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Short communication

Characterization of enterotoxigenic *Escherichia coli* strains isolated from the massive multi-pathogen gastroenteritis outbreak in the Antofagasta region following the Chilean earthquake, 2010

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ABSTRACT

In March 2010, a massive outbreak of gastroenteritis started in the region of Antofagasta (northern Chile). The outbreak was mainly attributed to Norovirus genogroup II although ETEC strains were also isolated with high frequency from clinical samples. We review this outbreak and determined that ETEC was an underestimated etiologic agent.

1. Introduction

ETEC are important etiologic agents of diarrhea, particularly in developing countries. This pathogen causes more than 400 million diarrheal episodes per year, resulting in 300,000–500,000 deaths, mostly in young children (World Health Organization - WHO, 2006). ETEC strains infecting humans produce a heat-labile toxin (LT) and/or heat-stable toxins (ST, including two subtypes, ST1a and ST1b). These strains can carry one or more of at least 22 different colonization factors (CFs) mediating adherence to the small intestinal mucosa (von Mentzer et al., 2014). Additionally, non-classical virulence genes such as *etpA*, and *eatA* are widely distributed and relatively conserved among ETEC strains (Del Canto et al., 2011; Luo et al., 2015).

On February 27, 2010, a magnitude 8.8 (Mw) earthquake struck Chile, affecting 2,671,556 people. This natural disaster claimed hundreds of lives, caused extensive material damage and affected health infrastructure and basic sanitation services (Gobierno de Chile, 2012). Routine distribution of chemicals used for water sanitation for the Metropolitan Region (Santiago city) as well as for northern and southern cities was limited during this period. On March 8, 2010, an outbreak of acute gastroenteritis started in the region of Antofagasta (northern Chile) mainly affecting the cities of Antofagasta (350.000 inhabitants) and Calama (150.000 inhabitants). As of April 28, 2010, 31,036 gastroenteritis cases were reported, diarrhea being the main symptom (97% of cases). Due to the increasing number of cases of gastroenteritis, a Rapid Response Team (RRT) was mobilized to the affected region by the Chilean Health Ministry in order to characterize the outbreak and mitigate its public health impact. The RRT performed an observational descriptive study, including epidemiological inquiries, environmental sample collection (drinking water and treated wastewater) and collection of stools from patients with gastroenteritis. Stool samples from 932 gastroenteritis cases were analyzed for viruses (norovirus I/II), bacteria (*Salmonella* sp., *Shigella* sp., diarrheagenic *Escherichia coli*, *Yersinia enterocolitica*, *Listeria monocytogenes*, *Vibrio parahaemolyticus*, *Aeromonas* sp. and *Campylobacter jejuni*) and parasites (*Cryptosporidium* sp. and *Giardia* sp.) at the Instituto de Salud Pública (ISP; 736 samples) and the Universidad de Chile (196 samples). Testing at ISP identified norovirus (NoV) genogroup II (GII) in 20/203 (10%) and diarrheagenic *E. coli* strains in 454/712 (64%) of samples. Additional bacterial pathogens were identified in lower proportions as was described for *Shigella* sp. (5,2%, 37/712) and *Vibrio cholerae* no O1 no O139 (1,5%, 11/712) (Díaz et al., 2012). At the Universidad de Chile,

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testing identified NoV in 68 (35%), ETEC in 70 (36%), and NoV-ETEC co-infection in 26 (13%) of 196 samples studied (Díaz et al., 2012). Additionally, NoV GII was studied and detected in 20 L of water samples from the Antofagasta city wastewater treatment plant which had decreased sodium hypochlorite concentrations. This contaminated water used to irrigate vegetable crops grown in the region, was deemed to be the main source for this massive outbreak (Díaz et al., 2012). The role of NoV GII was previously described; however, despite high isolation rates, further studies of the ETEC strains isolated during the outbreak and their role in the outbreak were lacking. In this study, we characterize the virulence and colonization factors of ETEC isolated in the 2010 gastroenteritis outbreak in Antofagasta.

2. Methods

2.1. Sample collections and growth conditions

We characterized 39 isolates of ETEC obtained from stool samples of patients who sought medical care for gastroenteritis occurring during the outbreak at four health centers in the cities of Antofagasta (Coordinates; latitude - 23.650000 and longitude - 70.400002) and Calama (Coordinates; latitude - 22.453712 and longitude - 68.925308), this last 201 km northeast of Antofagasta. In eight samples NoV was also detected (Díaz et al., 2012). These strains were stored in our −80 °C freezer at the Microbiology Program, faculty of Medicine, University of Chile. All strains were grown in Luria-Bertani (LB) broth (tryptone [10 g/L], NaCl [10 g/L], yeast extract [5 g/L]) at 37 °C for 18 h with no agitation.

2.2. Screening for ETEC virulence genes by polymerase chain reaction (PCR)

The identification of classical (enterotoxins and 20 CFs) and nonclassical virulence genes (*etpA*, *etpB*, *tia*, *tibA* and *eatA*) were performed by polymerase chain reaction (PCR) using previously described primers (Table 1).

2.3. Pulse field gel electrophoresis (PFGE)

Bacterial suspensions adjusted to optical density 0.8 at 420 nm were embedded in 1% SeaKem Gold agarose plug. Then bacteria were lysed, washed five times and a 2 mm thick piece was cut, equilibrated and digested with 50 U of *Xba*I enzyme at 37 °C for 3 h. PFGE was performed with the CHEF-DR III BIO-RAD in a 1% Pulse Field Certified Agarose gels (Ultrapure DNAgrade agarose) at 6 V/cm and 14 °C by 21 h. *Salmonella* serotype Braenderup H9812, previously digested with *Xba*I was used as a base pair marker and a run control. The separated PFGE fragments were visualized after staining with ethidium bromide using a UV transilluminator and the captured images were analyzed with the software BioNumerics GelCompar II 6.0 (Applied Maths, Belgium). The similarity between fingerprints was determined using Dice's correlation coefficient with a 1% tolerance between band positions. We defined pulsotypes as DNA banding patterns with similarity ≥95%. The cluster analysis and generation of dendrogram was performed using UPGMA.

2.4. Immunoblotting of secreted ETEC virulence antigens

Detection of the secreted virulence ETEC antigens was performed in the supernatants from overnight cultures, which were first precipitated with trichloroacetic acid (TCA) and then subjected to electrophoresis in polyacrylamide gel. Later, western blotting was performed using polyclonal rabbit antisera against recombinant proteins of EatA and EtpA, as previously described in (Luo et al., 2015), followed by detection using a secondary goat anti-rabbit-(IgG)-HRP conjugate (Santa Cruz Biotechnology, SC2004).

3. Results

Initially, distribution of classical (enterotoxins and 20 CFs) and non-classical virulence genes (*etpA*, *etpB*, *tia*, *tibA* and *eatA*) were studied by PCR using previously described primers (Table 1). The most common toxin profiles were ST1b (15/39; 38.5%), followed by LT + ST1b (14/39; 35.9%) and LT (5/39; 12.8%). Less common toxin profiles were

Table 1
List of primers used for detection of classical and nonclassical virulence genes.

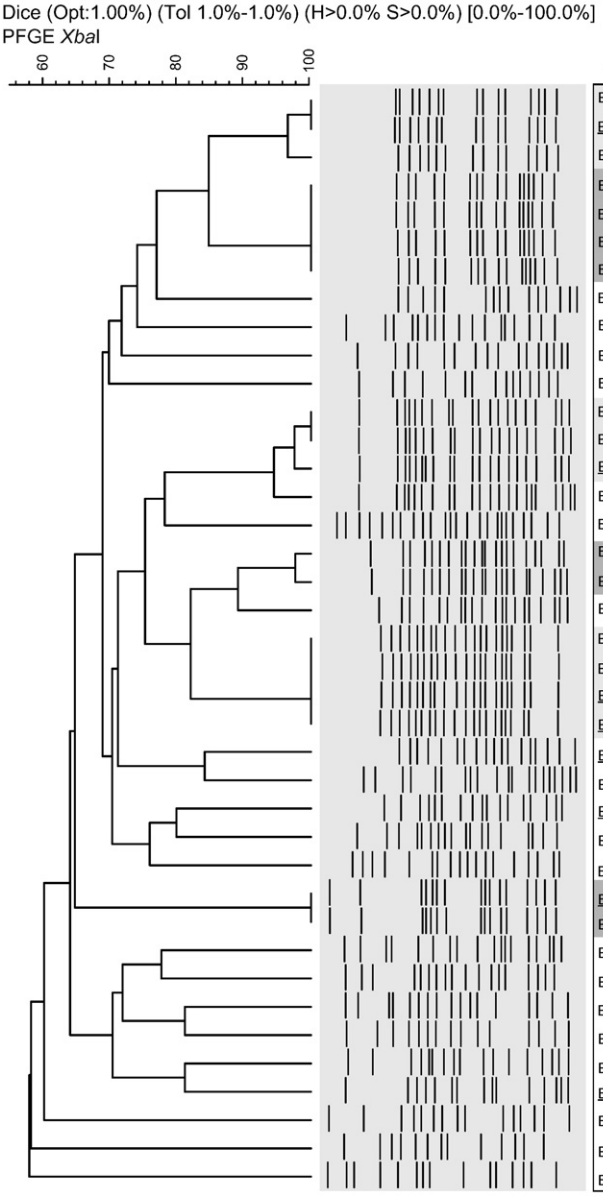
Virulence gene	Primer Sequence		Product size (bp)	Reference
	Forward (5′-3′)	Reverse (5′-3′)		
LT	GCACACGGAGCTCCTCAGTC	TCCTTCATCCTTTCAATGGCTTT	218	(Vidal et al., 2005)
ST1a	AAAGGAGAGCTTCGTCACATTTT	AATGTCCGTCTTGCGTTAGGAC	129	(Vidal et al., 2005)
ST1b	TTCTTTCTGTATTGTCTTTTTCACC	TAATAGCACCCGGTACAAGCAG	193	(Vidal et al., 2009)
CFA/I	ACTATTGGTGCAATGGCTCTGAC	CAGGATCCCAAAGTCATTACAAG	497	(Vidal et al., 2009)
CS1	GAGAAGACCATTAGCGTTACGG	CCCTGATATTGACCAGCTGTTAG	410	(Vidal et al., 2009)
CS2	ACTGTAACTGCTAGCGTTGATCC	TGCTTCCTGCATTAATAACGAGT	358	(Vidal et al., 2009)
CS3	CCCACTCTAACCAAAGAACTGG	CGTATTTCCAGCATTTTTATCCA	300	(Vidal et al., 2009)
CS4	ATTGATATTTTGCAAGCTGATGG	GTCACATCTGCGGTTGATAGAGT	242	(Vidal et al., 2009)
CS5	CAACCGTATCAGGTTCTGTTTTG	CAAATGTTACCGGAGCTACAAAG	558	(Vidal et al., 2009)
CS6	AAATGTATCCCAGGTAACGGTCT	TGTTGATTAGGCGTAACCTCTGT	165	(Vidal et al., 2009)
CS7	TGCTCCCGTTACTAAAAATAC	TAGATGTCGTATCACTACGT	203	(Del Canto et al., 2011)
CS8	ATCCGGATTATCAAGCTCCA	GAAGATGTTATTGCACCACCAA	166	(Sjöling et al., 2007)
CS12	GCGAATAACAATGATGCAAG	CCTGACTGGTTTACAAGATA	263	(Del Canto et al., 2011)
CS13	GGGACTGCCACAATGAATTT	CAGCACCACCTGCTGATTTA	178	(Sjöling et al., 2007)
CS14	TTTGCAACCGACATCTACCA	CCGGATGTAGTTGCTCCAAT	162	(Sjöling et al., 2007)
CS15	CGAAATTGGACAAGCGATG	TCCAGCAGGGATATTATTCG	130	(Sjöling et al., 2007)
CS 17	GGAGACGCTGAATACAACTGA	CTCAGGCGCAGTTCCTTGT	130	(Sjöling et al., 2007)
CS18	ATCCGTCAGGTGTTTGTGGT	CACCTGAATTCCTCGACAGG	362	(Rodas et al., 2009)
CS17/19	TAAACTTGATCTTCTGCAAGC	TCAGGCGCAGTTCCTTGTGTG	348	(Del Canto et al., 2011)
CS20	AGGTATCCAAATCCGCACTG	CATCAGCCAGCACATAGGAA	114	(Sjöling et al., 2007)
CS21	CCAGATTTTGTGGACCCATT	GTTAAAGCACCGCCAATAGC	158	(Sjöling et al., 2007)
CS22	ATTGGACAAGCGTCCAACAC	TTCCAGCAGGGATATTATCATTTT	127	(Sjöling et al., 2007)
CS23	CTGCTATGGCGTGGACTGTA	AGGTGAATAGGGGGTTCTCG	578	(Del Canto et al., 2012)
tia	GCTTCAGTTGTGATGCAGAC	CAGCATCCAGATAGCGATAG	535	(Del Canto et al., 2011)
tibA	CACGACAAATCAAACGTACC	CTTCCGCCGTAGAGATACAT	655	(Del Canto et al., 2011)
eatA	ATGTGCTTTGGCAGGTTAAT	ATATCCAGTCAGCACCCACT	1943	(Del Canto et al., 2011)
etpA	GGTTCAGGCAGTATCCAGAC	GGTGTAGCTGTCTGACCACA	999	(Del Canto et al., 2011)
etpB	CTTCGTGCCTGTATCGGTAG	GGTATTTGTCGGTCAGGAAG	760	(Del Canto et al., 2011)

ST1a, ST1a + ST1b, ST1a + LT and LT + ST1a + ST1b (Fig. 1). This toxin profile is consistent with other reports including Chilean ETEC strains in which ST is prevalent (Agüero et al., 1985) as well as reports from other countries (Qadri et al., 2005a). Notably, most of the isolates (36/39; 89.7%) carried one or more CFs genes, of which the CS21 pilin gene was most common (34/39; 84.6%). The majority of isolates were also PCR positive for one or more non-classical virulence genes, including *etpA* and *etpB* (28/39; 71.8%), which encode the secreted EtpA adhesin and its cognate two-partner secretion pore EtpB; while *eatA* which encodes the EatA mucin-degrading serine protease was present in more than half of the isolates (23/39; 59%). Production of EtpA and EatA was also confirmed by immunoblotting in the majority of genotypically positive isolates, suggesting that these antigens were widely distributed among the ETEC pathovar within the outbreak. Interestingly, production of EatA was confirmed by immunoblotting in five isolates in which the *eatA* gen was not detected (Fig. 1). This could be explained by cross-reactivity with a non-detectable allelic variant of *eatA* with the primers used.

Next, genetic relationship between ETEC isolates was studied by pulsed field electrophoresis (PFGE) using the *XbaI* restriction enzyme according to standard protocols. A total of 28 PFGE patterns (pulsotypes; DNA banding patterns with similarity ≥ 95%) were obtained. Isolates from different localities in Antofagasta and Calama were distributed among all of the dendrogram clusters. Nevertheless, there was good correspondence between isolates that shared the same pulsotype and the presence of classical and non-classical virulence genes. Interestingly, 6 pulsotypes (1, 2, 7, 10, 12 y 18) clustered two or more clonal isolates from different origin. Together, these findings strongly suggest that at least 6 different ETEC clonal groups were circulating throughout the Antofagasta region during the outbreak.

4. Discussion

A previous study described the role of norovirus in the 2010 Antofagasta outbreak (Díaz et al., 2012). In this study, we determined that ETEC played an important role as well, considering both its high



Strain ID[a]	Origin[b]	Pulsotype	Enterotoxins	Colonization Factors	Nonclassical Virulence Genes	EtpA	EatA
B49-ANT-40	B	01	ST1b	CS21	*etpA, etpB*		
B49-ANT-25	D	01	ST1b	CS21	*etpA, etpB*		
B49-ANT-86	B	01	ST1b	CS6, CS21	*etpA, etpB, eatA*		
B49-ANT-93	B	02	ST1b, LT	CFA-I, CS21	*etpA, etpB*		
B49-ANT-91	B	02	ST1b	CS21	*etpA, etpB*		
B49-ANT-33	B	02	ST1b	CS21	*etpA, etpB*		
B49-ANT-63	C	02	ST1b, LT	CS6, CS21	*etpA, etpB*		
B49-ANT-92	B	03	LT	CS6, CS8, CS21	*eatA*		
B49-ANT-03	B	04	ST1a	CS15, CS21			
B49-ANT-102	B	05	ST1a	CS15, CS21			
B49-ANT-67	C	06	ST1b, LT	CS1, CS3, CS21	*etpA, etpB, eatA*		
B49-ANT-103	B	07	ST1b	CFA-I, CS15, CS21	*etpA, etpB, tia, eatA*		
B49-ANT-44	B	07	ST1b	CFA-I, CS15, CS21	*etpA, etpB, tia, eatA*		
B49-ANT-57	B	07	ST1b, LT	CFA-I, CS15, CS21	*etpA, etpB, tia, eatA*		
B49-ANT-106	B	08	ST1b	CFA-I, CS15, CS21	*etpA, etpB, tia, eatA*		
B49-ANT-82	B	09	ST1b	CS21	*etpA, etpB, tia, eatA*		
B49-ANT-04	B	10	ST1b, LT	CS2, CS3, CS21	*etpA, etpB, eatA*		
B49-CAL-03	A	10	ST1b	CS2, CS3, CS21	*etpA, etpB, eatA*		
B49-ANT-73	C	11	ST1b, LT	CS2, CS3, CS21	*etpA, etpB, eatA*		
B49-ANT-22	D	12	ST1b, LT	CS1, CS2, CS3, CS21	*etpA, etpB, tia, eatA*		
B49-CAL-07	A	12	ST1b, LT	CS1, CS3, CS21	*etpA, etpB, tia, eatA*		
B49-CAL-08	A	12	ST1b, LT	CS1, CS3, CS21	*etpA, etpB, tia, eatA*		
B49-ANT-72	C	12	LT	CS1, CS2, CS3, CS21	*etpA, etpB, tia, eatA*		
B49-ANT-07	B	13	ST1b, LT	CS3, CS21	*etpA, etpB, eatA*		
B49-ANT-26	D	14	ST1b, LT	CS6, CS8			
B49-ANT-37	B	15	ST1a, LT	CS6, CS21	*eatA*		
B49-ANT-104	B	16	ST1a, ST1b	CS6, CS21			
B49-ANT-24	D	17	ST1a, ST1b	CS8, CS21			
B49-CAL-06	A	18	ST1b	CS21	*etpA, etpB, eatA*		
B49-ANT-47	B	18	ST1b		*etpA, etpB*		
B49-CAL-13	A	19	ST1b, LT				
B49-ANT-108	B	20	ST1b, ST1a, LT		*etpA, etpB*		
B49-ANT-74	C	21	ST1b, LT	CS8, CS21	*etpA, eatA*		
B49-ANT-59	C	22	LT	CS8, CS21			
B49-ANT-39	B	23	LT	CS8, CS17	*etpA, etpB, eatA*		
B49-CAL-14	A	24	ST1b		*etpA, etpB, tia, eatA*		
B49-CAL-17	A	25	ST1b	CS21	*etpA, etpB, eatA*		
B49-ANT-09	B	26	ST1b, LT	CS21			
B49-ANT-85	B	27	ST1b	CS2, CS15, CS21	*etpA, etpB, eatA*		

Fig. 1. Dendrogram showing the PFGE analysis and the molecular characterization of ETEC isolates. [a]Isolates obtained from patients where co-infection with calicivirus was reported are shown underlined. [b]Stool samples were taken from 4 different health centers. A, Hospital de Calama; B, Centro Asistencial Norte (Antofagasta); C, Consultorio Juan Pablo II (Antofagasta); D, Hospital Regional Leonardo Guzmán (Antofagasta). Production of EtpA and EatA that was confirmed by immunoblotting is showed in green squares. Red squares mean that the production of these proteins was not confirmed.

prevalence rate, and the fact that these strains harbored well recognized virulence genes. It is important to note that CS21 (*lngA*) and *etpA* were the most commonly detected virulence genes representing antigens that protect against ETEC colonization in murine models (Roy et al., 2009; Zhang et al., 2015). Given their broad distribution and immunogenicity, these protective antigens could complement existing canonical approaches to vaccine development centered on other colonization factors (Vidal et al., 2009).

Our study identified multiple ETEC stains, harboring diverse virulence repertoires, a common finding in outbreaks associated with this pathogen (Dalton et al., 1999; Qadri et al., 2005a). Taking into account the outbreak investigation report (Díaz et al., 2012), we consider the role of ETEC at the time of the outbreak was not well understood. ETEC is an important cause of diarrhea, not just in the developing but also in developed world, including the U.S.A. (Medus et al., 2016).

In consequence, the outbreak described in the Antofagasta region following the Chilean earthquake was a multi-etiology outbreak. The co-occurrence and co-infection with multiple enteric pathogens is common in gastroenteritis outbreaks especially when associated with massive contamination of water supplies (Charles et al., 2014; Ethelberg et al., 2010; Gonzaga et al., 2011; Qadri et al., 2005a, 2005b), as was the case in the Antofagasta region.

Finally, based on the frequency of isolation of ETEC during the outbreak, we estimate that at least 10,000 cases of gastroenteritis could have been attributable to this pathogen. To our knowledge, this would be one of the largest outbreaks associated with ETEC reported to date.

Conflict of interest

The authors declare that no competing interests exist.

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