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MOLECULAR CHARACTERIZATION OF *Aeromonas* spp. AND *Vibrio cholerae* O1 ISOLATED DURING A DIARRHEA OUTBREAK

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SUMMARY

This work aimed to assess pathogenic potential and clonal relatedness of *Aeromonas* sp. and *Vibrio cholerae* isolates recovered during a diarrhea outbreak in Brazil. Clinical and environmental isolates were investigated for the presence of known pathogenic genes and clonal relatedness was assessed by intergenic spacer region (ISR) 16S-23S amplification. Four *Aeromonas* genes (*lip*, *exu*, *gcat*, *fla*A/B) were found at high overall frequency in both clinical and environmental isolates although the *lip* gene was specifically absent from selected species. A fifth gene, *aer*A, was rarely found in *A. caviae*, the most abundant species. The ISR profile revealed high heterogeneity among the *Aeromonas* isolates and no correlation with species identification. In contrast, in all the *V. cholerae* isolates the four genes investigated (*ctx*A, *tcp*A, *zot* and *ace*) were amplified and revealed homogeneous ISR and RAPD profiles. Although *Aeromonas* isolates were the major enteric pathogen recovered, their ISR profiles are not compatible with a unique cause for the diarrhea events, while the clonal relationship clearly implicates *V. cholerae* in those cases from which it was isolated. These results reinforce the need for a better definition of the role of aeromonads in diarrhea and whether they benefit from co-infection with *V. cholerae*.

KEYWORDS: *Aeromonas; Vibrio cholerae*; Diarrhea; Virulence; PCR; Pathogenicity.

INTRODUCTION

Aeromonas are gram-negative bacilli from the Aeromonadaceae family found in aquatic environments: rivers and lakes and in both treated and raw sewage. They are known to be pathogenic to poikilothermic animals, causing ulcerative infections, and recently have been found to be associated with a variety of human extra-intestinal infections[17]. Their role as diarrhea causing agents is still controversial[12] as some studies have found aeromonads carried as transient flora in healthy asymptomatic individuals[2,15,17].

Although *Aeromonas* spp. produces virulence factors similar to other human enteropathogens[4], and despite the fact that there are some studies with mutant strains where lack of specific virulence factors may be associated with loss of pathogenicity[6,29], there is no animal model that reproduces the diarrhea so that the identification of virulence factors essential for its pathogenicity is therefore impaired. Hence, the role of *Aeromonas* in enteric infections have been mostly defined based on case reports, case-control studies and outbreak investigations associated with findings of the bacteria in diarrheic stools[19], thus linking the microorganism to the disease[3,17], although it has been shown that pathogenic aeromonads induces active Cl- secretion in the intestinal epithelium[10].

The *Aeromonas'* genomes harbor genes coding for putative virulence factors needed for different stages of infection, such as invasion, colonization and proliferation[11]: the *fla*A/B genes, coding for the polar flagellum, involved in the adhesion process which is essential for epithelium colonization[22]; the *exu* gene, coding for an extracellular DNase which blocks the antibacterial host defenses[5]; *lip* and *gcat* genes coding for extracellular lipases which make host cells membrane more susceptible to other toxins[26]; and the *aer*A gene, the most studied *Aeromonas* virulence factor, coding for a toxin, aerolysin, that induces pore formation in host cells membranes[17].

In 2004, a diarrhea outbreak occurred in São Bento do Una, Pernambuco, Brazil, with 2,170 cases and an attack rate of 4.65%. At that occasion, the supply of drinking water was precarious and people used water without microbiological control[16] suggesting that the water was the vehicle of infection. During the outbreak, 582 stool samples were analyzed and an enteric pathogen was isolated from 145 of these. *Aeromonas* spp. were the most frequent sole pathogen isolated from stools of patients suffering acute diarrhea (identified from 119 of the 145 samples where an enteric pathogen was recovered), with *Vibrio cholerae* O1 isolates also being recovered from a substantially smaller number of strains (18 out of 145)[16]. *V. cholerae* O1, the agent of cholera disease, is also a natural inhabitant of aquatic environments whose

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pathogenicity mechanisms are well defined[24]. Here, in an attempt to better characterize the pathogenic potential of the *Aeromonas* isolates, we have investigated the presence of putative virulence genes and assessed their clonal relatedness through ISR 16S-23S PCR analysis, performing parallel experiments with the *V. cholerae* isolates recovered during the same outbreak.

MATERIAL AND METHODS

Bacterial strains: One hundred and six *Aeromonas* spp. strains, out of a total of 119 originally isolated, and 18 *V. cholerae* O1 isolates from diarrheic stools and 19 *Aeromonas* spp. and seven *V. cholerae* isolates from aquatic environments were analyzed. Eighty two *Aeromonas* strains were kindly identified to the species level through 16S restriction fragment length polymorphism (RFLP) by Dr. Maria Jose Figueras from University Rovira i Virgili, Tarragona, Spain. Forty three strains were classified to the genus level, using biochemical tests[1], and are collectively called *Aeromonas* sp in this work. All *V. cholerae* O1 strains were identified by biochemical[25] and serological tests[30]. The differentiation between *Vibrio* and *Aeromonas* isolates was performed as previously described[13]. The reference strains *Aeromonas hydrophila* ATCC 7966^T, *Aeromonas veronii* biotype veronii ATCC 35624^T, *Aeromonas caviae* ATCC 15468^T, and *V. cholerae* 569B ^{T} were included as controls. The cultures were stored at -80 ºC in BHI plus 25% glycerol. DNA from the various strains was obtained as previously described[21].

PCR reactions: The presence of confirmed or putative virulence genes in the *Aeromonas* or *Vibrio* strains was assessed by PCR using primers described in the literature (Table 1). The *lip*, *exu*, *aer*A, *gcat* and *fla*A/B genes were investigated from the *Aeromonas* strains with the cholera toxin (*ctx*A), toxin co-regulated pilus subunit A (*tcp*A), accessory cholera enterotoxin (*ace*) and zonula occludens toxin (*zot*) genes being investigated from *V. cholerae*. The *Aeromonas* strains were also tested for *ctx*A to exclude the possibility of CTXΦ phage horizontal transfer between *V. cholerae* and *Aeromonas* strains. The Intergenic Spacer Region (ISR) between the 16S and 23S rDNA genes was amplified as previously described[9]. RAPD was performed with the primer CCGCAGCCAA as previously described[21]. PCR reactions were carried out in a Biometra T-3000 Genetic Analyzer thermal cycler using standard procedures and optimal annealing temperatures specific for each primer pair. PCR products were submitted to electrophoresis in agarose gels containing SYBR Safe DNA gel stain (Invitrogen) and photographed using the Kodak 1D Image Analysis software, version 3.5 (Digital Kodak Science).

Identification of PCR products: To confirm the identity of the amplified *Aeromonas* fragments, PCR products from one clinical (*A. hydrophila* ATCC 7966^T) and one environmental (*A. caviae)* isolates were sequenced on ABI Prism 3100 Genetic Analyzer, Applied Biosystems, Foster City, CA, using the same PCR primers. Sequences were aligned using the BLASTn program. The identity of the amplified *Vibrio* PCR gene fragments was confirmed by comparing their sizes with equivalent fragments predicted from the *V. cholerae* 569B reference strain.

Statistics: A chi-square or Fisher's exact test was used to compare

Table 1
Target, primer sequences, expected amplified fragments (bp), references and annealing temperature (AT) used to amplify *Aeromonas* putative virulence genes and *Vibrio cholerae* virulence genes

Genes	Nucleotide Sequence 5' → 3'	bp	References	AT
exu	(A/G)GACATGCACAACCTCTTCC GATTGGTATTGCC(C/T)TGCAA(C/G)	323	28	58 °C
lip	CA(C/T)CTGGT(T/G)CCGCTCAAG GT(A/G)CCGAACCAGTCGGAGAA	247	28	56 °C
*aer*A	CCTATGGCCTGAGCGAGAAG CCAGTTCCAGTCCCACCACT	431	28	58 °C
gcat	CTCCTGGAATCCCAAGTATCAG GGCAGGTTGAACAGCAGTATCT	237	28	56 °C
*fla*A/B	TCCAACCGT(C/T)TGACCTC G(A/C)(C/T)TGGTTGCG(A/G)ATGGT	608	27	55 °C
*ctx*A	CTCAGACGGGATTTGTTAGGCACG TCTATCTCTGTAGCCCCTATTACG	301	18	55 °C
*tcp*A El Tor	GAAGAAGTTTGTAAAAGAAGAACAC GAAAGGACCTTCTTTCACGTTG	471	18	55 °C
ace	AGAGCGCTGCATTTATCCTTATTG AACTCGGTCTCGGCCTCTCGTATC	600	21	55 °C
zot	GCTATCGATATGCTGTCTCCTCAA AAAGCCGACCAATACAAAAACCAA	900	21	55 °C

the virulence gene frequencies from clinical and environmental isolates. All conclusions are based on 5% significance level. The softwares Excel 2000 and R v2.10 were used.

RESULTS

***Aeromonas* species identification:** From a total of 125 (106 clinical and 19 environmental) *Aeromonas* isolates recovered during the 2004 diarrhea outbreak and available for this study, 57 were identified as *A. caviae* (51 clinical/6 environmental isolates, respectively), 13 were *A. veronii* (12/1), four were *A. hydrophila* (2/2), four were *A. media* (clinical isolates only), three were *A. trota* (also clinical isolates), one clinical isolate was *A. jandaei* and 43 (33/10) were identified only to the genus level and classified as *Aeromonas* sp (these represent the strains which could not be identified by RFLP, considered the gold standard method to *Aeromonas* identification[10,15]). *A. caviae* and *A. veronii* isolates comprise then 85% (70 of 82) of those classified to the species level.

Virulence gene frequencies for *Aeromonas* spp isolates: To compare the pathogenic potential of the different *Aeromonas* isolates, the presence of five different putative virulence genes was investigated (*lip*, *exu*, *gcat*, *fla*A/B and *aer*A) through conventional PCR amplification. Amplified fragments were recovered for all five genes from multiple isolates and their identity was confirmed through the sequencing of representative fragments, from one clinical and one environmental isolates for each gene, and the alignment of the resulting sequences with the corresponding genes deposited in the GenBank database. When all clinical and environmental isolates were considered, the fragment corresponding to the *gcat* gene was the only one amplified from all strains; the *fla*A/B genes was amplified from 87 (82.1%) of the clinical and 16 (84.2%) of the environmental strains; the *lip* gene from 90 (84.9%) of the clinical and 19 (100%) of the environmental strains; the *exu* gene from 91 (85.8%) of the clinical and 18 (94.7%) of the environmental strains, and the *aer*A gene was generated in 50 (47.2%) of the clinical and seven (36.8%) of the environmental strains (Table 2). The overall gene frequencies between clinical and environmental isolates were statistically similar ($p > 0.05$). All five genes were amplified in 33% of the clinical and 31.6% of the environmental *Aeromonas* strains and 60.3% of the clinical and 68.4% of the environmental strains amplified at least three genes (Table 3).

When the gene frequencies were evaluated only for those isolates identified to the species level, marked differences in frequency for the *lip* and *aer*A genes were observed between species. The *lip* gene, present in all environmental strains, was also found in most *A. caviae* (45 of 57) and in all *A. veronii* and *A. media* clinical isolates, however it was not detected in any of the *A. trota* or the *A. jandaei* isolates. In contrast, the *aer*A gene was found in only 15 of the 57 *A. caviae* isolates, despite being present in most of the isolates from the remaining species (20/25). Indeed the five genes were amplified from all four *A. media* and 10 of the 13 *A. veronii* isolates but only from 11 of the 57 *A. caviae* strains (Table 2).

ISR 16S-23S profiling for *Aeromonas* spp isolates: To evaluate the genetic relatedness of the various isolates and compare how these

Table 2
Distribution of putative virulence genes frequency in *Aeromonas* isolates.

Species	Nº		Gene frequency									
	Clin	Env	*fla*A/B		*exu*		*lip*		*aer*A		*gcat*	
			Clin	Env	Clin	Env	Clin	Env	Clin	Env	Clin	Env
Aeromonas caviae	46	6	35 76.1%	5 83.3%	38 82.6%	6 100%	39 84.8%	6 100%	12 26.1%	6 100%	46 100%	6 100%
Atypical* *Aeromonas caviae*	5	0	4 80%	0 0	4 80%	0 0	4 80%	0 0	0 0	0 0	5 100%	0 0
Aeromonas media	4	0	4 100%	0 0	4 100%	0 0	4 100%	0 0	4 100%	0 0	4 100%	0 0
Aeromonas hydrophila	2	2	2 100%	2 100%	2 100%	2 100%	1 50%	2 100%	2 100%	1 50%	2 100%	2 100%
Aeromonas veronii	7	0	7 100%	0 0	6 85.7%	0 0	7 100%	0 0	6 85.7%	0 0	7 100%	0 0
Atypical* *Aeromonas veronii*	5	1	4 80%	1 100%	4 80%	1 100%	5 100%	1 100%	4 80%	1 100%	5 100%	1 100%
Aeromonas trota	3	0	3 100%	0 0	3 100%	0 0	0 0	0 0	2 66.6%	0 0	3 100%	0 0
Aeromonas jandaei	1	0	1 100%	0 0	1 100%	0 0	0 0	0 0	1 100%	0 0	1 100%	0 0
Aeromonas sp.**	33	10	29 87.9%	9 90%	29 87.9%	9 90%	30 90.9%	10 100%	19 57.6%	2 20%	33 100%	10 100%

* Strains showing a discrete genotypic variation and a different band profile in RFLP; **Identified by genus level; Clin: clinical strains; Env: environmental strains.

Table 3
Association of putative virulence genes in clinical and environmental *Aeromonas* strains

Genes	Clinical strains		Environmental strains	
	Nº	%	Nº	%
gcat$^+$, *exu*$^+$, *lip*$^+$, *fla*$^+$, *aer*$^+$	35	33	6	31.6
gcat$^+$, *exu*$^+$, *lip*$^+$, *fla*$^+$	30	28.3	9	47.3
gcat$^+$, *exu*$^+$, *fla*$^+$	8	7.5	–	–
gcat$^+$, *exu*$^+$, *lip*$^+$	7	6.6	2	10.5
gcat$^+$, *aer*$^+$, *lip*$^+$, *fla*$^+$	5	4.7	–	–
gcat$^+$, *aer*$^+$, *exu*$^+$, *fla*$^+$	5	4.7	–	–
gcat$^+$, *lip*$^+$	5	4.7	–	–
gcat$^+$, *aer*$^+$, *exu*$^+$, *lip*$^+$	4	3.8	1	5.3
gcat$^+$, *lip*$^+$, *fla*$^+$	4	3.8	1	5.3
gcat$^+$, *aer*$^+$, *exu*$^+$	1	0.9	–	–
gcat$^+$, *exu*$^+$	1	0.9	–	–
gcat$^+$	1	0.9	–	–
Total	106	100	19	100

varies within and between species, and also within those classified only as *Aeromonas* sp., the amplification of the ISR 16S-23S from all 125 isolates was carried out. Nine different profiles were obtained (Fig. 1), herein called R1 to R9, all containing a common band, approximately 550 bp, plus others which varied according to each profile. Most of the strains (57.6%) fitted into the profile R1, 13.6% into R7, 8% into R5, 4.8% into both R8 and R4, 4% into R3, 3.2% into R2, 2.4% into R6 and 1.6% into R9. When the different profiles were compared to those isolates classified at the species level, no specific profile could be definitively linked to any particular species. Nine of the 13 *A. veronii* isolates fitted into the R7 profile, whilst 39 of the 52 *A. caviae* isolates were classified within R1, but no single profile was found in only one species and no species, with the exception of *A. jandaei* which was represented by only one isolate, was represented by only one profile.

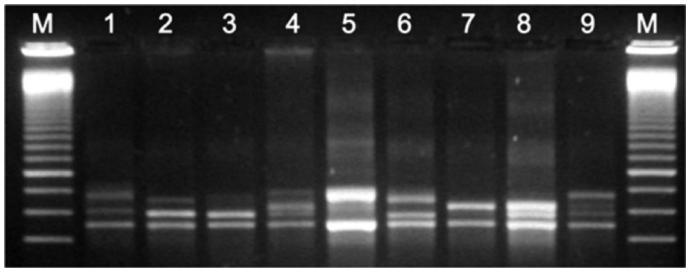

Fig. 1 - Intergenic Spacer Region (ISR) 16S-23S profiles (R1-R9) among *Aeromonas* isolates. Line M: 100 bp molecular marker; lines 1 to 9: R1 to R9 profiles.

Virulence gene frequency, RAPD and ISR 16S-23S profiling for *V. cholerae* isolates: To evaluate the virulence potential of the 18 clinical and seven environmental *Vibrio cholerae* isolates recovered during the same outbreak, all identified as *V. cholerae* O1 Ogawa, the presence of the known virulence genes *ctx*A, *tcp*A, *ace* and *zot* (for details see Material and Methods) was investigated through PCR and found in all isolates tested. Next, genetic relatedness was investigated through ISR 16S-23S amplification and, contrary to what was observed for the *Aeromonas* strains, even those from a single species, all *V. cholerae* O1 strains fitted into a single ISR 16S-23S profile (Fig. 2). All *V. cholerae* O1 isolates from the outbreak region showed the same RAPD profile observed in two strains from the 1993 outbreak, when cholera entered Brazil, suggesting the persistence of a clone in the environment. Only a non-toxigenic environmental strain from a second geographical region without human cases showed slight differences in profile when this technique was performed (data not shown).

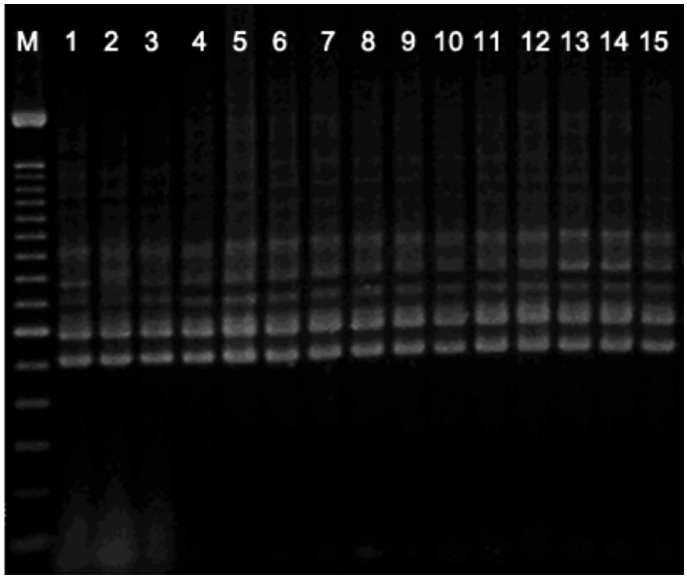

Fig. 2 - Homogeneity of the Intergenic Spacer Region (ISR) 16S-23S profile among different *Vibrio cholerae* O1 strains. Lines- M: 100 bp molecular marker; 1: non-toxigenic environmental *V. cholerae* O1; 2-12: toxigenic clinical *V. cholerae* O1 from 2004 diarrhea outbreak; 13-14: *V. cholerae* O1 from 1993 cholera outbreak in Brazil; and 15: *V. cholerae* O1 569B^T.

DISCUSSION

In spite of the high frequency of potentially virulent *Aeromonas* isolates in patient's feces during the evaluated diarrheal cases, the multiple species identified with distinct repertoires of virulence genes and heterogeneity in ISR 16S-23S sequences are not compatible with a single or related *Aeromonas* strain being responsible for the outbreak. On the other hand the *Vibrio* isolates analyzed revealed a homogeneous ISR and RAPD profile and high pathogenic potential associated with the presence of all searched virulence genes, clearly implicate *V. cholerae* as the etiological agent for those infections where it was found. Although it is not possible to rule out that the whole outbreak was due to an increased exposure of the target population to multiple enteropathogens, the possibility remains that some, if not most, of the cases where only *Aeromonas* strains were also isolated were also due to *V. cholerae*. It is possible that *Aeromonas* could be present as part of the patients' transient enteric flora and competed with *Vibrio in vitro* in the culture media, masking its presence and so, the real etiology of the disease. Alternatively,

a related explanation would be for the *Aeromonas* strain to succeed a preliminary *Vibrio* infection in patients debilitated by the primary event.

Although *Aeromonas* could not be recognized as the etiological agent of the diarrhea event in São Bento do Una, the high frequency of putative virulence genes suggest its pathogenic potential. The overall similarity of ISR 16S-23S profiles and frequency of virulence genes among clinical and environmental strains suggests environmental contamination by infected people feces and probably from animal carriers due to inadequate sanitation in that city. Various water environments constitute *Aeromonas* ecological niches[17] from where different bacterial lineages could spread to the city's inhabitants and proliferate, at least in immunocompromised individuals[11].

The gene *gcat*, which codes for a lipase that modifies the host cells permeability and raises its accessibility to toxins, was present in all *Aeromonas* strains investigated, regardless of their origin, confirming what was previously described and that this gene represents a marker to distinguish *Aeromonas* from other enteropathogens [7,8]. High frequency of *gcat* was also reported in another study[8] and in our analysis, despite the different species and genetic background, it was consistently amplified from all *Aeromonas* strains assayed. Hence, the presence of this gene could represent a marker to distinguish *Aeromonas* from other enteropathogens[7]. The *lip* and *exu* genes code for antibacterial host defense factors and were also detected at high frequency in the strains analyzed. For the *lip* gene, its absence from selected species could be due to a failure of the amplification reaction related to its polymorphic nature, however degenerate primer pairs were used for these reactions specifically to maximize gene amplification in cases of polymorphisms. It is also possible that lack of amplification may be a consequence of the small number of strains investigated for *A. trota* and *A. jandaei*. Nevertheless, considering the high frequency observed for the *lip* gene in strains from the remaining species (frequency rates varying from 50 to 100%) we are confident that this observation may reflect a real difference in virulence gene profile and which should further investigated in the future. Lipases also play a role on bacterial nutrition[26] and in the present study, the absence of the *lip* gene from clinical isolates of selected species, and its universal presence in the environmental isolates, may reflect more a role for survival in extracellular environment than in pathogenesis. The *fla*A/B genes essential for adhesion and epithelium colonization were found at high frequency in both clinical and environmental isolates. *Aeromonas* ability to form biofilms is directly related to the presence of the polar flagellum[20]. Therefore, the presence of *fla*A/B gene could be a virulence marker for *Aeromonas*.

Some authors associate the presence of high number of virulence genes with a higher pathogenic potential among *Aeromonas* strains[14,23]. *A. hydrophila* and *A. veronii* bv sobria showed a higher virulence potential compared to *A. caviae*[14]. Here, the frequency of *aer*A gene was indeed lower in *A. caviae* when compared with the remaining species, but still *A. caviae* was by far the most common species isolated from patients suggesting that this species remains virulent even in the absence of the aerolysin gene. Although the role of *Aeromonas* in diarrhea is not yet defined[17], the incidence of these bacteria in feces of patients with diarrhea has significantly increased and, in agreement with the concern raised with regards to *Aeromonas* within the scientific community, we therefore recommend the routine investigation for these bacteria in all coprocultures.

RESUMO

Caracterização molecular de *Aeromonas* spp. e *Vibrio cholerae* O1 isolados durante um surto de diarréia

O objetivo deste trabalho foi estabelecer o potencial patogênico e a relação clonal de isolados de *Aeromonas* sp. e *Vibrio cholerae* obtidos durante um surto de diarréia. Isolados clínicos e ambientais foram investigados quanto à presença de genes de virulência e sua relação clonal foi obtida através de amplificação da Região Espaçadora Intergênica (REI) 16S-23S. Quatro genes de *Aeromonas* (*lip*, *exu*, *gcat*, *fla*A/B) foram encontrados em alta frequência embora o gene *lip* tenha se mostrado ausente em algumas espécies. Um quinto gene, *aer*A, foi raramente encontrado em *A. caviae*, a espécie mais abundante. O perfil da REI revelou alta heterogeneidade entre os isolados de *Aeromonas* e nenhuma correlação com espécie. Em contraste, todas as amostras de *V. cholerae* amplificaram os genes investigados (*ctx*A, *tcp*A, *zot* e *ace*) e revelaram perfil clonal através de REI e RAPD. Embora *Aeromonas* tenha sido o principal patógeno isolado, o perfil da REI não é compatível como única causa para os eventos de diarréia, enquanto a relação clonal de *V. cholerae* aponta esse microrganismo como o provável agente do surto. Estes resultados reforçam a necessidade de definir melhor o papel de *Aeromonas* em diarréias e de que forma essas bactérias se beneficiam quando em co-infecção com *V. cholerae*.

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