilm drugs are inhibitors of quorum sensing pathways, such as isolimonic acid and quercetin [100, 101]. Another biofilm treatment is phage therapy, especially against chronic UTI caused by biofilm-producing UPEC strains [102, 103]. Antimicrobial peptides (AMPs) such as bactenecin and relacin can also be used as antibiofilm drugs against *Enterobacteriaceae* infections [26, 104, 105]. Some countries have already adopted the use of anti-biofilm drugs, but not yet in the West African sub-region. Therefore, there is a need to study the possibility of using these antibiofilm drugs in our area. There is also an urgent need to conduct larger-scale and more detailed studies on bacterial biofilms, especially in hospital settings. This could lead to the better prevention and eradication of biofilm-associated infections.

Whole genome sequencing would provide additional information that will help better assess the virulence capacity of our strains and their real impact on public health. Additionally, performing biofilm formation tests on 49 community-acquired strains would provide interesting information.

5. Conclusion

This study revealed that most of our ESBL-producing ExPEC strains had moderate biofilm-formation capacity and carried an average of nine virulence genes out of the 16 sought. The most frequently identified virulence genes were *fimH, mrkD, entB, fepA, ybtS, fyuA, iucA, iucB*, and *papC*. Eight strains carrying a combination of genes, including adhesins, iron acquisition, and toxin genes, may be hypervirulent. There is a need to carry out whole-genome sequencing to provide more valuable and broad information, such as mobile genetic supports and phylogenic information. The deepening of this kind of study on bacterial virulence and hospital bacterial biofilms could lead to the improvement of infections investigation, prevention and therapeutic protocols.

6. Recommendations

As a continuation of this study, we recommend future studies comparing the (*in vitro* and *in vivo*) biofilm formation capacities of hospital-acquired and community-acquired ExPEC strains. Additionally, we recommend studying the expression of these virulence genes in animal models to correlate their *in vitro* and *in vivo* pathogenicity.

Ethical Research Approval and Consent to Participate

This study has received the Ethical Research approval of the Research Ethics Committee (CER) of Cheikh Anta Diop University (UCAD) under the reference CER/UCAD/AD/MSN/051/2020.

Availability of Data and Materials

Additional data presented in this study are available from the corresponding author upon request.

Conflict of Interests

The authors have not declared any conflict of interests.

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