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Brief report

Virulence genes of *Aeromonas* spp. isolates from stool in Spain

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ABSTRACT

Introduction: The role of *Aeromonas* species in gastrointestinal disease is controversial. The aim was to analyze not only the virulence genes between different species of *Aeromonas* isolated from feces, but the distribution of these virulence genes between enterotoxigenic strains and co-pathogen strains.
Methods: Retrospective study of isolates of *Aeromonas* spp. in feces (2016–2021). The protocol included coproculture, identification by MALDI-TOF and confirmation by multiplex PCR. SPSS Statistics program was used.
Results: A total of 288 strains were studied for the virulence genes between different species of *Aeromonas*. To compare virulence genes between *Aeromonas* as co-pathogen and those isolated alone, 218 strains of the global set were used; 52 as co-pathogens compared with 166 *Aeromonas* without associated pathogen as controls.
Conclusions: We found no significant differences in the distribution of virulence genes versus co-existence of co-pathogens or not. *A. hydrophila* is the potentially most virulent species of our set.

Genes de virulencia de aislamientos en heces de *Aeromonas* spp. en España

Palabras clave:
Aeromonas spp.
Genes de virulencia
MALDI-TOF
Co-patógeno

RESUMEN

Introducción: El papel de las especies de *Aeromonas* en las enfermedades gastrointestinales es controvertido. El objetivo fue analizar no solo los genes de virulencia entre diferentes especies de *Aeromonas* aisladas de heces, sino también la distribución de estos genes de virulencia entre cepas enterotoxigénicas y co-patógenas.
Métodos: Estudio retrospectivo de aislamientos de *Aeromonas* spp. en heces (2016-2021). El protocolo incluyó coprocultivo, identificación por MALDI-TOF y confirmación por PCR multiplex. Se utilizó el programa SPSS Statistics.
Resultados: Se estudiaron un total de 288 cepas para los genes de virulencia entre diferentes especies de *Aeromonas*. Para comparar genes de virulencia entre *Aeromonas* como co-patógeno y los aislados únicos, se utilizaron 218 cepas del conjunto global; 52 como co-patógenos, comparados con 166 *Aeromonas* sin patógeno asociado como controles.
Conclusiones: No se encontraron diferencias significativas en la distribución de los genes de virulencia versus coexistencia de co-patógenos o no. *Aeromonas hydrophila* es la especie potencialmente más virulenta de nuestro muestreo.

Introduction

Aeromonas species are aerobic and anaerobic Gram-negative rods, belongs to the *Aeromonadaceae* family and comprises a group of Gram-negative bacteria widely distributed in aquatic environments, but its great capacity for adaptation also makes *Aeromonas* species able to colonize terrestrial environments. Some clinically

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isolated *Aeromonas* spp. are pathogenic to humans.[1] The genus *Aeromonas* comprises of two different groups of bacteria. One is non-motile psychrophilic *Aeromonas salmonicida* and the other group comprising of three mesophilic motile species: *Aeromonas hydrophila*, *Aeromonas caviae*, and *Aeromonas sobria*.[2]

Gastroenteritis is the most common type of *Aeromonas* infection in humans, but clinical presentation may include syndromes like skin and soft tissue infections, urinary tract infections, and central line associated bloodstream infections.[3]

Intestinal and extraintestinal infections by *Aeromonas* spp., remain controversial, due to the existence of healthy carriers of *Aeromonas* spp. In some studies, *Aeromonas* strains isolated from patients with enteritis was enterotoxigenic, while *Aeromonas* strains isolated from healthy people rarely showed enterotoxic characteristics forming part of the human gastrointestinal microbiota, varying the rates of frequency from 0% to 4% in people with no disease to 0.8%–7.4% in persons with diarrheal illness.[4] The prevalence in case of diarrhea in pediatric age is around 4.6%[5] and in other cases may even appear as a co-pathogen with other enteropathogen, especially in children, with a prevalence of 1.9%–57.7% in pediatric age.[6,7] In our health area *Aeromonas* spp. is the third most frequently isolated enteropathogen in feces after *Campylobacter* spp. and *Salmonella* spp. without finding significant differences regarding the distribution by sex (52.0% women) or age, with a mean age of infection of 34 years. There are 15.8% of co-infections, being more frequent in pediatric age (49; 84.5%) ($p = 0.01$) and mostly associated with *Campylobacter* spp. (75.9%).[8]

Under this context, the objective of this work was to identify the prevalence of five virulence genes in each species of *Aeromonas* studied, in this way we could advance the potentially most pathogenic species for humans. Also, the secondary objective of the work was to try to advance in clarifying which could be the potentially pathogenic species that affect humans, for this, we compared the presence of five virulence genes among those *Aeromonas* identified with another primary pathogen, considering as primary pathogen: *Salmonella* spp., *Yersinia* spp., *Shigella* spp. and *Campylobacter* spp., and *Aeromonas* isolated as a single pathogen in feces.

Methods

288 isolates of *Aeromonas* spp. were studied, only one isolation per patient was considered, isolated in stool between 2016 and 2020 in the Microbiology Service of the University Hospital Marqués de Valdecilla, Santander (313.040 census population). The protocol for these isolates included inoculated on Yersinia Selective Agar (CIN Agar; BD, Heidelberg, Germany) and incubated at 37 °C for 24 h. Identification was performed by MALDI-TOF system (Vitek-MS®, BioMerieux) and were confirmed by multiplex PCR developed by Perssons et al.,[9] and when there was not correlation between both systems, the rpoB gene sequencing was used. Also the presence of five virulence genes were studied by PCR: genes encoding heat-labile cytotonic enterotoxin (*alt*) and heat-stable cytotonic enterotoxin (*ast*), as well as genes encoding hemolysin (*hlyA*), aerolysin (*aerA*) and cytotoxic enterotoxin (*act*).[10] For the isolation of copathogens, feces were seeded in MacConkey, XLD, CIN agar plates (Thermo Fisher Diagnostics, Hemel Hempstead, United Kingdom) and selective enrichment broth (Selenite Broth, BioMerieux) at 37 °C for 24 h. In *Campylobacter* selective agar plates (CCDA selective medium, Thermo Fisher Diagnostics, Hemel Hempstead, United Kingdom) in microaerophilia ambient at 37 °C for 48 h, following standard laboratory procedures. Identification was performed by MALDI-TOF system (Vitek-MS®, BioMerieux). Enteropathogenic viruses were also ruled out by immunochromatography (CerTest, Biotec, Zaragoza, Spain). On the other hand, it was compared whether there are significant differences in the presence of the different virulence genes with the coexistence or not of other gastrointestinal pathogens. For this, those strains that had been isolated as co-pathogenic were selected from our population, comparing them with a control group from the same population randomly matched by species of *Aeromonas* isolated, age and sex of the patient.

The SPSS Statistics program was used for statistical analysis. The statistical test used for the normality study was Shapiro Wilks, and for the intergroup comparison an ANOVA test was performed with subsequent post hoc analysis for a group of non-parametric Kruskal–Wallis samples. Significant results were considered for p-value <0.05. For stratification by age, adults aged 16 and over are considered.

Results

The distribution of the strains studied was as follows: *A. caviae* (*n*: 230; 82%) *A. veronii* (*n*: 26; 9%) *A. hydrophila* (*n*: 16; 5%) *A. media* (*n*: 15; 5%) *A. salmonicida* (*n*: 1; 0.3%), with a distribution in children of *A. caviae* (*n*: 112; 98.2%), *A. veronii* (*n*: 2; 1.8%). And on the other hand we have studied the virulence genes of 52 *Aeromonas* spp. isolated as co-pathogens against 166 *Aeromonas* spp. isolated without associated pathogen. The 52 *Aeromonas* spp., recovered with an associated primary pathogen, were isolated in feces from 8 (15.4%) adults and 44 (84.6%) were isolated from children. Most

Table 1
Distribution of virulence genes in *Aeromonas* spp. strains.

Total strains (*n* = 218)	Virulence genes									
	act		*alt*		*ast*		*hlyA*		*aerA*	
Co-pathogen	Not-presence (%)	Presence (%)	Not-presence (%)	Presence (%)	Not-presence (%)	Presence (%)	Not-presence (%)	Presence (%)	Not-presence (%)	Presence (%)
Not (*n* = 166)	154 (76.2)	12 (75.0)	11 (68.8)	155 (76.7)	163 (76.2)	3 (75.0)	163 (76.2)	3 (75.0)	159 (75.7)	7 (87.5)
Yes (*n* = 52)	48 (23.8)	4 (25)	5 (31.3)	47 (23.3)	51 (23.8)	1 (25.0)	51 (23.8)	1 (25.0)	51 (24.3)	1 (12.5)
Total	202	16	16	202	214	4	214	4	210	8
Total strains (*n* = 114)	Virulence genes in children									
	act		*alt*		*ast*		*hlyA*		*aerA*	
Co-pathogen	Not-presence (%)	Presence (%)	Not-presence (%)	Presence (%)	Not-presence (%)	Presence (%)	Not-presence (%)	Presence (%)	Not-presence (%)	Presence (%)
Not (*n* = 70)	43 (38.0)	1 (100)	3 (100)	41 (36.9)	44 (38.6)	0	44 (38.6)	0	44 (38.6)	0
Yes (*n* = 44)	70 (62.0)	0	0	70 (63.1)	70 (61.4)	0	70 (61.4)	0	70 (61.4)	0
Total	113	1	3	111	114	0	114	0	114	0

Table 2
Presence of virulence genes by species.

	Virulence genes				
Species ($n = 288$)	*act*	*alt*	*ast*	*hlyA*	*aerA*
A. veronii ($n = 26$)	22 (84.6%)	4 (15.4%)	0	0	15 (57.7%)
A. hydrophila ($n = 16$)	6 (37.5%)	16 (100%)	16 (100%)	16 (100%)	6 (37.5%)
A. media ($n = 15$)	0	15 (100%)	0	0	0
A. salmonicida ($n = 1$)	1 (100%)	1 (100%)	0	1 (100%)	0
A. caviae ($n = 230$)	0	228 (99.1%)	0	0	0
Total	29	262	16	17	21

of them corresponded to the species *A. caviae* (47, 90.4%), while to a lesser extent they were *A. veronii* (4, 7.7%) and *A. hydrophila* (1, 1.9%). Also most of these *Aeromonas* spp. were isolated together with *Campylobacter* spp. (45, 86.6%) and lower percentage together with *Salmonella* spp. (6, 11.5%) and *Yersinia* spp. (1, 1.9%). *Aeromonas* spp. used as controls were isolated from patients with the same age ranges as the cases.

No significant differences were found in the distribution of virulence genes versus co-existence of co-pathogens with p value 0.911; 0.472; 0.444 for *act*, *alt*, and *aerA* respectively, while for *ast* and *hlyA* value was 0.957 in both cases (Table 1). On the other hand, differences were observed in the distribution of virulence genes and associated species $p < 0.01$. The most prevalent gene was *act* in *A. veronii* and *alt* in *A. caviae* for $p < 0.01$. All *A. hydrophila* studied were positive for the virulence genes *ast* and *hlyA*, while the *aerA* gene was only found in *A. veronii* and *A. hydrophila* although the difference found between these two species was not significant $p = 0.220$ (Table 2).

Conclusion

Since we found no significant differences between strains isolated with a primary pathogen versus those isolated alone, we should think that they are all potentially just as virulent strains and that they must be treated with equal importance when treating the patient. But it is also true that the most prevalent species is *A. caviae* that, as we demonstrated, that is the one in which fewer virulence factors were detected, therefore, it seems potentially less aggressive. The study of expression or cytotoxicity of the positive *alt* gene in 99.1% of our isolates would be missing to complete this comparison to definitively verify whether there are differences between co-pathogenic isolates and single-isolation strains.

As for the proportion of genes present in each of the species is not in line with some published studies as Soltan Dallal et al., 2016[11] or Pablos et al., 2010[12] because, in our opinion, identifying isolates methods have improved regarding when the mentioned studies were done. Our study is according with results of Silva et al., 2017,[13] who analyzed a greater number of strains and concluded that taking into account the number of virulence genes present in the majority of the isolates, the species *A. hydrophila* exhibit higher virulence potential among the species studied.

Not being able to clarify the controversy about the pathogenesis of *Aeromonas*, especially in pediatric patients, for not having been able to carry out a comparative study with strains isolated from healthy carriers, it would be prudent to analyze its appearance together with a compatible clinic. With all this, to say that antibiotic treatment would be indicated only in patients selected as some authors advised[14] and, in this case yes, taking into account those species with a greater virulent potential as has been demonstrated.

In conclusion, *A. hydrophila* is the potentially most virulent species studied. The role of *Aeromonas* as a co-pathogen in feces, especially in children, should be further studied. And it would be useful to study the possible association of virulence expression with clinical symptoms in patients and also with healthy carriers.

Transparency declarations

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Ethical approval

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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