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GENOTYPIC CHARACTERIZATION OF VIRULENCE FACTORS IN *Escherichia coli* STRAINS FROM PATIENTS WITH CYSTITIS

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SUMMARY

Adhesins (P-fimbriae, S-fimbriae, type 1 fimbriae and afimbrial adhesin), toxins (α-hemolysin and cytotoxic necrotizing factor type 1), iron acquisition systems (aerobactin) and host defense avoidance mechanisms (capsule or lipopolysaccharide) have been shown to be prevalent in *Escherichia coli* strains associated with urinary tract infections. In this work, 162 Uropathogenic *Escherichia coli* (UPEC) strains from patients with cystitis were genotypically characterized by polymerase chain reaction (PCR) assay. We developed three multiplex PCR assays for virulence-related genes *papC, papE/F, papG* alleles, *fimH*, *sfa/foc*, *afaE, hly, cnf-1, usp, cdtB, iucD,* and *kpsMTII*, all of them previously identified in UPEC strains. The PCR assay results identified 158 *fimH* (97.5%), 86 *kpsMTII* (53.1%), 53 *papC/papEF/papG* (32.7%), 45 *sfa* (27.8%), 42 *iucD* (25.9%), 41 *hly* (25.3%), 36 *usp* (22.2%), 30 *cnf-1*(18.5%) and 10 *afa* (6.2%) strains. No strain was positive for *cdtB*. In this work, we also demonstrated that adhesins may be multiple within a single strain and that several virulence genes can occur combined in association.

KEYWORDS: Uropathogenic *Escherichia coli* (UPEC); Polymerase chain reaction (PCR); Virulence factor (VF).

INTRODUCTION

Urinary Tract Infections (UTIs), including cystitis and pyelonephritis, are among the most frequent human extraintestinal infections. *Escherichia coli* is the major causative agent, and it also is the predominant facultative member of the normal human intestinal flora[23]. This agent primary of UTIs accounts for greater than 80% of these infections. The bladder is the primary site of infection in about 95% of all UTIs[23]. In most cases, uropathogenic clones are selected from the fecal flora and colonize the vaginal and periurethral tissue, and infected the urinary tract by the ascending manner.

Classification of UTIs depends on the part of the urinary tract which is colonized (lower or upper urinary tract), as well as on the individual predisposition. Cystitis (lower UTI) is characterized by pelvic discomfort, especially pre - and immediately post void, frequent or urgent voiding and suprapubic pain. Acute pyelonephritis (upper UTI) is, most of the times, a more serious problem and is clinically identified by the fever, flank pain, nausea and, sometimes, vomiting[19,22,23].

Uropathogenic *Escherichia coli* (UPEC) isolates are a genetically heterogeneous group that exhibit several virulence factors associated with colonization and persistence of the bacteria in the urinary tract. The virulent strains of UPEC that cause cystitis typically produce, at least, one adhesion system. Adhesins can also contribute to virulence, promoting colonization, invasion and replication within uroepithelial cells[22,23]. Besides bacterial adherence, several virulence factors may contribute to the pathogenicity of UPEC, facilitating the ability to adhere specifically to uroepithelial cells and the expression of other bacterial products to host tissues such as toxins, iron acquisition systems and host defense avoidance mechanisms[26].

Virulence factors (VFs) associated with UPEC include adhesins (P fimbriae, type 1 fimbriae, S and F1C fimbriae, afimbrial adhesin), toxins (hemolysin, and cytotoxic necrotizing factor), siderophores (the aerobactin system) and polysaccharide coatings (group II capsules)[1,29]. Recently, a new urovirulence factor gene, which was more frequently found in UPEC strains, was reported. This gene encoding a protein designated usp (uropathogenic-specific protein) was demonstrated significantly enhancing the infectivity of *E. coli* in the mouse UTI model[15,18,28].

The aim of this study was to detect the virulence genes of *E.coli* strains isolated from patients with clinical signs of cystitis in Brazil. PCR method was used for this purpose.

MATERIAL AND METHODS

Bacterial strains: A total of 162 *E. coli* strains collected in the mid-1990s, were isolated from each woman patient with recurrent UTI, attended at the Ambulatory sector of Medical School Hospital of the

Abbreviations: aerobactin (***iucD***), afimbrial adhesin (***afaB/C***), cytolethal distending toxin (***cdtB***), cytotoxic necrotizing factor type 1 (***cnf-1***), G adhesin classes of P fimbriae (***papG* alleles**), group II capsule (***kpsMTII***), α-hemolysin (***hly***), minor structural subunits of P fimbriae (***papE/F***), outer membrane protein of P fimbrae (***papC***), polymerase chain reaction (**PCR**), S fimbriae (***sfaC/D***), type 1 fimbriae (***fimH***), urinary tract infection (**UTI**), uropathogenic specific protein (***usp***), uropathogenic *Escherichia coli* (**UPEC**), virulence factors (**VFs**).

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Campinas State University (UNICAMP), Campinas, São Paulo. Subjects had no clinical history of severe complications such as vesicoureteral reflux, neurogenic bladder, or malignant neoplasm. The diagnosis of *E. coli* UTI was characterized by typical clinical symptoms of cystitis such dysuria, frequency, urgency, small-volume voids or lower abdominal pain and more than 10^5cfu *E. coli* per mL urine sample. All isolates were stored at room temperature in NB (Nutrient broth) 0.75% agar.

The reference *E. coli* strains FVL2 (*sfa, pap, iucD, hly, cnf-1*), FV35 (*afa, iucD, cnf-1*), ORN115 (*fimH*), FVL8 (*sfa, pap, papGII, hly, usp, iucD*, *cnf1*), J96 (*papGI* and *papGIII*), U9-41 (O2:K1:H4, *kpsMTII*), and CLDT7 (*cdtB*) belong to Laboratório de Antígenos Bacterianos II (Campinas State University) and were used as positive control. *E. coli* strain DH5α was used as a negative control.

Polymerase Chain Reaction (PCR): Primers for each VF genes were first established individually by the use of template DNA from appropriate positive and negative control strains. We examined through PCR, the presence of the following groups of genes: colonization factors, such as P fimbriae (*papC*, *papE/F* and *papG* alleles), type 1 fimbriae (*fimH*), S fimbriae (*sfaC/D*), afimbrial adhesin (*afaB/C*), group II capsule (*kpsMTII*), aerobactin (*iucD*), cytotoxic necrotizing factor type 1 (*cnf-1*), α-hemolysin (*hly*), cytolethal distending toxin (*cdtB*), and uropathogenic specific protein (*usp*). The appropriate primers sequences, annealing temperature, and size of amplified fragment (base pairs - bp) for the characteristics studied are shown in Table 1.

Bacterial DNA to be amplified was released from whole organisms by boiling, and a multiplex PCR assay was carried out in a total volume of 25 µL, containing 2 µL of template DNA, each of the primers at 60 ng, the four deoxynucleotide triphosphates (each at 200 µM), PCR buffer 1X and 1.5 U of Taq DNA polymerase[3,10]. For Multiplex PCR, amplifications

Table 1
Characteristics of oligonucleotide sequences used for PCR assays

Genes	Oligonucleotide sequences (5' → 3')	Size fragment (bp)	Concentration of each primer (ng/µL)	Reference	AT (º)
cnf1	CNF1: GAA CTT ATT AAG GAT AGT CNF2: CAT TAT TTA TAA CGC TG	543	90	3	63
hlyA	1: AAC AAG GAT AAG CAC TGT TCT GGC T 2: ACC ATA TAA GCG GTC ATT CCC GTC A	1,177	60	27	63
papC	1:GAC GGC TGT ACT GCA GGG TGT GGC G 2: ATA TCC TTT CTG CAG GGA TGC AAT A	328	60	5	63
papE/F	1:GCA ACA GCA ACG CTG GTT GCA TCA T 2:AGA GAG AGC CAC TCT TAT ACG GAC A	336	60	27	63
pap G classI	1:CAA CCT GCT CTC AAT CTT TAC TG 2:CAT GGC TGG TTG TTC CTA AAC AT	692	60	16	63
pap G classII	1:GGA ATG TGG TGA TTA CTC AAA GG 2:TCC AGA GAC TGT TCA AGA AGG AC	562	60	16	63
papG classIII	PG1:CAT GGC TGG TTG TTC CTA AAC AT PG2:TCC AGA GAC TGT GCA GAA GGA C	421	60	16	63
fimH	A: TGC AGA ACG GAT AAG CCG TGG B GCA GTC ACC TGC CCT CCG GTA	508	60	11	63
afaB/C	1: GCT GGG CAG CAA ACT GAT AAC TCT C 2: CAT CAA GCT GTT TGT TCG TCC GCC G	750	60	27	63
sfaD/E	1: CGG AGG AGT AAT TAC AAA CCT GGC A 2: CTC CGG AGA ACT GGG TGC ATC TTA C	410	90	5	63
iucD	A: TAC CGG ATT GTC ATA TGC AGA CCG T B:AAT ATC TTC CTC CAG TCC GGA GAA G	602	60	27	63
cdtB	A: TAT GAT AGC CTC TTT TAT CGT CGT CGT CTG B: AGA GGA GAG TTA GAG CCT ATG ATA GCC TCT	805	60	This study	50
usp	A: ATG CTA CTG TTT CCG GGT AGT GTG T B: CAT CAT GTA GTC GGG GCG TAA CAA T	1000	60	24	66
kpsMTII	A: GCG CAT TTG CTG ATA CTG TTG B: CAT CAG ACG ATA AGC ATG AGC A	272	60	11	63

AT: Annealing Temperature.

consisted of 25 cycles of 94 ºC for one min, specific annealing temperature for each primer for one min (Table1), 68 ºC for three min, and a final extension at 72 ºC for seven min in a Thermal Cycler (Gene Amp PCR System 9700/Perkin Elmer Corporation, Norwal CT/USA). Primers were then sorted in three pools according to annealing temperature and amplicon size: Multiplex A: *papC, papGI, papGII, papGIII*; Multiplex B: *kpsMTII, papE/F, iucD, hlyA;* Multiplex C: *sfaD/E, cnf-1*. The primers for *fimH*, *cdtB, usp* and *afaB/C* genes were used individually.

The amplified DNA product was visualized by standard submarine gel electrophoresis using 10 µL of the final reaction mixture on a 2% agarose gel in TAE buffer (1,6 M Tris-EDTA, 0,025 M acetic acid). Amplified DNA fragments of specific sizes were located by UV fluorescence, after staining with ethidium bromide. The 100-bp ladder was used as a standard for determining molecular size of PCR products.

RESULTS

A total of 162 UPEC strains from patients with cystitis were genotypically characterized by the use of PCR assay. The PCR assay results identified 158 *fimH* (97.5%), 86 *kpsMTII* (53.1%), 53 *papC/ papEF/papG* (32.7%), 45 *sfa* (27.8%), 42 *iucD* (25.9%), 41 *hly* (25.3%), 36 *usp* (22.2%), 30 *cnf-1* (18.5%) and 10 *afa* (6.2%) strains (Table 2). All strains were negative for *cdtB* and *papGI*.

A total of 76 strains shared DNA sequences related to, at least, two operons of adhesins, encoding *fimH* and other genes associated as *pap*, *sfa*, and/or *afa* (Table3).

FimH adhesin was the most prevalent virulence factor detected, having occurred in 97.5% of strains, and it was detected in 100% of operon *pap* (*papC/papEF/papG* alleles). Eighty two strains of the 158 *fimH* studied have only adhesin-encoding operon *fimH*, and in 48 of them it was not possible to detect any other VF gene.

Among the 53 *papC/papE/FpapG* strains, 29 were *papGII*, 23 were *papGIII*, one was *papGII/papGIII* and none was *papGI* (Table 2). Although *papG* alleles II and III were both associated with *sfa*, they exhibited associations with other VF genes. *PapGII* allele was associated with *iucD, kpsMTII, hly*, and negatively associated with *cnf-1*, whereas *papG* allele III was associated with *hly*, *cnf-1* and *kpsMTII*. S fimbrial adhesin was associated with *papG* alleles, capsule, cnf-1 and α-hly toxins. Table 3 shows the association between virulence factors and adhesins genes.

DISCUSSION

E. coli is the major causative agent in human UTIs, one of the most common bacterial infections. In most cases, uropathogenic clones are selected from the fecal flora, and the pathogenic potential of *E. coli* strains is thought to be dependent on the presence of VFs[19,23]. Based on their components and products called virulence factors, *E. coli* cells attach selectively to the mucosa uro-epithelium, promoting colonization and persisting in the urinary tract, inducing, then, a local inflammatory response and sometimes to promote tissue lesions[23,29].

We used, to detect and identify the presence of potential uropathogenic *Escherichia coli* virulence factors, a genotypic assay. PCR method is highly specific, informative and a powerful genotypic assay, used for detection of adhesin-encoding operons and other virulence factors that can also contribute to virulence in UTI. In this study, we confirmed the prevalence of *fimH* among UPEC strains[10,21,23]. *FimH* occurred in 158 strains. Eighty two strains presented only adhesin-encoding operon *fim*, and in 48 of these strains no other VF gene was detected. This result demonstrated that type 1 fimbriae is an important and relevant VF, and that it can also contribute to virulence in *E.coli* strains. Type 1-mediated adherence has been proposed to play a role in the induction of inflammation, enhancing *E. coli* virulence for the urinary tract. CONNELL *et al*., (1996)[4] reported that mice infected with a type 1-positive 01:K1:H7 isolate showed a higher urinary neutrophil influx into the urine than type 1- negative isolates. Furthermore, we demonstrated that 76 *fimH* strains, occurred with at least one adhesin, such as *pap*, *sfa*, or *afa*. This result corroborates the occurrences presented in the literature, where *fimH* was found in associations with P fimbriae and S fimbriae in UPEC[11].

P fimbriae, the principal mannose - resistant adherence organelles of extraintestinal pathogenic *Escherichia coli*, are known to contribute to pathogenesis by promoting bacterial colonization of host tissues and by stimulating an injurious host inflammatory response[17]. *E. coli* strains were examined for *pap* genotype and specific primers were utilized to detect genes associated with outer membrane protein (*papC*), minor structural subunit (*papE/F*) and papG allele's adhesin (*papGI/papGII/ papGIII*).

Table 2
Distribution (number and percentage) of virulence factor genes in uropathogenic *E. coli* isolates from patients with cystitis

Virulence factors (genes)	Number of positive strains (%)
fimH	158 (97.5)
kpsMTII	86 (53.1)
papC	53 (32.7)
papE/F	53 (32.7)
sfaD/E	45 (27.8)
iucD	42 (25.9)
hlyA	41 (25.3)
usp	36 (22.2)
cnf-1	30 (18.5)
papGII	29 (17.9)
papGIII	23 (14.2)
afaB/C	10 (6.2)
papGII/GIII	1 (0.62)
papGI	0 (0)
cdtB	0 (0)

Type 1 fimbriae (*fimH*), group II capsule (*kpsMTII*), outer membrane protein of P fimbrae (*papC*), minor structural subunits of P fimbriae (*papE/F*), G adhesin classes of P fimbriae (*papG* alleles), S fimbriae (*sfaC/D*), aerobactin (*iucD*), α-hemolysin (*hly*), *usp* (uropathogenic specific protein), cytotoxic necrotizing factor type 1 (*cnf-1*), afimbrial adhesin (*afaB/C*) and cytolethal distending toxin (*cdB*).

Table 3
Association between virulence factors and adhesins genes of uropathogenic *E. coli* isolates from patients with cystitis

Genes	*hly*	*cnf-1*	*iucD*	*kps*	*usp*	*iucD, kps*	*hly, iucD, kps*	*hly, cnf-1, kps*	*hly, kps*	*cnf-1, kps*	*cnf-1, iucD*	*iucD, cnf-1, kps*	*hly, cnf-1, iucD, kps*	*usp, hly*	*usp, cnf-1*	*usp, kps*	*usp, iucD*	*usp, hly, iucD, kps*	*usp, hly, kps*	*usp, hly, iucD*	*usp, iucD, kps*	*usp, hly, cnf-1, kps*	*usp, cnf-1, kps*	Negative	Total
fimH			6	10	5	5			2					1		4	1							48	82
fimH/papC/EF/GII				1		5	3									1					2			3	15
fimH/papC/EF/GIII								2	3										1	1		1			7
fimH/papC/EF/GII/sfa						1	4			1						1		3						1	12
fimH/papC/EF/GIII/sfa	1			1				4		3			1						1		1	4			16
fimH/papC/papGII/afa							1																	1	2
fimH/sfa		1						1		5	1		1		1	1		1				2	1		15
sfa	1																								1
fimH/afa				2	1	1															1			2	7
fimH/sfa/afa												1													1
fimH/papC/EF/GII/GIII														1											1
Negative				1		1	1																		3
Total	2	1	6	15	6	13	9	7	5	9	1	1	2	2	1	7	1	4	2	1	4	7	1	55	162

Type 1 fimbriae (*fimH*), group II capsule (*kpsMTII*), outer membrane protein of P fimbrae (*papC*), minor structural subunits of P fimbriae (*papE/F*), G adhesin classes of P fimbriae (*papG* alleles), S fimbriae (*sfaC/D*), aerobactin (*iucD*), α-hemolysin (*hly*), uropathogenic specific protein (*usp*), cytotoxic necrotizing factor type 1 (*cnf-1*), afimbrial adhesin (*afaB/C*).

The classes of *papG* genotype differed according to the type of receptor specificity and infection[22,23]. The class II G adhesin is associated with pyelonephritis and bacteremia, while the class III G adhesin sequence is associated with cystitis, although they have been found in pyelonephritis and bacteremia[6,9]. *PapGI* strains might have a larger prevalence among fecal isolates.

Fifty three strains (32.7%) were *papC/papE/F/papG*, demonstrating the presence of the intact operon, responsible for adhesin assembly. This frequency of *pap* in a cystitis strain agrees with literature data[26]. JOHNSON *et al*., 2005[11], reported that cystitis isolates differed from pyelonephritis and prostatitis isolates by their lower prevalence of *papA/C/EF/G*, *papG* allele II and the *papGII/III* alleles combination. We found 53 strains *papC* and all of them presented *papG*, demonstrated by PCR. There were 29 *papGII* strains, 23 *papGIII* strains and one strain carrying both sequences. The result that *papGIII* was less prevalent than *papGII* was not expected, since the strains were all derived from patients with cystitis.

PapGII and papGIII alleles are found in association with *hly*[2]. *E. coli* strains that produce Hly, frequently produce other two urovirulent factors: CNF-1 and P fimbriae. These three genes are found in the same pathogenicity island (PAI)[2,10]. In this study we detected 1 *papGII/GIII/hly* strain, 11 *papGII/hly* strains and 19 *papGIII/hly* strains. Our results are similar to that described in the literature[2], where the hemolytic gene (*hlyA*) is present in both *papG* alleles.

While *hly* was found in association with P fimbriae, *cnf1* was detected in 27 *sfa* strains. This result corroborates the occurrences presented in literature where, *cnf-1* was found in association with *sfa*[10,23].

Cdt-producing *E. coli* strains were first isolated from children with enteritis[12]. In other studies, *cdt* genes were detected in urosepsis and fecal *E. coli* isolates and from patients with a variety of diarrhea symptoms and encephalopathy[10,13]. CDTs produce giant mononucleated cells caused by an irreversible block in the cell cycle at the G2/M, occurring cellular death. In this study, *cdtB* was not detected by the PCR assay, indicating their relative unimportance in our *E.coli* collection. This finding suggests that cystitis can be caused by strains that are less virulent than those isolates that had *cdtB* and typically cause pyelonephritis, urosepsis and diarrhea.

Other VF found in association with pyelonephritis, meningitis and sepsis is S fimbriae[23]. S fimbrial adhesin recognizes surface sialic acid content, on receptors expressed by kidney epithelial and vascular endothelial cells, mediating bacterial adherence. Recently, it was identified sialic acid residues in the UP3, one of the four integral membrane uroplakin proteins, expressed on the bladder luminal surface, suggesting that S fimbriae may also have a role in cystitis[20]. We detected 45 *sfa* strains. *Sfa* was found in positive *cnf-1*, *hly* and *papG* alleles strains (Table 3). These results were similar to the ones described in the literature[2,10].

The concentration of iron (Fe) is limited in sites of extraintestinal

infection, in large part, due to host factors that reduce its availability. As a result, Fe acquisition is of critical need for pathogens that must grow within a host. One of the iron acquisition mechanisms is the biosynthesis of siderophore[7]. In the literature was reported that aerobactin system was found associated with *papG* allele II[2]. In this study, we detected 19 *papGII/iucD* strains and three *papGIII/iucD* strains, then, these results were similar to the ones described in the literature[2].

The majority of the UPEC strains present group II capsules (K1, K5) determined by *kps* operon[8]. Capsule is common in UPEC and is better known for contributing with pyelonephritis than other urinary tract infections[8]. In this study were detected 86 (53.1%) *kpsMTII* strains. All of them were associated with other VF searched. The literature reported the association among the VFs present in UPEC: capsule, papG allele II and aerobactin. An association of *kpsMTII* and *hlyA* was also observed. Our results corroborate those present in the literature[10], once we found 19 *papGII/kps/iucD* strains and only two *papGIII/kps/iucD* strains.

KURAZONO *et al.* (2000)[18] reported a putative pathogenicity island (PAI), which was more frequently found in UTI collections than in fecal *E. coli*. This PAI contains *usp* that encodes a 346-amino acid protein, which was designated as uropathogenic specific protein (usp). PARRET & DE MOT (2002)[25] hypothesized that the usp protein may represent a novel type of *E. coli* bacteriocin, acting against competing *E. coli* strains that occupy the same niche, thereby enhancing their infectivity in the urinary tract environment. Recently, it was demonstrated that usp significantly enhanced the infectivity of *E. coli* in the mouse UTI model[29].

In the study proceeding from Japan, *usp* gene occurred in 80% of 195 cystitis isolates, and 93% of the 76 pyelonephritis isolates[18]. It was noted that *usp* occurred more frequently in pyelonephritis than cystitis[14]. Herein, we detected 36 *usp* (22.2%) strains, and the reason for differences between these studies could be interpreted as being due to distinct women population analyzed or from the existence of different bacterial clones distributed between women from Brazil and Japan.

The VFs displayed distinctive and complex associations with each other. We demonstrated that *papG* allele II was found in positive *kps, iucD* and *hly* strains and was not found in positive *cnf1* strain, whereas *papG* allele III was associated with *kps, hly* and *cnf-1* genes. Both *papG* alleles were associated with S fimbriae. S fimbriae were positively associated with *cnf-1* and *hly*, *kpsMTII* and *papG* alleles.

The present study provides molecular epidemiological information about VF genes found in lower UTI (cystitis). We have rigorously established a multiplex PCR assay, capable of detecting VF highly prevalent among UTI isolates. We confirmed the prevalence of *fimH* genes among *E. coli* strains from cystitis. We showed that P and type 1 fimbriae are not sole adhesins on uropathogenic *E. coli* and that these adhesions, may be multiple within a single strain with several virulence genes occurring together in association.

RESUMO

Caracterização genotípica dos fatores de virulência em amostras de *Escherichia coli* isoladas de pacientes com cistite

Adesinas (Fímbria P, fímbria S, fímbria do tipo 1 e a adesina afimbrial), toxinas (α-hemolisina e o fator necrosante citotóxico do tipo 1), sistemas de captação de ferro (aerobactina), e mecanismos de defesa do hospedeiro (cápsula ou lipopolissacarídeo) são prevalentes em amostras de *Escherichia coli* associadas a infecções do trato urinário. O objetivo deste trabalho foi caracterizar genotipicamente 162 amostras de *Escherichia coli* uropatogênica (UPEC) de pacientes com cistite através do ensaio da reação em cadeia da polimerase. Foram realizados três ensaios de PCR multiplex para os seguintes fatores de virulência: *papC, papE/F,* alelos de *papG*, *fimH*, *sfa/foc*, *afaE, hly, cnf-1, usp, cdtB, iucD,* e *kpsMTII*. Os resultados da PCR identificaram, 158 amostras *fimH* (97,5%), 86 amostras *kpsMTII* (53,1%), 53 amostras *papC/papEF/papG* (32,7%), 45 amostras *sfa* (27,8%), 42 amostras *iucD* (25,9%), 41 amostras *hly* (25,3%), 36 amostras *usp* (22,2%), 30 amostras *cnf-1* (18,5%) e 10 amostras *afa* (6,2%). Nenhuma amostra foi positiva para o gene *cdtB*. Neste trabalho, demonstramos que podemos encontrar múltiplas adesinas em uma única amostra e que diferentes genes de fatores de virulência podem ser encontrados em associação.

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